Chapter

Extracellular Vesicles Released by *Leishmania*: Impact on Disease Development and Immune System Cells

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Abstract

Leishmania spp. release extracellular vesicles (EVs) containing parasite molecules, including several antigens and virulence factors. These EVs can interact with the host cells, such as immune cells, contributing to the parasite–host relationship. Studies have demonstrated that *Leishmania*-EVs can promote infection in experimental models and modulate the immune response. Although the immunomodulatory effect has been demonstrated, *Leishmania*-EVs can deliver parasite antigens and therefore have the potential for use as a new diagnostic tool and development of new therapeutic and vaccine approaches. This review aims to bring significant advances in the field of extracellular vesicles and *Leishmania*, focusing on their role in the cells of the immune system.

Keywords: extracellular vesicles, exosomes, microvesicles, *Leishmania*, immune response, leishmaniasis

1. Introduction

The host–parasite communication and the parasite's intercellular interactions are crucial in the life cycle of the *Leishmania* parasites [1, 2]. In addition, several bioactive molecules released by the parasites have shown an important role in the parasite's adaptation in the host [3]. In mammalian hosts, molecules released by *Leishmania* contribute to the parasite's infectivity and the physiopathology of the leishmaniasis, acting by several mechanisms, such as subverting the immune response and favoring the intracellular multiplication of the parasite [3].

Several works have demonstrated that *Leishmania* species can release proteins and other molecules in extracellular vesicles (EVs) [4–6]. EVs is a generic term used to describe particles spontaneously released by prokaryotic and eukaryotic cells [7]. Deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, lipids, and cellular metabolites are present in EVs that can deliver information from one cell to another [8]. Thus, EVs are now considered a new mechanism of intercellular communication [7].

Leishmania-EVs carry parasites molecules, such as small RNA, heat shock proteins (HSPs), and virulence factors (glycoprotein 63 - GP63 and lipofosfoglican - LPG) [4, 5, 9]. Functional studies showed immunomodulatory and signaling-inducing activities properties of the *Leishmania*-EVs [10]. They are present in the intestinal lumen of sandflies and are regurgitated along with promastigote forms during the blood meal [6]. In addition, these particles modulate the macrophage's activation and alter the course of the parasite infection [4–6, 11]. Although immunomodulatory properties have been demonstrated in experimental models, additional studies are necessary to better understand the role of EVs in the parasite–host relationship. Next, we describe an overview of the extracellular vesicles relevant to *Leishmania* infection and the main findings related to EVs released by *Leishmania* parasites.

2. Extracellular vesicles (EVs): an overview

EVs can be detected in body fluids, including urine, saliva, blood, plasma, amniotic fluid, breast milk, ascites, synovial fluid, and cerebrospinal fluid [7, 12]. Structurally, they present a spherical shape with a double layer composed of lipids and proteins and can be filled with biomolecules from the cell of origin [13]. EVs are classified based on their biogenesis, composition, and size, namely—exosomes, microvesicles (MVs), and apoptotic bodies (ABs) [8, 13]. Although MVs and exosomes show structural similarities, they are different in size, content, lipid composition, and biogenesis [7]. ABs are released by apoptotic cells and have specific characteristics [12] that will not be covered in this review.

Exosomes present sizes between 20 and 100 nm [14]. They are formed by the internal invagination of the endosomal membrane, originating the multivesicular bodies (MVBs) [8]. After maturation, exosomes are secreted by exocytosis via fusion of MBVs with the cell surface, or they may be digested by lysosomes [14, 15]. Exosomes are rich in lipids (mainly phosphatidylserine, cholesterol, and ceramides), nucleic acids, and proteins [8]. In addition, proteins such as endosomal sorting complexes required for transport (ESCRT), Alix, tumor susceptibility gene 101 (TSG101), heat shock cognate 70 (HSC70), HSP90β, HSP60 and HSP70, proteins from the annexin family, and tetraspanins (cluster of differentiation 63 - CD63, CD9, CD81, and CD82) participate in the process of formation of exosomes [8, 16]. These molecules are increased in exosomes, but they are not exclusive markers of these EVs types [7].

MVs are a group of EVs with a diameter between 100 and 1,000 nm [7]. They are originated from the protrusion of the cytoplasmic membrane, and they can carry molecules of cell surface such as membrane receptors, integrins, adhesins, and others [8]. Some studies have shown that structures such as actin and microtubules (cytoskeleton), kinesins and myosins, and soluble NSF attachment receptors (SNAREs) play a role in the formation of MVs [17]. However, the molecular pathway is not well understood [8, 13, 18], and specific markers of MVs have not yet been described. The releasing of MVs and exosomes occurs under physiological cell conditions, but the quantity and content can be altered after stimuli, such as low oxygen and nitrogen content, oxidative stress, among others [4, 5, 19].

Different vesicle isolation techniques have been performed; however, centrifugation/ultracentrifugation and size exclusion chromatography are the most commonly used [7]. Flow cytometry, Western blotting, nanoparticle tracking technique (NTA), mass spectrometry, and electron microscopy have been used to quantify and better characterize the isolated EVs (exosomes and/or MVs) [7]. The inclusion of new methodologies and the discovering of specific EVs markers will bring a new perspective to understand the role of these nanoparticles in the biology and the

pathophysiology of several diseases. In addition, there is a great expectation of the applications of EVs in diagnostics, treatments, and vaccine development.

Currently, there is a consensus that EVs play an important role in cell–cell communication being a vehicle for transporting molecules between cells, even cross-kingdom [8, 18, 20]. The effects on the recipient cells depend on the cell type, the origin of EVs, their content, and EVs can act locally and/or systemically. The changes in the recipient cells include modulation of the intracellular signaling pathways, gene regulation, post-transcriptional regulation, activation, or inhibition of different cell types [21–23]. After target cell recognition, EVs can interact with surface receptors, followed by fusion with the plasma membrane for releasing their content, and signaling different intracellular events. However, EVs can also be endocytosed by target cells or collapse after their secretion, delivering their contents into the intracellular space [8, 15].

In parasitic diseases, EVs have brought an exciting field to investigate since they can act as mediators in parasite–host interaction, allowing the transfer of virulence factors and effector molecules from the parasites to the host [24–26]. Parasites EVs are related to the pathogen adhesion, the spread of the parasites, and play a role in regulating the host's immune system. In addition, immune cells infected and/or stimulated with parasite components can release EVs [23] containing messenger RNA (mRNA), small noncoding RNAs (microRNA), chromosomal and mitochondrial DNA, retrotransposons, parasites antigens, and major histocompatibility complex (MHC) I and II [23, 27]. The effects in immunity are diverse, including modulation of innate immune response and antigen presentation.

The production and releasing of EVs by parasites or parasitized cells have been described and characterized in several parasitic infections [25]. For example, in *Leishmania*, several biological markers and virulence factors have been described in EVs released by the parasites [10, 28]. Thus, EVs released by these pathogens can have a role in the disease progression and the host's immune response to the parasite, contributing to the strategy to bypass the immune system.

3. EVs released by *Leishmania spp*

Leishmania species can release proteins and other molecules in EVs. Although the mechanisms for exosome/MVs secretion in *Leishmania* are still unclear, proteomics analysis of EVs has shed light on the functions and properties of these particles. Initial work showed that *Leishmania donovani* could use EVs as a protein transport vehicle [29]. Additional studies confirmed that *L. donovani* releases EVs. *Leishmania major*, *Leishmania mexicana*, and *Leishmania amazonensis* also used EVs as an important mechanism for protein secretion [4, 5, 30]. The presence of EVs in the intestinal lumen of sandflies and their release together with the parasites during the blood meal reinforce the hypothesis that these EVs contribute to the process of infection and development of leishmaniasis [6].

The release of EVs by *Leishmania* is related to the temperature. Promastigotes of *L. mexicana* and *L. donovani* increased the release of EVs after parasite cultivation at 37°C (mammalian host temperature), compared to the EVs obtained from parasites incubated at 26°C (vector temperature) [30]. Furthermore, to *L. donovani*, differences in the content of the EVs obtained at 37°C and 26°C [4] were also observed, suggesting a possible parasite strategy for establishment in the host. However, *L. amazonensis* showed a different pattern in EVs releasing since a higher number of particles were detected after cultivation at 26°C, compared to the parasite incubated at 34°C or 37°C [5]. Altogether these observations suggest that *Leishmania* species can adapt differently to the release of EVs.

Proteomic studies showed the presence of the metalloprotease GP63 in EVs released by *Leishmania* cultivated *in vitro* and by the parasite infecting sandflies. GP63 is the main surface glycoprotein of *Leishmania* and is considered a virulence factor since it contributes to the parasite escape of immune response [31–34]. Evaluating the proteomic profile of EVs released by *Leishmania infantum* in three different phases (logarithmic, stationary, and metacyclic stages) showed that the metacyclic phase had a higher abundance of GP63. In contrast, EVs of parasites in the logarithmic phase had the lowest abundance [35]. In a similar approach, higher concentrations of GP63 were detected in EVs released by *L. infantum* in the stationary phase while parasites in the logarithmic phase showed enrichment of ribosomal proteins [36]. However, proteomic analysis of EVs from *Leishmania infantum chagasi* showed no significant biological differences in EVs released by parasites in logarithmic or stationary phases [37].

Besides GP63, other proteins have already been identified in *L. donovani*-EVs, such as elongation factor-1α (EF-1α), fructose-1,6-bisphosphate aldolase FBA, HSP70, and HSP90 [4]. A comparative study of *L. infantum*-EVs from drug-resistance parasites identified differences in their morphology, size, distribution, and protein content. Identifying proteins related to drug resistance in EVs from resistant parasites can bring new possibilities to predict prognostics and treatments in leishmaniasis [38].

The presence of small noncoding RNAs was identified in EVs released by *L. donovani* and *Leishmania braziliensis*, suggesting the regulatory role of these EVs in the host cells [39]. Additional studies to address the EVs content from different *Leishmania* species may clarify the role of these particles in visceral and cutaneous leishmaniasis. Furthermore, these studies may provide the use of *Leishmania*-EVs in diagnostics, the development of a vaccine, and promising therapeutic alternatives.

4. *Leishmania*-EVs and immune response

Some evidence have pointed that *Leishmania*-EVs present immunomodulatory effects, altering the immune response and contributing to the disease progression. The treatment of human monocytes with *L. donovani*-EVs induced the production of interleukin 10 (IL-10) and inhibited the tumor necrosis factor-alpha (TNF-α) production, even after challenging with interferon-gamma (IFN-γ) [11]. Similar effects were observed in dendritic cells (DC) treated with these EVs since the production of cytokines IL-12p70, TNF-α and IL-10 were inhibited and there was impaired in the ability of these cells to stimulate the differentiation naive CD4 T lymphocytes into T helper 1 (Th1) profile [11]. On the other hand, EVs released by *L. amazonensis* increased the expression of IL-10 and IL-6 in bone marrow-derived macrophages (BMDM) [5]. In fact, EVs released by different *Leishmania* species seem to induce different responses in human macrophages [40]. EVs from *L. infantum* and *L. braziliensis* failed to induce an inflammatory response in human macrophages. However, *L. amazonensis*-EVs stimulated human macrophages to produce nitric oxide (NO), TNF-α, IL-6, and IL-10 via Toll-like receptor 4 (TLR4) and TLR2 (**Figure 1A**) [40].

Few studies have proposed mechanisms of intracellular signaling pathways activated by *Leishmania*-EVs into phagocytes cells. EVs released by *L. amazonensis* amastigotes containing DNA fragments were capable of inducing the CD200 expression in macrophages [41]. The high expression of this molecule leads to the inhibition of NO production, contributing to the parasite survival [41]. In addition, evidence suggests that the composition of EVs can influence the outcome of cell

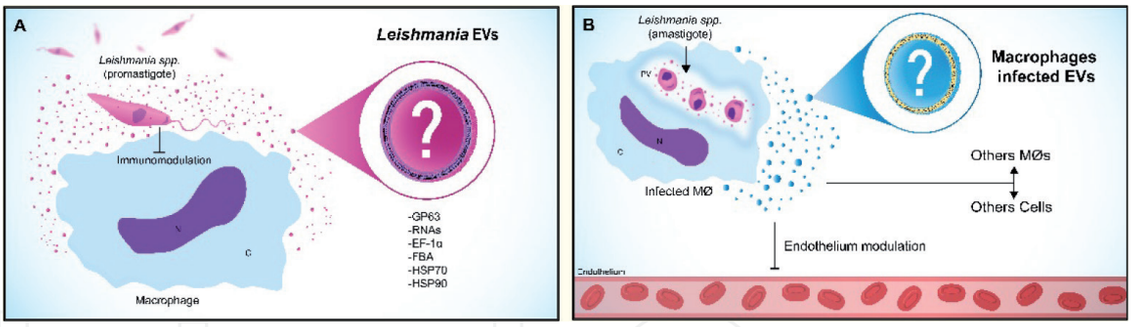

Figure 1.
Leishmania EVs and their influence on the modulation of immune and endothelial cells. (A) Macrophage modulation by EVs released by Leishmania spp. promastigotes. (B) Macrophages infected with Leishmania spp. release EVs with modulating activities. C - cytoplasm; N - nucleus; PV - parasitophorous vacuole; Mϕ(s) – Macrophages.

signaling. *Leishmania* EVs-containing *Leishmania* RNA virus (LRV1) released by *Leishmania guyanensis* trigger TLR3/TRIF (TIR domain-containing adaptor inducing interferon-β signaling), inducing inflammatory cytokines (pro-IL-1b, TNF-a, and IL-12), and the autophagy by impairing NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome network [42, 43] (**Figure 1B**). Thus, these initial studies demonstrated a refined and complex intracellular signaling pathway induced by EVs, which depends on the species and evolutionary form that is releasing the EVs and the presence or absence of *Leishmania* virus.

Besides macrophages and DCs, *Leishmania*-EVs can modulate other immune cells. EVs released by *L. infantum* inhibited the expansion of peripheral iNKT (Invariant Natural Killer T) cells and the production of IL-4 and IFN-γ by this cell type [44]. Experiments using CD1d specific ligands (glycolipid α-GalactosylCeramide (α-GalCer) suggest that lipids present in *L. infantum*-EVs and other exocomponents released by the parasites may compete for the CD1 binding site, inhibiting iNKT activation [44]. In addition, our group showed that murine B-1 cells (a subtype of B lymphocytes) stimulated with EVs released by *L. amazonensis* produced higher levels of NO, compared to non-stimulated B-1 cells [45]. The increase in the expression of TLR-9, TNF-α, and transcriptional factors related to the differentiation of B-1 cells to phagocytes are important changes observed in B-1 cells treated with *L. amazonensis*-EVs [45]. These data suggest that *Leishmania*-EVs participate in the modulation of different cells and different levels of the immune response. Interestingly, some mechanisms seem conserved between species, but some specifies are related to *Leishmania* species making comparative studies necessary.

In a mammalian host, *Leishmania* is an intracellular parasite. Thus, studying changes in infected cells can provide important information about the parasite's biology. Silverman et al. [4] showed *Leishmania* exosomes and exosomal proteins in the cytosolic compartment of infected macrophages. In addition, EVs released by macrophages infected with *L. mexicana* containing GP63, and this finding instigated the investigation to uncover the role of these EVs in immunity [46]. Naïve macrophages exposed to EVs from *L. mexicana*-macrophages infected cells induced the activation of mitogen-activated protein (MAP) kinases (except c-Jun N-terminal kinase - JNK) and the nuclear translocation of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) [46]. BMDM infected with *L. amazonensis* released EVs which were able to activate naive macrophages to produce proinflammatory cytokines IL-12, IL-1β, and TNF-α, contributing both to modulate the immune system in favor of a Th1 immune response and to the elimination of the *Leishmania*, leading, therefore, to the control the infection [47] (**Figure 1B**).

Thus, infected macrophages are able to release EVs that deliver information to activate naïve macrophages, contributing to activate an innate immune response.

Evidence suggests that EVs released by *Leishmania*-infected cells can stimulate different cells, promoting a response against the parasite. EVs released by macrophages infected with *L. donovani* stimulated endothelial cells to produce granulocyte colony-stimulating factor (G-CSF)/CSF-3, and vascular endothelial growth factor A (VEGF-A), promoted an increase in epithelial cell migration and induced endothelial cell tube formation [48] (**Figure 1B**). A study with EVs released by B-1 cells infected with *L. amazonensis* showed the impact of these EVs on naive macrophages activation and the protective effect on the experimental infection with the parasite [49]. Macrophages treated with EVs from infected peritoneal B-1 cells alter the expression of inducible nitric oxide synthase (iNOS), IL-6, IL-10, and TNF-α [49]. Overall, these studies demonstrated that *Leishmania* infection changes the content of EVs from infected cells and suggest that these EVs participate in the activation of immune and nonimmune cells, actively participating in the pathophysiology of the *Leishmania* infection.

5. EVs and leishmaniasis progression

Experimental models have contributed to better understanding the role of EVs in the leishmaniasis progression. The treatment of mice with *L. donovani*-EVs before the parasite infection exacerbated the infection and induced IL-10 production in the spleen [11]. Furthermore, mice treated with *L. major*-EVs before challenge with the parasite showed an increased frequency of IL-4-producing CD4+ T cells in both the spleen and lymph nodes, leading to disease exacerbation [11]. These findings suggest that *Leishmania* EVs are predominantly immunosuppressive and favor the parasite. In fact, our group demonstrated that *L. amazonensis* EVs co-injected with the parasite led to disease exacerbation with a predominance of Th2 response in BALB/c mice [5]. Similar results were observed for *L. major*, but the co-injection of the parasite and related EVs induced an increase in the expression of IL-17 and IL-4 [6].

Changes in the content of EVs may impact the immune response and disease progression [9, 11]. Studies performed with genetically modified parasites showed that in a mouse model of air pouch formation (murine air pouch injection) EVs derived from *L. major* GP63 knockout (KO) (*L. major* GP63$^{-/-}$) induced greater recruitment of inflammatory cells, compared to EVs derived from wild parasites [9]. Furthermore, EVs derived from *L. donovani* exhibited an immunosuppressive effect and exacerbated the disease in animals challenged with the parasite, but EVs derived from *L. donovani* HSP100 KO (*L. donovani* HSP100$^{-/-}$) were able to induce a pro-inflammatory response and did not exacerbate the disease [11]. Thus, the hypothesis that EVs derived from parasites with different virulence profiles (virulent and attenuated) present relevant alterations in their protein content and can induce distinct immune responses in an experimental immunization model cannot be discarded.

The relevance of EVs in *Leishmania* infection's biology was shown by the demonstration that *Leishmania* promastigotes release EVs in the sandflies [6]. The experimental infection with *L. major* in the presence of EVs released by the parasite in the vector led to higher lesion size and parasite load, associated with impaired effector immune response [6]. Taken together, the *in vivo* studies suggest that EVs released by *Leishmania* participate in the infection, favoring the establishment of the parasite and the progression of the disease.

6. Conclusions

The knowledge acquired studying EVs has allowed understanding that these particles are related to intercellular communication and cross-kingdom relationship. The release of these EVs by *Leishmania* is related to initial infection, modulation of the immune system, and disease progression in the host (**Table 1**). However, several aspects of the biology and physiology of these molecules still need to be better investigated. Would releasing these EVs into the vector be related to the parasite's adaptation to that environment? Can EVs contribute to parasite multiplication in the vector? Is there population regulation and/or transfer of resistance factors and immune response escape by EVs between different *Leishmania* species? Do these transfers occur in the vector and/or in the mammalian host? Can vesicles released by *Leishmania* be used for the development of vaccines and new diagnostic approaches? Thus, the field of EVs released by *Leishmania* and other pathogens is fascinating and, there will be significant advances and contributions to the area in the future with the discovery of new therapeutic targets and new players in the host–parasite relationship.

Leishmania **species**	**Biological function**	**Reference**
L. donovani	• Increased IL-10 and inhibited TNF-α production by human monocytes; • Inhibited IL-12p70, TNF-α, and IL-10 production by DC; • Impaired the ability of DC to drive T cells differentiation into Th1; • In experimental infection: exacerbated the infection; promoted IL-10 production in the spleen.	[11]
L. amazonensis	• Increased the expression of IL-10 and IL-6 in BMDM; • In experimental infection: led to disease exacerbation with a predominance of Th2 response in BALB/c mice;	[5]
	• Increased the production of NO, TNF-α, IL-6, and IL-10 via TLR4 and TLR2 by human monocytes;	[40]
	• In B-1 cells: increased NO production; increased expression of TLR-9 and TNF-α; induced the expression of factors related to myeloid commitment;	[45]
	• Increased the CD200 expression and inhibited the NO production (EVs released by amastigotes)	[41]
L. guyanensis infected with *Leishmania* RNA Virus (LRV1)	• Triggered TLR3/TRIF signaling; • Impaired NLRP3 inflammasome network	[42]
L. infantum	• Inhibited iNKT activation and production of IL-4 and IFN-γ by these cells;	[44]
L. major	• In experimental infection: • Increased the disease progression; • Increased the expression of IL-17 and IL-4	[10]

Table 1.
Biological effects of the EVs released by different Leishmania species.

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Conflict of interest

The authors declare no conflict of interest.

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