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Immune Evasion Strategies

M. Magdalena Aguirre-Garcia,
Alma R. Escalona-Montaña,
Arturo A. Wilkins-Rodríguez and
Laila Gutiérrez-Kobeh

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Abstract

Leishmania is the causative protozoan parasite of leishmaniasis. Distinct species provoke localized/diffuse cutaneous leishmaniasis or visceral leishmaniasis. *Leishmania* parasites have developed diverse strategies to evade the host immune response expressed through various cells, especially macrophages, NK cells, and dendritic cells. Participating in some of these strategies are *Leishmania* surface molecules, such as lipophosphoglycan (LPG) and protease gp63, which are thus considered virulence factors. LPG has been shown to modulate proinflammatory responses. For example, *L. major* LPG activates NK cells through toll-like receptor-2 (TLR2), while *L. mexicana* LPG elicits a differential production of cytokines in human dendritic cells and monocytes. Moreover, *L. mexicana* LPG activates MAP kinases in macrophages, which in turn enhance proinflammatory cytokine production through TLRs. Additionally, *Leishmania* exosomes have been found to strongly affect macrophage signaling and functions. Furthermore, proteins secreted by *Leishmania* promastigotes and amastigotes modulate the production of proinflammatory cytokines in human macrophages. Since *Leishmania* is an obligate intracellular parasite, its promastigotes utilize several mechanisms to survive and duplicate inside host cells, including the inhibition of apoptosis. It is now clear that MAPK p38, JNK, ERK 1/2, and PI3K/Akt participate in the inhibition of both natural and induced apoptosis of macrophages, neutrophils, and dendritic cells.

Keywords: *Leishmania*, cytokines, TLR, inflammasome, apoptosis, NO

1. Introduction

Leishmaniasis is a complex of neglected tropical diseases (NTDs) caused by protozoan parasites of the genus *Leishmania*. Epidemiological studies have revealed that 12 million people are

infected worldwide, with 2 million new cases each year. Approximately 350 million people are currently at risk of contracting leishmaniasis, mostly in developing countries.

Leishmania is a dimorphic protozoan parasite that completes its life cycle in two organisms: the sand fly vector and a mammalian host (a rodent, canid, or human). In the vector, the parasite enters the insect as aflagellated amastigotes through a blood meal from a mammal. In the sand fly midgut, this form changes into motile extracellular flagellated promastigotes that divide by binary fission. After being injected into the bloodstream of a mammalian host, the promastigotes are quickly engulfed by macrophages, where they differentiate into aflagellated intracellular amastigotes that can survive in this acidic environment. In macrophages, the amastigotes replicate by binary fission, causing the lysis of the cell and the invasion of other cells [1].

The outcome of a *Leishmania* infection depends on a wide array of factors, especially the species of the parasite and the host immune response. Species such as *L. major*, *L. mexicana* and *L. guyanensis* induce cutaneous leishmaniasis, while *L. amazonensis* and *L. braziliensis* cause the mucocutaneous form. *L. donovani* and *L. chagasi*, on the other hand, cause visceral leishmaniasis (VL) [2].

Whether *Leishmania* parasites manage to establish themselves in mammalian cells depends on their capacity to surpass host defense mechanisms. The survival strategies of the protozoan are based on the manipulation of distinct host cell functions, including modulation of cell signaling pathways through phosphorylation and dephosphorylation mechanisms [3]. The different strategies of *Leishmania* to evade the host immune response during the process of infection involve macrophages, NK cells, and dendritic cells. One of the most successful survival strategies displayed by *Leishmania* is the inhibition of apoptosis of host cells through the activation or silencing of proapoptotic or antiapoptotic signaling pathways [4, 5]. Among the *Leishmania* surface components participating in such evasion strategies is lipophosphoglycan (LPG), an abundant molecule that exerts its activity by binding to TLR2. Although the ability of *Leishmania* to inhibit inflammatory signaling pathways has been proposed as a virulence mechanism, the molecular events underlying this process have still not been fully elucidated [6].

2. Parasite molecules that regulate host cell signaling pathways

Two of the most studied molecules of *Leishmania* spp. are LPG and glycoprotein 63 (gp63), postulated as possible virulence factors for some species. LPG covers the surface of the parasite and the flagellum, forming a glycocalyx. The structure of LPG, which differs between the distinct species of *Leishmania*, is mainly constituted by repeating units of a disaccharide and a phosphate bound to the membrane by glycosylphosphatidylinositol (GPI). This molecule is more abundant in promastigotes than amastigotes [7]. Contrarily, gp63 is more frequently expressed in amastigotes than promastigotes. The absence of LPG in amastigotes emphasizes the relevance of gp63 in protozoan survival, as well as in the regulation of signaling pathways of host cells [8, 9].

Other important molecules for *Leishmania* are glycosylinositolphospholipids (GPIs), a class of glycolipids bound by GPIs and expressed 10 times more frequently than LPG. Their small size keeps them close to the parasite membrane [10, 11].

The term “secretome” was introduced for the first time in the global study of the genome of proteins secreted by *Bacillus subtilis*. The authors defined the secretome as a subset of the proteome consisting of secreted proteins and the components of the cellular machinery involved in protein secretion. They predicted all exported *B. subtilis* proteins by employing computational methods to search for signal peptides and cellular retention signals in protein sequences [12]. In *Plasmodium falciparum*, the secretome refers to all proteins exported to the host erythrocyte and mediated by an endoplasmic reticulum signal sequence, along with one export element of this parasite [13, 14]. Until very recently, there was very little information about the proteins secreted by protozoan parasites. Given the role of these proteins as virulence factors and their capacity to modulate host cells, this scant information represented an important scientific limitation.

Regarding trypanosomatids, the term secretome was introduced by Silverman in a proteomics approach used to identify a large number of extracellular proteins in a culture media conditioned by *L. donovani* [15]. Several studies on trypanosomatids have aimed to identify and characterize excreted/secreted factors due to their potential for the development of vaccines and/or new drugs [16].

Leishmania and other intracellular pathogens have developed strategies to invade and persist within the respective host cell. In some cases, the mechanisms entail the export of virulence factors to the cytosol of this same cell [15]. Almost a decade has passed since the first report of the secretion proteins by *L. donovani*. By means of a stable isotope label of amino acids in a culture called SILAC, the authors identified 151 proteins secreted by this species into the culture media. Interestingly, few of these are secreted through classical mechanisms [15]. Additionally, the bioinformatic analysis in the same study showed that none of the histidine phosphatase proteins result from the classical mechanism of secretion.

The authors found various proteins with several possible functions. For instance, some proteins take part in the vesicular transport process, essential for the survival of the parasite, and may thus be virulence factors. Among the proteins participating in signal transduction are those encoded by the gene LmjF 25.0750 of *L. major*, including a phosphatase serine threonine type phosphoprotein, a metal-dependent phosphatase (PPM) called PP2C [15]. This protein was cloned from the DNA genome of *L. major* and localized in the pocket and flagellum of the parasite through fluorescence microscopy assays and transmission electron microscopy [3].

Since the flagellum of *L. major* represents an important structure for differentiation in trypanosomatids, this location of PP2C suggests a role in a vital biological process of the parasite [3]. The same authors have shown that *L. mexicana* promastigotes and amastigotes secrete proteins with phosphatase activity into the culture medium. Such activity was more pronounced in the promastigote than amastigote secretion medium. Both media stimulated the production of various cytokines in human macrophages: tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-12p70, and IL-10 [17].

2.1. Exosomes secreted by *Leishmania*

Exosomes are organelles (30–100 nm) released by numerous mammalian cells, including reticulocytes, B cells, T cells, dendritic cells, and macrophages [18]. Bioactive exosomes are released

by cells infected with viruses and bacteria as well as some tumor cells [19, 20]. The release of exosomes also constitutes a mechanism for the secretion of proteins by *Leishmania*, and these vesicles allow for communication with the host cell [15]. Among the proteins released through exosomes, the metalloprotease 63 kDa and the elongation factor 1 alpha (EF-1 α) are known to have a substantial role in deregulating certain signaling pathways. These two molecules, contained in microvesicles, are responsible for the activation of tyrosine phosphatases (SHP-1) in the host cell [21]. A study of *L. mexicana* demonstrated a type of vesicle induced at a temperature of 37°C that did not correspond to exosomes, as evidenced by its size.

Other studies confirm a nonclassical route of secretion for *Leishmania* proteins. Two-dimensional electrophoresis displayed 270 secreted protein spots originating from *L. braziliensis*, of which 42 were identified. About 57% of these proteins presented non-classical secretion mechanisms [22].

In *L. infantum*, distinct proteins were observed in exovesicles of the parasites in the different phases of their growth. Ribosomal proteins were detected in the logarithmic phase of growth, thus indicating their crucial role in protein turnover. In the stationary phase, contrarily, there was a specific enrichment of vesicles with properties similar to apoptotic vesicles [23].

Diverse *Leishmania* species have been analyzed to explore the effect exerted by their secreted molecules on the host immune response. For example, in infection by *L. donovani* and interferon (IFN)- γ treatment, the exposure of human monocytes to the exosomes secreted by this parasite led to an alteration in the cytokine response. The resulting inhibition of IL-8 and TNF- α production combined with enhanced levels of IL-10 caused an anti-inflammatory effect. This immune suppression induced during *Leishmania* infection suggests that the secretion of exosomes by *Leishmania* likely plays a major role in the establishment of infection. Indeed, exosomes may be a mechanism of immune modulation used more generally by intracellular and extracellular pathogens [24].

3. *Leishmania* modulates proinflammatory cytokines, inflammasomes, and TLR expression

To detect *Leishmania* infections, the innate immune system utilizes different sets of germline-encoded receptors, including the TLRs found on cell membranes and in the endosome. Other receptors that have only been detected in the cytoplasm, such as the NOD-like receptor (NLR) family, also play a role in defending the host from a *Leishmania* invasion. Encompassing 34 members in all, NLRs sense pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively).

There is a subset of NLRs that assembles large multiprotein complexes known as inflammasomes. The latter trigger inflammatory caspase 1, which in turn promotes the conversion of pro-IL-1 β and pro-IL-18 into their bioactive forms. For instance, NLRP3 (the best-characterized inflammasome) consists of the NLRP3 protein, the bipartite adaptor protein ASC, and caspase 1 (in its minimum form). It has been demonstrated that this inflammasome, activated

by bacterial toxins, bacterial RNA, ATP, nigericin, uric acid, and silica crystals, is an essential component of the host immune response against bacterial and viral pathogens [25].

Leishmania parasites have developed diverse strategies to evade the immune response, especially in the form of macrophages, NK cells, and dendritic cells. Becker analyzed the interaction between *Leishmania* LPG and TLR2 receptors on human NK cells, finding that LPG purified from metacyclic and procyclic promastigotes of *L. major* stimulates these host cells. The consequent activation of NK cells leads to an upregulation of TLR2 expression, the nuclear translocation of NF- κ B, and an increased production of IFN- γ and TNF- α . Indeed, the activation of NK cells turned out to be greater with the infective metacyclic form than the non-infective procyclic form of LPG [26].

L. mexicana LPG elicits a differential production of proinflammatory cytokines, such as IL-12, TNF- α and IL-10, as well as the nuclear translocation of NF- κ B in monocytes and dendritic cells [27]. It also activates ERK and p38 MAP kinase in macrophages and induces proinflammatory cytokine production through TLR2 and TLR4 signaling [28].

After infecting the THP1 human cell line with *L. donovani*, the production of IL-10, TNF- α and IFN- γ was measured, and the expression of TLR2, TLR4, and TLR9 was determined in blood samples and the THP1 cell line. IL-10 levels were higher in controls positive to the leishmanin skin test (LST+) compared to patients with VL. TNF- α was moderately produced, exhibiting no variation between patients, controls, and THP1 cells. TLR4 and TLR9 expression was elevated in patients with VL. *L. donovani* increased the expression of TLR4 and TLR9 in patients with VL, and of TLR2 in THP1 cells, which suggests a link between TLRs and the generation of a mixed cytokine response [29].

Interestingly, other authors analyzed the expression of some components related to the inflammasome pathway in murine macrophages infected with *L. major*. At 6 and 18 h post infection, they evaluated the mRNA expression levels in control and infected macrophages of two NLRs (NLRP3 and NAIP5), the inflammasome adaptor molecule ASC, proinflammatory caspase-1, and proinflammatory cytokines IL-1 β and IL-18. The components related to the inflammasome pathway (NLRP3, ASC, IPAF, IL-1 β , and IL-18) were upregulated in murine macrophages infected with *L. major*. The activity of caspase-1 was more pronounced in infected than noninfected macrophages. Infected (versus uninfected) macrophages also showed significantly greater caspase-1 activity in harvested cells and a significantly higher concentration of IL-1 β in the supernatant of the cultured media [30].

It has been documented, based on *in vitro* (in macrophages) and *in vivo* studies, that a *Leishmania* infection activates the NLRP3 inflammasome and that the latter is key to the inhibition of parasite replication. For example, the capacity of inflammasome-deficient mice to resist infection with *L. amazonensis*, *L. braziliensis*, *L. infantum*, and *L. chagasi* was favored by IL-1 β production resulting from inflammasome activation. The mechanism involved in such activation was the increase in the level of nitric oxide (NO), which in turn was mediated by the elevated availability of nitric oxide synthase NOS2 resulting from signaling through the IL-1 receptor and MyD88. Lima-Junior et al. previously showed that the NLR3 inflammasome is vital for the host response to *L. amazonensis* infection, having proven to restrict

parasite replication in both isolated macrophages and *in vivo*. As can be appreciated, IL-1 β production is involved in the host resistance to infection. The signaling that triggers the production of this cytokine takes place through the IL-1 receptor and MyD88, contributing to elevated levels of NOS2. An increase in the latter enzyme leads to a greater generation of NO, a major host defense mechanism against *Leishmania* spp. [31].

In the case of *L. major*, secreted antigens suppressed the proliferation of BALB/c mice lymphocytes *in vitro*. After semi-purifying these secreted antigens, they were found to suppress 60% of lymphocyte proliferation and prevent the stimulation of lymphocytes. The fractions obtained decreased the production of IFN- γ and increased the level of IL-4 in lymphocytes, whereas they downregulated the formation of NO by activated macrophages. Hence, proteins secreted by *L. major* may function as immunosuppressive factors that downregulate the immune system [32]. On the other hand, the immunomodulatory effect of proteins excreted/secreted by *L. infantum* was described in the context of differentiation and maturation of human dendritic cells [33].

Regarding *L. donovani*, an immunomodulatory role has also been established for leishmanial excretory-secretory antigens (LESAs) released by promastigotes to the culture medium. The separation of fractions from LESAs revealed proteins of different molecular weights. Both fractions were highly immunogenic, as they significantly enhanced the activity of NADPH oxidase and SOD, as well as the production of NO, TNF- α , IFN- γ , and IL-12 in stimulated RAW 264.7 macrophages. These results strongly suggest the potential role of LESAs in the modulation of macrophage effector functions and Th1 responses, which could possibly be used in the development of a potent vaccine for visceral leishmaniasis [34]. Similarly, Kumar reported a potential immunostimulatory effect of soluble exogenous antigens of *L. donovani*, which may be instrumental in developing a subunit vaccine against VL [35].

4. *Leishmania* modulates L-arginine metabolism via NOS2 and arginase-1

Among other strategies developed by *Leishmania* parasites to avoid elimination by the host immune response, regulation of L-arginine metabolism via NOS2 and arginase-1 (ARG-1) enzymes has emerged as a crucial mechanism for parasite survival. Macrophages, the main host cells that battle *Leishmania*, can be instructed to kill or host intracellular amastigote forms of this parasite, depending on their ability to express NOS2 or ARG-1. The expression of these enzymes, which share L-arginine as a substrate, is regulated in macrophages by their perception of the environmental balance of cytokines.

Proinflammatory cytokines (e.g., TNF- α and IFN- γ) induce the classical activation of macrophages, upregulating NOS2 expression. This enzyme catalyzes the conversion of L-arginine into L-citrulline and NO, the latter molecule being considered the most potent leishmanicidal agent for the elimination of intracellular *Leishmania* parasites [36]. On the other hand, anti-inflammatory cytokines (e.g., IL-4, IL-10 and IL-13) elicit an alternative activation of macrophages that upregulates ARG-1 [37], which in turn catalyzes the conversion of L-arginine into

urea and L-ornithine. The latter is a basic source for the synthesis of polyamines, essential nutrients for the growth and surveillance of *Leishmania* [38, 39]. Hence, whether L-arginine metabolism takes place through ARG-1 or NOS2 is decisive for the life or death of *Leishmania* during infection [40].

To establish infection and avoid host surveillance, *Leishmania* parasites have developed different strategies to hijack L-arginine metabolism in order to promote the production of polyamines rather than NO. During its development inside the vector, for instance, the parasite generates a mucin-rich gel that sand flies deliver into the host skin when transmitting *Leishmania* promastigotes [41]. This gel, called promastigote secretory gel, is known to modulate L-arginine metabolism in macrophages [42]. Accordingly, promastigote secretory gel stimulates the recruitment of macrophages and promotes their alternative activation, causing an increased expression of arginase-1 along with its greater capacity to metabolize L-arginine to afford polyamines, which in turn enhance the growth of *Leishmania* [42].

The parasite-induced upregulation of arginase-1 can affect the production of NO through substrate (L-arginine) competition [41]. Additionally, the generation of some polyamines resulting from L-arginine metabolism via arginase-1 (e.g., spermine, spermidine and putrescine) inhibit NOS2 function [38, 39, 43]. The modulation of L-arginine metabolism is relevant not only during the onset of the infection but throughout the course the disease. The success of host immunity or the pathology of leishmaniasis depends mainly on the balance of the immune response. Since the formation of either NOS2 or arginase leads to the inhibition of the other, these two metabolic states are competitive and tightly regulated [44], determining the levels of NO and therefore the outcome of *Leishmania* infection (survival versus elimination) in the host.

Besides the production of polyamines and the resulting enhancement of *Leishmania* intracellular growth in alternatively activated macrophages, recently findings have shown that *Leishmania*-induced L-arginine metabolism via ARG-1 polarization is advantageous to the parasite in yet another way. A substantial accumulation of alternatively activated macrophages causes an elevated demand, consumption, and depletion of L-arginine in the microenvironment [41]. Since T lymphocytes are very sensitive to L-arginine starvation, a greater consumption of this amino acid via ARG-1 limits its availability to T cells, which in turn notably impairs the development and function of these cells that are required for the control of a *Leishmania* infection [45, 46].

5. Inhibition of apoptosis

Leishmania is an obligate intracellular parasite that invades a variety of host cells, but it is in dendritic cells and macrophages where it can survive and replicate inside the phagosome. The condition of being obligate intracellular parasites presupposes the utilization by *Leishmania* of mechanisms to manipulate host cells in order to evade the immune response and survive inside cells. Along the evolutionary history of this parasite, diverse survival strategies have been developed.

Although the inhibition of the phagosome-lysosome fusion comprises one such strategy, one of the most intriguing is the inhibition of apoptosis. The latter process is a type of programmed cell death characterized by a very orderly and immunologically silent dismantling of a cell [47, 48]. The activation or inhibition of several signaling pathways is required for apoptosis to occur [49–53]. Whereas the initiation of apoptosis involves gene activation and transduction pathways, the executioner phase requires the activation of the cellular machinery necessary for the dismantling of the cell.

Apoptosis is a crucial defense mechanism against intracellular pathogens [54]. However, many pathogenic microorganisms such as virus [55], bacteria [56], and protozoan parasites [57, 58] have developed mechanisms to persist within host cells without inducing apoptosis. It has been widely documented that *Leishmania* inhibits apoptosis of different cells such as macrophages [59–61], monocytes [64] and neutrophils [66].

Recently, it has been demonstrated that *L. mexicana* promastigotes and amastigotes also inhibit apoptosis in dendritic cells [62, 63]. In some of these studies, monocytes, dendritic cells, and macrophages were grown under apoptogenic conditions and infected with different species of *Leishmania*, resulting in the inhibition of normal apoptosis.

In particular, infection with *L. donovani* or a stimulus with its LPG inhibits apoptosis in macrophages. Cellular activation caused by infection increases the production of TNF- α , TGF- β , IL-6, and GM-CSF, while decreasing the secretion of M-CSF and IL-1 β [60]. Additionally, *L. major* delayed apoptosis by inhibiting the release of mitochondrial cytochrome C in infected macrophages grown in the presence of staurosporine [59]. Studies performed on other cell lines report a similar outcome, such as the inhibition of actinomycin D-induced apoptosis in the monocyte cell line U937 infected with *L. infantum* [64]. In macrophages from the cell line RAW 264.7 infected with *L. major*, apoptosis diminished even in the presence of cycloheximide [65]. Exposing neutrophils to *L. major* led to reduced caspase-3 activity, thus inhibiting spontaneous apoptosis [66]. Moreover, amastigotes and promastigotes of *L. mexicana* inhibited camptothecin-induced apoptosis in monocyte-derived dendritic cells [62, 63]. In the majority of reports, the antiapoptotic effect has been associated with a significant decline in caspase-3 activity in cells.

5.1. Signaling pathways involved in *Leishmania*-induced inhibition of host cell apoptosis

Although *Leishmania* infection is known to inhibit apoptosis in several cells, the mechanism(s) through which this process takes place in infected cells is not fully clear. One signaling pathway involved in apoptosis is that of MAPKs, a family of serine/threonine kinases. Four major pathways have been identified in mammalian cells for signaling by MAPKs: extracellular signal-related kinases (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38 (α , β , γ , δ), and ERK5 [67–70]. MAPKs respond to a wide variety of stimuli, such as proinflammatory cytokines, environmental stress, DNA damage, and growth factors [50, 71]. The p38 pathway is associated with cytokine production, inflammation, cell growth and differentiation, and cell death. JNK participates in the control of cell death and is encoded by three genes: JNK1/SAPK γ , JNK2/SAPK α , and JNK3/SAPK β . Contrarily, the signaling pathway of PI3K/AKT has an antiapoptotic role through the phosphorylation of PI3K/AKT that conducts to the downstream activation of multiple signaling pathways related to growth, development, and

cellular survival processes [72]. MAPKs [73, 74] and PI3K [75] are activated during *Leishmania* infections and participate in the apoptosis or survival cells [76–78].

Diverse signaling pathways have been implicated in the inhibition of apoptosis by *Leishmania*, such as NF- κ B, PI3K, and p38 MAPK PI3K. However, only the inhibition of PI3K resulted in the abrogation of the antiapoptotic phenotype, whose activation confers apoptosis inhibition in infected macrophages [79] and dendritic cells [5]. ERK1/2 is activated in neutrophils infected with *L. major* and modulates multiple apoptotic pathways [80]. Contrarily, p38 and JNK are deactivated to prevent apoptosis [4, 5]. Survival is mediated by signaling pathways (e.g., PI3K/Akt), as well as by the expression of antiapoptotic proteins of the Bcl-2 family (e.g., Bcl-2, Bcl-xL, MCL-1, and A1). We have demonstrated that *L. mexicana* promastigotes and amastigotes also inhibit apoptosis in dendritic cells [62, 63] through the downregulation of proapoptotic pathways (e.g., MAPK p38 and JNK), as well as the activation of antiapoptotic routes such as PI3K/Akt [4, 5].

6. Conclusion

Leishmania parasites have developed diverse strategies to evade the immune response: elicitation of a differential production of proinflammatory cytokines, upregulation of TLR2 and TLR4 expression, the nuclear translocation of NF- κ B, and modulation of inflammasome. These parasites have a dual relation with host cells. Whereas host cells provide them with nutrients and a place to survive and replicate, these same cells exhibit an immune response aimed at the destruction of the parasites. Hence, the latter must display a wide array of strategies to overcome host cells defense mechanisms. One of the most successful strategies utilized by *Leishmania* inside host cells is the inhibition of apoptosis.

Apoptosis is a type of programmed cell death involving a precisely orchestrated series of steps that culminate in the orderly dismantling of the cell. This process encompasses the activation and silencing of a wide variety of signaling pathways, among which a leading role is played by MAPK, PI3K/Akt and proapoptotic/antiapoptotic proteins of the Bcl-2 family. *Leishmania* has the capacity to inhibit apoptosis of different cells, especially macrophages, monocytes, neutrophils, and dendritic cells. Although the precise mechanisms have not been fully elucidated, it is now clear that MAPK p38, JNK, ERK 1/2, and PI3K/Akt participate in the inhibition of apoptosis in macrophages, neutrophils, and dendritic cells. The inhibition of apoptosis is a key strategy for the survival and replication of *Leishmania* in host cells and may have implications for its pathogenesis because of favoring the invasion of the host and the persistence of the parasite in host cells. Further research is needed on the mechanisms of activation and regulation of the inflammasome pathway to provide insights into the pathophysiology of chronic diseases and reveal new therapeutic targets.

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Author details

M. Magdalena Aguirre-García*, Alma R. Escalona-Montaña, Arturo A. Wilkins-Rodríguez and Laila Gutiérrez-Kobeh

*Address all correspondence to: maguirre@unam.mx

División de Investigación, Facultad de Medicina, Instituto Nacional de Cardiología Ignacio Chávez, Unidad Periférica de Investigación UNAM-INC, Ciudad de México, Mexico

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