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Inflammasomes and *Leishmania*: in good times or bad, in sickness or in health

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The inflammasomes are multi-molecular platforms that are activated in host cell cytoplasm when the innate immune cells are infected with pathogens or exposed to damage signals. Many independent groups reported that *Leishmania* infection trigger activation of the NLRP3 inflammasome in macrophages for restriction of intracellular parasite replication. Accordingly, *Leishmania* can dampen NLRP3 activation as an evasion strategy. *In vivo*, the NLRP3 inflammasome can promote parasite clearance, but the failure to eliminate parasites in the tissues together with sustained inflammasome activation can promote IL-1β-mediated disease pathology. In this review, we discuss the recent data regarding activation of the NLRP3 inflammasome in response to *Leishmania* and the beneficial and detrimental effects of the inflammasome during development of Leishmaniasis.

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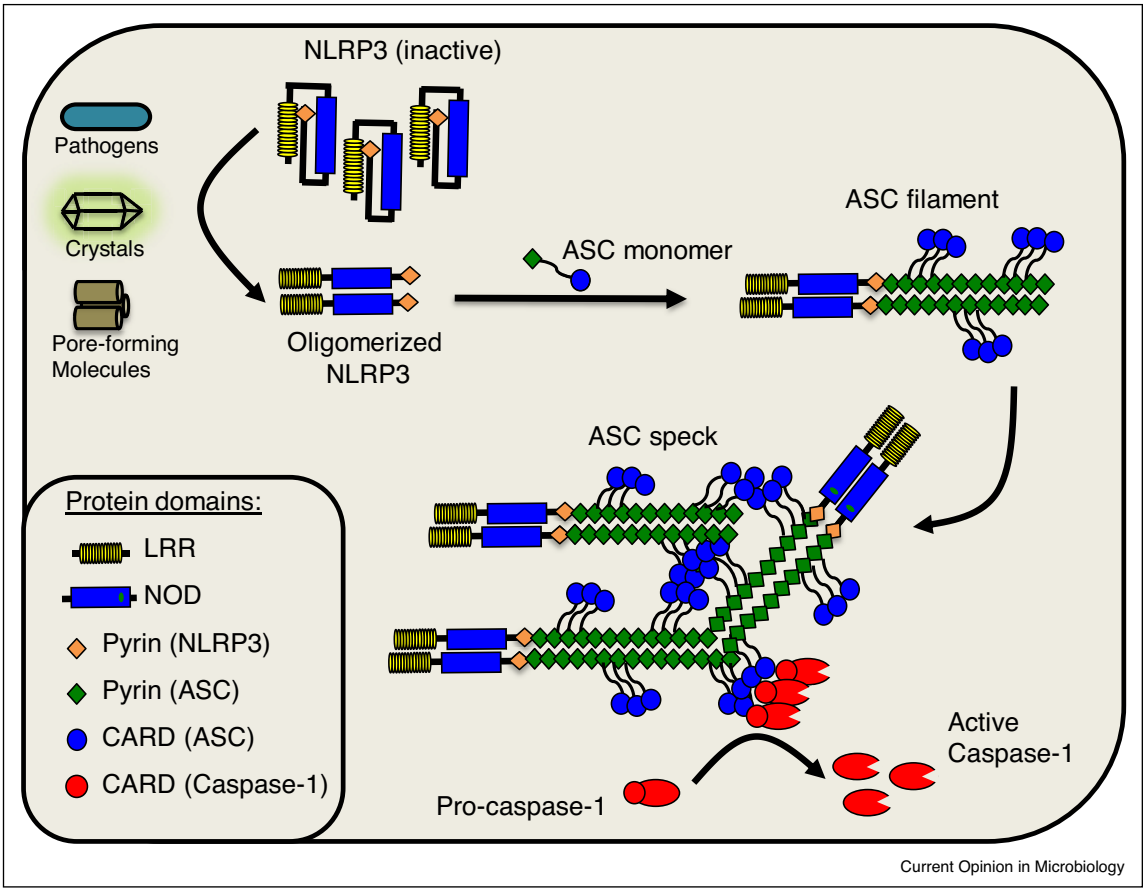
Introduction

The inflammasomes are multiprotein complexes that are assembled in the cytoplasm of innate immune cells when intracellular pattern recognition receptors detect pathogenic microorganisms or damage signals. The most studied inflammasome is the nucleotide-binding domain leucine-rich repeat protein (NLR) family member, NLRP3, which is activated in response to multiple pathogens and molecules that induce damage in the host cell membranes (Reviewed in Ref. [1]). Upon activation, NLRP3 oligomerizes and promotes the polymerization of ASC via Pyrin–Pyrin interaction, exposing multiple CARD domains of ASC in the filaments, which can recruit Caspase-1 via CARD/CARD interaction to promote caspase-1 activation (Figure 1). Importantly, two independent signals are required for activation of NLRP3. The first signal (also called priming) occurs when microbial components (or TNF-α) stimulate TLRs (or TNFR) leading to transcription of many inflammatory genes, including *Nlrp3*, *Casp11*, *and Il1b*. Although the first signal is essential, it is not sufficient, and a required second signal occurs with formation of pores or rupture in the macrophage membranes, which allows for the decrease in intracellular K^+. The induction of membrane pores by microbial toxins, the activation of K^+ channels or membrane lysis by crystals or noxious molecules allow K^+ efflux and the so called canonical activation of the NLRP3 inflammasome [1,2]. Besides the efflux of potassium, the production of ROS and lysosomal cathepsins is also important for canonical NLRP3 activation (reviewed in Ref. [1]). In contrast, activation of caspase-11 promotes the non-canonical activation of the NLRP3 inflammasome. Mechanistically, caspase-11 is activated when bacterial LPS is present in the cytoplasm and promotes proteolytic cleavage of Gasdermin-D. The active N-terminal domain of Gasdermin-D is further inserted in the macrophage membrane and forms a pore that allows K^+ efflux and the non-canonical activation of the NLRP3 inflammasome (reviewed in Ref. [1]). The requirement for the two signals provides a regulated control of the inflammasome activation that prevents unnecessary inflammation in the absence of infection or noxious molecules. Not surprisingly, intracellular pathogens have developed mechanisms to inhibit these signals, as discussed below in the context of *Leishmania*.

Leishmanial diseases represent a spectrum of neglected tropical diseases that are endemic in 88 countries worldwide, with over 1 million new cases each year and over 300 million people at risk. There are different forms of clinical disease in humans, ranging from self-limiting cutaneous lesions, to tissue destructive mucosal involvement, to visceral dissemination that is fatal in the absence of treatment. These diverse clinical outcomes are caused by different species of protozoan parasites in the genus *Leishmania* that are in each case transmitted to their mammalian hosts by the bite of an infected sand fly vector. *Leishmania* have a digenetic life cycle consisting of extracellular, flagellated promastigotes replicating in the sand fly, and intracellular aflagellate amastigotes replicating in phagocytic cells, primarily macrophages, in the mammalian host. There are no effective vaccines

Figure 1



Activation of NLRP3 inflammasome in response to pathogens, crystals and pore-forming molecules.
NLRP3 is activated when pathogens or specific molecules (crystals, toxins, purinergic receptors, etc.) promote perturbation of the host cell membranes. Once activated NLRP3 oligomerizes and promotes the polymerization of ASC via Pyrin–Pyrin interaction. Polymerized ASC forms a long filament exposing multiple CARD domains. This filament can bind to other ASC filaments to form the ASC speck. This platform recruits pro-caspase-1 via CARD/CARD interaction to promote caspase-1 activation and cleavage.

for use in humans, despite the fact that primary infection, especially those involving cutaneous strains, often result in strong, long lasting acquired immunity. As would be expected for immunity against pathogens that reside in phagosomes, T helper 1 (Th1) responses are a crucial component of the protective response, with strong T cell responses associated with healing, and weak T cell responses associated with high parasite burdens and disseminating infections. This dichotomy is not always so clear, however, as mucosal patients present with an exaggerated T cell response that controls the infection but drives the chronic tissue destruction. The severity of even localized cutaneous lesions reflects a balance between immunoprotective and immunopathologic processes that can be difficult to distinguish. This review focuses on central components of the innate response, NLRP3 inflammasome activation and secretion of IL-1β, that have been found to contribute to both protective and pathologic outcomes in experimental Leishmaniasis.

Activation of NLRP3 inflammasome and restriction of *Leishmania* replication in macrophages

The initial demonstration that *Leishmania* infection triggers the activation of inflammasomes occurred in 2013 when it was shown that bone marrow-derived macrophages infected with *Leishmania amazonensis*, *Leishmania major and Leishmania braziliensis* trigger Caspase-1 activation and IL-1β production. This process was abolished in the absence of NLRP3 or ASC, indicating that the NLRP3 inflammasome is responsible for caspase-1 activation in response to infection [3••]. This study also demonstrated that the inflammasome activation in macrophages favored the restriction of parasite replication in macrophages and in mouse by mechanisms dependent on IL-1R signaling. Another study published in the same year reported that *Leishmania infantum* induced Caspase-1 and IL-1β production through Dectin-1 and mannose receptor, which stimulated p47phox and arachidonic

acid-NADPH oxidase signaling for production of ROS and restriction of parasite replication via Caspase-1 and IL-1β [4••]. In agreement to these findings, it was demonstrated that *L. amazonensis* induced Dectin-1 activation, leading to activation of Syk kinase and ROS production by NADPH oxidase, a process that is critical for activation of NLRP3 in macrophages [5•]. Importantly, inhibition of ROS and NADPH oxidase during the early stages of infection was sufficient to abrogate inflammasome activation, indicating that the initial signals for inflammasome activation occurs during the phagocytosis of the parasites [5•]. These studies emphasize the participation of ROS in the activation of NLRP3 during *Leishmania* infection. More recently, lipophosphoglycan (LPG) from many *Leishmania* species was shown to trigger caspase-11 activation in macrophages [6••], demonstrating non-canonical activation of the NLRP3 inflammasome, although a caspase-11-independent pathway for inflammasome activation was also detected [6••]. The demonstration that caspase-11 is involved in activation of NLRP3 in response to *Leishmania* provided clues to explain the requirement of priming for inflammasome activation in BMDMs infected with *Leishmania in vitro*. Similarly to Pro-IL-1β, Caspase-11 is expressed in lower levels in steady state cells and transcriptional responses are required for protein expression [7]. According to these observations, a recent report demonstrated that because of the low levels of Caspase-11 and Pro-IL-1β in steady state BMDMs, priming is required for detection of Caspase-1 cleavage (assessed by western blot) and IL-1β activation (assessed by ELISA) in response to *L. amazonensis* infection [8]. However, priming was dispensable for assembly of the NLRP3 inflammasome (assessed by ASC specks), Caspase-1 activation (assessed by FAM-YVAD) and restriction of parasite replication via the NLRP3 inflammasome [8]. During *in vivo* infection, priming may occur via TNF-α, INF-γ or when bacteria contaminate the cutaneous lesions developed in patients' skins. It was also reported that gut bacteria from the sand fly may be egested into host skin with *Leishmania* parasites [9•], and may thus be the source of priming allowing robust inflammasome activation when the parasites infect the macrophages.

It was also reported that the mitochondrial phosphatase phosphoglycerate mutase family member five, a protein that plays a role in the restriction of *Leishmania* replication in macrophages, is important for IL-1β production in response to *Leishmania*, implying the participation of this protein in the activation of the NLRP3 inflammasome [10]. Compounds that trigger inflammasome activation and promote IL-1β production have also been shown to restrict parasite replication in macrophages, including nanoparticles of polyester poly (lactide-co-glycolide acid) loaded with an 11 kDa *Leishmania* antigen, [11], the antiprotozoal drug diterpene kaurenoic acid, and the leishmanicidal drug Amphotericin B [12]. Administration of anti-IL-1β reduced the parasite clearance by AmpB in a model of visceral *Leishmaniasis* [13•]. Collectively, these studies unequivocally demonstrate that the inflammasome can be activated in response to *Leishmania* infection and plays an important role in restricting *Leishmania* replication in macrophages.

Inhibition of NLRP3 inflammasome by *Leishmania* as an evasion strategy

Given its role as an innate immune defense against *Leishmania* infection, it is not surprising that *Leishmania* have evolved mechanisms to inhibit or limit the activation of the NLRP3 inflammasome in macrophages. Shio *et al.* demonstrated that *Leishmania mexicana* and *L. major* use the virulence factor GP63 to inhibit the production of IL-1β production in human THP-1 cells. The mechanisms of inhibition are still unclear but may involve inhibition of ROS production and also a direct cleavage of inflammasome components [14••]. *Leishmania donovani* inhibits the NLRP3 inflammasome by manipulating A20 (a negative regulator of NF-kB) and UCP2 (mitochondrial uncoupling protein 2). A20 was thought to inhibit the first signal (priming) whereas UCP2 inhibits of the second signal, involving the ROS production [13•]. Upregulation of A20 as a mechanism to inhibition inflammasome activation was also evident in experiments using *Leishmania guyanensis* [15]. Two recent studies involving *L. amazonensis* and *L. donovani* showed transcriptional inhibition of inflammasome components in infected macrophages [16,17]. Collectively, these findings do not necessarily conflict with the articles cited above reporting inflammasome activation in response to *Leishmania* infection. The inhibitory effect in certain conditions may reduce/limit the magnitude of inflammasome activation, but may not fully block the activation. The consensus is that the magnitude of inflammasome activation in response to *Leishmania* is reduced as compared to infection with bacteria or other protozoan parasites such as *Toxoplasma gondii* and *Trypanosoma cruzi* (reviewed in Ref. [18]). This may occur because *Leishmania* inhibits inflammasome activation and may explain why some groups fail to detect inflammasome activation in response to *Leishmania* infection.

Inflammasome activation and IL-1β can promote pathology and parasite growth *in vivo*

In vivo studies performed with *L. amazonensis* in mice indicated that the inflammasome were important for protective host response and restriction of parasite replication in the tissues. This was demonstrated using *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1/11*$^{-/-}$, *Casp11*$^{-/-}$ and *Il1r*$^{-/-}$ mice in C57BL/6 genetic background and using *Casp1/11*$^{-/-}$ mice in A/J background [3••,5•,6••]. However, studies performed with other *Leishmania* species reported a detrimental effect of the NLRP3 inflammasome. Infection of C57BL/6 mice with many *L. major* strains results in a healing lesion with minimal pathology at the site of inoculation in the skin. By

contrast, using a low dose infection with a strain of *L. major* (*Lm* Sd) isolated from a patient with chronic cutaneous lesions, C57BL/6 mice developed severe, non-healing dermal lesions and failed to effectively control tissue parasite loads despite a strong TH1 response [19]. Infection was associated with elevated early and sustained levels of IL-1β mRNA and IL-1β+ cells in the inoculation site, and a strong neutrophil infiltrate that persisted throughout the course of lesion development [20••]. Critically, mice deficient in IL-1R, IL-1β, ASC, caspase-1/11, and NLRP3, each showed minimal pathology and healed their *Lm* Sd infection. While a contribution of the inflammasome/IL-1β axis to the severe pathology might be expected, the pathology appeared to be secondary to the inability to control the infection, resulting in the persistence of organisms able to drive the inflammatory response. Similar observations have been made in mice lacking the NLRP3 inflammasome on a *Leishmania*-susceptible background (BALB/c), which exhibited better control over *L. major* growth in the footpad [21••], and in C57BL/6 and BALB/c mice treated with exogenous IL-1β, that in both cases led to increased *L. major* parasite burdens and lesion progression [22]. The severity of experimental visceral leishmaniasis following transmission of *L. donovani* by bites of infected sand flies has also been linked to the activation of the NLRP3 inflammasome and IL-1β production, thought to be exacerbated by gut microbiota from the fly that were egested along with the parasites into the skin [9•].

How might the NLRP3 inflammasome and IL-1β function to compromise host defense against *Leishmania*? Sustained recruitment of neutrophils is characteristic of each of these skin inoculations sites, and their ability to sequester the infection [23], or to be captured by macrophages and dendritic cells to suppress the microbicidal and antigen presentation function of these cells, has been described [24•,25]. IL-1β has also been shown to promote the expansion of Th2 and Th17 cells, at least in the mouse [26]. The inflammasome deficit in the *L. major* infected C57BL/6 and BALB/c mice resulted in diminished levels of Th2 cytokines, though in the later case IL-18 was thought to regulate the Th1/Th2 balance observed [21••]. IL-1β can also promote the proliferation and maturation of group 2 innate lymphoid cells (ILC2) [27], which could act by promoting Th2 development. The type 2 cytokines produced by ILC2 are also crucial to sustain eosinophils and alternatively activated macrophages in local tissue environments, such as adipose tissue [28]. This may explain the loss of the population of embryonic derived, alternatively activated, dermis resident macrophages in the inflammasome and IL-1β deficient mice, whose preferential infection by the *Lm* Sd strain was found to be essential to the development of the non-healing phenotype [29•]. Importantly, in contrast to the infected, monocyte-derived cells recruited to the site, the infected, dermis resident macrophages did not produce IL-1β.

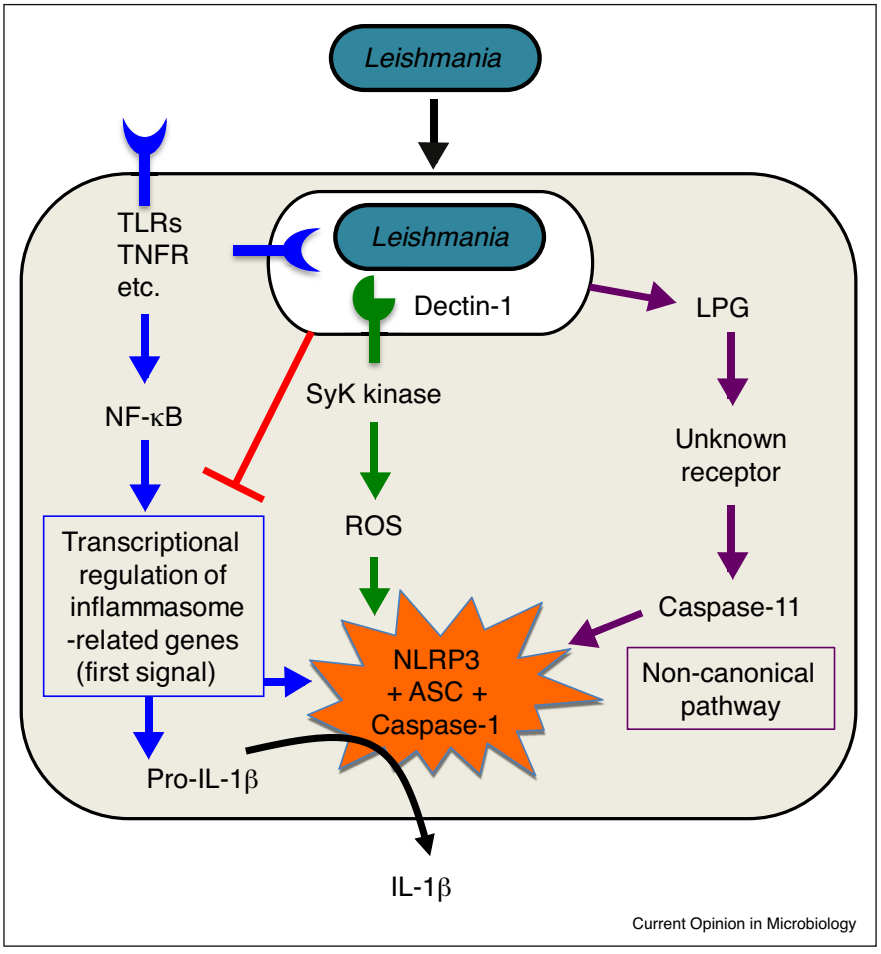
Clinically, IL-1β expression has been correlated with disease severity in patients with cutaneous leishmaniasis due to *L. mexicana* [30], and monocytes from patients with CL due to *L. braziliensis* expressed NLRP3, and secreted IL-1β in levels that correlated with areas of necrosis and lesion progression [31•]. More directly, blockade of the NLRP3 inflammasome using the small molecule inhibitor glyburide prevented IL-1β release in lesion biopsies of *L. braziliensis* patients [32•]. Transcriptionally, it was found that the levels of IL-1β expression in biopsied skin lesions positively correlated with genes involved in both inflammasome activation and cytotoxicity, including GZMB, GZMA, and PRF1 [32•,33]. A clear link between cytotoxic CD8+ T cells and NLRP3 inflammasome activation and IL-1β production was established in a mouse model of *L. braziliensis* infection, in which RAG deficient mice that were reconstituted with CD8+ T cells developed immunopathology that was ameliorated using CD8+ T cells from perforin knockout mice, or by pharmacological inhibition of NLRP3 or IL-1β [32•]. Thus, inflammasome activation by dead cells, or danger associated molecular patterns (DAMPS), in conjunction with the tissue parasites themselves, could augment the pathogenesis. Severe pathology in this model was again associated with the persistent recruitment of neutrophils, which were themselves a source of IL-1β, but importantly was not associated with increased tissue parasite loads.

Conclusions and perspectives

Work performed in the last five years by independent groups reported activation of the NLRP3 inflammasome in response to different *Leishmania* species. Inflammasome activation can be triggered as a protective host response and accordingly, *Leishmania* was reported to actively limit the inflammasome activation in macrophages. Nonetheless, when innate immune cells fail to clear parasites in the tissues, sustained inflammasome activation promotes exacerbated IL-1β production and inflammation, which can lead to tissue damage and aggravation of the disease. In experimental infections involving *L. major*, inflammasome activation can indirectly contribute to parasite growth and persistence, likely due to the ability of the inflammasome/IL-1β axis to promote Th2, ILC2, and/or neutrophil responses. The inability to appreciate an inflammasome driven host protective response in *L. major* infected mice might be explained by the relative strength of the T cell response that supersedes any direct effect of inflammasome activation on the innate response. Collectively, these observations highlight the importance of the inflammasome in the pathophysiology of Leishmaniasis and raises promising therapeutic approaches to reduce the clinical manifestations of the disease in certain cases.

Many other important aspects of NLRP3 inflammasome activation by *Leishmania* remain unclear, including at the most basic level how an intraphagosomal parasite triggers

Figure 2



Mechanisms of inflammasome activation in macrophages infected with *Leishmania*.
NLRP3 activation requires the first signal (or priming) that can be achieved when Toll-like receptors (TLRs) ligands, TNF-α (or other cytokines) induce transcriptional upregulation of inflammasome-related genes (indicated in blue). This process usually occurs via nuclear factor-κB (NF-κB)-mediated gene expression. *Leishmania* is able to inhibit the inflammasome activation at the priming stage by multiple mechanisms (indicated in red). *Leishmania* triggers Dectin-1, a C-type lectin receptor that signals via spleen tyrosine kinase (Syk) to induce ROS, which is critical for activation of the NLRP3 inflammasome (pathway shown in green). *Leishmania* Lipophosphoglycan (LPG) present in the macrophage cytoplasm triggers Caspase-11 activation indirectly. This pathway feeds in the non-canonical pathway for activation of the NLRP3 inflammasome (indicated in purple).

this cytosolic sensor. The production of ROS via NADPH oxidase and Syk kinase, as well as the efflux of K^+ appears to be important for the NLRP3 response to infection [4••,5•,13•,14••]. The involvement of the parasite glycoconjugate LPG in triggering Caspase-11 [6••] suggests that *Leishmania*-induced inflammasome activation occurs, at least partially, via the non-canonical pathway (Figure 2), although how LPG reaches the macrophage cytoplasm to trigger caspase-5/11 is still unclear. It is also possible that the release of ATP by dying macrophages favors the canonical activation the NLRP3 via purinergic receptors such as P2X7 and P2Y [34,35]. Finally, understanding the pathogenic effects of IL-1β/IL-1R in the tissues requires further investigation. The comprehensive understanding of the roles of NLRP3 inflammasome during Leishmaniasis is key for our understanding of the molecular basis of this important disease.

Conflict of interest statement

Nothing declared.

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