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Extracellular Vesicles

Role in Diseases, Pathogenesis and Therapy

Edited by Manash K. Paul

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Aims and Scope of the Series

Modern physiology requires a comprehensive understanding of the integration of tissues and organs throughout the mammalian body, including the cooperation between structure and function at the cellular and molecular levels governed by gene and protein expression. While a daunting task, learning is facilitated by identifying common and effective signaling pathways mediated by a variety of factors employed by nature to preserve and sustain homeostatic life. As a leading example, the cellular interaction between intracellular concentration of Ca+2 increases, and changes in plasma membrane potential is integral for coordinating blood flow, governing the exocytosis of neurotransmitters, and modulating gene expression and cell effector secretory functions. Furthermore, in this manner, understanding the systemic interaction between the cardiovascular and nervous systems has become more important than ever as human populations' life prolongation, aging and mechanisms of cellular oxidative signaling are utilised for sustaining life. Altogether, physiological research enables our identification of distinct and precise points of transition from health to the development of multimorbidity throughout the inevitable aging disorders (e.g., diabetes, hypertension, chronic kidney disease, heart failure, peptic ulcer, inflammatory bowel disease, age-related macular degeneration, cancer). With consideration of all organ systems (e.g., brain, heart, lung, gut, skeletal and smooth muscle, liver, pancreas, kidney, eye) and the interactions thereof, this Physiology Series will address the goals of resolving (1) Aging physiology and chronic disease progression (2) Examination of key cellular pathways as they relate to calcium, oxidative stress, and electrical signaling, and (3) how changes in plasma membrane produced by lipid peroxidation products can affect aging physiology, covering new research in the area of cell, human, plant and animal physiology.

Meet the Series Editor

Prof. Dr. Thomas Brzozowski works as a professor of Human Physiology and is currently a Chairman at the Department of Physiology and is V-Dean of the Medical Faculty at Jagiellonian University Medical College, Cracow, Poland. His primary area of interest is physiology and pathophysiology of the gastrointestinal (GI) tract, with a major focus on the mechanism of GI mucosal defense, protection, and ulcer healing. He was a postdoctoral NIH fellow at the University of California and the Gastroenterology VA Medical Center, Irvine, Long Beach, CA, USA, and at the Gastroenterology Clinics Erlangen-Nuremberg and Munster in Germany. He has published 290 original articles in some of the most prestigious scientific journals and seven book chapters on the pathophysiology of the GI tract, gastroprotection, ulcer healing, drug therapy of peptic ulcers, hormonal regulation of the gut, and inflammatory bowel disease.

Meet the Volume Editor

Manash K. Paul is a Principal Investigator and Scientist at the University of California Los Angeles. He has contributed significantly to the fields of stem cell biology, regenerative medicine, and lung cancer. His research focuses on various signaling processes involved in maintaining stem cell homeostasis during the injury-repair process, deciphering lung stem cell niche, pulmonary disease modeling, immuno-oncology, and drug discovery. He is currently investigating the role of extracellular vesicles in premalignant lung cell migration and detecting the metastatic phenotype of lung cancer via machine-learning-based analyses of exosomal signatures. Dr. Paul has published in more than fifty peer-reviewed international journals and is highly cited. He is the recipient of many awards, including the UCLA Vice Chancellor's award, a senior member of the Institute of Electrical and Electronics Engineers (IEEE), and an editorial board member for several international journals.

Contents

Preface

Extracellular vesicles (EVs) are attracting scientific interest, as an increasing number of clinical studies are evaluating exosomes for diagnostic and therapeutic uses. EVs are lipid bilayer-delimited nanoscale vesicles secreted by most cells and contain cargo, including proteins, nucleic acids, lipids, and metabolites from the parent cell and mediate a horizontal transport of the cargo to recipient cells. EVs facilitate cell-to-cell contact and communication under normal and pathological conditions and play a role in the pathogenesis of many diseases. Thus, EVs have inspired a new field of research in almost every aspect of biology, including developmental, host-pathogen interactions, tissue regeneration, and cancer. This comprehensive book presents current updates in EV biology and the relationship of EVs with disease diagnosis and treatment. It delves into the biogenesis of EVs, cargo loading, composition of EVs, their interactions with cell membranes, EV isolation, and future directions to overcome current hurdles associated with liquid biopsy. It further elaborates the scientific advances in characterizing and engineering EVs for biomarker discovery and disease diagnosis, prognosis, therapeutic application, and theranostics. The book also examines the role of EVs in the comprehension of inflammation, stress resistance, and vascular integrity. Chapters address the role of EVs in embryonic development, HIV-1, reproductive issues, and associated clinical translation. Additionally, the book examines the role of EVs produced from protozoan parasites in host immunomodulation, pathogenesis, and disease progression, and presents information on novel immunotherapeutic models.

Recent studies strongly emphasize the pathogenic and translational potential of EVs in cancer. This book describes the potential diagnostic implications and molecular characterization of EVs in various cancers and concepts for using exosomes as nanocarriers for therapeutic medicines. Cancer cells actively discharge EVs (tumor-derived EVs) into biological fluids, which mediate enhanced immunosuppression, angiogenesis, metastasis, and metabolic reprogramming. Liquid biopsy has enormous promise as a diagnostic and therapeutic monitoring tool and may soon replace invasively collected tissue samples-based diagnostics. The book highlights the significance of EVs in cancer treatment resistance, especially with radiotherapy and immunotherapy, and their potential role as prognostic and diagnostic biomarkers. It also discusses the status of EVs in clinical trials in multiple cancers like breast cancer and ovarian cancer. Finally, it also identifies the loopholes for clinical translation of EVs and points out potential future research directions for therapeutic translation and cancer therapy. This book is a useful resource for biologists, clinicians, and translational scientists.

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Chapter 9

The Role of Extracellular Vesicles in Immunomodulation and Pathogenesis of *Leishmania* and Other Protozoan Infections

Zeynep Islek, Batuhan Turhan Bozkurt, Mehmet Hikmet Ucisik and Fikrettin Sahin

Abstract

Extracellular vesicles (EVs) have lately emerged as crucial mediators in parasite infections. Recent research suggests that protozoan parasites, including *Leishmania*, employ EVs as transport vehicles to deliver biologically active effector molecules such as parasitic virulence factors to modulate the host immune system and their microenvironment. The immunomodulatory effects of EVs play an essential role in the formation and progression of parasitic diseases. The immunomodulatory strategies applied by EVs of protozoan origin have similarities to the development and progression of other infections or diseases such as cancer. In this chapter, we will provide recent insights into the role of EVs in host-pathogen interactions, intercellular-communication, immunomodulation and pathogenesis of *Leishmania* and other protozoan parasites, including *Plasmodium spp., Toxoplasma spp*. and *Trypanosoma spp.* In addition, biologically inspired by the immunomodulation strategies of protozoan parasites, new immunotherapeutic models are being currently investigated to implement EVs more intensively in both therapy and diagnostics. Therefore, besides highlighting the role of EVs in protozoan infections, this chapter sheds light briefly on new immunotherapeutic approaches utilizing the strategies of protozoan EVs in medicine.

Keywords: extracellular vesicles, immunomodulation, pathogenesis, protozoan, *Leishmania*, infectious disease

1. Introduction

Cellular communication is essential for all life forms to observe, comprehend and affect their surroundings [1–6]. One pathway that cells employ for the transfer of information is the use of extracellular vesicles (EVs) – lipid-bilayered secreted vesicles that carry lipids, nucleic acids and proteins that can cause physiological changes in other cells. The use of EVs for cellular communications is a highly conserved process

of life. The EV secretion was observed in all types of cells and organisms studied up to date, including plants [7–9], prokaryotes [3, 10, 11] and protozoans [12–21]. Moreover, evidence suggests that EVs can affect cells of different species, even across different kingdoms [10, 11, 13, 16, 20]. Cross-kingdom EV interactions were shown to take part in the pathogenesis of some parasitic diseases such as those caused by protozoan parasites [22, 23].

Protozoan parasites, also known as first animals, are single-celled organisms that display diversity among unicellular eukaryotic organisms with a complex life cycle on the host system [20]. They have developed many strategies not only to provide their survival and reproduction, but also to enable the invasion into the hosts by means of immune strategies including change in host antigens, development of self-tolerance, immune inactivation, immunosuppression and intervention of molecule-mimetic mechanisms between parasites and host antigens [16, 24, 25]. Recent studies propose that the parasites actually utilize the extracellular vesicles as one infection strategy [18, 20, 21, 26–31], where the questions are arisen on how EVs modulate the host immune system and ultimately cause the infection. Based on the cell of origin, the release mechanisms of EVs from different protozoan parasites, including Apicomplexa and Kinetoplastids such as *Leishmania* species (spp.) [22, 23, 26, 32–35], *Plasmodium spp.* [31, 36–41], *Toxoplasma spp.* [36, 42, 43] and *Trypanosoma spp.* [44–49] were described, where the parasitic infections were studied in detail for leishmaniasis, malaria, toxoplasmosis and Chagas disease independently.

Among the many species and subspecies of protozoa, *Leishmania* are digenetic intracellular protozoan parasite that cause leishmaniasis through the localization either in mononuclear phagocytes of vertebrates as amastigote form or in the sandfly vector as promastigote form. There are three main forms of leishmaniasis, including a localized form- cutaneous leishmaniasis (CL) or mucocutaneous leishmaniasis (MCL), and a life-threatening form – visceral leishmaniasis (VL) (also known as "Kala-azar") [50].

The EVs released from parasites or infected cells play a significant role in host-pathogen communications and thus contribute to pathogenesis [12, 13, 15, 16, 18–21, 51]. Studies indicated that *Leishmania* exosomes can modulate the host immune system through monocyte cytokine production occurring in response to *Leishmania* infection, which in return further exacerbates *Leishmania* infection [14, 21–23, 26, 32–35, 52–54]. Likewise, Evs' role in the occurrence of infection was also confirmed later for more protozoan family members such as *Plasmodium spp.* [31, 36–41], *Toxoplasma spp.* [36, 42, 43] and *Trypanosoma spp.* [44–49], which further directed the attention of researchers on protozoan EVs and their mechanism of action.

This chapter largely focuses on the role of EVs in *Leishmania*-host interaction, immunomodulation of the host immune system by *Leishmania* EVs, manipulation of the cellular microenvironment in favor of *Leishmania* species. In addition, the role of EVs in the pathogenesis of other protozoan parasites including *Plasmodium spp., Toxoplasma spp.* and *Trypanosoma spp.* are discussed and compared at the biological level to get a better insight on strategies in immunomodulation mechanisms. At the end of the chapter, novel and potential immunotherapeutic approaches utilizing the strategies of protozoan EVs are briefly discussed.

2. Extracellular vesicles (EVs)

Extracellular vesicles are nano-sized messengers secreted by all cell types. They consist of a lipid bilayer membrane, proteins, nucleic acids and other biomolecules,

which together make up the "message" to be conveyed to other cells. The composition of molecules that control the message differs in different cell types, and under different physiological conditions.

EVs' size ranges between 20 and 1000 nm in diameter, and they can be produced through a variety of different biogenesis pathways, with different physical and structural properties. Budding from the cellular membrane generally forms larger vesicles called microvesicles – however, this biogenesis pathway may also form vesicles that are smaller than 200 nm. Small extracellular vesicles can also be formed through the invagination of the cellular membrane into endosomes, collected and secreted together in multivesicular bodies (MVBs), or so-named exosomes [55]. However, it should be noted that most of the EV isolation methods used today cannot separate exosomes from small EVs formed through membrane budding, resulting in mixed populations of EVs in the working medium. The full extent of the biogenesis pathways remains to be unknown to researchers, and this is even more apparent in non-mammalian EVs [12]. However, evidence indicates that parasites secrete EVs through both the membrane budding and the multivesicular body pathways, mimicking the previously studied EV secretion pathways of mammalian cells [45].

3. Immunomodulation and pathogenesis by EVs from *Leishmania* species and other protozoan parasites

While the study of EVs in eukaryotes other than mammalians has been gaining momentum, the methods used in these studies were developed with mammalian EVs in mind. The International Society for EVs has listed the minimal requirements for categorizing a particle as an extracellular vesicle as reporting the size distribution of the population at a single-vesicle resolution, and detecting the presence of transmembrane and cytosolic proteins in the sample while testing for a non-vesicle related protein as negative control [6, 12]. While the physical characteristics of non-mammalian EVs do not differ greatly from their mammalian counterparts, the literature lacks the necessary amount of data to decide on protein biomarkers for most non-mammalian samples. These experimental results are also required for the characterization of *Leishmania* EVs and other protozoan parasites, including *Plasmodium spp., Toxoplasma spp.* and *Trypanosoma spp.*

3.1 *Leishmania species (spp.)*

Leishmania spp. are protozoan parasites belonging to the Trypanosomatidae family in the Kinetoplastidae order, belonging to the characteristics of a kinetoplast. They are obligated intracellular parasites that primarily infect macrophages in the mammalian through the transmission of the bite of an infected sand fly and cause leishmaniasis. Moreover, they are digenetic organisms that survive and replicate either as the promastigote, i.e., the extracellular form existing in the insect midgut or as the amastigote, i.e. intracellular form lodged within phagolysosome-like vacuoles inside the macrophages [50, 56].

The promastigote form of parasites inoculate in the dermis by the bite of a sandfly (*Lutzmoyia spp., Phlebotomus spp.*) are thought to infect macrophages and/or dendritic cells (DCs) of the skin where they transform into amastigotes and might protect their host cell from apoptosis [25]. Studies have shown that exosomes released from *Leishmania spp.* promastigote and amastigotes play a crucial role in host-pathogen

interactions and intercellular communication, leading to the development of infection (pathogenesis) and immunomodulation [14, 21–23, 26, 32–35, 52–54].

3.1.1 Leishmaniasis

Leishmaniasis is a neglected tropical disease caused by vector-borne parasites of the genus *Leishmania.* There are over 20 species of *Leishmania* that cause life-threatening disorders widely distributed in 98 tropical and subtropical regions including Asia, South America, Northern Africa, Southern Europe and the Middle East. According to the recent WHO report, more than 350,000 people are estimated at risk and 1.3 million new cases of leishmaniasis occur every year [50].

Leishmaniasis can be grouped into three main clinical forms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), also known as "Kala-azar", and mucocutaneous leishmaniasis (MCL), depending on which species is involved in the infection [50]. CL is a benign but often disfiguring condition that is caused by the multiplication of *Leishmania* in the phagocytes of the skin and has a tendency toward spontaneous resolution. The coexistence of these clinical forms in the same patient is rare. MCL is a metastatic form of localized CL infections occurring during the first episode of CL within 5 years. Lymphatic or hematogenous dissemination of the amastigotes from the skin to the naso-oropharyngeal mucosa results in the destruction of the nose and mouth to the pharynx and larynx. Untreated infections can result from severe disfiguration or even death. VL is a severe condition that results from the dissemination of *Leishmania* in the phagocytes, mainly macrophages, and is fatal in almost all cases if left untreated. VL is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver and serious anemia [50].

The outcomes of the infection are highly dependent on both host and pathogen factors involved in a molecular battle where the fittest survive and continue. In this context, it is well established that macrophages play an important role in defense against various parasites by regulating their invasion and progression within the potential host. However, like other pathogens, most *Leishmania* species have developed effective strategies to circumvent the innate immune response in the early moments of infection, provided by rapidly blocking the induction and regulation of major host cell functions including nitric oxide (NO) production, tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), radical oxygen species (ROS) [57–60].

Recent studies have investigated that EVs released from *Leishmania* can involve in the pathogenesis by delivering the virulence factors – GP63, Elongation Factor 1-alpha (EF-1α) and others – to mammalian host cells, modulating their microenvironment and inferring on host signaling pathways [26, 34, 61, 62].

3.1.2 Secretion of EVs containing Leishmania proteins

EVs carry biological messages in the form of the lipids, proteins and nucleic acids they are composed of. Both the cargo enclosed within the EV and the structural molecules of the EV itself can initiate cellular responses. The lipids and membrane proteins of EVs are capable of interacting with the surface receptors of a recipient cell, allowing the EV to initiate cell-to-cell contact-dependent responses by acting as a surrogate to their cell-of-origin. Cells tailor the cargo of their EVs for them to initiate the desired response on recipient cells [55].

Protein interactions are one of the primary ways for EVs to affect target cells. Hence, the proteomic analysis of protozoan EVs becomes crucial in determining

Evs' biological functions. Proteomic analysis indicate that parasite EVs are enriched in proteases [33, 45, 63–65], stress response proteins [45, 64, 66] and transcription factors [45, 67].

One of the most common types of proteins found in parasite EVs are proteases. Proteases are a large family of hydrolytic enzymes that take part in a large majority of biological processes. Through the breakdown of specific peptides, proteases allow the activation and removal of various proteins, regulating biological reactions associated with them [68]. Proteases are considered as one of the virulence factors of parasites increasing the infectivity by inactivating the complement system and cleaving transcription factors that aid macrophage activation. *Leishmania* parasites and other trypanosomatids employ *Leishmania* virulence factors, such as metalloprotease GP63 and other immunosuppressive proteins, as well as the ER/Golgi-mediated secretion pathway to exit the host cell post-transfection [21]. An example of this process was shown with *L. mexicana*, where cysteine proteases were sorted into lysosomes and subsequently released via the flagellar pocket when they reached the Golgi apparatus [21, 29].

Initial clues for the existence of EV-mediated non-conventional protein secretion in parasites came from a study of the *Leishmania* parasites, where hydrophilic acylated surface protein B (HSAPB) was found to be present on the parasites' membrane despite not having a signal peptide, transmembrane domain or GPI-anchor site [21]. A study by Denny et al. discovered a novel sequence of 18 amino acids that act as a "special" signal peptide, which allows the transfer of the protein to the cellular membrane [21]. The study also showed that the transfer of HSAPB continued even after the transfection of mammalian cells, with the protein being observed on the cell surface. This non-conventional secretion pathway of proteins is a characteristic feature of EVs and is crucial for the ability of parasite EVs in manipulating the hosts' microenvironment.

The evidence of *Leishmania* exosome secretion was demonstrated in the study of *L. mexicana* exoproteome associated with proteases [69]; however, the first report on the certain secretion of *Leishmania* exosomes was issued by Silverman et al. [54]. Also, proteomic analysis of parasite EVs reveals that different types of proteases are among the most abundant type of proteins in their proteome [62, 64, 65]. The enrichment of proteases in EVs occurs during the entire lifecycle of the parasites during the avirulent procyclic and virulent metacyclic phases [62]. However, metacyclic parasite EVs were shown to contain a higher concentration of proteases than EVs of avirulent procyclic parasites, suggesting a link between proteases and infectivity (34). Another study showed that *Leishmania* species can also hijack host proteases through plasminogen binding proteins that bind plasmin-precursor plasminogen to the parasite cell membrane. One such plasminogen binding protein, discovered in *Leishmania mexicana* EVs, is enolase, a highly conserved EV protein that may allow immune avoidance and parasite dissemination [63].

On the other hand, the EVs of different parasites have similar physical and biochemical properties with each other as well as with EVs of mammalian origin [54]. TEM micrographs captured the secretion of *Leishmania* exosomes through the fusion of MVBs with the parasite membrane [53] and orthologues to key proteins commonly associated with EV formation, such as Rab GTPases, Alix, and ESCRT proteins were found in the proteome of *Leishmania* EVs.

Another category of proteins commonly found in parasite EVs are stress-response proteins. Parasites face various stress conditions in both their insect and vertebrate hosts, and the proteomic profile of the parasite reflects that suitably. Oxidoreductase

proteins may protect the parasite from the free radicals of the immune system [45], while chaperone proteins such as the ER chaperone glucose-regulated protein (GRP), heat shock protein 70 (HSP70) are commonly reported as upregulated in parasite EVs [45, 66]. Their presence in the EVs may be due to the elevated expression of these proteins in the parasite itself, instead of an EV-specific sorting mechanism.

Transcription and translation factors detected in parasite EVs may also have roles in parasite infectivity and resilience against stress factors [45, 67]. While it is not clear whether or not if these factors are specifically packaged into EVs for a function, or present due to their abundance in the cytoplasm, studies note that proteins such as EF 1 or 2 were shown to be pro-infective in the parasite itself [70].

A recent study indicated that *Leishmania donovani* infection led to a quantitative and qualitative change in the protein profile of EVs released by the infected macrophages, confirmed by mass spectrometry and western blot analysis. Through the protein analysis, 59 parasite-derived proteins in EVs were found, which promote angiogenesis by inducing endothelial cells to release angiogenesis-promoting mediators [32].

EVs' role in exposed drug resilience of particular strains was also investigated. *L. infantum* strains resistant to various *Leishmania* drugs were found to secrete EVs with different physical and proteomic profiles and secreted more EVs than wild-type parasites [67]. Different histone and ribosomal proteins were found to be enriched in the EVs of drug-resistant strains, which might be a non-specific adaptation of the parasite to increase its fitness in general. This knowledge may be used to diagnose whether or not a patient is infected with a drug resilient strain of the parasite, and could potentially allow identification and prediction of the drug-resistance mechanism of the strain before starting the therapy [45, 67].

3.1.3 The evidence of the EVs released from Leishmania spp.

Leishmania parasites secrete EVs both *in vitro* and *in vivo* in the sandfly midgut [53] and these EVs display immunomodulatory and signal-triggering events on the host system, associating with the parasite virulence factors. Studies with mice and immune cells showed that EVs released from *Leishmania spp.* and infected cells may affect and contribute to the clinical form and severity of the disease regarding the multitude of factors [21].

Originally, the presence of exosomes-like vesicles secreted from *Leishmania* parasites was suggested in the supernatant of infected macrophage cultures by proteomic analysis of the secretome of *Leishmania donovani* [64]. Silverman and colleagues proposed that *L. donovani* utilizes the alternative non-classical secretion pathways and targeting mechanism rather than the classical secretion signal to direct the secreted protein export [64]. Based on this study, exosomes from *Leishmania* parasites are involved in the delivery of proteins into host target cells [54, 64].

On the other hand, the first report on the release of the exosomes from the protozoan pathogens and their use as a vehicle for protein secretion and uptake by macrophages was established by Silverman et al. [30]. This study demonstrated that L. *donovani* and *L. major* can release exosomes that were detected in cytosol of the infected macrophages and selectively induced secretion of IL-8 from macrophages [30]. Furthermore, exosome release was significantly detected in the culture supernatant of *L. donovani, L. mexicana* and *L. major spp.*, under high temperature (37°C) and low pH in which condition required for promastigote differentiation into amastigotes. In another study, using *Leishmania* expressing green fluorescent protein

(GFP), they found a release of *Leishmania* GFP+ vesicles into infected cells and an uptake fluorescence vesicles by non-infected cells, with the collection of GFP and parasite proteins in structures consistent with MVBs within the cytosol of infected macrophages [30].

In addition to studies on EVs from *Leishmania* within mammalian hosts, the secretion of EVs from *Leishmania* residing within the sandfly midgut was also demonstrated by Atayde et al. [53]. Moreover, the detailed characterization of EVs isolated from infected sandfly midguts was investigated. *Leishmania* EVs isolated from infected sandfly midguts were also compared with previously described *in vitro*-isolated *Leishmania* EVs.

3.1.4 Host manipulation and immunomodulation by EVs from Leishmania spp.

Leishmania inhibits normal macrophage functions and also interferes with the innate and acquired (both cell-mediated and humoral) immunity [60]. The uptake of promastigotes by the host-immune cells involves several different strategies that allow the parasite's protective mechanism to evade their immune systems [71]. To survive and evade the host defense mechanism, transmission begins with the differentiation of the intracellular amastigote form of *Leishmania* that replicates within macrophages in the vertebrate hosts to the extracellular promastigote form in the sandfly vector [60, 72].

Briefly, the life cycle of *Leishmania* begins with an infection of the female sandflies after ingesting blood meal in *Leishmania*-infected vertebrate hosts, as illustrated in **Figure 1**. In the sandfly vector, within the midgut, ingested amastigotes proliferate and then migrate to the foregut to differentiate into metacyclic promastigotes presented on the salivary glands of the sandfly vector. Once delivered to a vertebrate host

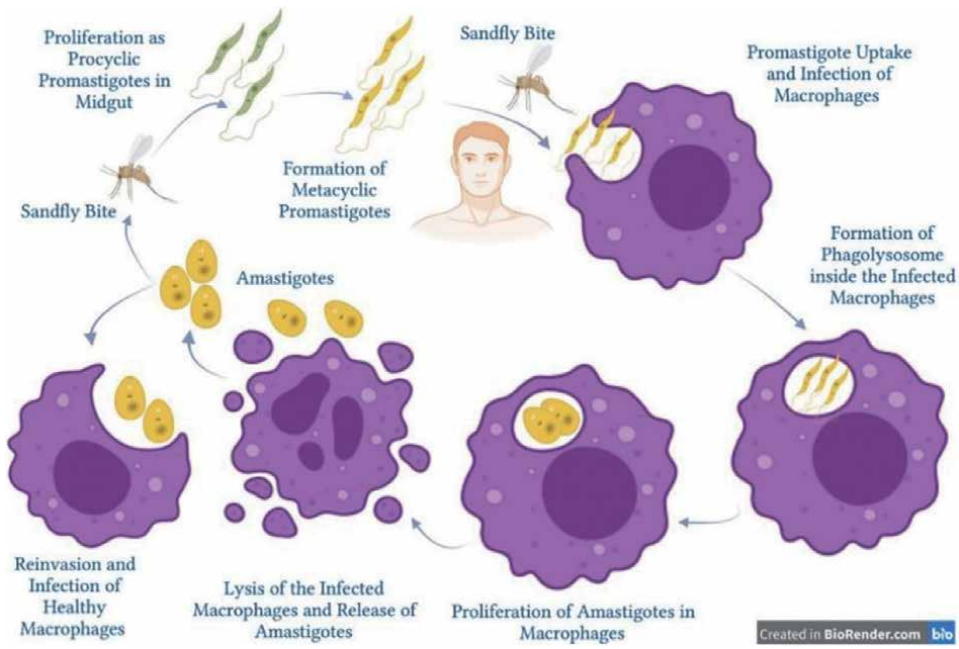

Figure 1.
The lifecycle of Leishmania parasites. Biorender software was used to create this figure under an academic license.

by the bite of an infected sandfly, promastigotes attach to phagocytic cells, macrophages, and are readily engulfed. Parasite-containing parasitophore vacuoles fuse with lysosomes forming a "phagolysosomes" in which promastigotes differentiate into the vertebrate stage, a flagellate form of amastigote [60, 73] (**Figure 1**). When a sandfly ingests a blood meal from an infected host, amastigotes differentiate back into promastigotes and become metacyclic. The metacyclic promastigotes that inoculate in the dermis by the bite of a sandfly (*Lutzmoyia spp*., *Phlebotomus spp*.) are thought to infect macrophages and/or DCs of the skin, where they transform into amastigotes into macrophages and might protect their host cell from apoptosis [74].

Once *Leishmania* metacyclic promastigotes (infective form) with sandfly saliva components are delivered into the mammalian hosts by an infected sandfly, promastigotes have to evade the complement-mediated cell-lysis before being eliminated by phagocytosis and must survive the impact of the innate immune system (**Figure 2**). For phagocytosis, macrophages are the main immune population involved in the elimination and clearance of the parasites. Although macrophages are the main host cell for *Leishmania* parasites, monocytes, DCs and neutrophils can be infected and contribute differentially to the immune response and the outcome of the infection [75] (**Figure 2**). As the first cell to be recruited to the infection site, neutrophils have delivered promastigotes to the macrophages through facilitating a silence entry, proposed as "Trojan Horse" [76] (**Figure 2**). Neutrophils infiltration and recruitment are contributed by various factors such as the leishmania chemotactic factor inducing IL-8 secretion by human neutrophils or interleukin-17 (IL-17), a hallmark of

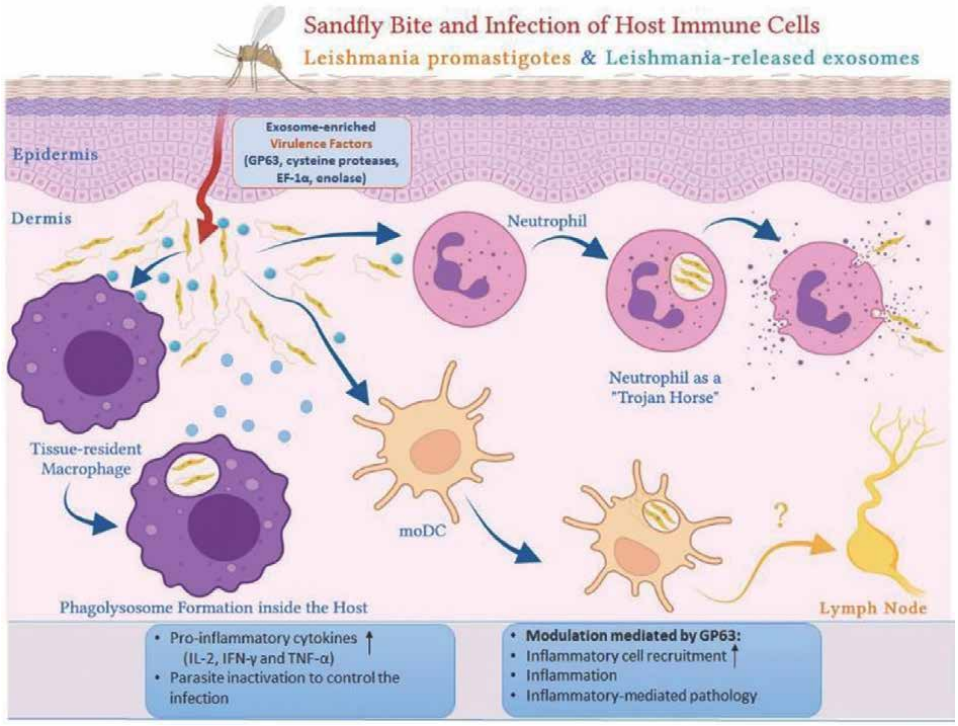

Figure 2.
The interaction of innate immune cells during Leishmania infection. Biorender software was used to create this figure under an academic license.

T helper 17 (Th17) inflammation in later phases of mucocutenous infection [77, 78]. Although parasites can readily be found in neutrophils, it is within mononuclear phagocytes that there is the best evidence for their replication and long-term survival. In a previous study, two-photon intravital imaging of mouse skin following needle injection of *L. major* has revealed that promastigotes were taken up by resident DCs like Langerhans within the first 4 h of infection and stimulating the activation of cytotoxic CD8-T cells [79]. DCs play a critical role in development of the immune response and coordinating an effector T helper 1 (Th1) adaptive immunity over the secretion of cytokines. Pro-inflammatory cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-γ) and TNF-α can activate the anti-parasitic mechanisms of the macrophages, leading to parasite inactivation and secretion of the cytokines such as IL-4, IL-5 and IL-13 to control the infection [71] (**Figure 2**). On the other hand, as the numbers of DCs and resident macrophages in the skin are too limited to sustain parasite multiplication, the progression of infection requires the recruitment of monocytes (**Figure 2**). DCs can become monocyte-derived DCs (moDCs) that express the major histocompatibility complex class II (MHC class II) molecules, which are critical for the secretion of IL-12 leading to the activation of a host-protective Th1- type response [80].

Several studies indicated that *Leishmania* exosomes can modulate monocyte cytokine production in response to *Leishmania* infection by influencing the innate and adaptive immune systems [22, 26, 30, 52, 54, 61] (**Figure 2**). Silverman and colleagues found that *L. donovani* exosomes could be predominantly immunosuppressive regarding cytokine responses on IFN-γ inhibition and IL-10 production by human moDCs [54]. In addition, exosomes released from heat shock protein 100 (HSP100) null *Leishmania donovani* in contrast to wild type *L. donovani* exosomes, are highly proinflammatory on immune cells, enabling the differentiation of naive CD4 lymphocytes into Th1 cells [54]. Similarly, pretreatment of mice with *L. donovani*- and *L. major*-released exosomes led to exacerbated infection and pathogenesis *in vivo*, related with IL-10 production and impaired generation of inflammatory Th2 cell response for parasite elimination and clearance [54].

In addition, studies on *Leishmania* EVs showed that EVs can involve in the pathogenesis by modulating the microenvironment of the mammalian hosts which is at a high temperature and a low pH than the midgut of the sandfly, and thus causing the disease [30, 61, 69]. Regarding the effect of the host microenvironment on *Leishmania* EVs, three independent studies have reported on temperature-dependent vesicle release from *Leishmania spp*. with different perspectives [30, 69, 81]. Accordingly, the release of *L. donovani* EVs was increased 3-fold by heat shocked-stationary phase promastigotes at a temperature mimicking the human body (37°C) [30]. In another study, increased temperature triggered the secretion of vesicles with the exposure of 4 h heat shocks [69]. However, contrary to temperature-induced vesicle release, Barbosa and colleagues indicated that the temperature shift (ambient temperatures of 25–26°C and 37°C) reduced the secretion of EVs from promastigotes and increasing temperature decreased parasite viability and morphology, hence affecting the release of EVs [81].

Up-regulation of EV secretion induced by infection-like temperatures suggested that these vesicles are released into the extracellular environment, before the invasion of a host such as macrophage, neutrophil, or DC occurs. These EVs may be secreted from either inoculated metacyclic promastigotes within the sand-fly salivary gland, free amastigotes in the mammalian hosts, or both [26, 32, 53, 64]. A study of Atayde et al. [53] demonstrated that *in vivo* secreted *Leishmania* EVs in the sand fly midgut

were egested by the sand fly during the bite, and these vesicles may have a role in the establishment and pathology of the CL [53]. Co-injection of mice footpads with metacyclic *L. major* promastigotes plus midgut-isolated or *in vitro*-isolated *L. major* EVs led to a significant increase in footpad swelling, and produce exacerbated lesions up to 6 weeks post-infection through over induction of inflammatory cytokines, in particular IL-17a (which is related to neutrophil infiltration) [53, 78]. On the other hand, a recent study indicates that *L. donovani* infection may promote angiogenesis by inducing endothelial cells to release angiogenesis promoting mediators including IL-8, G-CSF/CSF-3 and VEGF-A. This study shows the changes in the composition of EVs from infected cells resulted from *Leishmania* infection and suggests that EVs from infected cells could promote the vascularization in *Leishmania* infections [32].

3.1.5 Host manipulation and immunomodulatory properties of Leishmania EVs associated with parasite virulence factors

Protozoan parasites have developed numerous effective strategies to improve their protective mechanisms to escape from the immune system by modulation of the hosts' immune response and signaling pathways, as well as virulence factor secretion [20, 25, 71, 75, 82–84]. Moreover, they secrete EVs containing various parasitic factors and signaling molecules to modify the hostile microenvironment of their hosts to their benefit [26, 29, 33, 52]. By secreting EVs with proteases, parasites suppress the initial immune response raised at the point of infections for long enough to establish a foothold in their hosts [26, 29, 33, 52].

Leishmania utilizes multiple virulence factors including lipophosphoglycan (LPG) and surface acid proteinase (GP63), which trigger the modulation with the activation of protein tyrosine phosphatases (PTP), inhibition on pro-inflammatory transcription factors NF-κB, AP-1 and STAT-1 as well as other signaling molecules such as JAK-2, IRAK-1 and MAP kinases to successfully deactivate and infect on their host macrophages [52].

Together with the parasite surface molecules, multiple host cell receptors (complement receptor type 1 and type 3 (CRl, CR3), mannose-fucose-receptor, fibronectin receptor, macrophage receptor for advanced glycosylation end products) play a crucial role in the attachment and uptake of promastigotes by the immune cells [25].

Leishmania metacyclic promastigotes (infective) have to evade the complement-mediated cell-lysis via parasitic virulence factors such as GP63 and LPG, before being eliminated by phagocytosis. Moreover, they are resistant to complement activation in contrast to procyclic promastigotes (non-infective) that are extremely sensitive to the complement system, explained by the role of surface LPG. The surface LPG plays a central role in the parasite's entry and survival in host cells. In the metacyclic promastigotes, LPG is longer than non-infective procyclic forms and is almost completely absent in amastigotes, resulted in inhibiting the attachment of the C5b-C9 complement system subunits to the parasite surface [85]. In addition, surface protein kinases were indicated to phosphorylate the complement system, therefore, hampering the cascade. The surface protein, gp63, a zinc-dependent metalloprotease, is 10-fold less abundant than LPG, as an important *Leishmania* virulence factor that is expressed at the surface of the parasite via a glycophosphatidylinositol (GPI) anchor, or is directly secreted to the extracellular environment. GP63 promotes parasite survival by the stimulation of immunomodulation on the macrophages, and thus, plays a crucial role in pathogenesis. Previous studies on the action of GP63 in parasitic infections reported that GP63 can protect *L. amazonensis* and *L. major* against cell-lysis by

converting the C3b complement subunit into C3bi which accumulates on the surface of the parasites [85]. Fixation of C3 by the parasite increases the recognition of parasites by the macrophages' complement receptors 1 (CR1) and complement receptors 3 (CR3) allowing intracellular survival [86]. Thus, it appears that *Leishmania* not only inhibits activation of the lytic membrane attack complex (CSb-C9), but instead exploits C3 for "silent" invasion of host macrophages [25].

Experiments on mice and macrophages showed that these exosomes exhibit immunomodulatory activity, confirming the presence of parasite virulence factors in their content such as the surface metalloprotease GP63 [15, 26, 30, 33, 52, 54, 69, 87]. Hassani et al. previously showed that the contents of the macrophage exosomes undergo changes following LPS stimulation or *Leishmania* infection. Furthermore, they indicated that exosomes released from *Leishmania*-infected cells display unique signatures regarding composition and abundance of several functional groups of proteins such as plasma-membrane associated proteins, chaperons and metabolic enzymes [26]. In this study, surface metalloprotease GP63 was shown in the contents of the exosomes from *Leishmania*-infected macrophages, which could induce signaling molecules such as MAP kinases (except JNK) and immune-related gene expression like NF-kB associated with the immune system in naive macrophages [26]. The induction of phosphorylation of signaling proteins and translocation of activatory transcription factors into the nucleus was determined within 15 min and up to 1 h after treatment of exosomes isolated from LPS and *Leishmania*-induced macrophages and in particular in pro-inflammatory nuclear translocation of NF-kB and AP-1 and early tyrosine phosphorylation of MAP kinases ERK and P38. So, the overall effect of macrophage-infected exosomes in naive macrophages can be claimed as the down-regulation of pro-inflammatory genes and suppression of macrophage activation.

Another study comparing the EVs of wild-type and GP63-knockout *Leishmania* parasites showed the importance of GP63 in the modulation of macrophage responses [52]. While the wild-type EVs were capable of downregulating several genes associated with the immune response, GP63-knockout parasite EVs alteration of immune response genes occurred in a different pattern and had significantly reduced immunosuppressive capabilities. Furthermore, the lack of GP63 altered the proteome of EVs, suggesting that GP63 may have roles in the cargo-determinacy of parasite EVs [26, 52]. In addition, evidence suggests that exosomes secreted from *Leishmania*-infected cells containing GP63, may down-regulate the generation of specific host miRNAs and facilitate infection of the liver [87]. In one study, EVs secreted by *L. donovani* were shown to reduce miR-122 activity in hepatic cells, which reduced serum cholesterol levels and increased the infectivity of the parasite. The GP63 proteins of parasites EVs were suggested as the agent behind this alteration, as they could target the miRNA processor Dicer1 [87]. All these studies indicate that EVs from *Leishmania spp.* display a wide range of targets in mammalian hosts and, have an immune-hampering role.

3.2 Other protozoan parasites

3.2.1 Toxoplasma spp.

Toxoplasma gondii is a globally protozoan pathogen that uses felids (cats) as their primary host. When infecting other mammalians, the parasite infects the hosts' brain tissues, forming cysts. Infected rodents exhibit behavioral changes, such as reduced

aversion of felines [88]. The effects of the parasite in humans are less understood, however, studies link *T. gondii* infection with neural diseases such as Alzheimer's [89].

T. gondii EVs carry several virulence factors that aid their infectivity. In one study, complete mRNAs of neurologically active proteins, as well as various miRNAs were found in *T. gondii* EVs, which may have the capacity to affect the neural cells that they enter. The most enriched mRNA's belonged to various neurologically active proteins, Rab-13, eukaryotic translation EF 1-α1, thymosin beta 4 and LLP homolog [90]. One mRNA observed in the study, e.g. eukaryotic translation elongation factor 1, was also reported to be present in *Leishmania* EVs and associated with autism [90, 91]. Furthermore, immunoregulatory miRNA miR23-b was observed in the EVs, which regulates the secretion of IL-17. In addition to mRNA and miRNA components, *T. gondii* EVs were also shown to carry several proteins under the excreted/secreted antigens family, such as surface antigens, microneme proteins, dense granule antigens and rhoptry proteins, which are known to regulate the immune response of their hosts [42, 92].

3.2.2 Plasmodium spp.

Malaria is one of the deathliest protozoan parasitic diseases in the world and the leading cause of mortality in sub-Saharan Africa. It is caused by the family of *Plasmodium* parasites, which are spread through infected Anopheles mosquitoes, leading to fatal conditions such as cerebral malaria or severe malarial anemia. When passed to a human, the parasite infects red blood cells, allowing it to evade the immune response and penetrate deep tissues. The infected red blood cells increase vascular permeability and cause the apoptosis of endothelial cells, which both increase the severity of the disease and facilitate the spread of the parasite throughout the body.

As with other parasites, EVs secreted by malaria parasites modulate the hosts' immune system to increase the survivability of the *Plasmodium* parasite. When parasites were blocked from secreting EVs, they had reduced virulence and lessened symptoms in models of cerebral malaria [93]. Secretion of EVs continues after the infection of red blood cells. Studies show that the parasite hijacks the EV secretion in infected red blood cells, modifying their cargo. Infected red blood cells secrete EVs enriched in parasite surface antigens, and contain proteins associated with immunosuppression [94]. One study observed 120 plasmodial RNAs in infected red blood cells, which coded for proteins involved in drug resistance, as well regulatory small RNAs. The presence of these modified EVs can be used as a marker for the diagnosis of malaria [31]. In another study, infected red blood cells were shown to secrete EVs with parasite-specific proteins and RNA. Furthermore, proteins and miRNA that can alter gene expressions in endothelial cells, such as Ago2, were observed in these EVs. These infected EVs may explain malaria-associated vascular dysfunction [95].

3.2.3 Trypanosoma spp.

Trypanosomatids are insect-borne parasites that cause fatal diseases such as Chagas' disease [96] or African trypanosomiasis, "the sleeping sickness" [97]. EVs secreted by trypanosomes were shown to increase virulence in various studies. Proteins associated with metabolism, parasite survival and virulence were observed in parasite EVs [45]. In one study, EVs of *Trypanosoma brucei rhodesiense* were shown to carry serum resistance-associated protein – a key protein for human infectivity- as

well as flagellar proteins that increase virulence. Furthermore, the parasite EVs were shown to have the capacity to induce rapid erythrocyte clearance and anemia, suggesting a parasite-free pathogenesis pathway [44]. Another study observed that the parasite uses EVs to increase infectivity and survivability. Secreted vesicles enhanced parasite cyclogenesis, and lead to up to five times increased infection rates on susceptible cells [46].

4. EVs as diagnostic and therapeutic tools for protozoan parasitic infections

EVs offer exciting clinical opportunities in many diseases as diagnostic tools, drug delivery vehicles, or therapeutic agents – and parasitic infections are no exception. Both protozoan and host cell EVs are used in clinical applications against parasitic diseases. Moreover, immune cells infected with parasites also produce EVs that can induce inflammatory responses through the secretion of cytokines and chemokines *in vitro* and *in vivo* [21, 22, 54, 98, 99]. Considering their immunomodulatory effects, EVs could be potential vaccine candidates as components for infectious diseases [100–106].

EVs take part in the complex web of interactions that happen between immune cells. In particular, EV secreted by regulator immune cells like dendritic or T cells mimic the actions of their parental cell and prime the immune system against pathogens. When antigens of *L. major* are given to DCs, when administered, EVs secreted by those DCs were observed to protect mice from the parasite to great effect [100]. The EVs reduced footpad swelling and were capable of inducing antigen-specific T-cell responses [100]. A similar approach was also successful in inducing antigen-specific T-cell response against *T. gondii* [101, 102]. Using EVs instead of whole cells has several advantages, such as increased stability in freeze-thaw situations, and cannot alter their antigen-presentation, which may sometimes be the case with freeze-thawed DCs [103].

In addition to pulsing immune cells with protozoan antigens, protozoan EVs can also be used to induce the immune system, similar to vaccines. EVs from *Plasmodium yoelii*- infected reticulocytes were found to be capable of immunizing mice against the protozoan. Immunized mice were capable of producing IgG antibodies that could target the infected reticulocytes [39]. Similarly, EVs isolated from *L. amazonensis*-infected macrophages induce the production of the proinflammatory cytokines IL-12, IL-1b and TNF-α by neighboring macrophages, which contributes to modulate the immune system in favor of a Th1 immune response as well as the elimination of the *Leishmania*, and therefore, control of the infection [23].

As an image of the secreting cell, EVs have considerable potential as a diagnostic tool against parasitic diseases. The protein and miRNA cargo of EVs can allow a non-invasive biopsy of the parasite and may allow the determination of any drug resistance [104]. Regrettably, there are few examples of the use of EVs for the diagnosis of parasitic infections. One study of *Trigonoscuta cruzi* EV proteome revealed enrichment of antigen proteins used for the diagnosis of the parasite. Moreover, one category of proteins, retrotransposon hot spot proteins, do not cause any cross-reactivity with parasites of other diseases such as malaria, leishmaniasis or others, and may allow a definitive diagnosis of Chagas disease [105].

The natural ability of EVs to deliver cargo between cells gives makes them an attractive candidate for drug delivery applications. It has been shown that encapsulating

drugs within EVs may grant them cell-specific targeting, reduced toxicity, increased circulation times and increased biodistribution with the ability to pass through tissue barriers such as the blood-brain barrier. However, the field of EV-mediated drug delivery is still at its infancy [106], with few studies done on delivering anti-protozoan drugs. The one study available to the field showed that antimalarial drugs atovaquone and tafenoquine were more effective in inhibiting the growth of *P. falciparum* when loaded into vesicles isolated from malaria-infected red blood cells [38].

5. Conclusion

With the expansion of knowledge in parasitic diseases, the critical function of EVs became more evident in the development of the diseases. EVs applies many strategies not only to provide the survival and reproduction of *Leishmania* parasites inside the host, but also to enable the invasion by means of immune strategies including change in host antigens, development of self-tolerance, immune inactivation, immunosuppression and intervention of molecule-mimetic mechanisms between parasites and host antigens [16, 24, 25]. Recent studies propose that the parasites actually utilize the EVs as one infection strategy [18, 20, 21, 26–31], where the questions are arisen on how EVs modulate the host immune system and ultimately cause the infection. Based on the cell of origin, the release mechanisms of EVs from different protozoan parasites, including Apicomplexa and Kinetoplastids such as *Leishmania* species (spp.) [22, 23, 26, 32–35], *Plasmodium spp.* [31, 36–41], *Toxoplasma spp.* [36, 42, 43] and *Trypanosoma spp.* [44–49] were described, where the parasitic infections were studied in detail for leishmaniasis, malaria, toxoplasmosis and Chagas disease independently.

Several studies indicated that *Leishmania* exosomes can modulate monocyte cytokine production in response to *Leishmania* infection by influencing the innate and adaptive immune systems using parasitic virulence factors [22, 26, 30, 52, 54, 61]. Silverman and colleagues found that *L. donovani* exosomes could be predominantly immunosuppressive regarding cytokine responses on IFN-γ inhibition and IL-10 production by human moDCs [54]. In another study, macrophage-infected exosomes in naive macrophages were shown to downregulate the pro-inflammatory genes and suppression of macrophage activation [26]. Similarly, EVs secreted by the malaria parasite modulate the hosts' immune system to increase the survivability of the *Plasmodium* parasite. When parasites were blocked from secreting EVs, they had reduced virulence and lessened symptoms in models of cerebral malaria [93].

In addition to cytokine response, studies indicated that EVs can involve in the pathogenesis by modulating the microenvironment of the mammalian hosts which is at a high temperature and a low pH than the midgut of the sandfly and thus causing the disease [30, 61, 69]. Up-regulation of EV secretion induced by infection-like temperatures suggested that these vesicles were released into the extracellular environment, before the invasion of a host such as macrophage, neutrophil or DC occurs.

While EVs play such a multifaceted role in immunomodulation and disease development at protozoan diseases, the application potential of EVs as therapeutic agents or drug delivery vehicles in therapy or as a biomarker at diagnostics attracts the researchers' attention working on these fields. Considering their immunomodulatory effects, EVs could be potential vaccine candidates as components for infectious diseases [100–106] and the application of protozoan EVs in the clinic may be expected in the near future.

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Conflict of interest

No conflict of interest was declared by the authors.

Abbreviations

CRl	Complement receptor type 1
CR3	Complement receptor type 3
CL	Cutaneous leishmaniasis
DC	Dendritic cell
EF 1-α	Elongation factor 1-alpha
EVs	Extracellular vesicles
HSAPB	Hydrophilic acylated surface protein B
GFP	Green fluorescent protein
IL	Interleukin
IFN-γ	Interferon-gamma
L.	*Leishmania*
Leishmania spp.	*Leishmania species*
LPG	Lipophosphoglycan
MHC class II	Major histocompatibility complex class II
moDCs	monocyte-derived dendritic cells
MCL	Mucocutaneous leishmaniasis
NO	Nitric oxide
PTP	Protein tyrosine phosphatases
ROS	Radical oxygen species
Th1	T helper 1
Th17	T helper 17
TNF-α	Tumor necrosis factor-alpha
T. gondii	Toxoplasma gondii
VL	Visceral Leishmaniasis

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References

[1] Simons M, Raposo G. Exosomes – Vesicular carriers for intercellular communication. Current Opinion in Cell Biology. 2009;**21**(4):575-581

[2] Twu O, Johnson PJ. Parasite extracellular vesicles: Mediators of intercellular communication. PLoS Pathogens. 2014;**10**(8):E1004289

[3] Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual Review of Cell and Developmental Biology. 2014;**30**(1):255-289

[4] Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. Journal of Cell Biology. 2013;**200**:373-383

[5] Abels ER, Breakefield XO. Introduction to extracellular vesicles: Biogenesis, RNA cargo selection, content, release, and uptake. Cellular and Molecular Neurobiology. 2016; **36**(3):301-312

[6] Lötvall J, Hill AF, Hochberg F, Buzás EI, Di VD, Gardiner C, et al. Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. Journal of Extracellular Vesicles. 2014;**3**:26913

[7] You JY, Kang SJ, Rhee WJ. Isolation of cabbage exosome-like nanovesicles and investigation of their biological activities in human cells. Bioactive Materials. 2021;**6**(12):4321-4332. DOI: 10.1016/j.bioactmat.2021.04.023

[8] Savci Y, Kirbas OK, Bozkurt BT, Abdik EA, Tasli PN, Sahin F, et al. Grapefruit-derived extracellular vesicles as a promising cell-free therapeutic tool for wound healing. Food and Function. 2021;**12**(11):5144-5156

[9] Akuma P, Okagu OD, Udenigwe CC. Naturally occurring exosome vesicles as potential delivery vehicle for bioactive compounds. Frontiers in Sustainable Food Systems. 2019;**3**:23

[10] Kim KW. Visualization of extracellular vesicles of prokaryotes and eukaryotic microbes. Applied Microscopy. 2018;**48**:96-101

[11] Deatheragea BL, Cooksona BT. Membrane vesicle release in bacteria, eukaryotes, and archaea: A conserved yet underappreciated aspect of microbial life. Infection and Immunity. 2012;**80**: 1948-1957

[12] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. Journal of Extracellular Vesicles. 2018;**7**(1):1535750

[13] Coakley G, Maizels RM, Buck AH. Exosomes and other extracellular vesicles: The new communicators in parasite infections. Trends in Parasitology. 2015;**31**:477-489

[14] Lambertz U, Silverman JM, Nandan D, McMaster WR, Clos J, Foster LJ, et al. Secreted virulence factors and immune evasion in visceral leishmaniasis. Journal of Leukocyte Biology. 2012;**91**(6):887-899

[15] Silverman JM, Reiner NE. Exosomes and other microvesicles in infection

biology: Organelles with unanticipated phenotypes. Cellular Microbiology. 2011;**13**:1-9

[16] Barteneva NS, Maltsev N, Vorobjev IA. Microvesicles and intercellular communication in the context of parasitism. Vol. 4. Frontiers in Cellular and Infection Microbiology. 2013. p. 1-11

[17] Schorey JS, Cheng Y, Singh PP, Smith VL. Exosomes and other extracellular vesicles in host–pathogen interactions. EMBO Reports. 2015;**16**(1):24-43

[18] Bhatnagar S, Shinagawa K, Castellino FJ, Schorey JS. Exosomes released from macrophages infected with intracellular pathogens stimulate a proinflammatory response in vitro and in vivo. Blood. 2007;**110**(9):3234-3244

[19] Deolindo P, Evans-Osses I, Ramirez MI. Microvesicles and exosomes as vehicles between protozoan and host cell communication. In: Biochemical Society Transactions. 2013. p. 252-257

[20] Marcilla A, Martin-Jaular L, Trelis M, de Menezes-Neto A, Osuna A, Bernal D, et al. Extracellular vesicles in parasitic diseases. Vol. 3. Journal of Extracellular Vesicles. 2014. p. 1-15

[21] Atayde VD, Hassani K, da Silva Lira Filho A, Borges AR, Adhikari A, Martel C, et al. Leishmania exosomes and other virulence factors: impact on innate immune response and macrophage functions. Cell Immunol. 2016;**309**:7-18

[22] Nogueira PM, de Menezes-Neto A, Borges VM, Descoteaux A, Torrecilhas AC, Xander P, et al. Immunomodulatory properties of Leishmania extracellular vesicles during host-parasite interaction: Differential activation of TLRs and NF-κB translocation by dermotropic and viscerotropic species. Frontiers in Cellular and Infection Microbiology. 2020;**10**:1-9

[23] Cronemberger-Andrade A, Aragão-França L, de Araujo CF, Rocha VJ, Borges-Silva M d C, Figueiras CP, et al. Extracellular vesicles from Leishmania-infected macrophages confer an anti-infection cytokine-production profile to naïve macrophages. PLoS Neglected Tropical Diseases. 2014;**8**(9)

[24] Miller LH, Scott P. Immunity to protozoa. Current Opinion in Immunology. 1990;**2**(3):368-374

[25] Bogdan C, Röllinghoff M. The immune response to Leishmania: Mechanisms of parasite control and evasion. International Journal for Parasitology. 1998;**28**:121-134

[26] Hassani K, Olivier M. Immunomodulatory impact of Leishmania-induced macrophage exosomes: A comparative proteomic and functional analysis. PLoS Neglected Tropical Diseases. 2013;**7**(5)

[27] Montaner S, Galiano A, Trelis M, Martin-Jaular L, del Portillo HA, Bernal D, et al. The role of extracellular vesicles in modulating the host immune response during parasitic infections. Frontiers in Immunology. 2014;**5**(AUG)

[28] Soto-Serna LE, Diupotex M, Zamora-Chimal J, Ruiz-Remigio A, Delgado-Domínguez J, Cervantes-Sarabia RB, et al. Leishmania mexicana: Novel insights of immune modulation through amastigote exosomes. Journal of Immunology Research. 2020;**2020**:1-12

[29] Dong G, Filho AL, Olivier M. Modulation of host-pathogen communication by extracellular vesicles

(EVs) of the protozoan parasite Leishmania. Vol. 9. Frontiers in Cellular and Infection Microbiology. 2019. p. 1-9

[30] Silverman JM, Clos J, De'Oliveira CC, Shirvani O, Fang Y, Wang C, et al. An exosome-based secretion pathway is responsible for protein export from Leishmania and communication with macrophages. Journal of Cell Science. 2010;**123**(6):842-852

[31] Babatunde KA, Mbagwu S, Hernández-Castañeda MA, Adapa SR, Walch M, Filgueira L, et al. Malaria infected red blood cells release small regulatory RNAs through extracellular vesicles. Scientific Reports. 2018;**8**(1): 1-15

[32] Gioseffi A, Hamerly T, Van K, Zhang N, Dinglasan RR, Yates PA, et al. Leishmania-infected macrophages release extracellular vesicles that can promote lesion development. Life Science Alliance. 2020;**3**(12):1-16

[33] Marshall S, Kelly PH, Singh BK, Pope RM, Kim P, Zhanbolat B, et al. Extracellular release of virulence factor major surface protease via exosomes in Leishmania infantum promastigotes. Parasites and Vectors. 2018;**11**(1):1-10

[34] Santarém N, Racine G, Silvestre R, Cordeiro-da-Silva A, Ouellette M. Exoproteome dynamics in Leishmania infantum. Journal of Proteomics. 2013;**84**:106-118

[35] Pérez-Cabezas B, Santarém N, Cecílio P, Silva C, Silvestre R, AM Catita J, et al. More than just exosomes: Distinct Leishmania infantum extracellular products potentiate the establishment of infection. Journal of Extracellular Vesicles. 2019;**8**(1). 1541708. DOI: 10.1080/20013078.2018.1541708

[36] de Souza W, Barrias ES. Membrane-bound extracellular vesicles secreted by parasitic protozoa: Cellular structures involved in the communication between cells. Parasitology Research. 2020;**119**(7): 2005-2023

[37] Correa R, Caballero Z, De LLF, Spadafora C, Marti M. Extracellular vesicles could carry an evolutionary footprint in interkingdom communication. Frontiers in Cellular and Infection Microbiology. 2020;**10**(March):1-11

[38] Borgheti-Cardoso LN, Kooijmans SAA, Chamorro LG, Biosca A, Lantero E, Ramirez M, et al. Extracellular vesicles derived from Plasmodium-infected and non-infected red blood cells as targeted drug delivery vehicles. International Journal of Pharmaceutics. 2020;**587**:119627

[39] Martin-Jaular L, Nakayasu ES, Ferrer M, Almeida IC, Del Portillo HA. Exosomes from Plasmodium yoelii-infected reticulocytes protect mice from lethal infections. PLoS One. 2011;**6**(10): e26588

[40] Szempruch AJ, Dennison L, Kieft R, Harrington JM, Hajduk SL. Sending a message: Extracellular vesicles of pathogenic protozoan parasites. Nature Reviews Microbiology. 2016;**14**(11): 669-675

[41] Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, et al. Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell. 2013;**153**(5):1120-1133

[42] Silva VO, Maia MM, Torrecilhas AC, Taniwaki NN, Namiyama GM, Oliveira KC, et al. Extracellular vesicles isolated from Toxoplasma gondii induce host immune response. Parasite Immunology. 2018;**40**(9):e12571

[43] Długońska H, Gatkowska J. Exosomes in the context of Toxoplasma gondii – Host communication. Vol. 62. Annals of Parasitology. 2016. p. 169-174

[44] Szempruch AJ, Sykes SE, Kieft R, Dennison L, Becker AC, Gartrell A, et al. Extracellular vesicles from Trypanosoma brucei mediate virulence factor transfer and cause host anemia. Cell. 2016;**164**(1-2):246-257

[45] Bayer-Santos E, Aguilar-Bonavides C, Rodrigues SP, Cordero EM, Marques AF, Varela-Ramirez A, et al. Proteomic analysis of Trypanosoma cruzi secretome: characterization of two populations of extracellular vesicles and soluble proteins. Journal of Proteome Research. 2013;**12**(2):883-897

[46] Garcia-Silva MR, RFC d N, Cabrera-Cabrera F, Sanguinetti J, Medeiros LC, Robello C, et al. Extracellular vesicles shed by Trypanosoma cruzi are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. Parasitology Research. 2014;**113**(1):285-304

[47] Lovo-Martins MI, Malvezi AD, Zanluqui NG, Lucchetti BFC, Hideko Tatakihara VL, Mörking PA, et al. Extracellular vesicles shed By Trypanosoma cruzi potentiate infection and Elicit Lipid body formation and PGE2 production in murine macrophages. Frontiers in Immunology. 2018;**9**(APR):1-16

[48] de Souza W. Exosomes in the Pathogenic Protozoan Trypanosoma Cruzi. International Journal of Pathology Clinical Research. 2017;**3**(1):1-9

[49] Castillo C, Carrillo I, Liempi A, Medina L, Navarrete A, López P, et al. Trypanosoma cruzi exosomes increases susceptibility to parasite infection in human placental chorionic villi explants. Placenta. 2017;**51**:123-124

[50] Organization WH. Sustaining the drive to overcome the global impact of neglected tropical diseases. World Health Organization. 2013. 1-138 p.

[51] Schorey JS, Bhatnagar S. Exosome function: From tumor immunology to pathogen biology. Traffic. 2008;**9**: 871-881

[52] Hassani K, Shio MT, Martel C, Faubert D, Olivier M. Absence of metalloprotease GP63 alters the protein content of leishmania exosomes. PLoS One. 2014;**9**(4)

[53] Atayde VD, Aslan H, Townsend S, Hassani K, Kamhawi S, Olivier M. Exosome secretion by the parasitic protozoan Leishmania within the sand fly midgut. Cell Reports. 2015;**13**(5): 957-967

[54] Silverman JM, Clos J, Horakova E, Wang AY, Wiesgigl M, Kelly I, et al. Leishmania exosomes modulate innate and adaptive immune responses through effects on monocytes and dendritic cells. Journal of Immunology. 2010;**185**(9): 5011-5022

[55] Théry C, Zitvogel L, Amigorena S. Exosomes: Composition, biogenesis and function. Nature Reviews Immunology. 2002;**2**(8):569-579

[56] Arenas R, Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J. Leishmaniasis: A review. Vol. 6. F1000Research. 2017. p. 1-15

[57] Liew FY, Li Y, Millott S. Tumour necrosis factor (TNF-α) in leishmaniasis. II. TNF-α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology. 1990;**71**(4):556-559

[58] Samant M, Sahu U, Pandey SC, Khare P. Role of cytokines in experimental and human visceral Leishmaniasis. Vol. 11. Frontiers in Cellular and Infection Microbiology. 2021. p. 1-18

[59] Bacellar O, D'Oliveira A, Jerônimo S, Carvalho EM. IL-10 and IL-12 are the main regulatory cytokines in visceral leishmaniasis. Cytokine. 2000;**12**(8):1228-1231

[60] Novais FO, Scott P. Immunology of Leishmaniasis. In: Encyclopedia of Immunobiology. 2016. p. 114-124

[61] Silverman JM axwel., Reiner NE. Leishmania exosomes deliver preemptive strikes to create an environment permissive for early infection. Vol. 1. Frontiers in cellular and infection microbiology. 2011. p. 26

[62] Forrest DM, Batista M, Marchini FK, Tempone AJ, Traub-Csekö YM. Proteomic analysis of exosomes derived from procyclic and metacyclic-like cultured Leishmania infantum chagasi. Journal of Proteomics. 2020;**227**:1-10

[63] Figuera L, Acosta H, Gómez-Arreaza A, Dávila-Vera D, Balza-Quintero A, Quiñones W, et al. Plasminogen binding proteins in secreted membrane vesicles of Leishmania mexicana. Molecular and Biochemical Parasitology. 2013;**187**(1):14-20

[64] Maxwell MJ, Chan SK, Robinson DP, Dwyer DM, Nandan D, Foster LJ, et al. Proteomic analysis of the secretome of Leishmania donovani. Genome Biology. 2008;**9**(2):R35

[65] Lin WC, Tsai CY, Huang JM, Wu SR, Chu LJ, Huang KY. Quantitative proteomic analysis and functional characterization of Acanthamoeba castellanii exosome-like vesicles. Parasites and Vectors. 2019;**12**(1):1-12. DOI: 10.1186/s13071-019-3725-z

[66] Biyani N, Madhubala R. Quantitative proteomic profiling of the promastigotes and the intracellular amastigotes of Leishmania donovani isolates identifies novel proteins having a role in Leishmania differentiation and intracellular survival. Biochim Biophys Acta – Proteins Proteomics. 2012; **1824**(12):1342-1350. DOI: 10.1016/j.bbapap.2012.07.010

[67] Douanne N, Dong G, Douanne M, Olivier M, Fernandez-Pradaid C. Unravelling the proteomic signature of extracellular vesicles released by drug-resistant leishmania infantum parasites. PLoS Neglected Tropical Diseases. 2020;**14**:1-32

[68] Ward OP. Proteases. In: Comprehensive Biotechnology. 2011:571

[69] Hassani K, Antoniak E, Jardim A, Olivier M. Temperature-induced protein secretion by leishmania mexicana modulates macrophage signalling and function. PLoS One. 2011;**6**(5)

[70] Kushawaha PK, Gupta R, Sundar S, Sahasrabuddhe AA, Dube A. Elongation factor-2, a Th1 stimulatory protein of Leishmania donovani, generates strong IFN-$γ$ and IL-12 response in cured Leishmania-infected patients/hamsters and protects hamsters against Leishmania challenge. Journal of Immunology. 2011;**187**(12):6417-6427

[71] Gabriel ÁM, Galué-Parra A, Pereira WLA, Pedersen KW, da Silva EO. Leishmania 360°: Guidelines for exosomal research. Vol. 9. Microorganisms. 2021. p. 1-29

[72] Liévin-Le Moal V, Loiseau PM. Leishmania hijacking of the macrophage intracellular compartments. FEBS Journal. 2016;**283**:598-607

[73] Berman JD, Dwyer DM, Wyler DJ. Multiplication of Leishmania in human macrophages in vitro. Infection and Immunity. 1979;**26**(1):375-379

[74] Bates PA. Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. International Journal for Parasitology. 2007;**37**: 1097-1106

[75] Martínez-López M, Soto M, Iborra S, Sancho D. Leishmania Hijacks myeloid cells for immune escape. Vol. 9. Frontiers in Microbiology. 2018. p. 1-16

[76] Laskay T, Van Zandbergen G, Solbach W. Neutrophil granulocytes – Trojan horses for Leishmania major and other intracellular microbes? Trends in Microbiology. 2003;**11**: 210-214

[77] Pedraza-Zamora CP, Delgado-Domínguez J, Zamora-Chimal J, Becker I. Th17 cells and neutrophils: Close collaborators in chronic Leishmania mexicana infections leading to disease severity. Parasite Immunology. 2017;**39**(4)

[78] Boaventura VS, Santos CS, Cardoso CR, De Andrade J, Dos Santos WLC, Clarêncio J, et al. Human mucosal leishmaniasis: Neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. European Journal of Immunology. 2010;40(10):2830-2836

[79] Kaye P, Scott P. Leishmaniasis: Complexity at the host-pathogen interface. Nature Reviews Microbiology. 2011;**9**:604-615

[80] León B, López-Bravo M, Ardavín C. Monocyte-derived dendritic cells formed at the infection site control the induction of protective T Helper 1 responses against Leishmania. Immunity. 2007;**26**(4): 519-531

[81] Barbosa FMC, Dupin TV, Toledo M d S, Reis NF d C, Ribeiro K, Cronemberger-Andrade A, et al. Extracellular vesicles released by Leishmania (Leishmania) amazonensis promote disease progression and induce the production of different cytokines in macrophages and B-1 Cells. Frontiers in Microbiology. 2018;**9**:3056

[82] Rossi M, Fasel N. How to master the host immune system? Leishmania parasites have the solutions! International Immunology. 2018;**30**(3):103-111

[83] Gupta G, Oghumu S, Satoskar AR. Mechanisms of Immune evasion in Leishmaniasis. Advances in Applied Microbiology. 2013. p. 155-184

[84] Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. Cellular and Molecular Life Sciences. 2013;**71**(7): 1245-1263

[85] Puentes SM, Da Silva RP, Sacks DL, Hammer CH, Joiner KA. Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. Journal of Immunology. 1990;**145**(12):4311-4316

[86] Mosser DM, Edelson PJ. The third component of complement (C3) is responsible for the intracellular survival of Leishmania major. Nature. 1987;**327**: 329-331. DOI: 10.1038/327329b0

[87] Ghosh J, Bose M, Roy S, Bhattacharyya SN. Leishmania donovani targets dicer1 to downregulate miR-122, lower serum cholesterol, and facilitate murine liver infection. Cell Host & Microbe. 2013;**13**(3):277-288

[88] Tenter AM, Heckeroth AR, Weiss LM. Toxoplasma gondii: From animals to humans. International Journal

for Parasitology. 2000;**30**(12-13): 1217-1258

[89] Kusbeci OY, Miman O, Yaman M, Aktepe OC, Yazar S. Could Toxoplasma gondii have any role in Alzheimer disease? Alzheimer Disease and Associated Disorders. 2011;**25**(1):1-3

[90] Pope SM, Lässer C. Toxoplasma gondii infection of fibroblasts causes the production of exosome-like vesicles containing a unique array of mRNA and miRNA transcripts compared to serum starvation. Journal of Extracellular Vesicles. 2013;**2**(1):22484

[91] McLachlan F. Investigating the role of eukaryotic translation elongation factor eEF1A2 in autism, epilepsy and intellectual disability. The University of Edinburgh; 2020

[92] Quiarim TM, Maia MM, da Cruz AB, Taniwaki NN, Namiyama GM, Pereira-Chioccola VL. Characterization of extracellular vesicles isolated from types I, II and III strains of Toxoplasma gondii. Acta Tropica. 2021;**219**:105915

[93] Debs S, Cohen A, Hosseini-Beheshti E, Chimini G, Hunt NH, Grau GER. Interplay of extracellular vesicles and other players in cerebral malaria pathogenesis. Biochim Biophys Acta (BBA)-General Subj. 2019; **1863**(2):325-331

[94] Mantel P-Y, Hoang AN, Goldowitz I, Potashnikova D, Hamza B, Vorobjev I, et al. Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host & Microbe. 2013;**13**(5):521-534

[95] Mantel P-Y, Hjelmqvist D, Walch M, Kharoubi-Hess S, Nilsson S, Ravel D, et al. Infected erythrocyte-derived extracellular vesicles alter vascular function via regulatory Ago2-miRNA complexes in malaria. Nature Communications. 2016;**7**(1):1-15

[96] Rassi A Jr, Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010;**375**(9723): 1388-1402

[97] Brun R, Blum J, Chappuis F, Burri C. Human african trypanosomiasis. Lancet. 2010;**375**(9709):148-159

[98] Castelli G, Bruno F, Saieva L, Alessandro R, Galluzzi L, Diotallevi A, et al. Exosome secretion by Leishmania infantum modulate the chemotactic behavior and cytokinic expression creating an environment permissive for early infection. Experimental Parasitology. 2019

[99] Gupta A, Pulliam L. Exosomes as mediators of neuroinflammation. Journal of Neuroinflammation. 2014;**11**:1-10

[100] Schnitzer JK, Berzel S, Fajardo-Moser M, Remer KA, Moll H. Fragments of antigen-loaded dendritic cells (DC) and DC-derived exosomes induce protective immunity against Leishmania major. Vaccine. 2010;**28**(36): 5785-5793

[101] Aline F, Bout D, Amigorena S, Roingeard P, Dimier-Poisson I. Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. Infection and Immunity. 2004;**72**(7):4127-4137

[102] Beauvillain C, Ruiz S, Guiton R, Bout D, Dimier-Poisson I. A vaccine based on exosomes secreted by a dendritic cell line confers protection against T. gondii infection in syngeneic and allogeneic mice. Microbes and Infection. 2007;**9**(14-15):1614-1622

[103] Lener T, Gimona M, Aigner L, Börger V, Buzas E, Camussi G, et al. Applying extracellular vesicles based therapeutics in clinical trials–An ISEV position paper. Journal of Extracellular Vesicles. 2015;**4**(1):30087

[104] Wu Z, Wang L, Li J, Wang L, Wu Z, Sun X. Extracellular vesicle-mediated communication within host-parasite interactions. Frontiers in Immunology. 2019;**9**:3066

[105] Bautista-López NL, Ndao M, Camargo FV, Nara T, Annoura T, Hardie DB, et al. Characterization and diagnostic application of Trypanosoma cruzi trypomastigote excreted-secreted antigens shed in extracellular vesicles released from infected mammalian cells. Journal of Clinical Microbiology. 2017;**55**(3):744-758

[106] Elsharkasy OM, Nordin JZ, Hagey DW, de Jong OG, Schiffelers RM, Andaloussi S EL, et al. Extracellular vesicles as drug delivery systems: Why and how? Vol. 159. Advanced Drug Delivery Reviews. 2020. p. 332-343