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REVIEW

# Immune responses against protozoan parasites: a focus on the emerging role of Nod-like receptors

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**Abstract** Nod-like receptors (NLRs) have gained attention in recent years because of the ability of some family members to assemble into a multimeric protein complex known as the inflammasome. The role of NLRs and the inflammasome in regulating innate immunity against bacterial pathogens has been well studied. However, recent studies show that NLRs and inflammasomes also play a role during infections caused by protozoan parasites, which pose a significant global health burden. Herein, we review the diseases caused by the most common protozoan parasites in the world and discuss the roles of NLRs and inflammasomes in host immunity against these parasites.

**Keywords** NLR · Inflammasome · Parasites · Protozoa · NOD · NLRP3

## Introduction

Protozoa are unicellular eukaryotic microorganisms that can be free-living or parasitic. Although infection with most of the protozoa is harmless, some protozoan infections can be detrimental to human and animal health. In recent years, *Plasmodium* species, the causative agent of malaria, has garnered much attention because of the devastating effect of these protozoa in the infected host. Unfortunately, many other protozoan parasites that infect humans are still poorly understood and are categorized as neglected tropical disease by the World Health Organization (WHO). Although immune responses against some protozoan parasites have been relatively well studied, the roles of NLRs in regulating innate and adaptive immune responses against most of these parasites are only beginning to be understood. Here, we review immune responses and the roles of NLRs, inflammasomes and associated cytokines IL-1 and IL-18 in modulating adaptive immune responses during protozoan infections.

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## Nod-like receptors

Nucleotide-binding oligomerization domain receptors [Nod-like receptors (NLRs)] are cytoplasmic sensors that sense pathogen-associated molecular patterns (pathogens/foreign) or damage-associated molecular patterns (cells/self). To date, 22 NLRs in humans and 34 NLRs in mice have been characterized [1]. On the basis of the NLRs whose functions have been reported, NLRs can be classified into four major functional subgroups: (1) positive regulators of signaling pathways or inflammation, (2) negative regulators of signaling pathways or inflammation, (3) regulators involved in the formation of the inflammasome complex, and (4) regulators of transcription (Fig. 1). NLRC1 (NOD1) and NLRC2 (NOD2), which contain a caspase recruitment domain (CARD), were identified because of their ability to activate NFκB and mitogen-activated protein kinase (MAPK) in response to bacterial ligands [2–5]. NLRC3 [6], NLRC5 [7], NLRP6 [8], NLRP12 [9, 10], and NLRX1 (NOD5) [11, 12] negatively regulate the NFκB and MAPK signaling pathways. CIITA (NLRA) and NLRC5 are transcriptional activators of the major histocompatibility class II and class I molecules, respectively [13, 14]. Recent findings show that NLRP3 transcriptionally activates *T-helper 2* (*Th2*) genes in T cells [15].

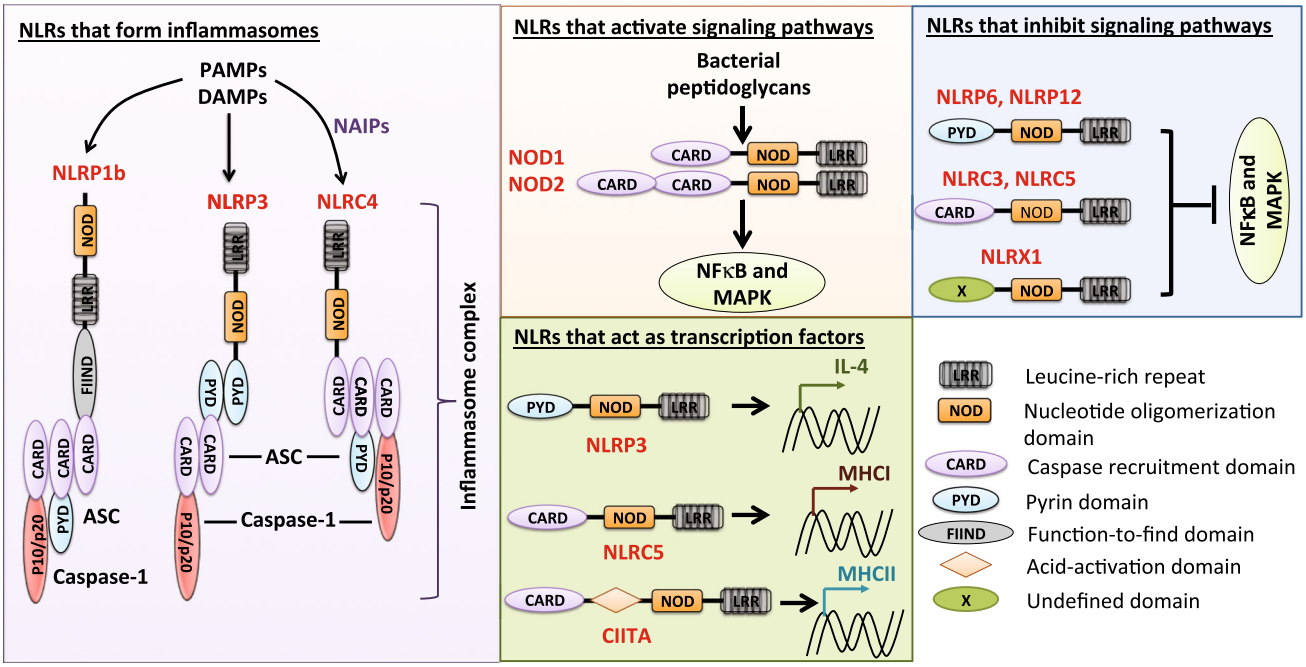


**Fig. 1** Diverse functions of NLR proteins. NLRs that form inflammasomes: NLRP1b, NLRP3 and NLRC4, upon sensing pathogen or damage signals, recruit ASC and caspase-1 to assemble the inflammasome complex. This inflammasome complex is crucial for activation of caspase-1 and ultimate cleavage of pro-IL-1β and pro-IL-18 into their mature forms. NLRs that activate signaling pathways: NOD1 (NLRC1) and NOD2 (NLRC2) recognize muramyl dipeptide and diaminopimelic acid from bacterial peptidoglycan and recruit downstream common adaptor receptor protein interacting kinase 2 to activate the NFκB and mitogen-activated protein kinase (MAPK) signaling pathways. NLRs that inhibit signaling pathways: NLRP6, NLRP12, NLRC3, NLRC5, and NLRX1 are negative regulators of NFκB and MAPK signaling pathways. NLRs that act as transcription factors: NLRP3 act as transcriptional regulator of IL-4. NLRC5 acts as a transcriptional activator of major histocompatibility complex (MHC) class I molecules, and CIITA has a well-established role as a transcriptional activator of MHC class II proteins

NLRP1b, NLRP3, and NLRC4 have well-established functions in forming the inflammasome, a multimeric protein complex that plays a central role in innate immunity [16–20]. More recently, NLRP6 and NLRP12 have also been implicated to form inflammasomes [21, 22], although additional independent and biochemical studies are needed to establish these findings. Upon sensing danger signals (self or foreign), these NLRs recruit caspase-1 and the apoptosis-associated speck-like protein containing a carboxy-terminal CARD (ASC) to form the inflammasome. Within the inflammasome complex, caspase-1 gets autocleaved and activated. Activated caspase-1 can then process pro-interleukin (IL)-1β and pro-IL-18 into their mature forms and induce pyroptotic cell death. Because of the known widespread functions of cytokines IL-1 and IL-18, NLRs that are associated with activation of the inflammasome complex are currently a major area of research.

## Amebiasis

Amebiasis is a worldwide health concern caused by the enteric protozoan parasite *Entamoeba histolytica* and affects more than 100 million people, causing 100,000 deaths per year [23]. Most infected individuals are asymptomatic, but diarrhea, dysentery, colitis, and liver abscesses can occur [24, 25]. The disease is transmitted mainly through the ingestion of *E. histolytica* cysts under conditions of poor sanitation and contaminated water [25]. The results of a longitudinal study of a cohort of children in Bangladesh suggest that infant malnutrition is a major factor that predisposes young children to amebiasis [26, 27]. The treatment of amebiasis is usually limited to pan antibiotics such as metronidazole, which are often associated with severe side effects. A comprehensive understanding of the immune responses that are generated against *E. histolytica* is lacking [28].

### Immune responses against *E. histolytica*

*E. histolytica* contains several virulence factors that allow it to persist and survive within the host. Although more than 90 % of *E. histolytica* infections are asymptomatic and remain as commensals within the host's gut environment, under certain circumstances, the parasite invades the host epithelium and becomes pathogenic [27]. Some of the well-known key virulence factors of *E. histolytica* are Gal-lectin (the 170-kDa surface Gal/GalNAc lectin of

*E. histolytica*), cysteine proteases, peroxiredoxin, and lipopeptidophosphoglycan (LPPG) [25, 29]. Although these virulence factors provide key survival advantages for *E. histolytica* in the infected host, they also activate the host's immune responses.

The intestinal tract provides the first line of defense, where mucus prevents ameba penetrance into the intraepithelial cells. Gal-lectin of *E. histolytica* binds mucosal matrix and uses its cysteine proteases to cleave mucin to gain access to the intestinal epithelial cells (IECs) [30, 31]. Mice deficient in mucin (MUC2 knockout mice) are indeed highly susceptible to *E. histolytica* infection, further demonstrating the importance of the intestinal mucin coating [32]. The intestinal tract is also filled with antimicrobial compounds and secretory immunoglobulin A (sIgA) that provide additional protection against *E. histolytica*. As such, deficiency in antimicrobial protein REG1 or sIgA renders the host highly susceptible to *E. histolytica* infection [33, 34].

Once the initial luminal barrier is breached, the IECs respond to *E. histolytica* to eliminate infection. IECs recognize LPPG associated with *E. histolytica* and produce several chemokines and cytokines, including TNF-α, IL-6, and GM-CSF, to recruit neutrophils and monocytes [35]. In addition to actively secreting inflammatory cytokines, IEC death also promotes inflammation [35]. Neutrophils are rapidly recruited to the site of infection to combat infection once intestinal epithelial barrier integrity is breached [36, 37]. Thus, neutrophil-depleted mice have more intestinal lesions and higher susceptibility to *E. histolytica* than do wild-type (WT) mice [38]. Neutrophils are also important for establishing early resistance to hepatic amebiasis [39].

Similar to neutrophils, macrophages also play an important role during *E. histolytica* infection. Macrophages express Toll-like receptors (TLRs) that can recognize *E. histolytica* virulence factors to initiate amebicidal immune responses. TLR2 and TLR4 recognize *E. histolytica* LPPG, whereas TLR9 recognizes *E. histolytica* DNA [40, 41]. Cytokines such as interferon (IFN)-γ further activate macrophages to produce nitric oxide synthase (NOS) and prevent *E. histolytica* infection [42, 43]. LPPG also activates dendritic cells through TLRs, increasing their expression of costimulatory molecules such as CD80, CD86, and CD40 and promoting cytokine production [44]. NK and NKT cells are not important for intestinal immunity but are necessary for protection against *E. histolytica* liver colonization [45, 46]. These cells secrete IFN-γ and tumor necrosis factor (TNF)-α, factors that are critical for activating innate immune cells, including neutrophils and macrophages [45, 46]. Mice lacking NKT cells are highly susceptible to *E. histolytica* colonization in the liver and subsequent liver abscesses [45]. Deficiency of either IFN-γ or NOS results in severe liver infection with *E. histolytica*, demonstrating the importance of these critical cytokines [42, 47, 48].

With regard to adaptive immunity, protection from or resistance to *E. histolytica* also seem to depend on Th1 responses. Patients who were infected with *Entamoeba* but were asymptomatic had higher levels of IFN-γ, suggesting a protective role for Th1 responses [49]. In contrast, patients with invasive amebiasis had increased levels of the Th2-associated cytokine IL-4 [49]. Consistent with these correlative studies, the results of studies in mice showed that high IL-4 production by T cells plays an important role in pathogenicity and susceptibility during *E. histolytica* infection [50]. Conversely, production of IFN-γ by T cells is associated with protection against *E. histolytica* infection [50]. More importantly, the protection observed in susceptible mice immunized with Gal-lectin was due to the production of IFN-γ and IL-17 by $CD4^+$ and $CD8^+$ T cells [51]. Taken together, these results demonstrate that protection against *E. histolytica* requires a balance of Th1 and Th2 responses.

## *E. histolytica* infection activates NLRP3 inflammasomes and IL-1 cytokine production

The role of NLRs, inflammasomes, and IL-1 and IL-18 during *E. histolytica* infection has not been thoroughly studied. The results of in vitro studies of cultured human epithelial and stromal cells as well as cell lines show that *E. histolytica* induces the robust production of IL-1α, which acts in a paracrine manner to induce strong cytokine production and inflammation [52]. The results of other studies show that the cysteine proteinase EhCP5 of *E. histolytica* can cleave and inactivate recombinant IL-1β and IL-18 [53, 54]. Furthermore, the monocyte locomotion inhibitory factor produced by *E. histolytica* downregulates *Il1b* expression [55]. Taken together, these results suggest a protective role for IL-1β and IL-18 in immunity against *E. histolytica*, which may be why this protozoan uses various strategies to neutralize these cytokines.

Although studies of NLRs are scarce, the results of studies by one group show that *E. histolytica* activates the inflammasome in vitro and in vivo (Fig. 2a). The Gal-lectin of *E. histolytica* induces robust caspase-1 activation and subsequent IL-1β and IL-18 production in mouse macrophages [56]. Similarly, the binding of *E. histolytica* to the integrin α5β1 recruits cysteine proteinase EhCP5, which activates the NLRP3 inflammasome and, subsequently, IL-1β and IL-18 in vitro and ex vivo [57]. Given that Gal-lectin induces inflammasome activation [56] and protective CD4 and CD8 T cell responses [51], the NLRP3 inflammasome may be involved in regulating adaptive immune responses. Further studies are needed to directly evaluate the effects of NLRP3, inflammasomes, and IL-1 and IL-18

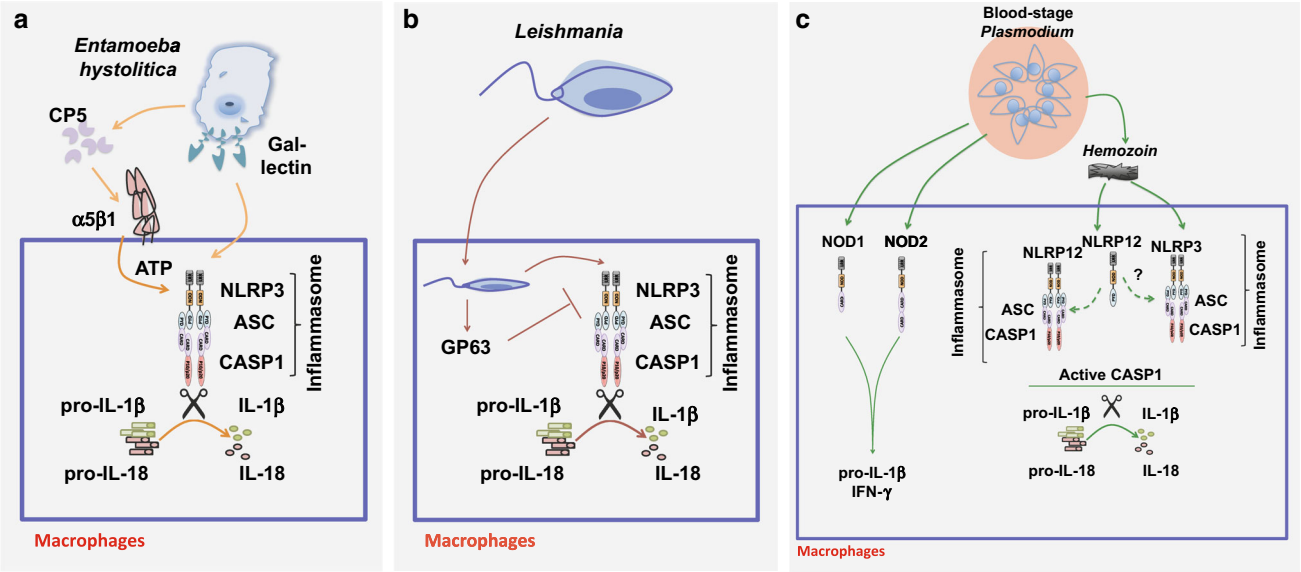


**Fig. 2** Role of the NLRP3 inflammasome during *E. histolytica*, *Leishmania,* and *Plasmodium* spp. infections. **a** The *Entamoeba hystolitica* Gal-lectin can directly activate the NLRP3 inflammasome through yet-unknown mechanisms. The *Entamoeba* protease CP5 can also engage the α5β1 integrin to modulate ATP and activate the NLRP3 inflammasome. **b** *Leishmania* activates the NLRP3 inflammasome to induce IL-1β and IL-18 production in vitro and in vivo. *Leishmania*-associated metalloprotease GP63 can inhibit NLRP3 inflammasomes by inhibiting ROS production and directly cleaving NLRP3. **c** *Plasmodium* spp. engage NOD1 and NOD2 to promote IL-1β and IFN-γ production. In addition, *Plasmodium* parasites can activate NLRP12 and NLRP3 inflammasomes through hemozoin. Whether NLRP12 is in complex with NLRP3 or forms independent inflammasomes is not known

on adaptive immune responses during *E. histolytica* infection.

## Leishmaniasis

Leishmaniasis, a disease caused by *Leishmania* species, is a major health concern in tropical and subtropical regions around the world. More than 300 million people are at risk of this parasitic infection, including 12 million people currently infected and 1.5 million new cases per year [58–60]. Mouse models of *Leishmania* infections have been extremely useful in furthering our understanding of Th1 (hallmark cytokine, IFN-γ) and Th2 (hallmark cytokine, IL-4) responses [61]. *L. major* infection of C57BL/6 mice induces powerful Th1 responses that are dominated by IFN-γ production, leading to resistance in mice. However, *L. major* infection of BALB/c mice induces strong Th2 responses that are dominated by IL-4 production, making these mice highly susceptible to these infections. Thus, resistance or susceptibility to *L. major* infections is highly correlated with Th1 (IFN-γ) or Th2 (IL-4) responses, respectively [61].

### Immune responses against *Leishmania*

The bite of an infected sandfly releases infectious *Leishmania* promastigotes in the skin, where they are taken up by phagocytic cells. The innate immune cells are critical for eliminating and clearing infections, but they also serve as a sanctuary for the obligate intracellular parasites. Neutrophils, macrophages, and dendritic cells play central roles in controlling and eliminating *Leishmania*. Neutrophils are one of the first cell types to be recruited to the site of infection and are constantly recruited to the parasitic lesions [62]. Depletion of neutrophils during *L. braziliensis* infection results in increased parasite load [63]. Similarly, infection of mice with *Leishmania* and neutrophils mixed together also leads to lower parasite burden both at the site of infection and within draining lymph nodes [63]. The neutrophil proteolytic enzyme elastase and neutrophil extracellular traps (NETs) have been proposed as mechanisms by which neutrophils kill and eliminate *Leishmania* [64, 65].

Similar to neutrophils, macrophages can phagocytize nascent *Leishmania* or *Leishmania*-infected dying cells. Macrophages are major effector cells that kill *Leishmania* parasites and, thus, are a major target for immune evasion by *Leishmania*. The inflammatory cytokines IFN-γ, IL-1, TNF-α, and type I IFNs activate macrophages to produce inducible nitric oxide synthase (iNOS) important for parasite killing. Indeed, mice deficient in iNOS are highly susceptible to *Leishmania* infection, even in a resistant C57BL/6 background [66].

Dendritic cells (DCs) are similar to macrophages in that they phagocytize *Leishmania* parasites and are critical

antigen-presenting cells responsible for priming adaptive immune responses. One of the major cytokines that DCs produce upon activation is IL-12 [67], which is critical for developing protective immunity and resolving infection [68]. Activating these innate immune cells requires recognition of *Leishmania*-associated antigens via TLRs. Indeed, *Myd88*$^{-/-}$ mice are highly susceptible to *Leishmania* infection and develop non-healing lesions, suggesting a strong role for TLR sensing [69–71]. *Leishmania* lipophosphoglycan (LPG), glycoproteins, and DNA activate TLR2, TLR4, and TLR9 signaling, respectively [72–76]. More importantly, activation of TLR2, TLR4, or TLR9 by their respective antigens results in significant protection from *Leishmania* pathogenesis and parasite burden [77]. Additionally, the results of recent studies show a surprising role for eosinophils and mast cells in inducing proper immune responses against *Leishmania* and in providing protection, mostly through the regulation of DCs and adaptive immunity [78, 79].

As described earlier, *Leishmania* studies have been instrumental in our current understanding of Th1/Th2 responses, with Th1 responses associated with IFN-γ production and protective immunity. Adaptive immunity that includes both humoral B-cell responses and T-cell-mediated immunity is critical in shaping immune responses during *Leishmania* infection. B cells and parasite-specific antibodies can be readily observed against *Leishmania*-induced skin lesions. Surprisingly, mice deficient in B cells are more resistant to *Leishmania* infections than are wild-type mice [80]. In contrast, T cell responses are critical in containing *Leishmania* infection, and infection of T cell-deficient nude or severe-combined immunodeficient (SCID) mice results in uncontrolled infection and death of the host [81, 82]. Both CD4$^+$ and CD8$^+$ T cells are important for protective immunity. *Leishmania* infection of either MHC class II-deficient (i.e., lacking CD4$^+$ T cells) mice or CD4-depleted mice results in severe lesions, demonstrating the importance of CD4$^+$ T cells [83, 84]. Interestingly, CD4-deficient mice clear infection similar to WT controls, owing to the generation of class II-restricted CD4$^-$αβ$^+$ T cells [85]. A similar supportive role of IFN-γ-producing CD8$^+$ T cells in providing protection during *L. major* infection has also been demonstrated using CD8-deficient mice or CD8-depeletion strategies [86].

## Roles of the IL-1 cytokine family in adaptive immunity against *Leishmania*

The roles of IL-1 and IL-18 in modulating adaptive immunity have been reported [87]. Both IL-1β and IL-18 promote Th1 and Th17 responses. Interestingly, during *L. major* infections, IL-1α, IL-1β, and IL-18 can be either protective or detrimental, depending on the genetic background of the host. *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice infected with *L. major* are slightly more resistant than are control BALB/c mice [88]. Consistent with this finding, a local IL-1β injection accelerates the progression of lesions in *L. amazonensis*-infected C57BL/6 mice [89]. Furthermore, IL-1 receptor antagonist-deficient (*Il1ra*$^{-/-}$) BALB/c mice, which have heightened IL-1α and IL-1β signaling, are highly susceptible to leishmaniasis [88, 90]. The resistance in *Il1a*$^{-/-}$ and *Il1b*$^{-/-}$ mice is correlated with a subsequent increase in IFN-γ and a decrease in IL-4-producing T cells [88, 90]. Similar results were observed in T cells from susceptible *Il1ra*$^{-/-}$ mice during *L. major* infections. IL-18 increases the susceptibility of BALB/c mice to *L. mexicana* and *L. major* infections [91, 92]. *Il18*$^{-/-}$ mice on a BALB/c background had slower lesion growth and a significantly lower parasite burden than did WT mice [91, 92]. Not surprisingly, the production of IL-4 by T cells was dramatically reduced and that of IFN-γ was increased in *Il18*$^{-/-}$ BALB/c mice. Furthermore, the Th1-associated antibody IgG2a significantly increased whereas the Th2-associated antibody IgG1 significantly decreased in these mice [91]. Taken together, these results support the notion that the inflammasome-associated cytokines IL-1α, IL-1β, and IL-18 promote a Th2 milieu during leishmaniasis and render the BALB/c host susceptible to these parasites.

In contrast, in C57BL/6 mice, both IL-1α and IL-1β are dispensable for protection against *Leishmania* infection [93], and *Il18*$^{-/-}$ mice are more susceptible to *Leishmania* infection than are WT mice, as demonstrated by a higher parasite burden and significantly increased lesion size [94–96]. The higher susceptibility of IL-18-deficient mice is associated with a concurrent decrease in IFN-γ production and an increase in IL-4 production by T cells. Taken together, these results suggest that IL-1 and IL-18 play a protective role in resistant C57BL/6 mice by promoting a Th1 environment.

## NLRP3 inflammasomes regulate immune responses during *Leishmania* infections

Inflammasomes are the major protein complexes that process pro-IL-1β and pro-IL-18 to their mature bioactive forms. Despite several studies of IL-1 and IL-18, the roles of NLRs (the major regulators of IL-1 cytokine family) during *Leishmania* infections remain largely unknown. Thus far, only the NLRP3 inflammasome has been shown to be involved in modulating immune responses against *Leishmania* infections [97–100]. The results of these studies suggest that the NLRP3 inflammasome is directly involved in processing and releasing IL-1β and IL-18 during *Leishmania* infection in vitro and in vivo (Fig. 2b). The NLRP3 inflammasome is similarly required for IL-1β and IL-18 production during *L. major* infection in both

C57BL/6 [98, 100] and BALB/c [97] mice. *Leishmania*-associated metalloprotease GP63 prevents NLRP3 inflammasome activation by inhibiting the production of reactive oxygen species and directly cleaving NLRP3 [99]. One of the first studies of NLRP3 inflammasomes showed that most *Leishmania spp*., including *L. amazonensis*, *L. braziliensis,* and *L. mexicana*, induce caspase-1 activation and IL-1β production upon infection of C57BL/6 macrophages [98]. Interestingly, IL-1β signaling promotes nitric oxide production, which promotes *Leishmania* killing in macrophages. As a result, C57BL/6 WT mice and mice deficient in components of the NLRP3 inflammasome (NLRP3, ASC, or caspase-1) have increased susceptibility to *L. amazonensis* infection and defects in clearance of the parasite. However, the authors of this study did not find any role for the NLRP3 inflammasome in clearing *L. major*, suggesting a species-specific role for the NLRP3 inflammasome. The results of a more recent study using a non-healing strain of *L. major* infection in C57BL/6 mice show that NLRP3 inflammasome-induced IL-1β promotes lesions through recruitment of neutrophils at the site of the infection [100]. Thus, mice deficient in the inflammasome components are significantly less protected from *L. major* infection in this model system. Similarly, the NLRP3 inflammasome promotes non-healing *L. major* infections in BALB/c mice, with NLRP3-dependent IL-18 directly promoting Th2 responses (IL-4 production) and inhibiting Th1 responses (IFN-γ production) [97]. Thus, the *L. major*-induced progression of disease or lesions in susceptible BALB/c WT mice can be partially rescued by neutralization of IL-18 in vivo. Mechanistically, IL-18 directly promotes the expression of GATA3 and cMAF, which are transcription factors for IL-4 [101, 102]. Whether increased IL-4 production results in a reduction in IFN-γ or whether IL-18 directly inhibits IFN-γ production by negatively regulating T-bet needs to be further examined. As reviewed elsewhere, these discrepancies in outcomes could be directly due to the differences in *Leishmania* strains or mouse genotypes tested [77].

## Malaria

Malaria is a deadly parasitic disease that affects more than half of the world's population. According to a WHO report in 2014, approximately 200 million cases of malaria were reported in 2013, resulting in approximately 0.5 million deaths [103]. Malaria is caused by protozoan parasites of the *Plasmodium* species, which are transmitted to humans and other mammals by mosquito bites. After its transmission into the host, the parasite undergoes two major stages to establish infection. First, the sporozoites (the infectious stage of *Plasmodium*) infect liver cells, where they divide and multiply and transform into merozoites. This stage is often referred to as the liver stage or pre-erythrocyte stage. After this cycle, the *Plasmodium* merozoites leave the liver to infect red blood cells (RBCs), and divide and multiply within these cells. This second stage of infection is known as the erythrocytic or blood stage. The periodic fever and symptoms of malaria are often associated with the cyclical bursting of merozoites from the infected RBCs [104].

### Immune responses against *Plasmodium* spp.

Infection by *Plasmodium* parasites is often lethal if left untreated. Immune responses against *Plasmodium* start as soon as the sporozoites are released in the skin by mosquito bites. In the dermis, the sporozoites can come in contact with innate immune cells, including neutrophils, macrophages, dendritic cells, mast cells, NK and NKT cells, and γδ T cells. As in other protozoan infections described in this review, neutrophils are one of the early responders to sporozoites and can be found in the dermis as early as 20 min after infection [105]. However, neutrophil depletion during sporozoite infection does not affect parasite distribution and development in the liver, suggesting redundant compensatory roles for other cell types [106]. Once in the liver, *Plasmodium* sporozoites infect hepatocytes and transition into merozoites. Kupffer cells (liver resident macrophages) play important roles in containing and clearing the parasites, and depletion of Kupffer cells increases the sporozoite invasion of hepatocytes [107]. Furthermore, other innate cells, such as neutrophils, eosinophils, monocytes, and macrophages, all infiltrate the liver in response to *Plasmodium* sporozoites, and this infiltration is strongly correlated with resistance of BALB/c mice to *P. berghei* and *P. yoelii* infection [108, 109].

Once the infection develops into the erythrocytic stage, key innate mechanisms for clearance rely on the ability of monocytes and macrophages to quickly phagocytize and clear the infected erythrocytes. Indeed, CD36 is an important receptor for direct clearance of infected erythrocytes [110]. More importantly, human populations that are deficient in CD36 are more prone to developing severe malaria [111]. In addition to their roles in phagocytizing and killing *Plasmodium*-infected cells, innate immune cells secrete critical cytokines, such as IFN-γ and IL-12, in response to *Plasmodium*-associated pathogen-associated molecular patterns (PAMPs), including glycosylphosphatidylinositol (GPI), hemozoin, RNA, and DNA [112]. Both IFN-γ and IL-12 promote resistance against *Plasmodium* infection. Although recognition of *Plasmodium* RNA through the MDA5-MAVS pathway induces type I interferon responses that provide protection in some experimental settings, TLR-MyD88-mediated recognition of *Plasmodium* PAMPs (GPI, hemozoin, and DNA)

promotes *Plasmodium* pathogenesis. Indeed, *MyD88*$^{-/-}$ mice are highly resistant to *P. chabaudi* and *P. berghei* infection, and TLR antagonists protect mice from cerebral malaria [112].

Both CD8$^+$ and CD4$^+$ T cells are critical for protective immunity following *Plasmodium* infection [113]. CD8$^+$ T cells are important for controlling and killing *Plasmodium* at the liver stage [114–117], whereas CD4$^+$ T cells are important at both the liver and blood stages [118–121]. CD4$^+$ T cells assist CD8$^+$ T cells in mounting an efficient immune response against *Plasmodium* parasites [122, 123]. However, a study has shown that CD4$^+$ T cells are critical in providing immunity whereas CD8$^+$ T cells are expendable [124]. Thus, the coordinated orchestration between T cells is essential for protective immunity against *Plasmodium* parasites.

### Role for NLRs during *Plasmodium* infection

Several cytoplasmic NLRs are involved during *Plasmodium* infection (Fig. 2c). The production of inflammatory cytokines, such as IL-1β and IFN-γ, is dramatically blunted in the absence of NOD1 and NOD2 in response to *P. berghei* ANKA infection, suggesting a role for these NLRs in sensing *Plasmodium* PAMPs [125]. However, mice deficient in NOD1 and NOD2 are comparable to WT mice and exhibit similar morbidity and mortality during *P. berghei* ANKA infection [125]. Future studies are needed to investigate the possible ligands from *Plasmodium* parasites that are recognized by NOD1 and NOD2 and their effects on protective immunity.

A major byproduct of the blood stage is hemozoin, a crystal of heme that is produced as a result of *Plasmodium* detoxifying the free heme present in the hemoglobin [126]. Hemozoin is perceived as a danger signal by the innate immune system and can directly activate inflammasomes in vitro and in vivo. When stimulated with hemozoin, WT macrophages induce caspase-1 activation and subsequent IL-1β and IL-18 production that are dependent on NLRP3 and ASC, suggesting the involvement of the NLRP3 inflammasome [127–129]. However, activation of the NLRP3 inflammasome and production of IL-1β during *Plasmodium* infection in vivo has detrimental effects. Mice deficient in NLRP3, ASC, caspase-1, or IL-1β demonstrate significant resistance to *Plasmodium* infection when compared to WT mice [127, 129]. The NLRP3 inflammasome-deficient mice have a similar parasitic burden, suggesting an immunopathologic role for the NLRP3 inflammasome and its associated cytokines. However, other studies have found no role for components of the inflammasome in *Plasmodium* disease pathology in vivo [130, 131]. In a separate model of *Plasmodium*-bacterial coinfection, both NLRP3 and NLRP12 promote immunopathology [132]. Specifically, WT mice infected with *P. falciparum* or *P. vivax* produce high levels of IL-1β in an NLRP3- and NLRP12-dependent manner. WT mice infected with *P. faclipurum* are highly sensitive to secondary bacterial infection or low-dose lipopolysaccharide injection (which mimics bacterial infection). In agreement with these results, mice deficient in NLRP3, NLRP12, or components of the inflammasome (ASC and caspase-1) have higher survival rates than do WT mice after *Plasmodium*-bacterial coinfection.

A recent publication analyzing single nucleotide polymorphisms in symptomatic human patients with *P. vivax* malaria demonstrated that polymorphisms associated with NLRP1 gene was associated with increased *Plasmodium* pathogenesis [133]. Although these studies are highly correlative at this time, it highlights a possible role for NLRP1 in modulating immune responses during *Plasmodium* infection.

## Toxoplasmosis

Toxoplasmosis is caused by *Toxoplasma gondii,* an obligate intracellular protozoan that infects humans and animals. At least one third of the world's population is infected with *T. gondii*, but only immunocompromised individuals are at high risk for developing complications such as pneumonia, organ failure, and encephalitis [134–137].

### Immune responses against *T. gondii*

Both innate and adaptive components are important in providing immunity against *T. gondii* infections. Innate immune responses are critical for protection against *T. gondii* infections, as shown by the results of studies in mice lacking critical cytokines and molecules, including IFN-γ [138], IL-12 [139], and iNOS [140]. Mice lacking these innate effector molecules are highly susceptible to *T. gondii* infections. Innate immune cells that produce and respond to these cytokines include neutrophils, macrophages, DCs, and NK cells. *T. gondii* causes increased morbidity and mortality in mouse depleted of these innate cell populations [141, 142].

TLRs play an integral role in innate recognition of *T. gondii*-associated PAMPs and in initiating cytokine responses by the innate immune cells. As such, *MyD88*$^{-/-}$ mice are highly susceptible to *T. gondii* infection. Several TLRs, including TLR2, TLR4, TLR7, TLR9, and TLR11, are involved in innate recognition of *T. gondii* and promote production of IL-12 and IFN-γ [143]. More recently, the *T. gondii*-secreted protein profilin was shown to be the specific ligand for TLR11, suggesting that TLR11 is the

principal innate sensor [144]. However, deficiency in individual TLRs does not render mice more susceptible to *T. gondii* infection than WT controls are [143]. These results suggest a possible redundancy between TLRs in recognizing and clearing *T. gondii* infection in vivo.

T cells are essential for providing complete protection against *T. gondii,* which is confirmed by the finding that mice deficient in T cells are highly susceptible and die as a result of uncontrollable proliferation of the parasite in various organs, including the brain [145, 146]. Both $CD8^+$ and $CD4^+$ T cells are important for controlling *T. gondii* infection, and IFN-γ production by these cells is critical for protection [147–149]. *T. gondii* infection of IFN-γ-deficient mice or WT mice with neutralized IFN-γ results in severe acute inflammation and development of necrotic lesions in the brain [150, 151]. Unlike its role in other protozoan infections, IL-4 is protective against *T. gondii* infection. IL-4-deficient mice are highly susceptible to *T. gondii,* and all mice succumbed to the infection [152]. IL-4-deficient mice have significantly fewer IFN-γ-producing T cells than did WT mice, supporting the role for IL-4 in promoting Th1 cell differentiation.

### Role for NLRs during *T. gondii* infection

Several NLR molecules are involved in providing protective immunity against *T. gondii* (Fig. 3a). NOD2 was one of the first NLRs shown to be important in providing protective immunity against *T. gondii*., with all WT mice infected with *T. gondii* surviving and all NOD2-deficient mice succumbing to infection by day 21 [150]. Interestingly, NOD2-mediated protection against *T. gondii* is independent of its adaptor protein RIPK2, as RIPK2-deficient mice are completely protected [150]. Although NOD2 deficiency in antigen-presenting cells is not important for immunity, NOD2 expression is critical in T cells and required for optimal IFN-γ production. Mechanistically, NOD2 in T cells is required for optimal IL-2 production and proliferation. In T cells, NOD2 binds to c-Rel to promote T-cell activation and IFN-γ production. As a result, c-Rel translocation in NOD2-deficient T cells is severely blunted. Thus, NOD2 has a T-cell-intrinsic role in promoting proliferation and the production of effector cytokines during *T. gondii* infection [150]. However, these findings have yet to be substantiated by independent groups. Indeed, one study has found no significant role for NOD2 in T cells and shown that NOD2 is dispensable for protection during *T. gondii* infection of mice [153]. This discrepancy in findings from two independent studies might be due to the differences in mouse genetic backgrounds or differences in microbiota.

In addition to NOD2, both NLRP1b and NLRP3 are also involved in protection against *T. gondii*. Single-nucleotide polymorphisms in the *NLRP1* gene are associated with increased susceptibility to toxoplasmosis in humans [154]. These findings were further confirmed via RNA interference-mediated knockdown of NLRP1 in human monocytic cell lines. Activation of the inflammasome during *T. gondii* infection in human monocytes was determined via short hairpin RNA-mediated knockdown of ASC and caspase-1 [155]. The involvement of the NLRP1 inflammasome during *T. gondii* infection has also been confirmed in both murine and rat models [156]. These studies' results show that the activation of caspase-1 and subsequent production of IL-1β and IL-18 in human cells in response to *T. gondii* infection are mediated by the NLRP1 inflammasome. More recent in vitro and in vivo studies of *T. gondii* infection in mice suggest a dual role for NLRP3 and NLRP1 inflammasomes [157]. NLRP3-, NLRP1b-, ASC-, caspase-1-, or caspase-11-deficient mice are much more susceptible to *T. gondii* infection than are WT mice. Although IL-1β production is detectable during in vitro stimulation of macrophages with *T. gondii*, only IL-18 is measurable in the serum of *T. gondii*-infected mice in vivo. Interestingly, both IL-1 and IL-18 signaling are required to protect mice during *T. gondii* infection because IL-1R- and IL-18-deficient mice are much more susceptible than are WT mice.

Altogether, the results of these studies show that several NLRs function in both innate and adaptive immune cells to recognize and combat *T. gondii* infection. Whether other NLRs are also involved in various immune cell types should be an active research area for future studies.

## Trypanosomiasis or Chagas disease

Trypanosomiasis or Chagas disease is caused primarily by the obligate intracellular parasite *Trypanosoma cruzi*. More than 10 million people worldwide are infected with *T. cruzi*, of which approximately 30 % develop cardiac diseases and complications [158–160]. The precise molecular mechanisms involved in providing protection against *T. cruzi* are largely unknown because of the paucity of studies in this field. However, both innate and adaptive immune responses are necessary for protective immunity and resistance to *T. cruzi* in vivo [161–163].

### Immune responses against *T. cruzi*

Innate immune cells, including neutrophils, monocyte/macrophages, DCs, and NK cells, play an important role in controlling *T. cruzi* infection in the host. Recognition of *T. cruzi* by the innate immune cells is important for production of protective factors such as IL-12, TNF-α, IFN-γ, and nitric oxide. Indeed, mice deficient in IL-12, IFN-γ, and iNOS are highly susceptible to *T. cruzi*

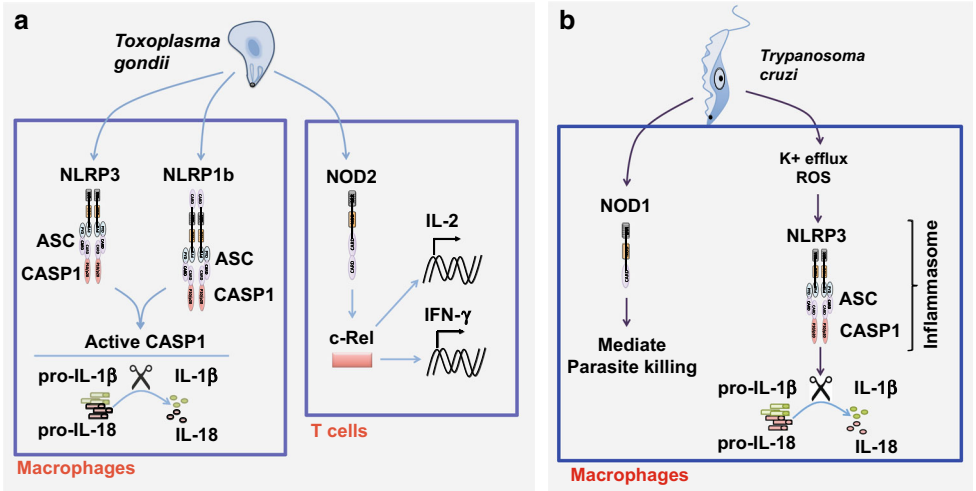


**Fig. 3** Role of NLR in modulating immune responses during *Toxoplasma gondii* and *Trypanosoma cruzi* infection. **a** *Toxoplasma gondii* activates both NLRP1b and NLRP3 inflammasomes to induce the robust production of IL-1β and IL-18. In addition, NOD2 in T cells directly interacts with c-Rel to induce the production of IFN-γ and IL-2 during *T. gondii* infection and is required for resistance. **b** NOD1 promotes *T. cruzi* killing within the macrophages. *T. cruzi* also activates the NLRP3 inflammasome through mechanisms that involve K+ efflux and reactive oxygen species

infection [164–166]. Depletion of neutrophils and macrophages in BALB/c mice is detrimental during *T. cruzi* infection, demonstrating these molecules' importance, and similar important roles for NK cells have also been shown by depletion studies [167, 168]. NK cells are not only directly cytotoxic but are also the major source of IFN-γ at acute time points. The IFN-γ signaling is critical for macrophages to effectively kill intracellular *T. cruzi*. The TLR signaling axis is critical in priming these immune responses by innate immune cells. Mice lacking MyD88 are highly susceptible to *T cruzi* infections as a result of failure to produce proinflammatory cytokines such as IL-12 and IFN-γ. Attempts to identify upstream TLRs that recognize *T. cruzi* ligands have identified TLR2, TLR4, TLR7, and TLR9 as possible innate receptors [169]. TLR2 recognizes *T. cruzi* glycophosphatidylinositol, and TLR4 recognizes glycoinositolphospholipid. TLR7 and TLR9 recognize *T. cruzi*-derived RNA and DNA fragments respectively. Although deficiency of any individual TLR results in increased susceptibility to *T. cruzi*, the disease in such deficient mice is less severe than that in *Myd88*$^{-/-}$ mice, suggesting partial redundancy between the TLRs [169].

Adaptive T cells play an extremely important role in providing protection against *T. cruzi* infection. Mice deficient in either CD4$^+$ or CD8$^+$ T cells are more susceptible to *T. cruzi* infection than are WT mice and quickly succumb to infection [170, 171]. Upon activation, CD4$^+$ and CD8$^+$ T cells produce IFN-γ that induces further activation of phagocytic cells, including macrophages, to promote parasite killing [172–174]. CD8$^+$ T cells, in particular, can directly kill infected cells. Unfortunately, the adaptive immune response is not sufficient to achieve sterilizing immunity, and the parasite can establish chronic infections in most individuals.

## Roles of NLRs during *T. cruzi* infection

Evidence for the roles of NLRs during *T. cruzi* infection is limited, and the few studies conducted on the roles of NLRs during *T. cruzi* infection are discussed here (Fig. 3b). NOD1 and NLRP3 play a protective role during *T. cruzi* infections. The results of one study showed that NOD1, but not NOD2, is important in providing protection against *T. cruzi* in mice in vivo [175]. Mice deficient in NOD1 bear a significantly increased parasite load and have higher mortality rates than NOD2-deficient or WT mice. Interestingly, NOD1 does not seem to affect overall serum cytokine levels during *T. cruzi* infection but is required for IFN-γ sensitivity in macrophages to clear *T. cruzi*.

The results of two independent studies suggest a role for the NLRP3 inflammasome in response to *T. cruzi* infection [176, 177]. *T. cruzi* infected-macrophages induce caspase-1 activation and subsequent IL-1β and IL-18 production. The activation of the NLRP3 inflammasome in response to *T. cruzi* is dependent on established activators, such as potassium efflux, ROS production, and lysosomal damage. *T. cruzi* infection of mice deficient in NLRP3, ASC, and caspase-1 results in severe illness, high parasitic burden, and death. It is unclear whether IL-1β and IL-18 are involved in these protective effects in vivo. Of note, the in vitro protective effects of WT macrophages (killing of *T. cruzi*) are not dependent on IL-1β or IL-18 [176]. However, the in vivo protection in mice could be due to IL-1β- and

IL-18-mediated effects on priming the adaptive immune responses, especially $CD8^+$ T-cell responses.

## NLRs in modulating adaptive immune responses during protozoan infection: a perspective

NLRs are novel players in the regulation of innate immunity during protozoan infections. As discussed earlier in the review, NLRs can function directly as either positive or negative regulators of pro-inflammatory signaling in various innate immune cell types in response to pathogenic insults. Overall, protozoan PAMP sensing by NLRs results in the production of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-12, IL-1β, and IL-18; the production of reactive oxygen and nitrogen species; and inflammatory cell death. Although our understanding of NLRs in regulating innate immunity against protozoan infections is beginning to unfold, several important findings suggest that NLRs can also modulate adaptive immune responses. Here, we have presented a perspective on how these NLRs are involved in modulating adaptive immune responses against protozoan infection, with specific examples wherever applicable.

NLRs could use one or more of the following mechanisms to regulate T cell immune responses during protozoan infections: (1) NLR-mediated production of pro-inflammatory cytokines by innate immune cells may regulate T cell responses, (2) cell-intrinsic roles of NLRs in innate immune cells may ultimately affect T cells, (3) inflammasome-activation-induced IL-1β and IL-18 and inflammatory cell death may affect adaptive immunity, and (4) NLRs may have cell-intrinsic roles in T cells.

NOD1 and NOD2 are activated in response to bacterial peptidoglycans. The result of this recognition is activation of NFκB and MAPK signaling pathways and subsequent production of pro-inflammatory cytokines. In the context of protozoan infections, both NOD1 and NOD2 are involved in the recognition of an unknown *Plasmodium* ligand and promote production of pro-inflammatory cytokines such as IL-1β and IFN-γ. Given that IL-1β drives T cell proliferation and survival [87] and that IFN-γ promotes Th1 differentiation [178], it could be posited that NOD1 and NOD2 play an important role in regulating adaptive immunity during *Plasmodium* infections.

Similarly, both NOD1 and NOD2 are required for activation of NFκB and MAPK signaling induced by *T. cruzi* infection in macrophages. As a result, production of cytokines such as IL-12, IFN-γ, and TNF-α are blunted in *T. cruzi*-stimulated *Nod1*- and *Nod2*-deficient macrophages. Interestingly, only $Nod1^{-/-}$ mice are susceptible to *T. cruzi* infection. Although the $Nod1^{-/-}$ mice had similar levels of IL-12 and IFN-γ as WT mice, they had lower NOS levels. NOS-induced production of nitric oxide by macrophages is known to promote Th1 immune responses and, thus, could be a mechanism of NOD1-mediated protection against *T. cruzi* [179]. More interestingly, $Nod1^{-/-}$ mice die 15 days post infection, strengthening the argument that NOD1 has a role in regulating adaptive immune responses during *T. cruzi* infection.

Cell-intrinsic roles for NLRs in macrophages and DCs have been reported, but this area needs further investigation. NOD2 has been shown to regulate DC function and survival. Specifically, $Nod2^{-/-}$ DCs exhibit reduced expression of CD80 and CD86 costimulatory molecules and defects in survival compared to WT DCs [180]. As a result, $Nod2^{-/-}$ DCs are less efficient in priming T cell responses during influenza virus infection. A similar intrinsic function for NLR molecules has not been observed for macrophages or DCs during protozoan infections. However, NOD1 is required for IFN-γ-induced killing of *T. cruzi* in macrophages, suggesting a possible macrophage intrinsic role for NOD1 in protozoa killing [175]. Although there are no current studies on the roles of CIITA and NLRC5 during protozoan infection, these two molecules regulate MHCI and MHCII transcription, both of which are important for antigen presentation and activation of T cell responses [181].

The NLRP3 inflammasome is activated in response to all protozoan parasites discussed in this review. In addition to the NLRP3 inflammasome, *Plasmodium* hemozoin also activates the putative NLRP12 inflammasome, and *Toxoplasma gondii* activates the NLRP1b inflammasome. The functional consequence of inflammasome activation in the cell is release of cytokines IL-1β and IL-18, leading to pyroptosis, an inflammatory form of cell death. Both IL-1β and IL-18 promote T cell responses. In the context of *Leishmania* infection, NLRP3 inflammasome-induced IL-18 promotes Th2 immune responses, rendering mice susceptible to infection [97]. Of interest, inflammasome activation is also detrimental for *Plasmodium* infection [127, 129]. Whether IL-1β and IL-18 prime Th2 responses in these settings is not known, and future studies are needed to understand these molecular and cellular underpinnings. In contrast, inflammasome activation during *E. histolytica*, *T. gondii*, and *T. cruzi* infection are all protective [56, 57, 157, 176]; thus, it could be posited that the IL-1β and IL-18 cytokines may prime a protective Th1 immune response in these infectious settings.

A major consequence of inflammasome activation is pyroptotic cell death, which is often overlooked when studying immune responses. The results of previous studies show that DCs that phagocytize activated dead cells or necrotic dead cells upregulate CD80/CD86 and produce higher levels of IL-12 cytokines [182]. Pyroptosis is an inflammatory cell death and thus it could be hypothesized

that macrophages and DCs that uptake these pyroptotic dead cells will have upregulated expression of co-stimulatory molecules and increased IL-12 production that could ultimately affect T cell activation. In contrast, higher rates of pyroptosis in macrophages and DCs could directly limit the source of antigen presenting cells available for T cell activation.

Although NLRs can regulate T cell responses through their role in macrophages and DCs as discussed in this review, NLRs also have T cell-intrinsic functions. NLRP3 acts as a transcriptional factor for IL-4 (in transactivation with Interferon regulatory factor 4 (IRF4)) in T cells and drives Th2 responses [15]. More recently, NLRP12 was shown to negatively regulate NFκB and ERK signaling in T cells and to promote T cell hyperproliferation and IL-4 production during experimental autoimmune encephalitis in mice [183]. Additionally, inflammasome-induced pyroptosis of $CD4^+$ T cells is suggested to be the major reason for T cell loss in HIV infected patients [184]. With regards to protozoan infections, NOD2 has T cell-intrinsic functions and drives T cell proliferation and IFN-γ production during *T. gondii* infection [150]. Mechanistically, NOD2 directly interacts with the transcription factor c-Rel to drive T cell proliferation and IFN-γ production [150]. Future studies should reveal more information on the potential role of NLRs in T cells during several protozoan infections.

## Conclusion

Despite the high morbidity and mortality associated with the protozoan infections discussed in this review, viable treatment options for these diseases are largely lacking. Most of the treatments for these protozoan parasites are non-specific and often associated with severe side effects. With the exception of malaria, the research, attention, and funding required to develop effective therapies against these protozoan infections are severely lacking. As a result, the WHO continues to categorize most of these diseases as neglected tropical diseases.

Studies of the roles of NLRs during protozoan infections are still in their infancy. Moreover, even for the NLRs that have been shown to be involved in these protozoan infections, their exact roles in different cell types and whether they affect adaptive immunity remains unknown. With only a few of the known NLRs being tested thus far, there remains a large knowledge gap in our understanding of how various NLRs are involved in providing or modulating immunity during these protozoan infections. Thus, immediate research on the role of NLRs during protozoan infections is warranted to design new immunotherapies and vaccines.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing financial interests.

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