Exosome Secretion by Leishmania infantum modulate innate and adaptive immune responses and create an environment permissive for early infection.

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Highlights

- Exosomes are nanovesicles secreted from different cell types
- Characterization of *Leishmania infantum* exosomes are conducted in this study.
- This study demonstrates the capacity of exosomes to regulate the immune system
- *L. infantum* exosomes create an environment permissive for early infection.

Abstract

In recent years, several studies demonstrated the role of exosomes in intercellular communications, several *Leishmania* species belonging to subgenera *Leishmania* and *Viannia* have been demonstrated to release exosomes, and their role in parasite-macrophage interactions and in leishmaniasis development has been investigated. However, the release of exosomes by *Leishmania infantum* has not been studied so far. The aim of this study was to isolate and characterize *L. infantum* exosomes, and to investigate the biological activity of these exosomes in macrophage cultures. To this end, exosomes were collected from both amastigote and promastigote *L. infantum* conditioned medium by ultracentrifugation. Exosomes were then characterized by monitoring the presence of HSP70, HSP83/90 and acetylcholinesterase activity. Moreover, nanoparticle-tracking analysis revealed that promastigote and amastigote exosomes had mean diameter of 122± 56 nm and 115 ± 65 nm, respectively. Human monocytic cell line U937-derived macrophages treated with promastigote and amastigote exosomes showed an increase in motility and an overproduction of interleukin (IL)-10 and IL-18 reduction, involved in immune response. Since *L. infantum* exosomes demonstrated the capacity to regulate the immune system, they could contribute in the disease establishment and may be considered an appropriate candidate for a vaccine therapy in prophylaxis and treatment.

Keywords: *Leishmania infantum*, exosomes, cytokines production

1 Introduction

Leishmaniasis, caused by the intracellular protozoan Leishmania, is a wide spread disease in tropical and subtropical areas. The parasites occur in most parts of the world and the infection is growing also in non-endemic areas. Different Leishmania species can cause four different clinical presentations of the disease: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and diffuse cutaneous leishmaniasis (DCL) [1].

Leishmaniasis causes significant morbidity and mortality worldwide and it is an important public health problem. In the absence of effective human and canine vaccines, the only feasible way to treat and control leishmaniasis is through the use of affordable medications.

Recently, the exosomes secreted by the parasites have been proposed as one of the strategies used by Leishmania to orchestrate changes in the host environment ensuring a successful infection. Exosomes are nanovesicles secreted from different cell types, including pathogens and infected cells. In the classical pathway, exosomes are formed via the invagination of endocytic compartments generating multivesicular bodies (MVBs), and are released to the extracellular space after fusion of the MVBs with the plasma membrane [2, 3]. Despite the abundant knowledge obtained through studies using exosomes purified *in vitro* or from various biological fluids [4] observation of their formation and release *in vivo* remains a major challenge. As a consequence of their origin, exosomes contain endosome-associated proteins and proteins involved in the MVB biogenesis, such as Rab GTPases, and PDCD6IP (ALIX) or Tsg101, respectively [5]. In addition, many groups of proteins are consistently present in exosomes: heat shock proteins (HSP60, HSP70 and HSP90), tetraspanins (CD81, CD63 and CD37), annexins (I, II, V and VI), cytoskeletal proteins (actin and tubulin), metabolic enzymes, proteins involved in translation (Elongation Factors 1 and 2) and signaling proteins [6, 7, 8, 9, 10, 11]. Importantly, also mRNAs and microRNAs (miRNA) are present in exosomes, and are transferred to target cells [12, 13].

Exosomes from *Leishmania mexicana*, *L. donovani*, *L. major* and *L. braziliensis* have been shown to play a crucial role in host-pathogen interactions and were able to induce modifications in non-infected neighboring cells or act as antigen presenters for the immune system [14]. In fact, experiments performed with mice and macrophages have shown that these exosomes possess immunomodulatory and signaling-inducing activities, corroborating the presence of parasite virulence factors in their content such as the surface metalloprotease GP63 [15, 16, 17, 18].

Thus, it is postulated that these vesicles contribute to the multitude of factors determining the form and severity of the leishmaniases, a spectrum of diseases that ranges from self-healing

cutaneous to fatal visceral forms and represents a major public health problem worldwide. The aim of this study was to isolate, characterize *L. infantum* exosomes and to investigate the biological activity of these exosomes in macrophage cultures.

2 Materials and Methods

2.1 *Parasite cultures*

Leishmania infantum promastigotes (MHON/TN/80/IPT1, MON1) taken by stock archive of the OIE Reference Laboratory National Reference Center for Leishmaniasis (C.Re.Na.L.) located in Palermo (Italy) and cultured at 25°C and pH 7.18 in RPMI-PY medium, which consisted of RPMI 1640 (Sigma R0883) supplemented with equal volume of Pepton-yeast medium [19], 10% decomplemented and ultracentrifuged fetal bovine serum (FBS), 1% glutamine, 250 μg/mL gentamicin and 500 μg/mL of 5-fluorocytosine [20]. Temperature, differentiation time, and acidification of the medium were used as variables for preconditioning of the promastigote cultures. The influence of temperature was evaluated by incubating the promastigotes from 25°C at 37°C. The conditioning time for the promastigotes varied from 24 h to 72 h and the pH was acidified with 1 N HCl to 5.4 to obtained amastigote parasites.

2.2 *Exosomes isolation*

Exosomes were isolated as described by Thery et al., (2009) [9] with modifications. To collect exosomes, after 24 h, promastigotes and amastigotes forms (~10.0×10^6 cells/mL) were removed from RPMI-PY culture media supplemented with 10% FBS by differential centrifugation. Briefly, culture medium was centrifuged subsequently for 5 min at 300 × g, 15 min at 3,000 × g, 30 min at 10,000 × g and ultracentrifuged 90 min at 100,000 × g in a Type 70 Ti, fixed angle rotor. Pelleted exosomes were washed and then resuspended in PBS. To further verify the identity of vesicles as exosomes, we isolated exosomes on a 30% sucrose/D2O cushions as described by Lamparski and

colleagues [21]. Vesicles contained in the cushion were recovered, washed several times, ultracentrifuged for 90 min in PBS and collected for use. Exosome total protein content was determined with the Bradford assay.

2.3 *Exosomes characterization*

Five µg of total promastigotes and amastigotes forms or exosome lysates were loaded on 8 % SDS-PAGE gels for western immunoblotting. After separation, the proteins were transferred to a Nitrocellulose membrane. The nitrocellulose membrane was then blocked with 5 % milk in 1× Tris-buffered saline with 0.05 % Tween 20 (TBST) for 1 h at room temperature. Antibodies against *Leishmania* HSP70 and HSP83/90 (kindly provided by Prof. Requena, Universidad Autonoma de Madrid) were then added and incubated at 4 °C overnight. The membrane was washed in TBST and incubated with Anti-rabbit IgG, HRP-linked secondary antibody for 1 h at room temperature. An enhanced chemiluminescence (ECL) system (ThermoFisher) was used to detect the blots. The size distribution and quantification of exosome preparations were analyzed by measuring the rate of Brownian motion with a NanoSight LM10 system (NanoSight, Malverne, United Kingdom) equipped with fast video capture and particle-tracking software. Purified exosomes from amastigotes and promastigotes were diluted in 500 µl of 1× PBS, 5 mM EDTA and injected into a NanoSight sample cubicle. The mean ± SD size distribution of exosomes was determined. The activity of acetylcholinesterase, an exosome marker protein, was determined as described by Savina et al. Briefly, a total of 10 µg exosomes in 100 µl of PBS and 10 µg total cell lysate were resuspended in a solution of 1.25 mM acetylthiocoline and 0.1 mM 5,5'-dithiobis (2-nitrobenzoic acid) in a final volume of 1 mL. The incubation was carried out in cuvettes at 37 °C, and the change in absorbance at 412 nm was detected at different time points (from 0 to 90 min).

2.4 *Motility assays*

Migration assays were performed in transwell chemotaxis chambers with 8 μm pore filters (NeuroProbe, Cabin John, MD, USA) [22]. Specifically, U937 cells and U937 cells differentiated into macrophages (0.5×10^6cells/ well) were resuspended in 500 μl of RPMI 1% FBS and exposed, as chemoattractant, to amastigote and promastigote exosomes (10 μg/mL) for 6 h at 37 °C. After this time, filters were removed, fixed in methanol and stained with Diff-Quick (Medion Diagnostics GmbH, Dudingen, Switzerland). Each test group was tested in three independent experiments; the number of migrating cells in five high-power fields per well were counted at 400× magnification.

2.5 ***Evaluation of cytokines production***

To evaluate the main cytokines production in cultures of macrophages infected with *Leishmania infantum* treated with *L. infantum* exosomes, the infection protocol described by Sharma et al. (2011) [23] was used, with some modifications. U937 monocytic cells (1 x 10^5 cells/mL) in the logarithmic phase of growth were plated onto chamber Lab Tek culture slides in 2.5 mL of RPMI 1640, 10% FBS, 1% glutamine medium containing 25 ng/mL of phorbol 12-myristate 13-acetate (PMA-Sigma) for 18 h to induce differentiation [24]. After incubation, the medium was removed by washing twice with RPMI-1640 medium. Non adherent cells were removed, and the macrophages were further incubated overnight in RPMI 1640 medium supplemented with 10% FBS. Then, adherent macrophages were infected with *L. infantum* promastigotes at a parasite/macrophage ratio of 5:1 for 24 h at 37 °C in 5% CO_2. After 24 h free promastigotes were removed by three extensive washing with RPMI-1640 medium, and infected macrophages were either incubated 24 h in media alone (infection control) or in media containing 10 μg/mL amastigote and promastigote exosomes. The production and release of macrophage cytokines (IL-10, IL-12, IFN-γ, IL-18, IL-1β, IL-1α, IL-4, TNF-α) with and without exosomes treatment was investigated by using Human ELISA Kits for cell culture supernatants (Sigma) following the manufacturer's protocol. The samples were read using a microplate reader Spectrostar Nano (BMG Lab Tech) 450 nm immediately.

2.6 *Evaluation of cytokines expression*

Genes expression of IL-4, IL-10, IL-1α, IL1-β, IL-12, IL-18, IFN-γ and TNF-α, were quantified with real-time PCR and β-actin was used as reference gene. After 24 h treatment with 10 µg/mL of amastigote and promastigote exosomes, total RNA was isolated from U937 cells and U937 cells differentiated, using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed by the iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, California, CA). Quantitative real-time PCR was performed using the SoFastTM EvaGreen® Supermix (Bio-Rad, Hercules, California, CA) in the StepOnePlusTM Real-Time PCR System (ABI Applied Biosystems, Foster City, CA). According to Stordeur et al. (2002), Giulietti et al. (2001) and Ramakrishna et al. (2017) [25, 26, 27] primers and taqman Probes were used to test samples in triplicate and the gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantification method (Table 1). Results were expressed as fold-increase with respect to control and plotted as mean ± SD of three independent experiments.

2.6 *Statistical analysis*

All exosomal treatment assays were performed by two observers in three replicates samples and repeated with three new batches of parasites. The mean and standard error of at least three experiments were determined. The differences between the mean values obtained for experimental groups were evaluated by the Student's t test. P-values of 0.05 or less were considered significant. Data were plotted with Microsoft Excel (Microsoft).

3 Results

3.1 *Characterization of Leishmania infantum exosomes*

Microvesicles from promastigotes and amastigotes form of *L. infantum* cells were purified on a 30% sucrose/D2O cushion. They were characterized by analysis of particle size, distribution, concentration, and presence of well-established exosomal protein markers. NanoSight nanoparticle tracking analysis revealed a mean particle diameter of 122 ± 56 nm with a mode of 94 nm for amastigotes and a mean particle diameter of 115 ± 65 nm with a mode of 76 nm for promastigotes vesicles. The presence of exosomal markers HSP70, HSP83/90 was also revealed by Western blot analyses (Fig. 1).

Acetylcholinesterase activity, a characteristic enzyme localized in exosomes, was found associated with the exosome fraction while negligible amounts were found in cells (Fig. 2). All these data demonstrated that *L. infantum* released exosomes.

3.2 *Leishmania exosomes promote migration of U937 cells and macrophages derived from U937*

Leishmania parasites are obligate intracellular pathogens that survive and replicate into host macrophages. After the initial infection, both neutrophils and macrophages migrate to the site of infection. During the infection, the migration of macrophages play an important role in spreading the disease due to the capacity of these cells to reach several sites, such as skin, mucous membranes or internal organs. In order to understand if exosomes may have a role in promoting motility of these cells, we performed a transwell motility assay. The addition of 10 µg/mL of promastigote and amastigote exosomes to the bottom wells caused a significant increase in the motility of U937 and U937 cells differentiated into macrophages, in particular with amastigote exosomes, compared to control (medium without exosomes) (Fig. 3). This data demonstrate that the chemotactic effects of exosomes on U937 and U937 cells differentiated into macrophages can facilitate the progression of infection.

3.3 *Leishmania exosomes modulate production of cytokines*

The ability of exosomes to modulate the production of cytokines involved in the immune control of leishmaniasis was investigated in U937 cell line as follow: U937 monocytes and U937 differentiated into macrophages, were incubated with 10 μg/mL exosomes for 24 h. Supernatants were collected and cytokines assayed by ELISA. Both EXOa and EXOp exosomes induced in monocytes significant increases in the production of IL-10 (3,6 pg/mL and 3,7 pg/mL respectively, fig. 4A). Another cytokine varied in culture medium was IL-18, in fact both EXOa and EXOp induced in monocytes significant reduction in the IL-18 production (CTL= 155,9 pg/mL, EXOa=40,7 pg/mL, EXOp=15,7 pg/mL, fig 4 C). In macrophage cells, a basal production of IL-10 already occurred (CTRL=164,7 pg/mL), while the treatment with the exosomes determined an increase in the production of IL-10 both for EXOp and in a more marked way for EXOa (EXOa=338,4 pg/mL, EXOp=216,5 pg/mL, fig 4 B). There was no marked difference in IL-18 production in macrophages treated with exosomes compared to CTRL (fig 4 D). Finally, EXOa and EXOp did not cause any modification in IL-12, IFN-γ, IL-4, TNF-α, IL-1 α and IL-1 β production in the cell line used. In contrast, treatment with 10 μg/mL of amastigote and promastigote exosomes for 24 h did not result in significant changes in the mRNA expression of the cytokines discussed in materials and methods (data no showed).

4 Discussion

Leishmaniasis is a zoonotic vector-borne spectrum of diseases that can manifest from self-healing ulcers to deadly hepatosplenomegalia. The manifestation of the disease is mostly dependent on the infecting parasite species but also on the host immune system and environmental factors. Successful parasitism of the host requires effective modulation of its signaling and functions. *Leishmania* lives inside the macrophage, the professional microbe killer of the immune system, operating a complex immunomodulation and immunosubversion for successful parasitization. Alteration of macrophage signaling and function occurs at various levels, from blocking signaling pathways by activating inhibitory PTPs, to degradation of key signaling molecules such as kinases,

transcription factors, translation regulators and to release different forms of membrane vesicles. These signaling modulations allow for inhibition or interference with many key macrophage functions such as production of nitric oxide (NO), reactive oxygen species (ROS), cytokines and antigen presentation, resulting in perseverance of *Leishmania* in the macrophage phagolysosome.

Pro- and anti-inflammatory cytokines play different roles in the immunopathogenesis of Leishmania infection. The cytokines production, such as IL-12, IL-1, IFN-γ, TNF-α, and/or IL-2, belonging to a subset of T helper 1 cytokine response, allow a eradication and a resistance to Leishmaniasis [28].

Recently, exosome release was investigated in different Leishmania species (i.e. *L. mexicana*, *L. donovani*, *L. major* and *L. braziliensis*). These exosomes were found to affect macrophage cell functions and were shown to be pro-inflammatory, showing the capacity to recruit neutrophils at their inoculation site [29]. However, to the best of our knowledge, exosomes released by *L. infantum* have not been studied yet. The findings reported in this study indicate that *L. infantum* release microvesicles possessing the known biochemical and morphological characteristics of mammalian exosomes. These Leishmania organelles contain exosomal markers (HSP 70, HSP 89/90), display exosome morphology (a mean particle diameter of 122 ± 56 nm with a mode of 94 nm for amastigotes and a mean particle diameter of 115 ± 65 nm with a mode of 76 nm for promastigotes vesicles) and migrate through a linear sucrose gradient in exactly the same manner as classical exosomes. Furthermore, with transwell-motility assay, we have shown the significant ability to increase the motility of U937 and U937 cells differentiated into macrophages. These data demonstrate the chemotactic effects of exosomes on U937 and U937 cells differentiated into macrophages, suggesting a role of exosomes in facilitating the progression of infection. Moreover, *L. infantum* exosomes (from both amastigotes and promastigotes) lead to a significant increase of IL-10 production in the U937 cell line (both monocytic and macrophage). There was also a significant reduction in IL-18 production in monocyte cells.

IL-10 is a cytokine produced by numerous cells of the immune system (e.g. Th1, Th2, CD8+ T, B cells, Th17 and Treg cells). The association between IL-10 and Leishmaniasis susceptibility has been confirmed in several studies. Patients with visceral Leishmaniasis (VL), showed higher IL10 serological levels as well as increases in mRNA in the target organs (e.g. spleen and liver) [30].

Belkaid et al. (2001) showed that treatment of *L. major*-infected mice with anti-IL-10 receptor antibodies led to sterile cure and parasite clearance [31]. During VL, IL-10 could cause disease promotion mainly by conditioning the host macrophages, increasing the parasite's growth and survival. In fact, the macrophage is "silenced" by the action of the cytokine, inhibiting the amastigote killing and the down regulation the production of TNF-α and NO [32, 33]. IL-18 is a pleiotropic cytokine secreted by activated macrophages, DCs and Kupffer cells whose role is not well known. IL-18 acts with IL-12 to induce the development of Th1 cells. On the other hand, IL-18 is also able to induce Th2 responses: Monforte et al. (2000) observed that in C57BL/6 mice IL-18 gene deficient (IL-18-/-) produced strong Th1 responses and therefore a high resistance to infection from *L. major* [34]. Also in other studies, the treatment of *L. major*-infected BALB/c mice with recombinant IL-18 promotes Th2 responses in the absence of IL-4 and leads to exacerbated disease in comparison with untreated animals [35]. Moreover, during L. *major* infection in BALB/c mice, which produce a Th2 response probably due to the lack of persistent IL-12 stimulation, IL-18 contributes to an increase in Th2 responses [36]. Indeed, during infection IL18 seems to carry out a Th1 or Th2 responses action based on the cytokine environment generated and host genetic background.

In conclusion, we showed that exosomes could regulate the immune system, adding to the repertoire of virulence factors involved in vector-transmitted infection. In particular at first by promoting the recruitment of the monocyte-macrophage line, and simultaneously performing a suppressive action against the immune system, inducing a IL-10 production and IL-18 suppression,

triggering a Th2 environment that allowed phagocytosis and the permanence of Leishmania parasite in the host. Since exosomes are able to contribute in the disease establishment, further studies are necessary for understanding the mechanism of infection, as they could represent a fundamental stage in preventing leishmaniasis or they could be considered an appropriate candidate for a vaccine therapy in prophylaxis and treatment.

[Abbreviations]

VL, visceral leishmaniasis; **CL**, cutaneous leishmaniasis; **MCL**, mucocutaneous leishmaniasis, **DCL**, diffuse cutaneous leishmaniasis; **MVB**, generating multivesicular bodie; **HSP**, heat shock proteins; **L.**, Leishmania; **C.Re.Na.L.**, National Reference Center for Leishmaniasis; **FBS**, fetal bovine serum; **min**, minutes; **g**, g-force; **PBS**, phosphate buffered saline; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis; **°C**, Celsius; **h**, hour; **µl**, microliter**;** **min**, minute, **µg**, microgramme; **ml**, milliliter; **mM**, millimolar; **µm**, micrometer; **PMA**, phorbol 12- myristate 13-acetate; **IL**, interleukin; **IFN**, Interferon; **TNF**, Tumor necrosis factor; **nm**, nanometer; **EXOa**, amastigote exosomes; **EXOp**, promastigote exosomes; **pg,** picogramme; **CTRL**, control; **PTP**, Protein-tyrosine Phosphatase; **SD**, standard deviation**;** **NO**, nitric oxide; **ROS**, Reactive oxygen species; **Th**, Type T helper cell, **Treg**, regulatory T cells.

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