Trends in Parasitology

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Review

Inflammasome Activation in Response to Intracellular Protozoan Parasites

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Inflammasomes are cytosolic complexes that assemble in response to cellular stress or upon sensing microbial molecules, culminating in cytokine processing and an inflammatory form of cell death called pyroptosis. Inflammasomes are usually composed of a sensor molecule, an adaptor protein, and an inflammatory caspase, such as Caspase-1, which cleaves and activates multiple substrates, including Gasdermin-D, pro-IL-1β, and pro-IL-18. Ultimately, inflammasome activation promotes inflammation and restriction of the microbial infection. In recent years, many studies have addressed the role of inflammasomes during fungal, bacterial, viral, and parasitic diseases, revealing sophisticated aspects of the host–pathogen interaction. In this review, we summarize recent advances on inflammasome activation in response to intracellular parasites, including *Leishmania* spp., *Plasmodium* spp., *Trypanosoma cruzi*, and *Toxoplasma gondii*.

Highlights

Inflammasomes are activated in response to many intracellular parasites and induce inflammation, cell death, and restriction of infection.

Leishmania spp. induce Caspase-11-mediated noncanonical activation of the NLRP3 inflammasome, a process that is important for the outcome of leishmaniasis.

Plasmodium spp. trigger inflammasome activation and a strong inflammatory response mediated by IL-1β, a critical inflammatory mediator involved with the pathogenesis of the disease.

Trypanosoma cruzi induces NLRP3 activation and production of IL-1β, which is critical for the clinical outcomes of Chagas disease.

Toxoplasma gondii triggers both NLRP3 and NLRP1, which leads to parasite restriction and resistance to toxoplasmosis.

Inflammasomes: An Overview

The term Inflammasome was first used in 2002 to nominate a cytosolic complex formed by **Nod-like receptor (NLR)** (see Glossary) Pyrin domain 1 (NLRP1), **apoptosis-associated speck-like protein containing a CARD (ASC)**, and the cysteine protease **Caspase-1 (CASP1)** [1]. Currently, a quick search using the word 'inflammasome' hits more than 10 000 articles, illustrating the massive expansion of the inflammasome field over the last 20 years. Together with the expansion of the field, our understanding of the mechanisms and functions of inflammasomes has advanced significantly [2] (Box 1).

Although NLRP3 (Box 2) is the most-studied NLR (Figure 1), several independent inflammasomes have been characterized (Box 3). While the role of NLRC4, AIM2, and NLRP12 during protozoan infections remain poorly known, NLRP1 and NLRP3 have been more studied in the context of parasitic infections [3]. Human NLRP1 is a single protein composed of a PYD, CARD, NACHT, and an LRR domain, while mouse encodes three paralogs (NLRP1a, b, and c) that do not express PYD [4]. Although the *Bacillus* anthrax lethal toxin has been described as the activator of this receptor, the mechanisms leading to its activation were poorly understood until two elegant papers revealed the mechanisms of NLRP1 activation [5,6]. It was shown that proteosomal degradation of the amino-terminal domain of NLRP1 is essential to its activation. Whether this mechanism explains NLRP1 activation by protozoan parasites remain to be addressed.

Inflammasome Activation by *Leishmania* spp.

Inflammation is a hallmark of leishmaniasis and the initial modulation of the host innate immunity upon infection is critical to the different clinical outcomes of this complex disease (Box 4). Although the induction of inflammasome-dependent cytokines upon infection, such as interleukin (IL)-1β and IL-1α, has been reported in early papers [7], a study published by our group in 2013 was the first to describe the molecular mechanisms of inflammasome activation upon *Leishmania* infection [8]. By using different species of *Leishmania*, it was demonstrated that all these species are capable of inducing IL-1β release by macrophages via NLRP3, ASC, and CASP1.

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Box 1. Inflammasome Components and Activation

The inflammasomes are composed of a sensor protein (usually a NLR), an adaptor protein (usually ASC), and an inflammatory caspase, usually CASP1 [80]. Activation of certain inflammasomes (including NLRP3) requires two signals: the initial step (known as 'first-signal', or 'priming') occurs upon TLR activation or cytokine signaling (e.g., TNF-α, IFN-γ, IFN-β). The first step promotes transcriptional regulation of specific genes, culminating in robust protein expression of NLRs and inflammatory molecules, such as pro-IL-1β, pro-IL-18, pro-caspase-1, and CASP11. The second step, called the 'second signal' or 'trigger', induces specific-NLR activation and the assembly of the inflammasome platform. NLRs are typically composed of three different domains: the N terminal leucine-rich repeat (LRR) domain, responsible for the regulation of the NLR oligomerization; the NOD or NACHT domain, responsible for receptor oligomerization; and the C terminal effector domain, which may be a Pyrin-domain (PYD) in the case of the NLRPs, a CARD domain (caspase-associated recruitment domain) in the case of the NLRCs, or three BIR domains (bacilovirus-IAP repetitions) in the case of the NAIPs [2]. Differences in these effector domains structurally distinguish one NLR from another. Upon activation, NLRs (except NLRP1) oligomerize to recruit the adaptor molecule, which in turn recruits and activates CASP1, an enzyme capable of cleaving its many substrates in the cytosol, into their active forms. These include the IL-1 family cytokines pro-IL-1β and pro-IL-18, which are secreted and play a potent inflammatory role, locally or systemically [2]. Recent studies have demonstrated that the gasdermin (GSDM) family of proteins are also substrates of CASP1 and CASP11 [81]. The cleaved N terminal fragment of Gasdermin-D act as a pore-forming molecule to induce cell death by **pyroptosis**, which is a typical consequence of inflammasome activation [81–83]. Therefore, either through cell death or release of inflammatory mediators, the inflammasome ultimately leads to inflammatory responses that may help to clear invading pathogens and/or contribute to the generation of chronic inflammatory diseases [83].

Importantly, it was shown that the NLRP3 inflammasome assembles in response to infection in a process dependent on **potassium efflux** and cathepsins [8]. Moreover, it was demonstrated that production of IL-1β via this platform induces IL-1R/MyD88 signaling to trigger nitric oxide (NO) production, enabling parasite killing and resistance to disease in C57BL/6 mice. More recently, new studies have advanced the understanding of the molecular pathways of inflammasome activation [9]. It has been reported that upon phagocytosis, parasites trigger the C-type lectin receptor Dectin-1. This triggers a Syk-dependent signaling pathway that results in reactive oxygen species (ROS) production, which partially contribute to NLRP3 activation upon *Leishmania amazonensis* infection [10]. Recently, it was shown that the *Leishmania* **lipophosphoglycan (LPG)** is capable of activating **Caspase-11 (CASP11)** and the noncanonical NLRP3 inflammasome [11]. Using macrophages genetically deficient for CASP11, highly purified LPG and parasites lacking a transferase that is important for LPG anchoring in the membrane (*Leishmania major Lpg1*$^{-/-}$), it was demonstrated that CASP11 is activated by several

Box 2. General Characteristics of the NLRP3 Inflammasome

NLRP3 is possibly the most studied inflammasome. It is composed of NLRP3, the adaptor protein ASC, and CASP1 [84]. Recent work has demonstrated that this molecular platform also contains **NEK7** [85] and **DDX3X** [86] and that oxidized mitochondrial DNA could act as a direct agonist of this NLR [87]. NLRP3 activation is largely dependent on **damage-associated molecular patterns (DAMPs)**, such as potassium efflux, ROS, cathepsins, ATP, and crystals, which assemble the platform to induce Gasdermin-D (GSDMD)-dependent pyroptosis and cytokine release [84]. It is still unknown whether NLRP3 is a *bona fide* receptor or a molecule that signals downstream of a putative unidentified receptor that senses the signals that trigger the NLRP3 inflammasome [84]. Given that cellular stress is a major component involved in almost all inflammatory diseases, activation of the NLRP3 inflammasome has been described as a hallmark of many clinical outcomes, such as cancer, autoinflammatory, autoimmune, and infectious diseases [88,89], becoming an important putative target to treat several pathological conditions [2,84]. Noteworthy, NLRP3 inflammasome can be activated via the canonical and noncanonical pathways [84]. The first is triggered by molecules that induce potassium efflux (via membrane damage or pore formation) or intracellular ROS production induced by cellular stress (heat, pressure, or infectious stimuli). By contrast, the noncanonical pathway was described in the context of gram-negative bacteria infection, as LPS gets recognized by CASP11 in the cytosol [90,91]. Once activated, CASP11 cleaves GSDMD, which induces potassium efflux and, consequently, NLRP3 activation [82,90–92]. Recently, additional PAMPs and also DAMPs have been associated with CASP11 and, therefore, noncanonical NLRP3 activation. Kagan's group has demonstrated that oxidized phospholipids act as a direct agonist of CASP11 in live dendritic cells [93], while our group has shown that the *Leishmania* LPG indirectly activates CASP11 in macrophages [11]. Likewise, lipoteichoic acid has also been shown to trigger CASP11 activation during gram-positive bacterial infection [94]. Taken together, these evidences expand the importance of the CASP11-mediated noncanonical NLRP3 activation independently of gram-negative bacteria.

Glossary

Apoptosis-associated speck-like protein containing a CARD (ASC): small adaptor protein that is a component of many inflammasomes. ASC is composed of a Pyrin and a Card domain. The former binds to the Pyrin domain of certain inflammasome sensors (NLRP3, AIM2, etc.), while the latter binds to Card domain of Caspase-1, promoting inflammasome assembly and caspase activation.
ASC specks: polymerized monomers of ASC that occur when inflammasomes are activated. ASC filaments trigger caspase-1 recruitment and cleavage, amplifying the caspase-1 activity upon inflammasome activation. ASC specks are formed inside the cells, but can be extracellular upon cell death to propagate inflammation.
Caspase-1 (CASP1): cysteine protease that is activated upon inflammasome assembly. Active caspase-1 cleaves and activates multiple substrates, including pro-IL-1β, pro-IL-18, and Gasdermin-D.
Caspase-11 (CASP11): inflammatory caspase involved in the sensing of bacterial LPS, *Leishmania* LPG, and oxPAPCs. Upon activation, Caspase-11 triggers Gasdermin-D activation, noncanonical activation of NLRP3 inflammasome, and pyroptosis.
Damage-associated molecular patterns (DAMPs): host-derived molecules that are structurally conserved and are released during host cell stress, injury, and/or infection.
DDX3X: ATP-dependent RNA helicase, one of the major stress granule proteins.
Hemozoin (Hz): insoluble crystal produced by *Plasmodium* during the intracellular cycle in the erythrocytes. Produced as a result of hemoglobin's digestion.
Lipophosphoglycan (LPG): abundant glycoconjugate found in the surface of *Leishmania* parasites. Highly abundant in promastigote forms of the parasites and present at lower concentrations in amastigote forms.
Mammalian target of rapamycin (mTOR): protein kinase that regulates different cellular processes, including cell growth, proliferation, motility, survival, protein synthesis, autophagy, among others.
NEK7: protein required for microtubule nucleation activity of the centrosome, playing an important role during mitosis.

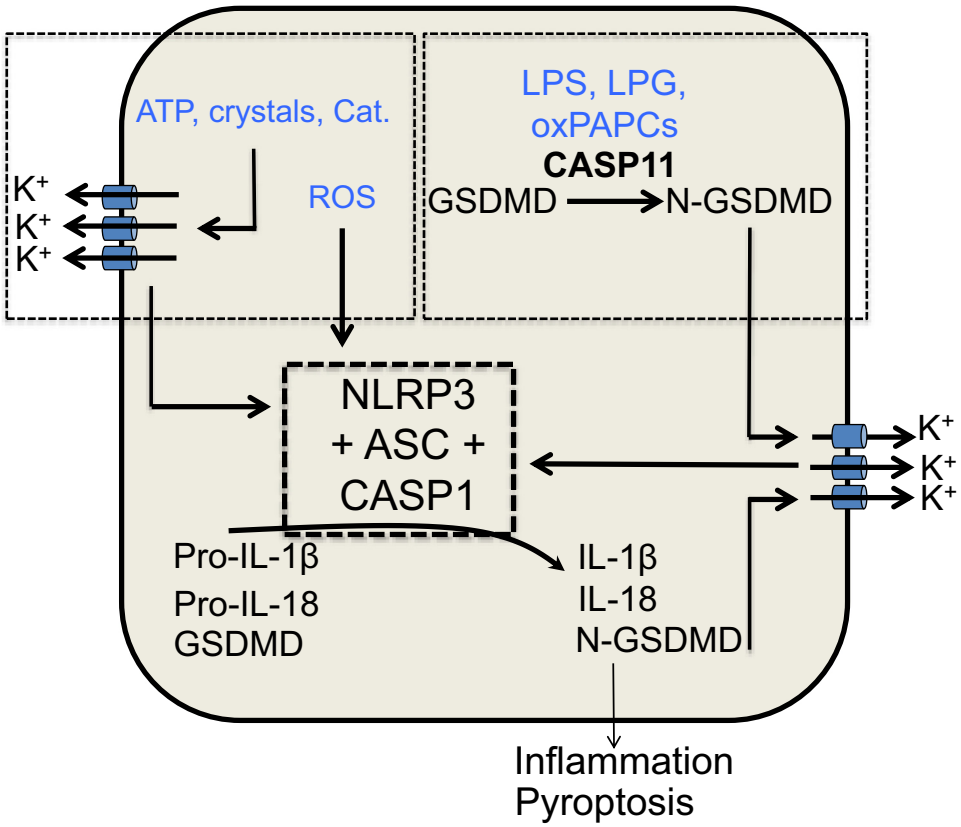


Figure 1. Canonical and Noncanonical Activation of the Nod-like Receptor Pyrin Domain 3 (NLRP3) Inflammasome. NLRP3 is the most studied inflammasome. Many different damage-associated molecular patterns (DAMPs) and membrane damage triggers NLRP3 activation in a process that requires reactive oxygen species (ROS) and potassium (K^+) efflux. The NLRP3 inflammasome is composed of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and Caspase-1 (CASP1). This platform has been associated with a wide variety of diseases, ranging from infectious, neurodegenerative, and autoimmune disorders, as well as cancer. The canonical NLRP3 inflammasome is activated upon cellular stress and perturbations in the cellular homeostasis, which generates damage in the plasma membrane and allows efflux of potassium from the cell. The main signals that have been described to activate NLRP3 are ATP (via P2X7 receptor), crystals, cathepsin (Cat.), ROS, and pore formation, which may be induced in different contexts. Potassium efflux can be induced upon pore formation, via specific K^+ channels and membrane rupture and also through the activation of Caspase-11 (CASP11) by bacterial lipopolysaccharide (LPS), *Leishmania* lipophosphoglycan (LPG), and oxidized lipids (oxPAPCs), which in turn cleaves Gasdermin-D (GSDMD) and releases the N terminal fragment (N-GSDMD), capable of forming pores, thus allowing K^+ efflux and cell death. NLRP3 inflammasome assembly cleaves its different inactive substrates [pro-interleukin (IL)-1β, pro-IL-18, Gasdermin-D (GSDMD)] in their active forms, promoting inflammation and cell death by pyroptosis.

Recently reported to associate with NLRP3 for inflammasome activation.
Nod-like receptors (NLRs): family of cytosolic proteins that senses PAMPs and DAMPs in the host cell cytoplasm and oligomerize to promote activation and specific responses.
Opsonization: process of antibody or complement coating of pathogens, normally enhancing phagocytosis and inflammation.
Pathogen-associated molecular patterns (PAMPs): conserved molecules present in microbes that contain molecular motifs that can be recognized by pattern-recognition receptors.
Pattern-recognition receptors (PRRs): germline-encoded receptors that specifically recognize conserved molecular patterns such as PAMPs and DAMPs.
Potassium efflux: a process in which intracellular potassium is released to the extracellular milieu. Occurs upon pore formation, activation of specific ion channels, or plasma membrane damage.
Pyroptosis: inflammatory form of cell death induced upon activation of inflammatory caspases. Pyroptosis occurs through the formation of pores into the plasma membrane and culminates with the release of intracellular contents and inflammation.
Toll-like receptor: germline-encoded pattern-recognition receptors that are activated by PAMPs, promoting transcriptional regulation of many inflammatory genes.

Leishmania species and their respective LPGs promote pore formation, cell death, NLRP3 activation, and IL-1β release when delivered in the cytoplasm of macrophages [11]. This study also demonstrated that the amastigote form of the parasite, which displays very low levels of LPG in its surface, induces less inflammasome activation compared with promastigote forms. Besides, a recent study has shown that amastigotes subvert NLRP3 activation in macrophages by targeting histone H3 [12], but whether this pathway also decreases CASP11 activation remains to be investigated. These findings are particularly important since the amastigote is the highly infective form of the parasite that perpetuates infection *in vivo*, suggesting that in later time points, when infection is established, *Leishmania* might modulate inflammasome activation to avoid

Box 3. NLRC4, NLRP12, and AIM2 Inflammasomes

NLRC4 is a particular type of NLR that has been involved with bacterial sensing, being composed of a NACHT, LRR, and CARD domain [95]. However, different than previously expected, NLRC4 is not a sensor, but an adaptor that is activated in response to activation of a NAIP receptor. The mouse genome encodes multiple NAIPs (NAIP1 to NAIP6), whereas humans encode only one NAIP (hNAIP). NAIPs recognize specific bacterial PAMPs secreted by virulent bacteria expressing type III or IV secretion systems (T3SS and T4SS, respectively) [95]. The secretion of needle proteins from T3SS activates hNAIP and NAIP1, while NAIP2 recognizes rod proteins from T3SS. Interestingly, mNAIP5 (and with a lesser activity mNAIP6) bind to bacterial flagellin in the cytosol [95]. Therefore, sensing of specific bacterial molecules by NAIPs triggers the recruitment of NLRC4, which recruits ASC and CASP1, forming the NLRC4 inflammasome. Although ASC is constantly found in the NAIP/NLRC4 inflammasomes, it is dispensable for its activation since NLRC4 has an N terminal CARD domain that binds to the CASP1 CARD domain, promoting its activation [95]. This pathway has been described as the main mechanism to clear bacterial infections, such as *Legionella pneumophila* and *Salmonella typhimurium*, but remains poorly characterized in the context of parasitic infections. One study reported that NLRC4 is dispensable during *T. cruzi* infection [56].

NLRP12 is another NLR that has been described to play an important role in inflammatory processes, yet the nature of its activator is still a matter of concern [58]. The NLRP12 inflammasome is composed of NLRP12, ASC, and CASP1, but differently from the conventional inflammasomes, such as NLRP1 and NLRP3, this receptor has been suggested to act as a negative regulator of NF-kB signaling, since recent reports found that *Nlrp12*$^{-/-}$ mice exhibit signs of increased inflammation in the context of ulcerative colitis [58].

Absent in melanoma (AIM2)-like receptors (ALRs) have also been described as examples of inflammasomes that assemble in the absence of NLRs [2]. Among this group, the AIM2 inflammasome is the best characterized inflammasome. It is composed of an AIM2 sensor (a DNA helicase composed of a PYD and a HIN200 domain), ASC, and CASP1. AIM2 is activated directly by double-stranded DNA (dsDNA) in the host cell cytoplasm [96]. Recent evidences have shown that nuclear, exogenous, and mitochondrial DNA bind to the HIN200 domain and trigger AIM2 oligomerization and inflammasome activation and this pathway has been involved in many diseases [2].

killing. Interestingly, another recent study has demonstrated that *Leishmania* engage the host cell secretory pathway to export virulence factors, such as GP63 and LPG [13], suggesting that this could be a possible mechanism of CASP11 activation by this lipid. The findings that CASP11 account for noncanonical activation of NLRP3 in response to *Leishmania* were corroborated by a study from an independent group showing that both Leukotriene B4 and the ATP

Box 4. Leishmaniasis: Overview and Early Innate/Adaptive Immune Events

Leishmania is the causative agent of leishmaniasis, a parasitic disease that affects 12 million people worldwide in 98 different countries, with over 1 million cases being diagnosed every year [35]. The disease has a wide variety of clinical manifestations that range from cutaneous leishmaniasis (CL), with self-healing lesions, to the devastating/disfiguring mucocutaneous leishmaniasis (MCL) that affects naso-pharyngeal mucosal tissues [35]. Another manifestation of the disease is the visceral leishmaniasis, in which the parasites disseminate systemically and reach the spleen, bone marrow, and liver, thus representing the most aggressive form, eventually leading patients to death [35].

Parasite transmission occurs through the bite of sand fly vectors such as *Phlebotomus* spp. or *Lutzomyia* spp., which inoculates the highly infective metacyclic promastigotes of *Leishmania* spp. into the skin of the mammalian host [58]. There, parasites get internalized by phagocytes, such as neutrophils, dendritic cells (DCs), and mainly macrophages, the main subset of cell in which the parasite resides. Once intracellular, the parasites differentiate into the amastigote forms and establish its replicative niche within the parasitophorous vacuole [35,58]. Once infected, macrophages activate innate immune pathways in order to clear infection. However, parasites manipulate the host's intracellular signaling to establish infection, dictating the development of protective or deleterious immune responses that will directly influence the clinical outcome of the disease [97] (Figure 1).

Several studies successfully addressed the role of some important immune receptors during the pathogenesis of leishmaniasis, mainly by using murine models of infection. While C57BL/6 mice develop strong Th1 responses that are important to clear infection via production of IFN-γ, BALB/c mice are more prone to develop a Th2 profile, in which the main cytokines produced are IL-4, IL-13, and IL-10 [97], making these genetic backgrounds of mice resistant and susceptible to the infection, respectively. Among innate immune receptors, endosomal TLRs, such as TLR9, were shown to be triggered by *Leishmania* [35,58,97], culminating in the induction of protective mechanisms, such as autophagy [98], and a diverse set of co-stimulatory molecules and inflammatory mediators, such as TNF-α and IL-12, which are critical to the development of effective Th1 cell responses [97].

receptor P2X7 induce noncanonical inflammasome activation upon *L. amazonensis* infection [14]. Further studies are necessary to correlate lipid mediators, ATP, and LPG to better understand how LPG gets access to the host cytosol and induce CASP11 activation.

It is well accepted in the *Leishmania* field that NLRP3 activation is critical to the outcome of the disease. However, while some studies suggested a protective role for NLRP3 during leishmaniasis, others have shown a deleterious role for this inflammasome to the host. These differences could be explained by the use of different parasite species, strains, and different mouse backgrounds [9]. For example, by using *L. amazonensis* infection in C57BL/6 mice, it was demonstrated that NLRP3 plays an important role in parasite elimination and lesion healing [8,11,14,15]. In contrast, studies using C57BL/6 and *Leishmania braziliensis* [16] or the nonhealing *L. major* Seidman strain [17] reported a deleterious role for NLRP3, favoring parasite persistence and lesion development. Likewise, studies performed in patients infected by *L. braziliensis* have shown a positive correlation between inflammasome activation and the severity of the disease [18,19]. Differently from these two reports, studies performed in C57BL/6 mice have shown a protective role for NLRP3 [8] and IL-1β [20] upon *Leishmania infantum* infection. For *L. major* LV39 strain, NLRP3 is dispensable, possibly because the C57BL/6 mouse background is highly resistant to LV39 strain of *L. major*, suggesting that additional restrictive host mechanisms control the infection regardless of the inflammasome [8]. However, a study conducted in BALB/c mice using the same strain of *L. major* indicated that NLRP3 is deleterious to the host [21]. In this study, IL-18 was shown to be involved in the generation of T helper 2 (Th2) responses that do not contribute to parasite elimination, exacerbating lesion development [21]. In the case of *Leishmania donovani*, an important species that causes visceral leishmaniasis in humans, it was demonstrated that in a model of natural sand fly infection in C57BL/6 mice, the microbiota egested upon phlebotomine bites is essential to induce upregulation of inflammasome components (priming) and to promote inflammasome activation [22]. Mechanistically, the authors demonstrate that inflammasome activation promotes neutrophil recruitment, which is critical to parasite dissemination and exacerbation of the disease [22].

Regardless of the detrimental or protective role of the inflammasome during leishmaniasis, these studies highlight a key role of inflammasome-derived cytokines (IL-1β and IL-18) during the pathogenesis of the disease. In the case of IL-18, studies have suggested that this cytokine plays a protective role against the infection by inducing protective T helper 1 (Th1) responses in C57BL/6 mice [23]. However, IL-18 production upon infection in BALB/c mice skews the adaptive immune response towards a Th2 profile, favoring *Leishmania mexicana* [24] and *L. major* [21,25] replication. With regards to IL-1, studies conducted with IL-1R antagonist (IL1-RA) and IL-1α- or IL-1β-deficient mice showed a deleterious role for these cytokines in BALB/c mice [26], whereas in C57BL/6 background, the result was the opposite: deficiency of any of these molecules led to a delay in lesion resolution, supporting a protective role for these cytokines [8,27]. Therefore, differences in the profile of inflammatory mediators and T cell responses elicited, mouse genetic background and microbiota, and different parasitic species/strains may contribute to explain these different outcomes observed regarding the role of inflammasome in the pathogenesis of leishmaniasis.

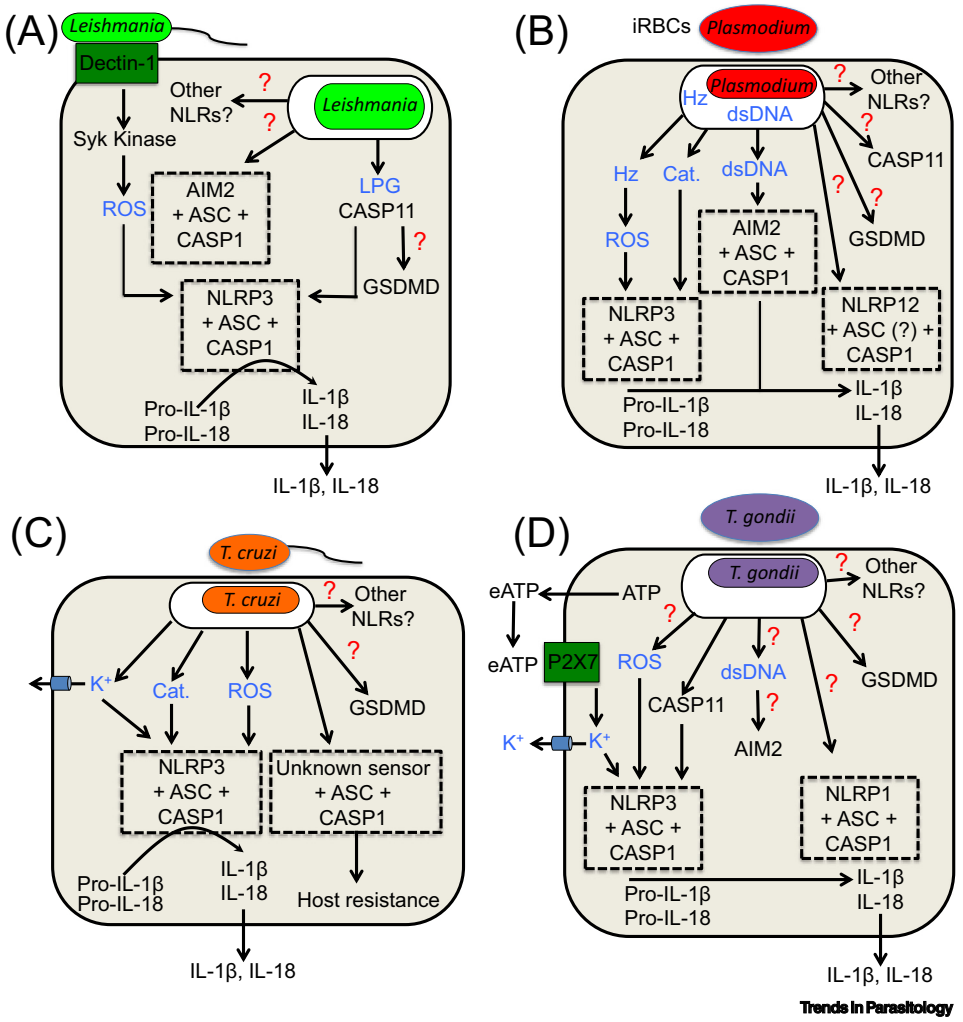
In line with the evidences that NLRP3 is key to the outcome of leishmaniasis, some studies have reported that *Leishmania* can negatively regulate inflammasome activation. The first study to report such a phenomenon demonstrated that the *Leishmania* zinc-metalloprotease GP63 blocks PKC signaling in human monocytes, avoiding ROS production and, consequently, inflammasome activation and IL-1β production [28]. Later, other studies reported that *L. donovani* [29] and *Leishmania guyanensis* [30] block inflammasome activation by inducing

A20, a protein that suppresses NLRP3 activation and restricts ubiquitination and degradation of pro-IL-1β [31]. More recently, it was reported that *Leishmania* RNA virus (LRV), a double-stranded RNA endosymbiont of parasites of the *Viannia* subgenus, such as *L. braziliensis*, *Leishmania panamensis* and *L. guyanensis*, is involved in the modulation of innate immune signaling against the parasite. Fasel's group has postulated that LRV+ parasites, that were previously associated with the development of mucocutaneous leishmaniasis (MCL), trigger a **Toll-like receptor** 3 (TLR3)-dependent hyperinflammatory immune response, which favors parasite dissemination and the severity of the disease [32,33]. Recently, a study from our group has advanced in the mechanisms of LRV-driven pathogenesis downstream of TLR3 signaling. We reported that IFN-β induction via TLR3/TRIF induces autophagy, that in turn targets NLRP3 and ASC for degradation, limiting inflammasome activation by the parasites [34]. Taken together, these studies demonstrate that *Leishmania* can actively inhibit NLRP3 activation, which argues in favor of its protective role to the host. In line with these observations, it was reported that loss-of-function polymorphisms in the human *Il1b* gene have been associated with more severe forms of the disease [8], but studies evaluating polymorphisms in inflammasome genes are still missing in the literature. Interestingly, our study evaluating the mechanisms triggered by LRV to exacerbate disease was the first to demonstrate the magnitude of inflammasome activation in different outcomes of the disease in patients. Analyses of cervical brushes from 49 patients with cutaneous leishmaniasis (CL) or MCL revealed that CASP1 p20 and IL-1β levels are inversely correlated with the severity of the disease, suggesting a protective role for the inflammasome in patients with leishmaniasis [34]. Nonetheless, the deleterious role for IL-1 was attributed to the development of cytotoxic CD8 T cells responses [16], neutrophil recruitment [17,22], and impairment of Th1-protective responses [17], while in macrophages and *in vitro* conditions, IL-1R signaling was associated with parasite control [8,11,14,15]. A study from our group has shed light into the molecular requirements for inflammasome activation upon *Leishmania* infection in macrophages. Specifically, we demonstrated that *L. amazonensis* infection induces robust activation of the NLRP3 inflammasome. This was demonstrated measuring CASP1 activation and formation of **ASC specks** without the need of previous priming by lipopolysaccharide (LPS) or TLRs agonists. Priming is only required to achieve robust protein levels of pro-IL-1β and CASP11, which are necessary for efficient cytokine release. Nonetheless, residual quantities of IL-1 cytokines are sufficient to induce IL-1R-mediated parasite restriction [15].

In summary, despite several studies regarding the role of inflammasomes during *Leishmania* infection, there are still many open questions, including how inflammasome is modulated by infection. This is particularly important given that the majority of the studies indicate that NLRP3 activation is a key event determining the outcome of the disease (Figure 2A). More recently, it was proposed that not only NLRP3, but also the AIM2 and NLRP1 inflammasomes, are involved with the pathogenesis of leishmaniasis in patients and in mice [18]. These findings lead us to speculate that future therapies targeting inflammasome activation in leishmaniasis should take into consideration many different factors, like the outcome of the disease and parasite species. Factors affecting the patient's immune response, such as co-infections with HIV [35], microbiota, and polymorphisms in important immune genes, should also be taken into account for future therapies targeting inflammasome or its mediators during leishmaniasis.

The Role of Inflammasomes during Malaria Pathogenesis

Innate immunity to parasites of the *Plasmodium* genus is key to determine the degree of inflammation during malaria, a complex infectious disease characterized by intense fever, anemia, and organ failure, that leads to more than 0.4 million deaths yearly, mainly in children under 5 years of age [36]. Different species are responsible for the different clinical outcomes: *Plasmodium vivax* and *Plasmodium falciparum* are the main causative agents of the disease, with the latter inducing



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cerebral malaria, a particular type of the disease characterized by intense brain inflammation, blood–brain barrier dysfunction, and death [37]. These parasites are transmitted by *Anopheles* mosquitos, which inoculate the highly infective sporozoite forms into the mammalian host. Malaria has a characteristic disease cycle in which sporozoites reach the blood and the liver, where the parasites multiply and differentiate into merozoites. Upon lysis of infected hepatocytes, these forms infect red blood cells (RBCs) and transform into trophozoites, forming mature schizonts that lysis RBCs to perpetuate infection [36]. Interestingly, this process coincides with the high-fever episodes presented by patients, caused by a cytokine storm that includes IL-1β, TNF-a, IL-12, among others [37,38].

The release of these inflammatory mediators is reported to be caused by recognition of the parasite's **pathogen-associated molecular pattern (PAMP)**, which include **hemozoin (Hz)**, a molecule that was described as a TLR9 activator [39]. Later, it was shown that DNA contamination of purified Hz was responsible for this phenomenon [40]. More recently, Hz was also described by independent groups as an activator of NLRP3, inducing IL-1β release and contributing to

the strong inflammation observed upon infection by these parasites [41]. In addition, Hz has been shown to trigger AIM2 inflammasome because Hz is often associated with DNA [42]. A study from Olivier's group suggests that NLRP3 plays a deleterious role during infection, since inflammasome-deficient mice demonstrated a better survival and more effective control of parasitemia upon infection by a lethal dose of *Plasmodium chabaudi* [41]. Likewise, Tschopp's group has advanced towards the understanding of the molecular mechanisms driving NLRP3 activation, demonstrating that potassium efflux and NADPH oxidase are both required for efficient inflammasome activation and peritonitis induction by Hz [43]. Moreover, their study concludes that infection with *Plasmodium berghei* ANKA sporozoites triggers robust NLRP3 activation, pointing to this molecule as a key player during cerebral malaria in mice, despite not affecting parasitemia control. Another group has shown that activation of the inflammasome by Hz induced NLRP3 activation via uric acid, culminating in IL-1β and IL-6 production, which accounts to neutrophil accumulation in the infected tissues [44]. A recent report has demonstrated that treatment of *P. berghei* ANKA-infected mice with IL-33 protected them from cerebral malaria by reducing Hz-mediated NLRP3 activation and IL-1β release by microglia and intracerebral monocytes [45]. However, other groups have indicated that, despite activation of NLRP3 by Hz, the inflammasome is not important for cerebral malaria in mice, neither affecting parasitemia nor mortality [46]. Importantly, one of these studies suggested that Caspase-12 regulates NF-κB signaling and inflammation in an inflammasome-independent manner [47]. However, further studies have indicated that activation of the inflammasome during human malaria is indeed a relevant process during the disease. In patients infected by *P. falciparum*, it has been shown that **opsonization** induces inflammasome activation in human macrophages upon phagocytosis [48]. Furthermore, circulating anti-DNA antibodies present in patients form immunocomplexes capable of activating the inflammasome in monocytes [49]. In another study involving human and animal models [50], CASP1 activation was detected in different subsets of monocytes obtained from patients infected with *P. vivax* or *P. falciparum* [50]. Moreover, upon infection with *P. chabaudi*, both NLRP3 and NLRP12 activation were reported to occur in mice; while these events were not critical for parasitemia, the animals challenged with a secondary

Figure 2. Inflammasome Regulation by Protozoan Parasites. (A) Upon phagocytosis by macrophages, *Leishmania* parasites trigger dectin-1 activation and a signaling pathway involving the participation of Syk kinase, which generates reactive oxygen species (ROS) and promotes NLRP3 activation. In addition, *Leishmania* lipophosphoglycan (LPG) induces Caspase-11 (CASP11) activation, enabling efficient Nod-like receptor Pyrin domain 3 (NLRP3) assembly by the noncanonical pathway. Recently, it has been suggested that the absent in melanoma (AIM2) inflammasome is also activated during *Leishmania* infection, albeit the mechanisms remain elusive. The involvement of other Nod-like receptors (NLRs) remains to be investigated. (B) In the vacuole, some *Plasmodium*-specific molecules, like red blood cell (RBC)-derived hemozoin (Hz) and the parasite's double-stranded DNA (dsDNA), are released and eventually get access to the host cell cytosol upon lysosomal rupture and cathepsins (Cat.) release. In the cytoplasm, both cathepsins and hemozoin trigger NLRP3 inflammasome. The latter was demonstrated to trigger NLRP3 activation via the production of ROS and membrane damage. In addition, the dsDNA shed within the cytoplasm (and complexed with Hz) was suggested to trigger AIM2 inflammasome activation. Another inflammasome that can also be engaged upon *Plasmodium* infection is NLRP12, but the mechanisms by which this platform assembles remain to be determined, as well as the participation of CASP11 and other NLRs. Inflammasome-derived interleukin (IL)-1β and IL-18 effectively account for induction of fever and other clinical symptoms present in malaria. (C) *Trypanosoma cruzi* lyses the parasitophorous vacuole to reach the host cell cytoplasm, triggering NLRP3 inflammasome activation. Both potassium (K^+) efflux, Cat. Release, and ROS were shown to be involved with NLRP3 activation upon infection, albeit the role of other NLRs, CASP11, and Gasdermin-D (GSDMD) is still a matter of concern. IL-1β and IL-18 were shown to be important for parasite elimination through the induction of nitric oxide. (D) Although *Toxoplasma gondii* is considered to be a silent parasite, recent studies have shown that the AIM2 and NLRP3 inflammasomes are triggered upon infection. The mechanisms of NLRP3 activation only recently began to be elucidated. The P2X7 receptor, which is activated by extracellular ATP (eATP), was shown to be activated upon *T. gondii* infection. Additionally, ROS production is also achieved upon sensing parasites, but how this event is induced remains to be determined. As reported for *Leishmania*, CASP11 and the noncanonical inflammasome were also demonstrated to be activated by *T. gondii*. NLRP1 was also shown to play a role in toxoplasmosis, albeit the mechanisms are still not fully understood. As already mentioned for the other parasites, the role of GSDMD and the participation of additional NLRs are a matter for future studies.

challenge of LPS were more susceptible to septic shock, shedding light into the clinical observations of septic-shock-like syndrome in postmalaria patients [51].

Recently, a study focusing on the pathology of sterile hemolysis induced by heme has shown that in its free state, heme triggers ROS production, Syk kinase and NADPH oxidase activation, all events important to inflammasome assembly [52]. Whether this pathway is involved in malaria pathogenesis remains to be determined. A previously unappreciated role for the inflammasome in regulating type I IFN production has been shown to impact malaria pathogenesis. Using a murine model of *Plasmodium yoelii* infection, the authors demonstrated that NLRP3, AIM2, and CASP1 negatively regulates type I IFN by negatively regulating MyD88 and IRF7, both known to be important in the production of these antiviral cytokines, which they also report to play an antimalarial effect [53].

Although studies on inflammasome-related polymorphisms in malaria have not been extensively performed, a recent report selected single-nucleotide polymorphisms (SNPs) in the NLRP3, NLRP1, AIM2, IL-1β, IL-18, CARD8, and MEFV genes from *P. vivax*-infected patients, correlating important mutations in NLRP1, IL-18, and IL-1β with important clinical aspects, such as anemia and fever [54]. Altogether, these studies suggest that different NLRs contribute to CASP1 activation and IL-1β release during malaria pathogenesis in mice and humans (Figure 2B). Despite the controversies, a more comprehensive analysis involving different mice backgrounds, *Plasmodium* species, and human data would help to clarify the molecular mechanisms of inflammasome regulation upon malaria infection.

Inflammasome Activation by *Trypanosoma cruzi*

Chagas disease is endemic in Latin America and is caused by the protozoan parasite *T. cruzi*. It is transmitted by different species of hematophagous triatomine insects [55]. Although many studies have addressed adaptive immunity to *T. cruzi*, fewer studies have addressed the innate immune pathways related to the pathogenesis, specifically in terms of inflammasome activation. *T. cruzi* is able to lyse the vacuolar membrane and reach the host cell cytoplasm, where it replicates. This supports the key role of intracellular receptors and inflammasomes in recognition of this parasite. However, only a few studies have evaluated the role of different NLRs and IL-1β in Chagas disease models. The first report to identify the upregulation of different inflammasome-related genes, such as *Casp1*, *Asc*, *Casp11*, *Il1b*, and *Il18*, but not *Nlrp3*, in the heart tissue of infected mice was performed in 2013 [56]. Interestingly, *T. cruzi* infection in ASC-deficient macrophages, or treatment with inhibitors of ROS, potassium efflux, and cathepsins abolished CASP1 activation and IL-1β production in response to infection [57]. The involvement of ROS, K^+ efflux, and cathepsins on cytokine production highly suggests that NLRP3 is activated in response to infection, as supported by studies using macrophage infection *in vitro* [57,58]. However, *Il1r1*$^{-/-}$, *Casp1/11*$^{-/-}$, and *Asc*$^{-/-}$, but not *Nlrp3*$^{-/-}$ mice are more susceptible to infection, as shown by survival assays [56]. Taken together, these findings suggest that another ASC-dependent inflammasome may also play a relevant role in disease pathogenesis *in vivo*. Whether it is Aim2, Nlrp1, Naip/Nlrc4, Pyrin, or another NLR remains to be determined. Another 2013 study [57] reported that both NLRP3 and CASP1 activation occur *in vitro* and *in vivo* upon *T. cruzi* infection. In both studies, NLRP3 activation was shown to be critical for NO production and protective immunity against the parasite, similar to that observed for *Leishmania* parasites [8,11,15,20]. Other groups have shown that NLRP3 is indeed engaged in response to *T. cruzi* in macrophages [59] and plays important roles in T cell activation and parasite control [60]. Therefore, these studies suggest that the NLRP3 inflammasome plays an important role in restriction of parasite replication by inducing protective levels of NO, considered a key effector molecule to target *T. cruzi*. Another recent *in vivo* study has shown that IL-6 is an important modulator of IL-1β production and,

consequently, NO production. The authors showed that IL-6 deficiency led to increased amounts of circulating IL-1β, NO, and inflammatory monocytes, resulting in increased mortality upon infection, suggesting an important role for IL-6 in modulating excessive inflammation [61].

Recently, work from Bortoluci's lab shed light on downstream mechanisms of NLRP3 activation that leads to parasite control. They demonstrated that engagement of NLRP3 by *T. cruzi* culminates in autophagosome formation, putting autophagy as an important mechanism downstream of the inflammasome involved with parasite control [62]. The crosstalk between autophagy and the inflammasome was suggested to occur in many different outcomes [63], and another recent study has shown that inhibition of **mammalian target of rapamycin (mTOR)** signaling induced by rapamycin increased NLRP3 expression, mitochondrial ROS production, and IL-1β release, resulting in more efficient parasite control in macrophages [64]. The authors did not discuss a possible direct effect of autophagy in the phenotypes observed upon rapamycin treatment. This is particularly important because rapamycin is a potent inducer of autophagy and has been widely used as a positive control in LC3 conversion in many studies [34]. Therefore, it is possible that autophagy acts as a negative regulator of inflammasome assembly, as already demonstrated in *Leishmania* infection studies [34].

While specific questions regarding the role of the inflammasome upon *T. cruzi* infection have already been addressed using murine models (Figure 2C), it remains to be determined whether the participation of NLRs and IL-1β are also important in human cells and whether this pathway accounts for disease pathogenesis in patients. One study has correlated SNPs in inflammasome genes (*Card11* and *Nlrp1*) with chronic Chagas cardiomyopathy, suggesting that variations in these genes are associated with severity of the disease [65]. A more comprehensive analysis using a bigger cohort of patients may be required to definitively correlate specific inflammasome components with different stages of Chagas disease.

Regardless of the advances in understanding specific NLRs and downstream effector mechanisms during *T. cruzi* infection in cells and disease progression, the precise mechanisms and contributions of each individual NLR in Chagas disease remain to be determined. A better understanding of the mechanisms regulating inflammasome effector functions could lead to the development of more efficient therapeutic approaches against this parasite, expanding the possibilities of pharmacological interventions to people suffering from Chagas disease.

Toxoplasma gondii and Inflammasomes

T. gondii is an obligate intracellular parasite that is the causative agent of toxoplasmosis. Nearly 40% of the world's population is infected with this parasite, which can also infect other mammals and birds [66]. In healthy individuals, *T. gondii* is efficiently controlled and there is no symptomatic disease, but reactivation can occur in immunosuppressed individuals. In this case, cysts appear in the brain and other tissues, eventually causing a devastating disease that may, ultimately, become fatal if not properly treated [67]. Thus, the induction of optimal innate and adaptive immunity against *T. gondii* is crucial to avoid disease. These involve different myeloid [neutrophils, macrophages, dendritic cells (DCs)] and lymphoid (T, NK, and B cells) subsets, at different time points after infection [58]. A hallmark of the beginning of the immune response is the production of IL-12 by DCs, which prime IFN-γ-mediated effective NK and T cell responses. Several efforts have been made to understand the **pattern-recognition receptors (PRRs)** involved with IL-12 production by DCs, suggesting an essential role for TLRs, such as TLR9, TLR11, and TLR12, and the adaptor proteins MyD88 and UNC93B1 [68,69].

With regards to NLRs, the first sensor reported to be involved with *T. gondii* pathogenesis was NLRP1. It was described that susceptibility alleles related to NLRP1 were directly associated with human congenital toxoplasmosis and monocytes silenced for NLRP1 had a differential parasite killing and cell death [70,71]. A further study confirmed this assertion, showing that CASP1 and ASC are necessary for efficient IL-1β release [72], but the nature of the NLR involved with parasite's sensing was only demonstrated in a different report, where NLRP1 was pointed out as the NLR involved in parasite restriction in mice and rat cells [73]. In the same year, NLRP3 was also appointed to be involved with *T. gondii* sensing by inducing IL-18, which was shown to be important for restriction of parasite infection [74]. However, only recently the mechanisms of NLRP3 activation by this protozoan began to be elucidated. A study by Coutermarsh-Ott *et al*. [75] demonstrated that activation of CASP11 by *T. gondii* is crucial to enhance host protection, contributing to inflammation and parasite control, a finding similar to that recently observed by our group [11] with *Leishmania* parasites. Moreover, two different groups have shown that ATP generated upon infection is important to trigger canonical NLRP3 activation, generating IL-1β and contributing to infection control [14,76]. Consistent with these findings, another study reported that potassium efflux is an important event for NLRP3 activation in human monocytes during *T. gondii* infection [77].

While the mechanisms responsible for NLRP3 activation within the host cells are now being more clearly elucidated, the mechanisms by which NLRP1 is activated in response to *T. gondii* infection remain to be determined (Figure 2D). Additionally, whether specific components from *T. gondii* modulate inflammasome assembly also deserves further attention. One study reported the role of a molecule from this parasite in modulation of CASP1 activation [78]. The redox enzyme peroxiredoxin (rTgPrx), known to be important for the parasite's oxidative balance, was shown to be involved with M2 macrophage polarization, increasing arginase and other M2 markers, while decreasing CASP1 activation and IL-1β release. These effects led to an increased parasite survival within these macrophages, supporting the protective role of the inflammasome in infection control. Taken together, the literature from *T. gondii* and inflammasomes support a protective role for the inflammasome in restriction of parasite replication.

Concluding Remarks

The last 20 years of research in innate immunity has shed light into the importance of the IL-1 family of cytokines and inflammasomes in the pathogenesis of many infectious diseases, including those caused by protozoan parasites. Although several groups have advanced toward global

Outstanding Questions

Which protozoan molecules are released in the host cell cytoplasm, leading to activation of cytosolic sensors?

Does Caspase-11 have a role during *T. cruzi* and *Plasmodium* infection, as reported for *T. gondii* and *Leishmania*?

How do intracellular parasites inhibit pyroptosis despite the robust activation of the inflammasome?

What is the role of gasdermins in parasitic diseases?

Do parasites modulate the magnitude of inflammasome activation to balance inflammation and to promote chronic disease?

Table 1. Specific Inflammasomes Triggered by Intracellular Parasites

Inflammasomes	Protozoan parasites	Activators (DAMPs/PAMPs)
NLRP1	*Toxoplasma gondii*	Unknown
NLRP3	*Leishmania* spp.	K^+ efflux, ROS, Cat.
	Plasmodium spp.	K^+ efflux, ROS, Cat., Hz
	Trypanosoma cruzi	K^+ efflux, ROS, Cat.
	Toxoplasma gondii	K^+ efflux, ROS, ATP
NLRP12	*Plasmodium* spp.	Unknown
AIM2	*Plasmodium* spp.	dsDNA
Caspase-11	*Leishmania* spp.	LPG
	Toxoplasma gondii	Unknown

Abbreviations: Cat., Cathepsin; DAMP, damage-associated molecular pattern; dsDNA, double-stranded DNA; Hz, hemozoin; LPG, lipophosphoglycan; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species.

understanding of the mechanisms of NLR activation in response to *Leishmania*, *Plasmodium*, *T. cruzi*, and *T. gondii* (Table 1), there are still many open questions regarding innate recognition of intracellular parasites (see Outstanding Questions). The specific PAMPs and microbial components involved with inflammasome activation and inhibition, and whether these parasites can effectively manipulate or evade immune detection in order to establish disease, remain to be determined. Importantly, for pathogens such as *Leishmania*, it is still unknown how parasites actively translocate their virulence factors to the host cell cytosol in order to subvert host cell functions and allow cytosolic PRR activation. The new era of CRISPR-mediated genome editing may allow identification of parasite-specific components involved in pathogenesis and activation of the host innate immunity. This knowledge may promote the development of new therapeutic approaches to treat these challenging protozoan diseases, since potent small molecule inhibitors for the inflammasome are now undergoing clinical trials for a wide variety of diseases [79]. This will possibly allow its use also in the treatment of protozoan infections, since the drugs currently used to treat people infected by these protozoan diseases are often toxic and poorly effective. Thus, a better understanding of the molecular mechanisms governing host–parasite interactions may bring new hope to people suffering from these debilitating and devastating parasitic diseases.

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