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Review

Manipulation of Macrophages: Emerging Mechanisms of Leishmaniasis

Devki Nandan[1,*,†], Harsimran Kaur Brar[1,†], Neil Reiner[1]

[1]Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, BC V6H 3Z6, Canada
*Correspondence: dnandan@mail.ubc.ca (Devki Nandan)
[†]These authors contributed equally.

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Abstract

As professional phagocytes, macrophages represent the first line of defence against invading microbial pathogens. Various cellular processes such as programmed cell death, autophagy and RNA interference (RNAi) of macrophages are involved directly in elimination or assist in elimination of invading pathogens. However, parasites, such as *Leishmania*, have evolved diverse strategies to interfere with macrophage cell functions, favouring their survival, growth and replication inside hostile and restrictive environments of macrophages. Therefore, identification and detailed characterization of macrophage-pathogen interactions is the key to understanding how pathogens subvert macrophage functions to support their infection and disease process. In recent years, great progress has been achieved in understanding how *Leishmania* affects with critical host macrophage functions. Based on latest progress and accumulating knowledge, this review exclusively focuses on macrophage-*Leishmania* interaction, providing an overview of macrophage cellular processes such as programmed cell death, autophagy and RNAi during *Leishmania* infection. Despite extensive progress, many questions remain and require further investigation.

Keywords: macrophages; *Leishmania*; host-pathogen interactions; effector mechanisms

1. Introduction

1.1 Macrophages

Macrophages, commonly abbreviated as "Mϕ", are essential cells of innate or non-specific immunity. They are among the first responders of the immune system that engulf and destroy the pathogen in a process called "phagocytosis". The tissue macrophages originate from either embryonic yolk sac and fetal liver or bone marrow-derived hematopoietic stem cells [1] (Fig. 1). Therefore, in the tissues, the macrophage population is usually a mix of embryonic and bone marrow-derived macrophages. However, in adult humans, the majority of the macrophages found in the tissues are recruited from circulating monocytes [2,3].

The introduction to macrophages in this review is mainly in the context of immunity and infection.

1.1.1 Functional Classification of Macrophages

Depending on various environmental cues, resting macrophages (M0) can adopt distinct activation states, M1 (classically activated) and M2 (alternatively activated). M0 macrophages, when activated by proinflammatory cytokines like interferon(IFN)γ and tumor necrosis factor (TNF)α or proinflammatory stimuli like lipopolysaccharide (LPS), can differentiate into M1 macrophages [4,5]. They can also be differentiated into M1 macrophages by growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) [6]. M1 macrophages release proinflammatory cytokines like interleukin (IL)-1β, IL-6, TNFα [7,8], and IFNγ [9] in response to phagocytosed microbes. Additionally, they produce nitric oxide (NO) and reactive oxygen species (ROS), which are potent killers of intracellular pathogens [4]. CD16, CD32, CD64, CD80/86 and Human Leukocyte Antigen-DR isotype (HLA-DR) are some of the cell surface molecules or markers associated with M1 phenotype [5,10,11]. On the other hand, M2 macrophages are anti-inflammatory in nature and are induced by immunosuppressive cytokines and growth factors like IL-4, IL-10, IL-13, transforming growth factor-beta (TGF)-β and macrophage colony-stimulating factor (M-CSF) [6,12]. M2 macrophages perform tissue repair and remodelling and are integral to immune response suppression. These cells produce anti-inflammatory cytokines like TGF-β, IL-10 and IL-6 [12] and are associated with molecular markers CD200R [13], CD206, CD163 and Dectin-1 [12]. M2 macrophages are further classified into three subtypes: m2a, m2b and m2c [14]. However, this classification oversimplifies the complex spectrum of macrophage activation statuses observed *in vivo*, where macrophages exhibit a vast overlap between M1 and M2 extremes, as highlighted in recent studies [14].

1.1.2 Macrophage Effector Functions

Macrophages, strategically placed in almost all body tissues, can respond to injury, pathogens and antibody opsonized pathogens, by performing either effector or sentinel functions. As effector cells of innate immunity,

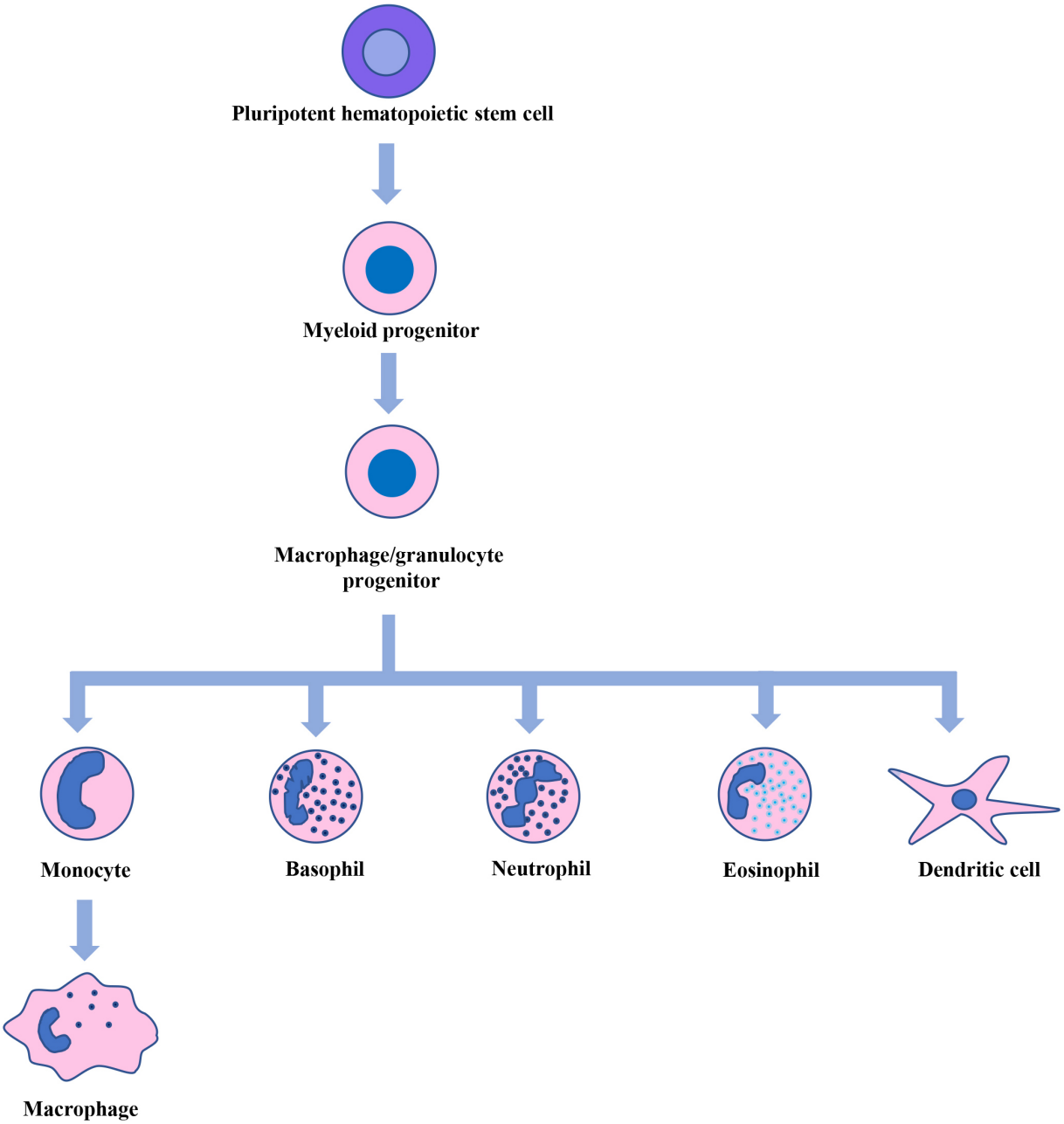

Fig. 1. Hematopoiesis. Pluripotent hematopoietic stem cells in the bone marrow generate myeloid, lymphoid, and erythroid progenitor cells. From myeloid progenitor cells, macrophage/granulocyte progenitor cells are formed, which give rise to monocytes, basophils, neutrophils, eosinophils, and dendritic cells. The circulating monocytes then move into different tissues and differentiate into macrophages.

macrophages eliminate microbial pathogens by phagocytosis, secretion of proinflammatory cytokines, antimicrobial mediators and initiation of apoptosis to limit intracellular pathogen load [15,16]. Pathogen elimination begins with pathogen recognition—mainly involving pathogen-associated molecular patterns (PAMPs)—which are recognized by pattern recognition receptors (PRRs) found on the surface of innate immune cells. Some PRRs, like Toll-like receptors (TLRs), play a central role in host cell recognition of invading microbial pathogens. Other receptors, like complement receptors can also detect foreign pathogens. Interaction between host and pathogens triggers phagocytosis, starting with macrophage membrane protrusions surrounding pathogens and resulting in phagosomes, which ultimately fuse with lysosomes to transport pathogen into phagolysosomes for elimination. In the phagolysosomes, nicotinamide adenine dinucleotide phosphate (NADH)-oxidase produces ROS, and nitric oxide synthase produces NO. These reactive molecules play an important role in effectively killing phagocytosed pathogens [17–19]. Active

NADH oxidase increases oxygen consumption, referred to as an "Oxidative burst" leading to the production of H_2O_2 by superoxide dismutase [17,20].

In addition, macrophages can act as sentinel cells to commence innate or adaptive immunity activation by secreting chemokines and proinflammatory or anti-inflammatory factors or by presenting antigens on their surface to recruit adaptive immunity cells [16]. Moreover, they are also involved in the clearance of damaged/dead cells and tumor cells, thus promoting homeostasis.

1.1.3 Other Macrophage Functions Related to Innate Immunity

In addition to phagocytosis, there are other mechanisms through which pathogen neutralization in macrophages can occur. Macrophage autophagy—a lysosomal degradation pathway for intracellular components—plays a key role in innate immunity, especially in the context of infection and inflammation [21,22]. In most situations, activation of autophagy has antimicrobial effects by directly targeting pathogens to lysosomal degradation. The breakthrough in deep sequencing and other technologies have revealed the key role played by non-coding RNAs in shaping the immune response. In recent years, ample evidence has accumulated that microRNAs (miRNAs) are associated with innate immune processes through post-transcriptional regulation of proteins either by degradation of mRNAs or by translational interference [23–26].

This review provides an overview of innate immunity-related macrophage functions, such as apoptosis, autophagy and RNA interference (RNAi), in the context of intracellular infections using *Leishmania* as a paradigm. The following sections highlight recent advances in understanding how *Leishmania* employs various complex strategies to regulate the numerous functions of host macrophages to survive the defence mechanisms/innate immunity rather than a detailed overview of all the literature in the field.

2. The Macrophage Paradox: Regulation of Macrophage Biology by Intracellular Pathogens

At the site of infection, macrophages get recruited, leading to phagocytosis of invading pathogens. Accordingly, macrophages have microbicidal arsenals to directly kill or coordinate the eliminating pathways. Paradoxically, many intracellular pathogens preferentially reside and replicate inside the hostile environment of macrophages. These pathogens have evolved to exploit the fundamental biology of macrophages to grow and proliferate in unique cellular and metabolic environment. An excellent example is *Leishmania*, an obligate intracellular protozoan parasite. Macrophages act as the primary host cells for *Leishmania*, where they reside, grow and proliferate. Over the years, studies related to *Leishmania*-macrophage interactions have provided critical information on host defence mechanisms against intracellular pathogens and how *Leishmania* has evolved to regulate numerous aspects of macrophage biology, ensuring its survival inside infected macrophages.

3. Leishmaniasis

Various human pathogens are transmitted by blood-sucking arthropods, collectively called vector-borne diseases. Leishmaniasis is a spectrum of diseases caused by around 20 *Leishmania* species transmitted worldwide by infected female sandflies [27]. Out of almost 1000 sandfly species described thus far, only 10% of them are demonstrated or suspected vectors of *Leishmania* parasites [28]. Disproportionally affecting humans in resource-poor countries in the tropical and subtropical regions, leishmaniasis is considered a Neglected Tropical Disease (NTD) [29]. According to recent estimates, around 350 million people in 98 countries are affected by leishmaniasis [30–32]. Leishmaniasis most commonly occurs in tropical, subtropical and Mediterranean regions of the world [33]. However, there is a real risk of leishmaniasis spreading to non-endemic areas of the world facilitated by migration, globalization, changes in weather patterns, and war/conflicts in the endemic regions. Leishmaniasis is on the rise in endemic areas due to a lack of approved prophylactic human vaccines, drug resistance and a lack of interest among big pharmaceutical industries to develop new drugs against leishmaniasis [34–36].

Digenetic Life Cycle of Leishmania Parasite

Like many protozoan parasites, *Leishmania* has a complex digenetic life cycle that involves both vertebrate and invertebrate hosts and two morphologically distinct forms: the extracellular motile promastigotes (sandfly vector) and non-motile intracellular amastigotes (mammalian hosts). The promastigote form is found in the midgut of sandflies whereas the amastigote form occurs intracellularly, primarily in phagolysosomes of macrophages. The bite of an infected sandfly transmits *Leishmania* by injecting infective stage promastigotes into the dermis of a mammalian host during a blood meal. Infective promastigotes are ingested by host macrophages and transformed into non-motile amastigotes, which then multiply, leading to clinical manifestation. Sandflies become infected when they take a blood meal from an infected mammalian host. In the sandfly gut, amastigotes are transformed into promastigotes, multiply and move to proboscis [37]. Most *Leishmania* species that are pathogenic to humans have zoonotic transmission involving dogs, jackals, and rodents, serving as reservoir hosts for *Leishmania* pathogens. Leishmaniasis in nature is maintained by the complex interactions among *Leishmania* parasites, sandfly vectors, mammalian hosts (including humans) and zoonotic reservoirs. Interactions between *Leishmania* parasites and sandflies have re-

cently been reviewed [38,39] and won't be reviewed here. The current review is mainly restricted to macrophage-*Leishmania* interactions.

4. Early Interaction of *Leishmania* with Mammalian Host at the Site of Sandfly Bite

It is becoming increasingly clear that *Leishmania* tropism/virulence is not solely associated with disease pathogenesis. Complex interactions between *Leishmania* and its host determine infection and disease progression. As discussed above, leishmaniasis is initiated by the entry of promastigotes into mammalian hosts' dermis during an infected sandfly's bite. Promastigotes are inoculated into the pool of blood created by the bite of a sandfly, where they interact with leukocytes. Many deposited *Leishmania* are quickly ingested by neutrophils, recruited due to substantial tissue damage and inflammation [40,41]. It is also gaining traction that sandfly microbiota is critical to leishmaniasis development and transmission by host IL-1β which promotes neutrophil recruitment [40]. *Leishmania* then exploits the acute neutrophilic response to promote infection [41]. Neutrophils are short-lived cells and can either kill parasites or could serve as intermediate host cells for *Leishmania*. The surviving *Leishmania* uses these infected apoptotic neutrophils to gain "silent entry into macrophages". Thus, neutrophils serve as a vector for parasite entry into macrophages. This supports the "Trojan Horse" style infection model [42,43]. In a more recent study using confocal Intravital Microscopy (IVM), it was clearly shown that infected neutrophils transfer parasites to dermal macrophages. This cell-to-cell contact involves Tyro3, Axl and Mer (TAM) receptor tyrosine kinases and sustains the anti-inflammatory properties of dermal macrophages [44]. This early neutrophil and macrophage interaction plays a critical role in the outcome of infection. Taken together, it strongly suggests that dermis macrophages are the predominant phagocytes that take up the parasites within the early hours of infection post-bite. Macrophages destroy the internalized parasites or become the final host for parasite replication. Here, it is worth mentioning that dendritic cells in the skin also play an essential role in regulating immune response against *Leishmania* [45–47]. Some studies have shown that infected dendritic cells likely migrate to lymph nodes for antigen presentation [48,49].

In addition to *Leishmania* interaction with macrophages, neutrophils and dendritic cells, other cells such as fibroblasts [50] and B-1 cells [51] can also be infected with *Leishmania* spp. Interestingly, B-1 cells can be differentiated to phagocytic cells and have been shown to phagocytose *L. major* promastigotes [51]. Further, it was shown that internalized parasites transformed into amastigotes and were able to secrete pro-inflammatory and anti-inflammatory cytokines after stimulation [51]. These cells primarily used mannose receptors for phagocytosis of *Leishmania*. Despite the seemingly important role played by B-1 cells, few studies attempted to evaluate role of B-1 cells during *Leishmania* infection. Recently, these studies have been reviewed in the context of the role played by B-1 in disease progression during infection with *Leishmania* spp [52].

5. Regulation of Host Macrophage Apoptosis by *Leishmania*

5.1 Apoptosis

It is a conserved physiological process of non-lytic, programmed cell death which occurs in all multicellular organisms. It is mainly characterized by cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation, and plasma membrane blebbing [53]. Apoptotic cells also showcase cell surface markers like phosphatidylserine (PS), usually present on the inner surface of the cell's plasma membrane. PS is recognized by the phagocytic cells, like macrophages, as an "eat me" signal [54,55]. Apoptosis is considered 'immunologically silent', whereas other forms of cell death, such as pyroptosis, necroptosis, and ferroptosis, are considered relatively 'violent' types of cell death [56]. Apoptosis is mediated by the caspase enzymes, which have proteolytic activities to cleave proteins at aspartic acid. So far, twelve caspases have been described in humans [57]. Based on the type of stimulants, apoptosis can be carried out via—the intrinsic or mitochondrial pathway, extrinsic or death receptor pathway and the caspase (aspartate-specific peptidases, dependent on cysteine)-independent pathway [58]. Extrinsic apoptosis is triggered by the binding of a ligand such as Fas ligand (FASL), TNF, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), and TNF-like weak inducer of apoptosis (TWEAK) to members of the TNF receptor superfamily, leading to receptor oligomerization and the recruitment of adaptor proteins containing death domains such as tumor necrosis factor receptor 1 (TNFR1)-associated death domain protein (TRADD) and Fas-associating protein with death domain (FADD). These complexes activate caspase-8, further activating caspase-3 and caspase-7, resulting in cell death. The intrinsic pathway is induced by cellular stress, which can originate from DNA damage, oxidative stress, radiation, hypoxia, and nutrient deprivation. Regardless of how this pathway is induced, it always leads to mitochondrial outer membrane permeabilization (MOMP) and is intricately regulated by proteins that belong to the B-cell lymphoma 2 (BCL-2) family. The BCL-2 family of proteins contains both pro-apoptotic and anti-apoptotic members. Ultimately, the intrinsic pathway leads to the membrane potential dissipation, release of cytochrome c and other toxic proteins into the cytoplasm and inhibition of the respiratory chain. The cytochrome c released from the mitochondria also interacts with apoptotic peptidase activating factor 1 (APAF1) to form apoptosomes, activating the initiator caspase-9. In the caspase-independent pathway, release of

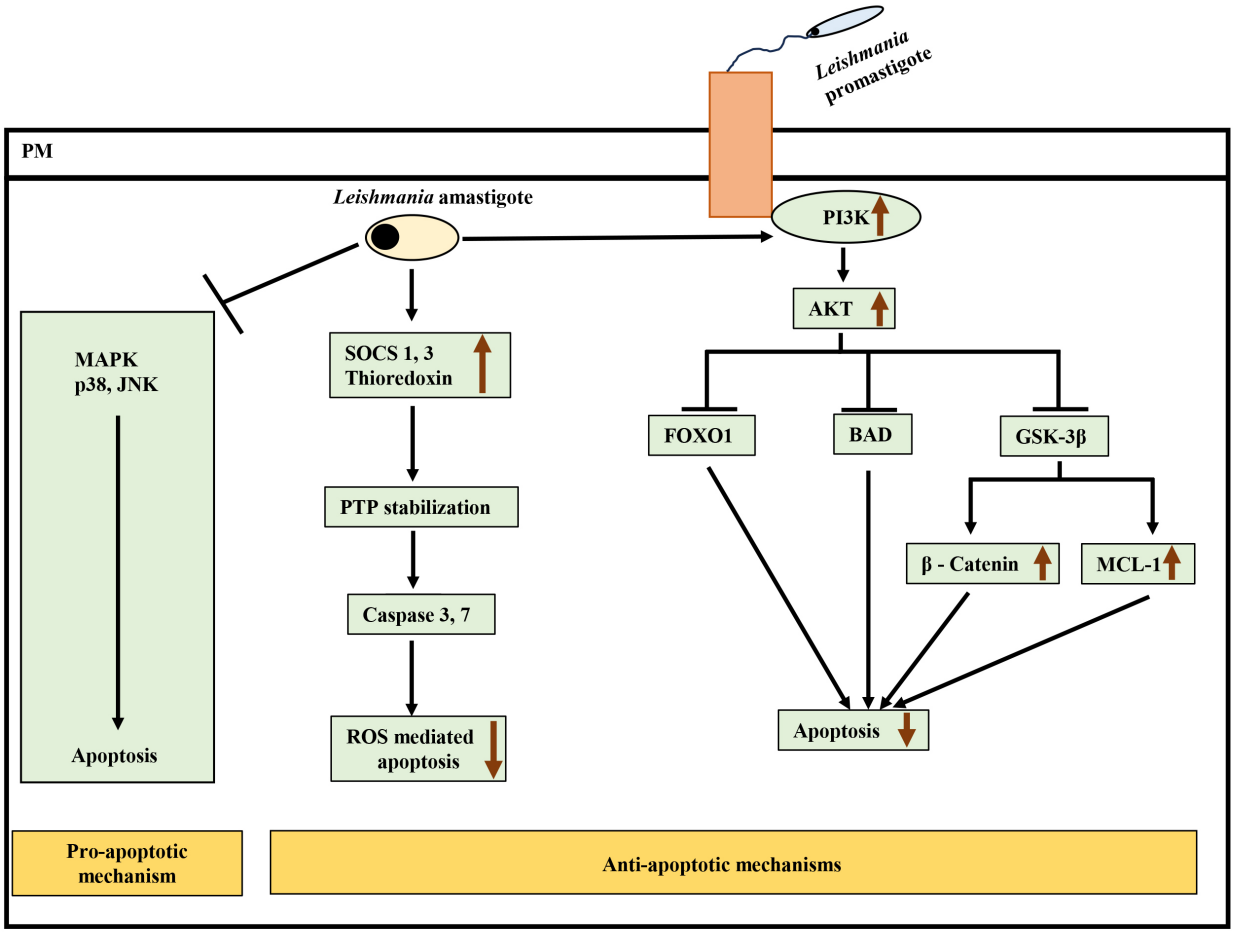

Fig. 2. Interplay of pro-apoptotic and anti-apoptotic pathways leading to inhibition of apoptosis after *Leishmania* infection. *Leishmania* attenuates pro-apoptotic MAPK p38 and JNK signaling and simultaneously activates anti-apoptotic PI3K-Akt pathway to inhibit apoptosis. Active Akt negatively regulates forkhead box protein O (FOXO1), Bcl-2 associated agonist of cell death (BAD), and glycogen synthase kinase-3β (GSK-3β) to inhibit apoptosis. Inhibition of GSK-3β upregulates β-Catenin and MCL-1. *Leishmania* infection also inhibits ROS-mediated apoptosis through suppressors of cytokine signaling (SOCS) pathway. Amastigotes upregulate SOCS 1 and 3 and thioredoxin, which stabilizes protein tyrosine phosphatases (PTP). PTP then downregulates caspase 3 and 7, which leads to decrease in apoptosis. PM, Plasma membrane; MAPK, Mitogen-activated protein kinase; ROS, Reactive Oxygen Species; PI3K, Phosphoinositide 3-kinase; AKT, Ak strain transforming; MCL-1, Myeloid Cell Leukemia 1; JNK, Jun N-terminal kinase.

toxic proteins from the intermembrane space of the mitochondria to the cytoplasm due to mitochondrial damage can induce apoptosis and does not include caspases [58–60].

5.1.1 Regulation of Apoptosis

Apoptosis plays a crucial role in many biological processes, including cellular defence, and cellular and tissue remodelling, which are essential for the homeostatic balance of an organism. Due to this, apoptosis is tightly regulated by various proteins and furthered by an intricate network of signalling pathways. Depending on the situation, they can activate or deactivate apoptosis [61]. The PI3K/AKT signalling pathway is one of the key pathway which negatively regulates apoptosis, mainly by activating anti-apoptotic genes and deactivating pro-apoptotic genes [61]. Active AKT can inhibit apoptosis by phosphorylating BCL-2 associated agonist of cell death (BAD) protein, a member of the BCL-2 family, and promoting its degradation. It can also inhibit glycogen synthase kinase-3β (GSK-3β) or phosphorylate the transcription factor, forkhead box protein O (FOXO), which is then degraded by the proteasome pathway. Inhibition of GSK-3β in turn upregulates MCL-1, an anti-apoptotic protein, and β-Catenin, which upregulates expression of anti-apoptotic proteins, both leading to downregulation of apoptosis. FOXO is involved in the synthesis of pro-apoptotic genes [61]. Mitogen-activated protein kinases [includes p38, Extracellular Signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs)] signalling pathways also regulate apoptosis. For instance, induction of JNK can promote the ac-

tivation of pro-apoptotic genes in the nucleus. It is also involved in the release of cytochrome c from mitochondria into the cytoplasm, activating the intrinsic pathway of apoptosis [61,62]. Additionally, JNK phosphorylates BCL-2, BCL-XL and Bcl-2 Interacting Mediator of cell death (BIM) favouring apoptosis [63]. Similarly, p38 can phosphorylate proteins, including pro-apoptotic members of the BCL-2 family, like BIM, Bcl-2–associated X protein (BAX), BAD and BIM extra-long (BIM(EL)), leading to apoptosis activation [61,64–67]. To favor apoptosis, p38 also inhibits anti-apoptotic ERK and Akt pathways [68]. Contrary to JNKs and p38, ERK mainly favours cell survival but under certain circumstances, it can favor apoptosis [69]. Thioredoxin and members of suppressors of cytokine signalling (SOCS) family are also known negative regulators of apoptosis, ROS-mediated apoptosis in particular [70], impacting outcome of infection.

5.1.2 Manipulation of Host Apoptosis by Leishmania

Apoptosis has been shown to play a key role in the development, regulation and function of the immune system. Induction of cell death during infection has been demonstrated in several bacterial, viral and parasitic infections that significantly impact pathogenesis [71–73]. The death of an infected cell often results in the death of an infecting agent, thus promoting pathogen clearance. Therefore, the initiation of cell suicide in infected cells is promoted to limit infection. In addition, phagocytosis of a dying infected macrophage by dendritic cells results in enhanced antigen presentation to T cells for adaptive immunity [74–76]. As already mentioned, apoptosis is an important host defence mechanism against intracellular pathogens. However, some pathogens employ a range of strategies to inhibit apoptosis and survive and reproduce [77–81]. *Leishmania* is an excellent example of an intracellular pathogen that promotes its survival inside host cells by employing numerous smart strategies to counteract this host cell defence mechanism. The following section mainly overviews the manipulation of host macrophage apoptosis by *Leishmania*.

Several protozoan parasites are known to regulate apoptosis for their survival [82]. Moore and Matheshewski in 1994 [83] were the first to demonstrate that *L. donovani* or lipophosphoglycan (LPG), the major surface molecule of *L. donovani* promastigotes, can inhibit apoptosis in bone marrow-derived macrophages (BMDMs) activated by deprivation of M-CSF. They could also downregulate apoptosis when treated with supernatants of the *L. donovani*-infected macrophages, indicating the involvement of soluble factors. The cytokine profiling of the infected cells showed increased gene expression for granulocyte-macrophage CSF (GM-CSF), TNFα, TGF-β, and IL-6, but not M-CSF or IL-1β. BMDMs, when induced using recombinant TNFα and GM-CSF, showed inhibition of apoptosis. Infection with *Leishmania major* in the absence of M-CSF also showed inhibition in apoptosis and delays apoptosis in the presence of staurosporin. This delay was attributed to a decrease in MOMP, downregulation of mitochondrial cytochrome c release, and inhibition of caspase-3 activation [84].Infection of *L. major* in RAW 264.7 cells in the presence of cycloheximide also downregulated the frequency of apoptosis [85]. Additionally, the infection of U937 cells with *L. infantum* repressed actinomycin D-mediated apoptosis [86].

It has been shown that *L. donovani* evade the mitogen-activated protein kinase (MAPK) pathway in naïve macrophages by not triggering phosphorylation of p38, JNK and ERK1/2, ensuring parasite survival [87]. Later, in another study, it was also shown that upon inhibiting p38 MAPK by pharmacological inhibitor SB203580, the number of infected macrophages and parasite survival in BMDMs increased [88]. *Leishmania* can also prevent programmed cell death by upregulating anti-apoptotic pathways like the PI3K/AKT pathway. For example, *L. donovani* induced AKT phosphorylation, inhibiting FOXO-1 and, therefore, the transcription of pro-apoptotic genes. Activated AKT also inhibited GSK-3β, which led to the upregulation of β-Catenin, which in turn upregulated the transcription of anti-apoptotic genes (Fig. 2). Furthermore, attenuation of the AKT pathway resulted in an increase in apoptosis in *L. donovani*-infected murine macrophages. In parallel, the inhibition of AKT also reduced IL-10 production and increased IL-12 production, correlating with reduced parasite survival [89]. In another study, activation of the PI3K/AKT pathway in BMDMs infected with *L. major* or *Leishmania pifanoi* reduced the pro-apoptotic action of BAD protein, thereby reducing apoptosis [90].

Leishmania can also interact with other BCL-2 family members to promote its survival. For example, *L. donovani* upregulated MCL-1 (anti-apoptotic protein of the BCL-2 family) in murine macrophages to prevent BAK-mediated mitochondrial apoptosis [91]. The activation of BCL-2 in *L.donovani*-infected macrophages led to NO production inhibition, enhancing parasite survival. In monocytes derived from the blood of visceral leishmaniasis (VL) patients, BCL-2 expression was observed to be increased significantly with reduced nitrites [92].

Interestingly, *L. donovani* infection in RAW264.7 cells has been shown to take advantage of the SOCS pathway to resist ROS-mediated apoptosis. Infection induced SOCS proteins 1 and 3 and thioredoxin, downregulating downstream caspases 3 and 7, leading to inhibition of ROS-mediated apoptosis [70] (Fig. 2). This study also reported downregulation of p38 activation upon infection.

In addition to the exploitation of host factors to inhibit apoptosis, proteins from *Leishmania* parasites can also inhibit host macrophage apoptosis. For instance, a structural ortholog of macrophage inhibiting factor (MIF) produced by *L. major*, when transfected into murine macrophages, was shown to activate ERK1/2 MAPK to inhibit macrophage apoptosis *in vitro* [93]. In another study,

L. amazonensis was shown to delay apoptosis or prevent ATP-mediated cytolysis of murine macrophages by releasing nucleoside diphosphate kinase (NDK) [94].

Recently, in addition to the involvement of host/parasite protein factors, the role of host miRNAs in regulating apoptosis upon infection with protozoan parasites like *Leishmania* has only begun to emerge. In *L. donovani*-infected macrophages, various miRNAs like miR-155, miR-335, miR-143, miR-221, miR-93, and let7c have been associated with negative regulation of apoptosis [95]. In *L. major* infected RAW264.7 cells and BALB/c mice, miR-155 inhibitor and miR-15a mimic increased apoptosis, reducing parasite burden [96]. In another study, *L. infantum* infection in macrophages also upregulated miR-155a expression [97]. miR-155a is known to destabilize caspase 3 mRNA, resulting in decreased apoptosis [98,99]. On the other hand, miR-15a negatively regulates *Bcl-2* and *Mcl-2* gene expression and cell survival [100]. The expression of another miRNA, miR-24-3p, was also upregulated in *L. major* infected macrophages rapidly post-infection. Further, bioinformatic analysis revealed the anti-apoptotic effect of miR-24-3p by repressing pro-apoptotic genes like caspases 3 and 7 [101] (Fig. 3).

The studies reviewed in this section show that host apoptosis plays an important role in leishmaniasis. However, further investigation delineating the detailed molecular mechanisms of *Leishmania*-mediated regulation of host apoptosis is needed to understand the pathophysiology of leishmaniasis fully. The knowledge generated will provide the basis for novel discoveries about leishmaniasis.

Although this section is mainly restricted to exploitation of apoptosis by *Leishmania.* It is of interest to point out that other trypanosomatid pathogens such as *T. cruzi* also take advantage of apoptosis to facilitate parasite spreading. It has been shown that *T. cruzi* infection triggers activation-induced cell death of T-lymphocytes during the acute phase of infection [102,103]. Interaction of apoptotic, but not necrotic, T-lymphocytes with *T-cruzi*-infected macrophages promoted parasite growth. This interaction triggered the release of TGF-α, which further suppresses the release of pro-inflammatory cytokines from macrophages, thus creating a pro-parasitic environment [102]. The relevance of leukocyte apoptosis and associated immunomodulatory mechanism in the pathogenesis of Chagas' disease has recently been reviewed [104].

6. Regulation of Host Macrophage Autophagy by *Leishmania*

In this section, we overview a limited number of studies regarding the interaction of *Leishmania* with host macrophage autophagy that could play critical roles in the outcome of infection.

Autophagy

It is an evolutionarily conserved pathway that enables cells to digest their cytoplasmic contents, aiding in recycling and maintaining homeostasis. In eukaryotic cells, there are three mechanistically distinct types of autophagy—macroautophagy, microautophagy, and chaperone-mediated autophagy. In this review, we focus on macroautophagy—the most important type of autophagy—and will be referred to as autophagy herein. Autophagy is characterized by the active degradation of cytoplasmic constituents that are engulfed by double-membrane structures, known as autophagosomes. These distinctive structures ultimately fuse with lysosomes to form autophagolysosomes. It is at this stage that the intravesicular contents are degraded [105]. Autophagosome biogenesis is a complex process involving multiple proteins and lipids [106–108]. Central to autophagy are evolutionarily conserved proteins called autophagy-related proteins or ATGs. Among more than 30 autophagy-related (ATG) proteins identified thus far, the lipid-conjugated protein marker, microtubule-associated protein 1 light chain 3b (LC3-II)/ATG8, associates with the autophagosome double membrane, has been used extensively as an indicator of autophagy in a wide variety of cells and tissues [109].

Autophagy can be regulated via multiple signalling pathways. Broadly, the two most commonly defined pathways are either mammalian target of rapamycin (mTOR)-dependent or mTOR-independent [105,110]. The mTOR-dependent pathway involves PI3K-AKT activating mTOR, which leads to the inhibition of cellular autophagy. This is considered to be the classical pathway of autophagy regulation. mTOR-independent regulation of autophagy has also been identified [111]. Inositol-lowering agents, such as lithium, induce autophagy independent of any change in mTOR activity [112]. Previously, it was assumed that autophagy was exclusively a bulk process involving a non-selective pathway. However, recent evidence has clearly established that through the use of autophagy receptors and adaptors, autophagy can be selective and exclusively degrade specific cellular constituents and better fulfil the catabolic needs of the cell [113,114]. It is well established that LC3-II plays a vital role in the biogenesis of autophagosomes. In addition, it also seems to play a key role in selective autophagy by tethering cargo to the sites of engulfing autophagosomes and by serving as docking sites for adaptor proteins [115–119].

Regulation of Macrophage Autophagy by Leishmania

Due to its role in many physiological processes, it is unsurprising that the dysregulation/manipulation of autophagy is implicated in various diseases [22] and infections [120–123]. In addition, autophagy has been involved in defence against intracellular pathogens [22,124]. Notably, macrophage autophagy attenuates the survival of numerous pathogens, such as *Mycobacterium tuberculosis*, *Shigella*

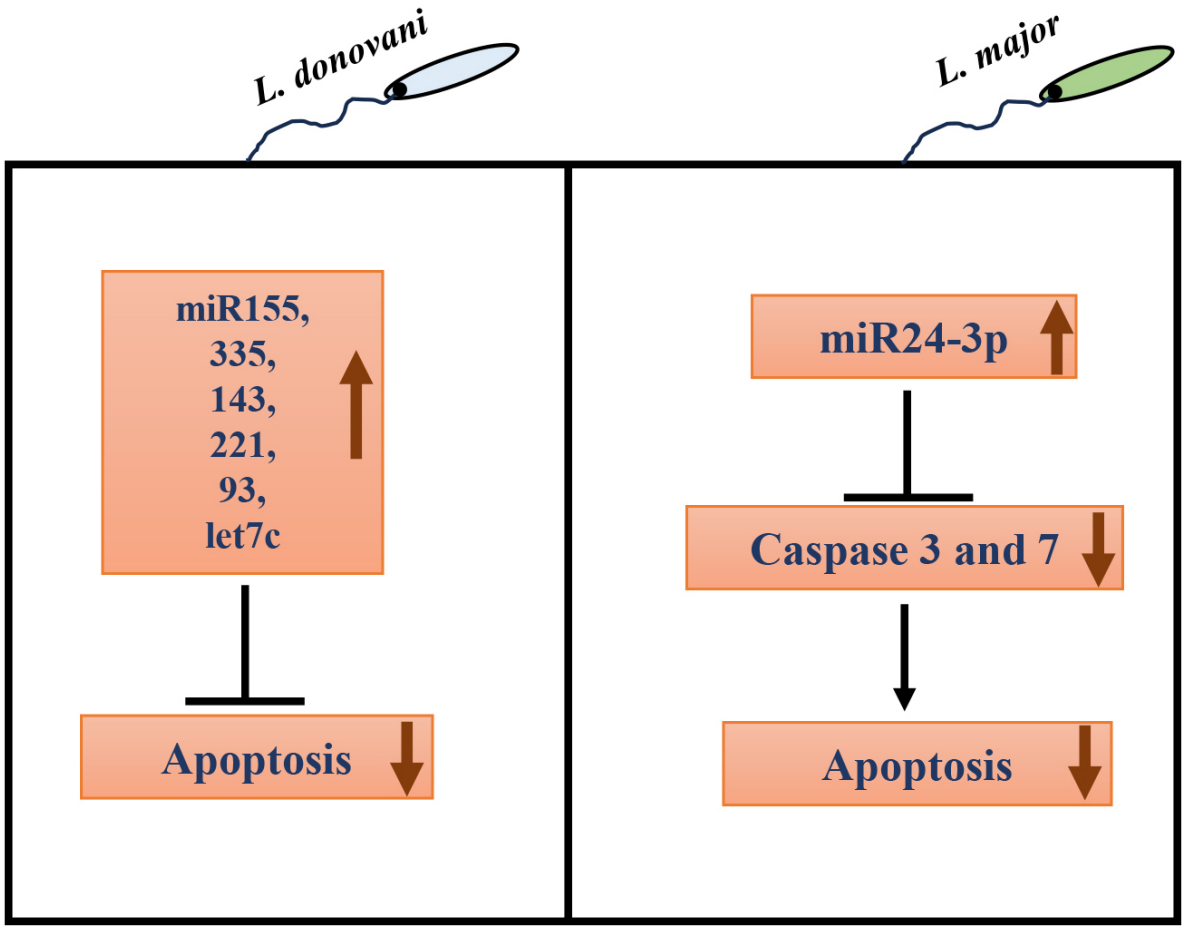

Fig. 3. Role of host microRNAs (miRNAs) in autophagy regulation in *Leishmania* infected macrophages. In *L. donovani* infection, many host miRNAs like miR-155, miR-335, miR-143, miR-221, miR-93, and let7c have been associated with negative regulation of apoptosis. In *L. major* infected macrophages, miR24-3p increases, which decreases pro-apoptotic caspase 3 and 7 gene expression, inhibiting apoptosis.

flexneri, among others [22]. On the other hand, certain intracellular pathogens such as *Toxoplasma gondii* [123], Hepatitis C virus [125] and *Coxiella burnetiid* [126] appear to have evolved to regulate host autophagy to their advantage. Several species of *Leishmania* have been observed to induce macrophage autophagy [127–131], but the molecular mechanism(s) involved in the autophagy response are yet to be established. Understanding the regulatory mechanisms and key players involved in autophagy will provide critical insights into *Leishmania*-macrophage interactions. Our group recently showed bidirectional regulation of macrophage autophagy by *L. donovani* to promote their survival in human mononuclear phagocytes [132]. *L. donovani* uses the PI3K/AKT/mTOR pathway to actively attenuate host autophagy in infected hosts at early and late-stage infection. Continuous *L. donovani*-mediated activation of host AKT sustains mTOR activity, which, in turn, suppresses autophagy at early and late-stage infection. However, once infection becomes established, *L. donovani* promotes mTOR-independent autophagy selectively [132] (Fig. 4A). Disruption of autophagy by downregulating macrophage ATG5 or ATG9A (essential autophagy proteins) resulted in a marked decrease in *L. donovani* survival [132], leading to the discovery that autophagy promotes intracellular survival. These exciting findings regarding the regulation of macrophage autophagy by *L. donovani* indicate that pathogen uses dual strategies to exert countervailing effects on host autophagy. Based on these findings, it is tempting to hypothesize that *L. donovani* fine-tunes autophagy to suit its catabolic needs to promote optimal parasite survival. Another recent study from our group [133] further explored the role of autophagy in *Leishmania*-infected cells. This study identified the protein constituents of *L. donovani*-induced autophagosomes. The proteomic content of autophagosomes upon *Leishmania* infection was compared with the proteome of autophagosomes of THP-1 cells induced with known autophagy inducers like rapamycin and starvation. This study revealed that 146 proteins were significantly modulated in *L. donovani*-induced autophagosomes compared to the rapamycin-induced au-

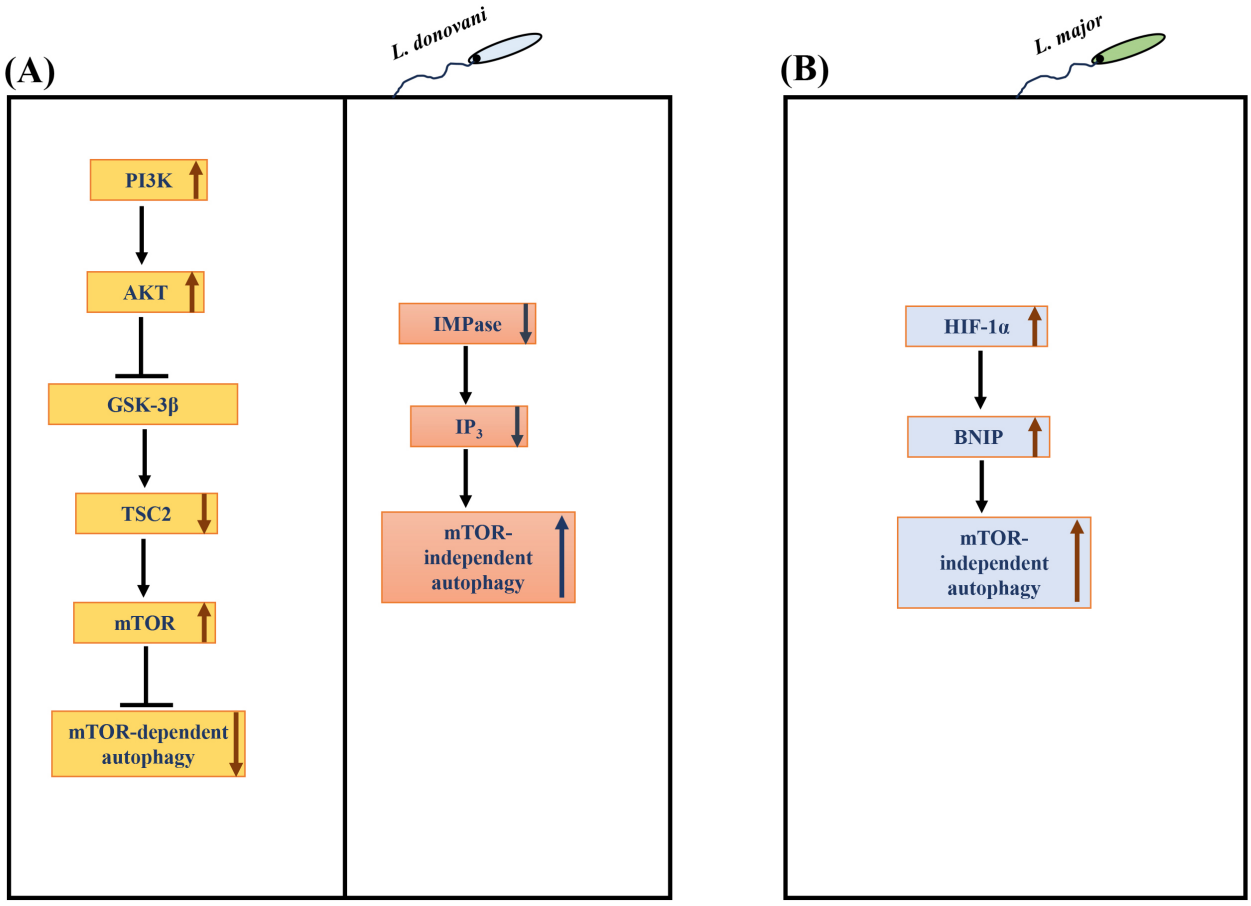

Fig. 4. Regulation of autophagy in *Leishmania* infected macrophages. (A) *L. donovani* hijacks the PI3K-AKT-GSK3β-TSC2-mTOR axis to promote mTOR activity leading to inhibition of mTOR-dependent autophagy. Host Akt is kept activated by *L. donovani* throughout the infection which upregulates mTOR and downregulates mTOR-dependent autophagy. At later stages of infection, *L. donovani* decreases inositol monophosphatase (IMPase) activity and inositol 1,4,5-trisphosphate (IP_3) concentration in host cells to induce autophagy independent of mTOR. (B) *L. major* infection increases BCL2/adenovirus E1B 19 kDa protein interacting protein 3 (BNIP) in BMDMs, which induces autophagy independent of mTOR. HIF-1α, an up regulator of BNIP, was also increased here. TSC2, Tuberous Sclerosis complex 2; mTOR, mammalian target of rapamycin; HIF, Hypoxia inducible factor.

tophagosomes, whereas 57 were significantly modulated compared to starvation-induced autophagosomes. Therefore, it showed that the composition of *Leishmania*-induced autophagosomes was more similar to starvation- rather than rapamycin-induced autophagosomes, which suggests the non-selective nature of *L. donovani*-induced autophagy. Interestingly, 23 *Leishmania* proteins were also detected in the proteome of *L. donovani*-induced autophagosomes.

Infection with *L. major* has also been reported to induce host autophagy. Elevated levels of LC3-II increased the number of autophagosomes [131]. An elevated level of ubiquitin, an adaptor protein important for autophagy, was also upregulated in *L. major* infection in BMDMs. Interestingly, the autophagy was not mTOR-dependent; rather, hyperphosphorylation of mTOR and ribosomal protein S6 was seen. This mTOR-independent autophagy was verified by elevated levels of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP) in *L. major* infected cells [131]. BNIP is known to have a role in autophagy-mediated elimination of *L. major*. Hypoxia inducible factor (HIF)-1α, a positive regulator of BNIP was also activated in macrophages infected with *Leishmania* [134] (Fig. 4B). As a consequence, *Leishmania* got eliminated in infected cells after induction of autophagy [131]. In some cases, autophagy activation results in a higher load of *L. amazonensis* in macrophages from Bagg Albino mice/c (BALB/c) mice but not C57 black 6 (C57BL/6) mice [128].

Despite several contradictory studies in *Leishmania* infected cells, it is evident that autophagy induction is important for survival. However, in-depth studies are needed on how autophagy is regulated during intracellular infection and how this contributes to microbial pathogenesis and influences host defence mechanism(s).

7. Regulation of Macrophage Non-Coding RNAs and Argonaute Proteins by *Leishmania*

The literature review in this section represents accumulating evidence that host and pathogen non-coding RNAs play critical roles in the outcome of infection. The main focus is how *Leishmania* regulates macrophage non-coding RNAs (ncRNAs) and the associated RNA interference (RNAi) components to promote its survival.

7.1 Non-Coding RNAs

For many years, scientists have focused on proteins as main effector molecules used by pathogens. Recent progress in deep sequencing and other technologies has revealed that the majority of the genome (90–95%) is transcribed to RNAs, which are not translated into proteins. These RNAs are known as non-coding RNAs (ncRNAs) and constitute about 90% of total cellular RNAs [135–137]. Based on their size, ncRNAs can be classified into two groups: the small ncRNAs (<200 bp) and long ncRNAs (>200 bp). Small non-coding RNAs (sncRNAs) are a heterogeneous group of transcripts that include micro RNAs (miRNAs), small interfering RNAs (siRNAs), tRNA, tRNA fragments (tRFs) and rRNA [138]. sncRNAs are highly adapted to their specific roles in interacting with other types of RNA and DNA via base pairing. The most abundant sncRNA is miRNA, identified three decades ago, and to date, over 2654 human miRNAs have been annotated in miRbase (v22.1) [139]. Classical biogenesis of sncRNA includes transcription in the nucleus, which is then exported to the cytoplasm for maturation and further steps leading to functional RNAs. Classically, sncRNAs are involved in gene regulation by targeting complementary mRNAs, leading to their degradation or translational repression. For this action, sncRNAs need to interact with argonaute (AGO) protein, an integral part of the functional RNA-induced silencing complex (RISC) [140]. In addition, evidence is accumulating that ncRNAs can play a role as intercellular communicating molecules through their secretion in extracellular vesicles or act similarly to hormones [141,142]. Interestingly, miRNAs can target TLRs like known ligands [143], leading to induction of immune response [144–146]. Over the years, sncRNAs have been implicated in several diseases like cancer, cardiovascular diseases, neurogenerative diseases [135,147–149] and many infectious diseases such as acquired immunodeficiency syndrome (AIDs) and tuberculosis [150–152]. Several ncRNAs are involved in the functioning of immune cells, including macrophages, dendritic cells and T lymphocytes [153].

Regulation of Macrophage sncRNAs by Leishmania

It is known that *Leishmania* modulates macrophage transcription to its advantage. Previous studies have investigated global changes in gene expression in several *Leishmania*-host cell models, including primary human macrophages [154], patient's lesions [155] and human and murine macrophage cells [156–159]. The majority of the above studies are related to transcripts that encode proteins. However, due to recent advancements in RNAseq, the involvement of ncRNAs in leishmaniasis has begun to emerge. The main focus of these studies is restricted to host miRNAs [95,160–164] playing roles during *Leishmania*-macrophage interactions either favoring parasite survival or enhancing effector functions against *Leishmania* persistence. For instance, host miR-210 was upregulated during *L. donovani* infection of macrophages, enhancing parasite survival by targeting p50 of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), leading to attenuation of TNFα and IL-12 [165]. miR-294 and miR-721 have been shown to promote intracellular survival of *L. amazonensis* by targeting nitric oxide synthase, leading to decreased NO production [166]. A recent study investigated the miRNA profile of human THP-1 cells infected with *L. donovani* isolated from VL and Post-Kala-Azar dermal leishmaniasis. This study revealed the differential expression of many macrophage miRNAs in infected cells, which are predicted to be involved in various biological processes such as PI3 kinase activation [95]. A previous study identified 940 miRNAs in *L. donovani*-infected macrophages, out of which 85 miRNAs were modulated during infection. Functional characterization of select ten differentially regulated miRNAs revealed their potential involvement in regulating macrophage effector functions such as apoptosis inhibition, phagocytosis, differential cytokine production and cell cycle regulation [167].

Regarding the role of host macrophage miRNAs as regulator of gene expression in cutaneous leishmaniasis (CL) caused by *L. braziliensis*, there are three recent elegant studies. First study demonstrated that level of miR-361-3p and miR-140-3p were significantly elevated in CL lesions compared to normal skin from the same patient. Interestingly, miR-361-3p was correlated with failure of antimonial therapy and, consequently, longer healing time for cutaneous ulcers [168]. In another study, the expression of miRNAs related to the TLR/NF-kB pathway in human macrophages infected with *Leishmania* isolates from three clinical forms of disease caused by *L. braziliensis*: cutaneous leishmaniasis (CL), mucosal (ML) and disseminated (DL) leishmaniasis was investigated. Interestingly, significant differential expression of miRNAs in macrophages infected with *Leishmania* isolated from ML and DL forms of leishmaniasis was observed. Some of these miRNAs were found to be correlated with parasite loads [169]. In a more recent focused study, same group investigated the functional role of miR-155a-5p in CL pathogenesis caused by *L. braziliensis*. They showed that miR-155a-5p is correlated with increased ROS and impaired apoptosis in human macrophages infected with *L. braziliensis*. Together, this study suggests a role of host miR-155 in regulating ROS production and apoptosis [170] in leishmaniasis.

Recently, our group demonstrated that *L. donovani* downregulated a broad subset of miRNAs in human primary macrophages [162]. This *Leishmania*-mediated miRNA inhibition occurred at the level miRNA gene transcription and was transcription factor c-Myc-dependent. c-Myc itself was upregulated markedly by infection, and inhibition of c-Myc activity, either by using a specific inhibitor (10058-F4) or downregulation by short interfering RNAs (siRNAs), restored macrophage miRNA levels to normal. Moreover, and of special interest in terms of infection biology, c-Myc silencing also brought about a dramatic reduction (approximately 90%) in the intracellular survival of *Leishmania*. Taken together, this investigation found c-Myc not only acts to bring about genome-wide repression of host miRNAs but also plays an essential role in promoting *Leishmania* survival. This identifies c-Myc as a novel virulence factor by proxy, contributing to *Leishmania* pathogenesis. Thus, this study identified one mechanism of how the miRNA machinery is targeted during *Leishmania* infection [162]. It will be of interest to extend this study since identification of c-Myc sensitive factor(s) in infected cells will have potential to be developed as novel therapeutic targets for leishmaniasis. In addition to the role of miRNAs in *Leishmania* pathogenesis, the role of long ncRNAs has begun to emerge. A recent study has implicated long ncRNAs in regulating macrophage functions [171]. This study has shown that the repression of 7SL RNA promotes a pro-parasitic environment.

There is a lack of information about the contribution of pathogen-related ncRNAs to host immune evasion and disease outcomes. An in-depth analysis of the *L. major* genome has revealed 1884 unique ncRNAs in the parasite [172]. The contribution of these parasite-derived ncRNAs to the outcome of parasite-host interactions needs to be investigated. As discussed above, ncRNAs need to be loaded onto Ago proteins to perform their function in RNAi. *Leishmania* seems to target this important class of molecules to regulate host RNAi.

7.2 RNAi

RNAi is a conserved biological response to endogenous/exogenous double-stranded RNA that regulates the expression of protein-coding genes in a wide variety of organisms, including plants, animals and fungi [173–175]. In most cases, double-stranded RNA is diced into small fragments, approximately 21–25 base pairs, by Drosha and Dicer. These tiny fragments of double-stranded RNAs bind to Argonaute proteins, an integral constituent of effector complex RISC. Double-stranded sncRNA unwinds during RISC assembly, followed by the degradation of one strand (known as passenger strand). The remaining mature/guide strand hybridizes with a complementary mRNA, leading to either slicing of mRNA or translation inhibition due to RISC stuck on the target mRNA [173].

7.3 Argonaute (AGO) Proteins

AGO proteins are central components of RISC in addition to sncRNAs and/or accessory proteins directly or indirectly interacting with them. This includes glycine/tryptophan repeat-containing 182 protein (GW182 protein), and heat shock proteins (HSP70/HSP90), among others [176–179]. GW182 is a vital scaffolding protein that directly binds to Ago proteins and bridges its interaction with additional factors that coordinate all downstream steps of RNAi [177].

Humans have four AGO proteins, AGO1-4, which share a high sequence identity [180]. Despite sharing the same catalytic (Asp-Glu-Asp-His) tetrad, only AGO2 has been shown to possess slicing activity when the target mRNA sequence has complete complementarity to the guide sncRNA strand [181,182]. All four AGO proteins have four distinct domains: N-terminal, Piwi-Argonaute-Zwille (PAZ), Middlle (MID) and P-element induced wimpy testis (PIWI) domain [183,184]. AGO proteins have a bilobed configuration connected by Linker 1 and Linker 2 involved in structural rearrangements after binding to sncRNAs. A recent review nicely describes common features and target specificities across the four AGO proteins [180].

It is known that the majority of AGO functions are restricted to cytoplasm. However, several recent studies have reported non-canonical functions of the AGO proteins in the nucleus, where they remodelled chromosomes, and played roles in alternate splicing and DNA repair processes [185–187]. Thus, eukaryotic AGO proteins have a spectrum of functions involving many cellular processes. In recent years, AGO proteins have been implicated in several human diseases, such as viral infections, autoimmune diseases, cancer, neuronal diseases and metabolic deficiencies as reviewed by Pantazopoulou *et al*. [188]. Thus, evidence has emerged showing the role of AGO proteins in both physiological and pathological conditions.

Leishmania Regulates Macrophage Ago1 to Promote Its Survival

As described above, several studies have implicated macrophage miRNAs in the pathogenesis of *Leishmania* infection. However, the role of AGO proteins in *Leishmania* infection and disease outcome has begun to emerge. Recently, our group showed the selective involvement of macrophage AGO1 in the survival of *Leishmania* in infected cells [189]. *Leishmania* infection selectively upregulated the abundance of macrophage AGO1 in infected cells. Interestingly, an increased level of AGO1 in infected cells positively correlated with the enhanced level of AGO1 in functional RISC, indicating a preference for AGO1 in regulating RNAi in infected cells. In virus-infected mammalian cells, it has recently been shown that sncRNAs other than miRNAs were selectively loaded onto AGO1 but not AGO2 [190]. Similarly, in *Drosophila*, it has been

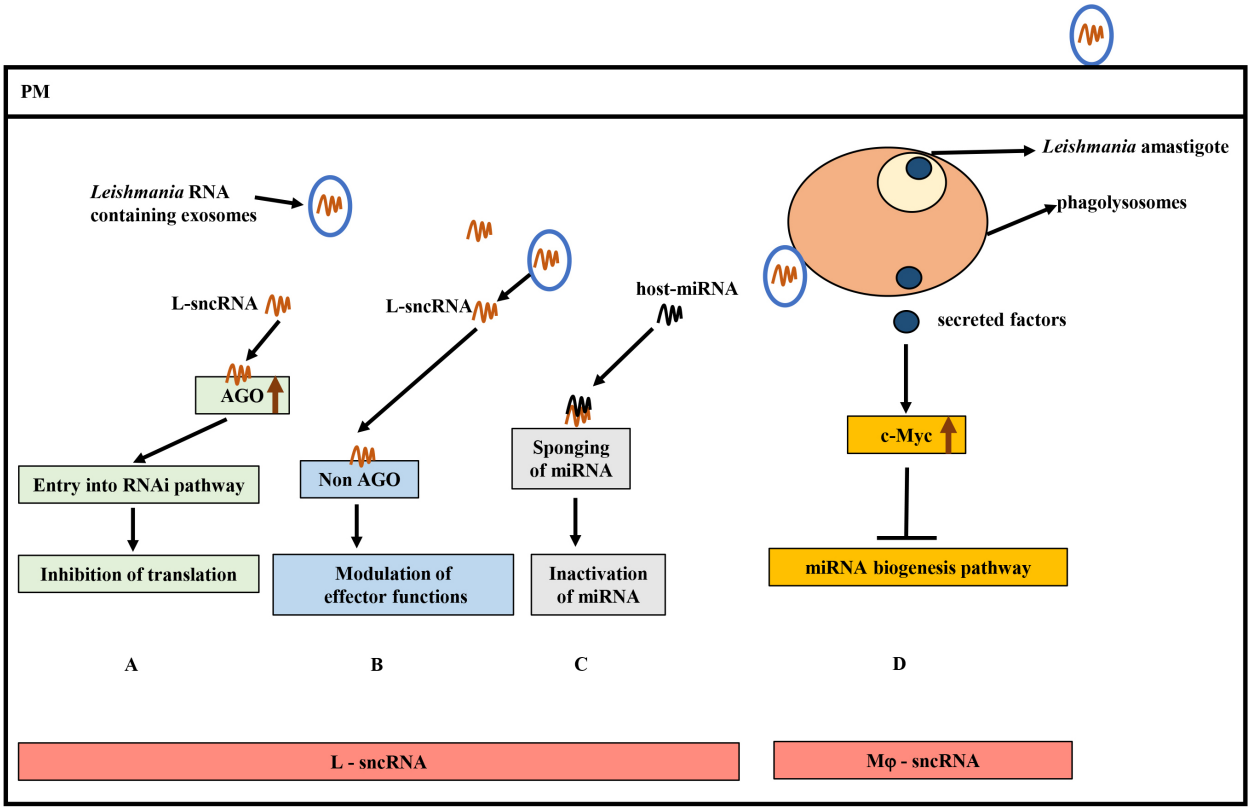

Fig. 5. Hypothetical model of small non-coding RNAs (sncRNAs) and contributions to *Leishmania* pathogenesis. (A) Hijacking of host RNA interference (RNAi) pathway by *Leishmania* sncRNAs (L-sRNA) by interacting with host Ago proteins (critical component of RNA Induced Silencing Complex (RISC)) to inhibit translation. (B) Binding of L-sncRNA to host non-AGO proteins, leading to modulation of host effector functions. (C) Inactivation of host-microRNA (miRNA) function by L-sncRNAs by complementary base-pairing. (D) Inhibition of host miRNA expression by *Leishmania* secreted effectors by upregulating c-Myc levels. Increased c-Myc levels target the miRNA biogenesis pathway at the transcription level. AGO, Argonaute; miRNA, MicroRNA.

demonstrated that perfectly matched sncRNA duplexes are loaded onto AGO2, whereas non-perfectly matched sncRNAs are loaded onto AGO1 [191]. Deliberate knock down of AGO1 using siRNAs attenuated *Leishmania* survival in infected cells. Moreover, the expression of several *Leishmania* pathogenesis-related proteins seems to be dependent on the optimal level of AGO1 [189]. Interestingly, 53 of the 71 proteins are related to the pathogenesis of other intracellular pathogens [189]. This study could provide a framework for further analysis of the role of RNAi in *Leishmania* pathogenesis in humans (Fig. 5). A deep understanding of inter-species RNAi in the context of *Leishmania* infection could offer novel therapeutic strategies to control and treat leishmaniasis and may have implications for other intracellular pathogens. We note that this research is restricted to proteomic analysis, and it will be of interest to complement these findings with relevant biological assays. Further, identification and characterization of *Leishmania* ncRNAs and their host targets and host ncRNAs relevant to leishmaniasis could add to the molecular understanding of *Leishmania* pathogenesis.

8. Conclusions

Given the knowledge summarized in this review, it is clear that leishmaniasis provides an excellent paradigm of immune evasion in several ways. Over the years, we have learned that *Leishmania* has evolved to acquire the ability to regulate the cell biology of its host macrophages through complex mechanisms to reside, grow and proliferate inside inhospitable and restricted environment of parasitophorous vacuoles (PVs) of infected cells. The evasion strategies include inhibition of apoptosis, regulation of host autophagy and targeting of host ncRNAs. Exploitation of macrophage autophagy and RNAi are emerging themes in many intracellular pathogens, including *Leishmania*. The role of host ncRNAs during *Leishmania* infection is mainly restricted to miRNAs. There is a need to investigate the role of other ncRNAs during *Leishmania* infection. In RNAi, how *Leishmania* targets RISC to regulate host gene expression is an important question that needs to be answered. The molecular and functional characterization of virulence factors and investigating their effects on host ncRNAs and autophagy is likely to provide new insights into pathogen-

esis. This knowledge could be the key to identifying new targets for intervention to combat and prevent leishmaniasis. We note that host immune response and *Leishmania* pathogenesis are highly variable and influenced by infecting *Leishmania* species, their virulence factors and choice of the host. Extension of current knowledge of *Leishmania*-macrophage interactions with all diseases causing *Leishmania* species will be very helpful in designing new approaches to this neglected disease.

Author Contributions

DN and HKB were involved in planning and collecting the data for this review and drafting/editing the manuscript. NR was involved in the conceptualization, interpretation of data, fund acquisition, and critical review of figures. NR planned and acquired the funding. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors have approved the final draft of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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