The impact of extracellular vesicles on parasite-host cell interactions: Searching for biomarkers and new anti-parasite strategies.

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ABSTRACT

Extracellular Vesicles (EVs) are released by a wide number of cells including blood cells, immune system cells, tumor cells, and adult and embryonic stem cells. EVs are an heterogeneous group of vesicles (~30-1000 nm) known by several different names including: microparticles, microvesicles, ectosomes, shedding vesicles or exosomes. The various roles of EVs during parasite-host cell interaction have been described recently in several diseases, bringing to the fore a novel concept in cell communication between parasites and host cells. The physiological release of EVs represents a normal state of the cell raising a metabolic equilibrium between catabolic and anabolic processes. Moreover, when the cells are submitted to stress with different inducers or in pathological situations (malignances, chronic diseases, infectious diseases.), they respond with an intense and dynamic release of EVs. The EVs released from stimulated cells versus those that are released constitutively may themselves differ, both physically and in their cargo. EVs contain protein, lipids, nucleic acids and biomolecules that can alter cell phenotypes or modulate neighboring cells. In this review, we have summarized ~~most of the~~ findings involving EVs in certain ~~specific~~ protozoan diseases. We have commented on strategies to study the communicative roles of EVs during parasite host cell interaction and hypothesized on the use of EVs for diagnostic, preventative and therapeutic purposes in infectious diseases. This kind of communication could modulate the innate immune system and reformulate concepts in parasitism. Moreover, the information provided within EVs could provide alternatives in translational medicine.

KEY FINDINGS

Extracellular vesicles (exosomes and microvesicles) have different biogenesis and can be released constitutively or under stimulation during host pathogen interactions.

Extracellular vesicles contain protein, lipids, nucleic acids and biomolecules that participate in cellular communication with other cells.

Extracellular vesicles have a potential diagnostic value.

INTRODUCTION

Neglected diseases, including leishmaniasis and Chagas disease, cause many thousands of deaths per annum, and are prevalent in several regions of the world, but mainly in underdeveloped regions, associated with poverty, in Asia, Africa and the Americas (Hotez, 2017). The World Health Organization (WHO) have implemented various strategies to combat these diseases, including treatments, surveillance, improvement of housing, and vector control. These have reduced significantly the prevalence allowing prediction and may result in the elimination of some diseases, such as human African trypanosomiasis, in the next years (WHO, 2016).

Chemotherapy against protozoan parasites is a field that needs to improve and different current challenges are being considered. Several drugs have been used for decades, but they have limited efficiency, because of the development of drug resistance, toxicity issues for patients, and because their administration needs supervision thus incurring high costs for the health system (Klokousas et al, 2003; Hart et al, 2017; Menegon et al, 2016). Hopefully, new antiprotozoan drugs will be made available in the next few years to follow the success of Artemisinin for malaria.

Efforts have been made in the post-genomic era to elucidate metabolic pathways in parasites with the aim of discovering specific targets that may be important for putative

chemotherapies and also to improve current drugs used as treatments (Lechartier et al, 2014; reviewed by Weigelt, 2010).

The challenge for improving therapies is a better understanding of host-parasite interactions with an improved view on parasite adaptation and how the pathogen is able to manipulate its host environment. In recent decades, the research in this area has largely focused on the biomolecules secreted by parasites, many of which down-modulate host immune responses (reviewed in Evans-Osses et al, 2015). Despite the progress in this field and the description of secreted products in different parasitic infections (Kaur et al, 2001; Coakley et al, 2017; Grébaut et al, 2009), little is known about the mechanism(s) that regulate their interaction during host-parasite interaction,.

The current decade has seen immense activity in extracellular vesicle research, with some suggesting that EV release is the main process for modifying the phenotype of neighboring cells. (Szempruch et al, 2016; Kim et al, 2016; Buck et al, 2014). Physiological release of EVs represents a normal state of the cell ~~raising a metabolic equilibrium or steady state between catabolic and anabolic processes.~~ When the cells are submitted to stress with different inducers or in pathological situations (malignances, chronic diseases, infectious diseases, etc), they response with an intense and dynamic release of EVs (Cocucci & Meldolesi, 2015).

Although there is a plethora of literature describing the cargo or content of EVs, there are very few reports giving an exact description of the mechanism of their release or providing a clear understanding of the role of EVs in cell-cell communication.

The concept that EVs represent intercellular communicative vectors is based on the idea that the cells release a compartmentalized cargo with proteins, lipids, nucleic acids, and biomolecules for uptake and integration into other cells. The intense flux between cells of EVs has been described intensively in recent years in many biological systems and we have summarized the findings in host-parasite interactions in table 1. With the increasing research in this field, more information has been obtained in characterization of EVs by the proteomic analysis of EVs and description of microRNAs contained in

exosomes or microvesicles (MVs) from a variety of cell types (Zhang et al, 2015; Eirin et al, 2014; Alegre et al, 2014). An integrated platform with the data obtained from proteomics results is available in Vesiclepedia (Kalra et al, 2012).

During host-pathogen interaction, protozoan cells employ a vast set of evasion mechanisms to resist the attack of the immune system to penetrate into the organism and establish the infection. In recent years EVs have been raised as a new element of pathogens' evasion mechanisms and modulate parasite-host cell interaction (reviewed by Evans-Osses et al, 2015; Coakley et al, 2017). In this review, we summarize the role of EVs in host-parasite interaction in parasite diseases caused by protozoan parasites. However, our focus is to discuss the application of the knowledge learned in EVs in different models to develop alternatives to diagnostic, vaccine and translational medicine.

EXTRACELLULAR VESICLES HAVE DIFFERENT MODES OF BIOGENESIS

Exosomes and Microvesicles ~~(also called microparticles, ectosomes)~~ are released from cells by energy dependent processes display differences in biogenesis, size, function and cargo. Exosomes (30–100 nm in diameter) are vesicles formed upon inward budding of endosomes resulting in intraluminal vesicles, within multivesicular bodies; exosomes are then released by exocytosis within the secretory pathway (reviewed by Evans-Osses et al, 2015). Exosomes contain proteins derived from their cell of origin enriched for MHC class I and II, as well as heat-shock proteins and other proteins. MVs however are not derived from endocytosis, forming instead from a budding of the plasma membrane, occurring in response to activation of cellular processes (Silva et al, 2017), in a Ca^{2+} -dependent manner , and their size varies (100 nm–1 μm). Briefly, external stimuli, such as interaction with pathogen membranes, or some kind of cell damage result in Ca^{2+}influx to the cytoplasm or its release from internal sources. The rise in intracellular Ca^{2+} activates the calpain-mediated cleavage of the actin cytoskeleton. Flippase and floppase are then inhibited and scramblase is activated transporting the

negatively charged phospholipids from the inner to the outer leaflet of the plasma membrane (Fujii et al, 2015), resulting in MV formation with phosphatidylserine exposure. MV content is a reflection of the cellular state (Stratton et al, 2015) and of the topographic region of the plasma membrane where it was formed (Figure 1). Isolation of EVs involved in host-pathogen interaction can be performed from several sources, such as cell-protozoan *in vivo* or *in vitro* interaction/infection and also from parasite axenic culture. In addition to the *in vitro* approaches, vesicles can be obtained from patients' and from laboratory animals' biofluids. Researchers use several techniques for the isolation of EVs, the most common and best accepted being differential centrifugation and size exclusion chromatography (Ramirez et al., 2017). Detection of EVs is based on their biophysical properties and marker identification. The most common detection procedures are Western blotting, nanoscale tracking analysis and electron microscopy (Gardiner et al, 2016). In addition, protein quantification assays can be used to estimate the protein concentration of EVs.

During the long evolution of protozoan species, many pathogens have evolved to invade a wide range of hosts. Some of these needed to infect host cells to complete the life cycle and produce an infection. Pathogens need to interact with the immune system using an array of mechanisms to avoid host immune recognition and effector systems. The production of EVs is one of these strategies. Protozoan EVs could be involved in invasiveness, innate recognition, such as the complement system, immunomodulation and other processes, and this vesicle flux is essential to understand its pathogenicity. While there is much to be learned about host-parasite interactions, exosomes and MVs are involved in the persistence of parasite populations within the host.

INTRACELLULAR PARASITES

Leishmania sp.

Leishmania parasites,the causative agents of leishmaniasis, are spread by the bit of phebotomine sand flies and the disease manifests itself differently in people. The first

158 suggestion of the release of exosome-like vesicles by *Leishmania* was a description of
159 a large number of known eukaryotic exosomal proteins in *Leishmania* conditioned
160 medium, suggesting a vesicle-based secretion system (Silverman et al. 2008). Later,
161 other authors confirmed these findings (Silverman et al. 2010) showing that the release
162 of leishmanial exosomes is upregulated by infection-like stressors (37°C; ± pH 5.5), also
163 altering the quantity of vesicles and their protein composition. In the same work, the
164 uptake of GFP+ exosomes by infected and non-infected macrophages was observed,
165 and Leishmania exosomal proteins HSP70 and HSP90 detected in the cytosol of
166 infected macrophages with specific antibodies. The selective induction of IL-8 secretion
167 in a dose-dependent manner in macrophages treated with exosomes points to an
168 exosomal delivery of molecular messages to infected as well as neighboring uninfected
169 macrophages. It has also been shown that , the protein content of purified exosomes
170 released by macrophages infected with *Leishmania mexicana* promastigotes displays a
171 unique composition and abundance of functional groups of proteins, such as plasma
172 membrane-associated proteins, chaperones and metabolic enzymes compared to the
173 exosomal content of macrophages exposed to LPS and exosomal-free medium (Hassani
174 & Olivier 2013). Macrophages exposed to *Leishmania* release exosomes containing
175 parasite surface protease GP63 that can modulate macrophage protein tyrosine
176 phosphatases and transcription factors in a GP63-dependent manner, playing a notable
177 role in dampening the innate inflammatory response (Hassani et al. 2014). With a
178 different focus, total *Leishmania* RNA was compared with exosomal RNA (Lambertz et
179 al. 2015) and it was shown that exosomes are selective and specifically enriched in small
180 RNAs derived almost exclusively from non-coding RNAs, which could have regulatory
181 functions in cells, influencing host-parasite interactions. The expression and function of
182 an *L. major* phosphatase, LmPRL-1, that participates in the intracellular survival of the
183 parasites inside macrophages was characterized recently (Leitherer et al. 2017) and it
184 has been shown that this protein is secreted mostly inside exosomes .
185

186 *Trypanosoma cruzi*

187 Recognized by WHO as one of the world's 13 most neglected tropical diseases, Chagas
188 Disease is caused by the protozoan *Trypanosoma cruzi* and represents a relevant social
189 and economic problem mainly in Latin American countries. *T. cruzi* is able to invade most
190 eukaryotic cells and has a complex life cycle involving mammalian hosts and insect
191 vectors (WHO, 2002). The release of EVs has been described in epimastigote,
192 metacyclic and tissue-derived trypomastigote stages of *T. cruzi*. These EVs may be
193 releasedspontaneously or upon activation and they are able to interact with host cells
194 (Gonçalves, M. F. 1991; Neves, 2014; Silveira, 1979; Cestari, 2012; Bayer-Santos,
195 2013). Ramirez et al (2017) showed that vesicles obtained by the interaction of parasites
196 with THP-1 monocytes cells contain components of mutual origin . Analysis of purified
197 vesicles isolated from the supernatant of infected VERO (African Green Monkey kidney
198 fibroblast-like) cells indicated that only ~10% of the total proteins detected were of *T.*
199 *cruzi* origin. Also, it was described that cells infected with metacyclic trypomastigotes
200 shed vesicles containing GP82, transialidases, gp63, and other parasite proteins
201 (Bautista-lópez et al. 2017; Bayer-santos et al. 2013). It has been suggested a relevant
202 role of EVs in interfering with host cell dynamics even before parasite-cell contact is
203 established. Besides the fact that vesicles isolated from trypomastigote cultures induce
204 different levels of proinflammatory cytokines and nitric oxide by macrophages in a
205 heterogeneous manner (Nogueira et al, 2015), the effect of host modulation was shown
206 in several studies. Microvesicles can also act as an immune evasion mechanism and
207 result in increased parasite survival. For example, vesicles were shown to form a
208 complex with C3 convertase, the central enzyme of the complement system, leading to
209 the decay of the enzyme on the parasite surface. To escape the immune system, *T. cruzi*
210 could use microvesicles containing TGF-β, promoting an increase in the number of
211 intracellular parasites per cell (Cestari et al, 2012; Ramirez et al, 2017). Interestingly, the
212 phenomenon of increased infectivity and inhibition of the complement system appears

Comment [JI1]: This sentence needs complete rewording. It does not make sense.

Comment [JI2]: Please reword. As above.

to be class specific, since vesicles derived from parasites of one class did not alter complement resistance and the invasion process of parasites from the other class (Wyllie & Ramirez, 2017). In vivo, it has been shown that mice previously inoculated with EVs derived from pathogen- host cell interaction from some metacyclic forms, upon receiving a challenge with metacyclic trypomastigotes forms from the same strain of *T cruzi,* the parasitemia increases in mice (Cestari et al, 2012; Ramirez et al, 2017). However when mice were given an injection of a vesicle fraction prior to *T. cruzi* infection with tissue culture-derived trypomastigotes, this led to increased death and development of a more severe pathology compared to controls (Trocoli Torrecilhas et al, 2009). This consolidates the concept that vesicles induced by parasites contribute to a pro-parasitic environment.

Comment [B3]: Professor, favor modificar.

Plasmodium sp.

Malaria is a disease transmitted by *Anopheles* mosquitoes caused by protozoan parasites of the genus *Plasmodium* that infect erythrocyte and hepatocyte cells and was responsible for 212 million new infections and 429 000 deaths worldwide only in 2015. Although malaria can be a deadly disease, illness and death from malaria can usually be prevented (CDC, 2015). *Plasmodium falciparum* parasites directly communicate with other parasites and host cells using exosome-like vesicles that carry parasite proteins and antigens (Martin-Jaular et al, 2011). These are able to deliver genes, as verified by their capacity to transfer drug resistance to parasites (Regev-Rudzk et al, 2013). Extracellular vesicles promote differentiation of gametocytes, the sexual parasite stage for disease transmission to mosquito vector (Regev-Rudzk et al, 2013; Mantel et al, 2013).

Extracellular vesicles in *Plasmodium* infection can activate host immune cells and induce macrophage to produce proinflammatory cytokines IL-6, IL-12, and IL-1 βand the anti-

inflammatory cytokine IL-10 in a dose-dependent manner (Mantel et al, 2013). Red blood cells parasitized with *Plasmodium* produce 10 times greater numbers of EVs than unparasitized cells (Nantakomol et al, 2011) and infected patients have higher frequencies of plasma circulating vesicles compared to healthy controls (Campos et al, 2010). The pathogenic role of EVs was studied *in vivo* in mice during *Plasmodium* infection, where the peak of plasma EVs coincided with the appearance of neurological manifestations of cerebral malaria (CM). In this model, fluorescently labelled EVs from mice with CM were transferred into infected mice and were shown to be attached to the endothelium of brain vessels. EVs transferred from activated endothelial cells into healthy recipient mice could induce CM-like histopathological anomalies in brain (El-Assaad et al, 2014). The relation between EV levels and pathology was demonstrated in patients by Nantakomol et al (2011), whose group showed that plasma red blood cell–derived 'microparticle' concentrations were increased in patients with falciparum malaria in proportion to disease severity. Another study found increased numbers of circulating endothelial-derived vesicles in children with coma and severe malaria. In this work it was also noticeable that during convalescence of the infection the number of endothelial-derived vesicles was significantly less than in the acute stage among patients with cerebral malaria or coma and severe anemia (Combes et al, 2004).

Toxoplasma gondii

Toxoplasmosis is caused by *Toxoplasma gondii*, an intracellular protozoan that infectsmost nucleated cells found worldwide. *T. gondii* infection may be asymptomatic in immunocompetent individuals, but may cause severe, life-threatening illness in immunocompromised individuals and fetal complications if the mother experiences primary infection during pregnancy (Beauvillain et al. 2009). Knowledge about exosomes secreted by *T. gondii*, or by cells infected by the protozoan is still very limited (Dlugonska & Gatkowska, 2016; Wowk et al, 2017). Pope & Lässer (2013) hypothesize that

exosome-like particles derived from *T. gondii*-infected fibroblasts could participate in the pathogenesis of the parasite, and could be responsible for increasing mRNA levels associated with neurological activity (Rab-13, thymosin) and their transfer to uninfected cells. The first proteomic profile from EVs of *T. gondii,* obtained from infected human foreskin fibroblast, reveals biomolecules already described in other models, such as CD63, HSP 70, and calcium-binding proteins (Wowk et al, 2017). Kim et al. (2016) observed changes in the proliferation of myoblasts treated with exosomes derived from infected cells (increase in the S phase).

EXTRACELLULAR PARASITES

Giardia intestinalis

G. intestinalis is one of the most prevalent gastrointestinal pathogens on the planet that produce a diarrhoea with low inflammation and in some cases a chronic infection. The replicative form trophozoites need to adhere to the small intestine mucosa for survival. Secretory products involved in the communication between leukocytes by parasites was reported by Lee et al (2012): Trophozoites of *G. intestinalis* stimulated the production of IL-8 which wase responsible for the recruitment of neutrophils. As reviewed by Evans-Osses *et al* (2015), it is suggested that the EVs represent an important form of communication and immunomodulation. Evans-Osses et al. (2017) identified the release of MVs from *G. intestinalis*, for the first time. The release of MVs may vary at different pH conditions (7.0 being the best condition) and time, and their release increases the adhesion capacity of the protozoa in Caco-2 (Caucasian colon adenocarcinoma cells) and their uptake and maturation by human dendritic cells. The pathogenesis of *Giardia* is not yet fully understood. Kho et al (2013) demonstrated that HCT-8 (ileocecal colorectal adenocarcinoma cells) infection could induce apoptosis, with signs of chromatin condensation and activation of caspase-3. It was demonstrated that extracts

of parasites in contact with Caco-2 induced apoptosis, showing that the presence of the parasite is not necessary to start the process and suggesting that it could be dependent on EVs.

Trypanosoma brucei

Nten et al (2010) identified for the first time that secretomes of *T. brucei* (*T. brucei brucei*, *T. brucei gambiense*) are associated with the pathway of exosomal biogenesis, being composed of proteins associated with pathological processes and the evasion of the immune system (metalopeptidases, Gp63 protease). Eliaz et al. (2017) demonstrated that exosome secretion was inhibited in *T. brucei* when transcription is inhibited (*Vps 36*), but production of exosomes continued in nanotubes. In addition, EVs could interfere in the social motility of parasites, repelling individuals from unfavorable conditions. Using time-lapse microscopy, it was demonstrated that exosomes were secreted for the transmission of stress signals. Szempruch et al (2016) concluded that exosomes contain mostly flagellar and membrane proteins, such as surface glycoproteins and HSP-70. These particles merge with erythrocytes, causing a decrease in circulation, and possibly resulting in host anemia. Furthermore, *T. brucei rhodesiense* can transfer vesicles containing serum resistance-associated proteins to non-human trypanosomes, allowing immune system evasion. A number of proteins contained in exosomes of *T. brucei gambiense* (HSP-70, RAB protein, α and β tubulins, heavy chains of clathrin, histamine) have been identified (Geiger et al. 2010), with physiological functions not only for survival in the host, but also for immunomodulation and intercellular communication. Enzymes involved in nucleotide metabolism were also identified, which could influence the inflammatory process.

Trichomonas vaginalis

Trichomonas vaginalis colonizes the human urogenital tract producing sexually transmitted disease known as trichomoniasis. Twu et al. (2013) identified for the first time the participation of *T. vaginalis* exosomes in immunomodulation and host-parasite communication, allowing an improvement of adhesion when exosomes from highly adhesive parasites were incubated with a less adhesive parasite. Olmos-Ortiz et al (2017) observed the immunomodulatory effects of *T. vaginalis* exosomes on murine macrophages. These vesicles induced an increase of IL-10, Il-6, TNF-α expression, and nitric oxide production (cytotoxic and immunomodulatory activity). In addition, infected mice treated with exosomes increased IL-10 production and decreased IL-17 levels, resulting in the diminution of the inflammatory process without a reduction in parasitic load.

WHAT IS THE NEXT STEP: TRANSLATIONAL APPLICATIONS.

The apparent role of EVs in a large number of biological processes, along with many of their intriguing features, forms the basis of extending EV analysis beyond basic research and into the clinical and therapeutic context (Revenfeld et al, 2014). EV isolation methods should be more specific, to ensure safe therapeutic possibilities (Momem-Heravi et al, 2014; Alvarez-Erviti et al, 2011; Zhou et al, 2013). In this regard, the development of portable point-of-care diagnostic tools for detecting circulating exosomes as biomarkers, should be important in the future. Although much effort has been employed to understand the biology of EVs, we still need more experimental advancements to develop applied methods for the community. The need for such diagnostic tools in developing countries are very important as many are burdened by parasitic diseases, and lack professional and material resources.

While naturally secreted exosomes may mediate beneficial effects in certain disease conditions, targeted exosomes loaded with therapeutic molecules may optimize efficacy while also reducing off-target delivery (Barile & Vassali, 2017; Moore et al., 2017) The

lipid composition of their membranes may also increase antigenic stimulation (Zitvogel et al, 1998; Escudier et al, 2005). It is now imperative that the findings from basic research are translated into biotechnological applications with greater urgency (Fontana et al, 2012).

Vaccines

Among numerous biomedical applications, the use of EVs in immunization may be explored in the future. The way EVs interact in antigen presentation allows the possibility of its use in developing a T cell-dependent response. In the past decade, it is laudable that philanthropic initiatives have been involved in the production of vaccines, since diseases caused by unicellular eukaryotes are a major burden to tropical developing countries.

The genome of many protozoa has already been studied, and others have been initiated for the study of transcriptomes (Birkeland et al, 2010; Rastrojo et al, 2013; Morse et al, 2016). However, for ethical reasons, it is not possible to use live cell lines in an immunization protocol in humans (Beauvillain et al, 2009), especially considering the contraindication of live attenuated vaccines for immunocompromised patients.

Exosomes could therefore provide a new method for communication and for the exchange of antigenic information between cells in the immune system (Aline et al, 2004). Since they are of the same size as viruses, a similar uptake by antigen-presenting cells may be observed. Interestingly, the use of exosomes as a versatile tool for signal delivery compared with soluble molecules is gaining support due to their double-layered membrane (Trelis et al, 2016). In eukaryotic pathogens, both immuno-stimulatory and immuno-inhibitory functions have been reported for exosomes (Atayde et al, 2016). The ability of exosomes, especially those derived from dendritic cells (DCs), to induce protective immune responses offers an alternative to DC-based vaccines (Beauvillain et

al, 2007). Dendritic cells, presenters of antigens that participate in the onset of the adaptive response, are able to secrete EVs carrying MHC class II antigens, allowing the development of a specific T cell response; these EVs would be antigen-presenting vesicles (Zitvogel et al, 1998; Escudier et al, 2005).. Intracellular parasites, bacteria, and viruses that enter cells via an endocytotic pathway are prime candidates for DC-based exosome Immunogens. Vaccines consisting of exosomes will both preserve the positive aspects of live parasite vaccines and avoid their inherent risks (del Cacho et al, 2012).

Aline et al (2004) demonstrated for the first time the participation of exosomes in protection against pathogens. They developed a vaccine composed of exosomes of DCs stimulated by *T. gondii*, capable of eliciting a TH1-mediated response. This protective capacity may be associated with DCs or exosome trafficking. Dendritic cells stimulated *in vitro* with *T. gondii* antigens secreted exosomes capable of inducing a significant humoral responses in syngeneic and allogeneic mice, with a greater participation of IgA and reduction of cysts in the brain. Due to the low safety of administering an attenuated *T. gondii* vaccine to humans (Beauvillain et al, 2007), the use of antigen-presenting vesicles is a possible way of stimulating T cells.

Exogenous derivatives of DCs, stimulated with *Leishmania major* antigens, created a protective response with TH1 activation against cutaneous leishmaniasis (Schnitzer et al, 2010).

Martin-Jaular et al (2011) verified increased IgG in mice by treating them with exosomes of reticulocytes infected with *Plasmodium yoelli*. Antibodies were able to recognize erythrocytes infected with the protozoan. A summary of EVs involved in immunity is shown in Table 2. A summary of EVs involved in immunity is shown in Table 2 and some of possible strategies are illustrated schematically in Figure 2 and 3.

However, although the use of exosomes allows for a cell-based vaccine, there are both conceptual and practical issues that need to be addressed before this potential application can become a reality. These include obtaining exosomes with the correct mix

of antigens that provide protection, with the risks of introducing 'non-self' human molecules into a vaccinated individual (Schorey et al, 2015).Through EVs, protozoa are able to initiate proinflammatory responses from target cells, such as increased production of interleukins. Therefore, it is suggested that the vesicles may have the added benefit of acting as adjuvants. Exosomes have also been shown to induce anti-tumor immunity in the absence of adjuvants or heat treatment (Greening et al, 2015; Morishita et al, 2016) thus suggesting the use of exosomes as adjuvant carriers because of the ability of these structures to act as molecule carriers. Due to their physical properties, EVs could enhance the immunogenicity of antigens. A satisfactory response must activate specific arms of the immune system, such as the cell-mediated response, and possibly constitute an effective immunomodulatory effect for diseases that lack an effective immunogen.

Diagnostic

The level of EVs in human serum could be a marker of disease status (Pisitkun et al, 2004; Wekesa et al, 2014; Kim et al, 2017). As EVs reflect the proteomic content of the cells from which they are derived, it is possible to use them for disease detection, as first investigated in tumors (Santiago-Dieppa et al, 2014). EV cargo can also be involved in disease prognosis. The nature of the vesicles allows a means of transport, free of blood degradation. This has proven to be particularly significant for the use of miRNA as valuable biomarkers because most RNA in blood exists within EVs (Revenfeld et al, 2014). Recently, Melo et al (2015) detected cancer-cell derived exosomes containing a high concentration of cell surface proteoglycan, glypican-1. It was showed that glypican-1 is a pan-specific marker of cancer exosomes, specifically detected in the serum of individuals with pancreatic cancer. Many parasitic diseases have parasite-host interactions with poorly studied pathogenesis. As EVs participate in these processes, clinical investigation should consider the detection of EVs derived from parasites or hosts

in biological fluids. When the *T. cruzi* secretome was analyzed after 16 hours of ultracentrifugation, proteins involved in the pathogenicity of the protozoa, such as GP63 and Aminopeptidase P, were found to be increased within the EVs compared to the supernatant fraction (Bayer-Santos et al, 2014). According to Geiger et al. (2010) metallopeptidase thimet oligopeptidase A (M3 family) is the first of the group to be identified in Protozoa, resulting in a potential marker for diagnosis in *T. brucei*. The presence of proteins on the EV lipid bilayer such as the tetraspanins, could present targets for detection by monoclonal antibodies .

It is also possible that nucleic acid ,present in EVs has some diagnostic potential (Melo et al, 2015;, Zhang et al, 2015; Eirin et al, 2014). Such a molecular diagnosis of parasitic diseases offers high sensitivity and specificity, compared to microscopic examination, reducing shortcomings and it can be standardized (Stensvold et al, 2011). As microscopy remains labour intensive and highly observer-dependent, molecular detection techniques, such as real-time PCR, are excellent alternatives, in particular in settings where the number and range of parasitic infections is low and personnel costs are substantial (Lieshout & Roestenberg, 2015). Serological assays also have limitations. For example, conventional serological assays for *T. cruzi* may lead to unspecific reactions (false positives) due to cross reactivity with antibodies elicited by other pathogens, e.g., *Leishmania* spp. and *Trypanosoma rangeli*. Because of a lack of a single test with high sensitivity/specificity, the WHO recommends positive results from two different serological tests for a confirmation of *T. cruzi* infection (Zingales, 2017). In that context, diagnostic techniques based on molecular targets are becoming more popular.

Accordingly, as reviewed by Inamdar et al (2017), expression profiling can be useful as a diagnostic tool in diseases that lack definitive biomolecular biomarkers. The detection of nucleic acids in EVs obtained from clinical samples, like feces, could become a regular procedure . It was shown by Liu et al, (2016) that is possible to obtain a suitable Fecal

Suspension Particle size Distribution by NanoSight and EM Feces from mice with a combination of dilution of feces in PBS , spun down at 10,000 ×g for 5 min by centrifugation and then filtered to remove the debris. Moreover, EVs were isolated from feces homogenized in PBS, using exoEasy Maxi Spin Columns (Qiagen), followed by RNA isolation using miRCURY RNA isolation kit (Exiqon) with on-column DNase treatment (Qiagen).

Comment [JI4]: ??

Other types of analytes that could be studied in the exosomal space, such as lipids, might represent good biomarkers to investigate in the near future. All these provide an important base to continue research in parasite-derived exosomes as diagnostic targets and demonstrating their utility as clinical biomarkers (Sánchez-Ovejero et al, 2016).

Therapeutics

Extracellular vesicles participate in cellular traffic thereby influencing pathophysiological conditions and it is this capacity to be delivered that could be extrapolated for therapeutic purposes as well (Moore et al., 2017). Mammalian stem cells EVs have been shown to be involved in cell proliferation and improved renal function of cisplatin-induced acute kidney injury in rats (Zhou et al, 2013). For the same model, it was also demonstrated that exosomes could deliver miRNA for up-regulation of anti-apoptosis genes, and reduce mortality caused by cisplatin (Bruno et al, 2012). Intranasal delivered exosomes were taken up by microglial cells, which are key mediators in neuro-inflammatory diseases; delivery of curcumin-loaded exosomes resulted in a reduction in activated microglial cells in both encephalitis and LPS-induced brain inflammation models (as reviewed by Inamdar et al, 2017).

There are different ways to explore the potential of therapeutic EVs. For example, macrophage-derived MVs may represent a way of converting an autologous intrinsically biocompatible sub-cellular entity into a drug delivery system able to carry both nanoparticles and drugs. In this regard, it would be of interest to demonstrate that cellular

MVs might be loaded with different drug molecules while simultaneously enclosing nanoparticles that enable spatially controlled drug delivery.

Silva et al (2015) encapsulated different drugs with magnetic nanoparticles within MVs. The magnetic properties of the nanoparticles could influence the uptake of the loaded MVs, and such a combination could represent a model for the delivery of different types of drugs, as for example in cancer-therapy. The possibility of producing MVs from different cell populations could improve such association, resulting in a specific delivery method. Strategies for loading molecular cargo in exosomes and related efficacies differ based on the chemistry of the loaded molecule (as reviewed by Inamdar et al, 2017). In this regard, it is possible to explore the loading capacity of EVs for different antiprotozoal drugs.

Besides drug delivery, nucleic acids have also been involved in therapeutic purposes. The specificity imparted by targeted exosomes, the ability to load exogenous genetic cargoes, the ability to systemically administer the gene therapy and immune evasion by exosomes are valuable properties for future oligonucleotide therapy applications (Alvarez-Erviti et al, 2011). In addition, exosome-transferred miRNAs are emerging as novel regulators of cellular function (Alexander et al, 2015). Momem-Heravi et al (2014) loaded exosomes with microRNA (miR-155, with electroporation) or inhibitor to be uptaken by hepatocytes or macrophages, respectively. Exosome-mediated inhibition was superior to conventional transfection models. Alvarez-Erviti et al (2011) through *in vivo* administration of exosome-loaded siRNA were able to specifically knockdown BACE 1 (protease involved in Alzheimer´s Disease).

Delivery of drugs or nucleic acids though EVs to treat parasitic diseases have not been explored yet. However, mammalian models shows promise. We speculate that if EV-loaded microRNAs inhibit genes involved in the pathogenesis of protozoa, it will significantly affect parasite-host interactions. MicroRNA could specifically regulate protozoan virulence through inhibition of invasiveness-related genes, or improve the host

Comment [IR5]: Especulate

immune system by up-regulating immune-related genes. Another promising strategy relies on gene-editing tools, like CRISPR/Cas 9. CRISPR/Cas 9 is a technology based on a known mechanism from bacteria and archaea that enable the organisms to respond to and eliminate invading genetic material. The CRISPR/Cas9 system consists of the Cas9 nuclease and a single guide RNA, which are used to guide effector endonucleases that target interested DNA sequence based on sequence complementarity (Chira et al, 2017). While *in vivo* delivery of this system has a low efficiency, the use of exosomes loaded with CRISPR/Cas9 showed a promising result in cancer. Kim et al (2017) demonstrated a reduced apoptosis in ovarian cancer by suppressing poly (ADP-ribose) polymerase-1, with CRISPR/CAs9 loaded exosomes. While there is a long way in medical approaches concerning the application of this tool, a CRISPR/Cas9 system editing pathogen genes will allow a better understanding about host-pathogen interaction. This knowledge could provide the possibility of designing novel targets for therapeutic interventions .

CURRENTL REMARKS

The strategies debated in this work are speculations of what is to come from the rapidly expanding field of EVs. It is clear that “trial-and-error” research is necessary to expand applications in the routine medical laboratory. Since parasitic diseases are common in developing countries, translation into clinics must involve low-cost strategies. If participation of EVs in cell communication models are becoming highly proven, it is only a matter of time until biotechnology is able to deliver accessible procedures.

Ar EV-detection methods going to emerge as markers for next-generation diagnostics of protozoa?

Are EVs a satisfactory alternative for the immunization of poorly responsive populations?

Can EVs be considered a delivery system for drugs to reduce off-target effects on parasitological diseases?

Is Clinical parasitology going to translate extracellular vesicle-based research in the future?

Can gene-editing systems interfere in host-parasite communication, acting as a therapeutic alternative?

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Comment [JI6]: Some Refs have d.o.i. Others do not. I guess it is more likely that this is not required.

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963 Figure Legends

964 Figure 1. Schematic representation of the dymamic flux of extracellular vesicles

965 between host cells and parasites.

966 Figure 2. Different strategies to use EVs as vaccines and trigger antibodies production.

967 Figure 3.Loading surface molecules into nanovesicles.Vesicles coupled with antigens or

968 carrying specific antibodies acting as vaccines or neutralyzing pathologic effects.

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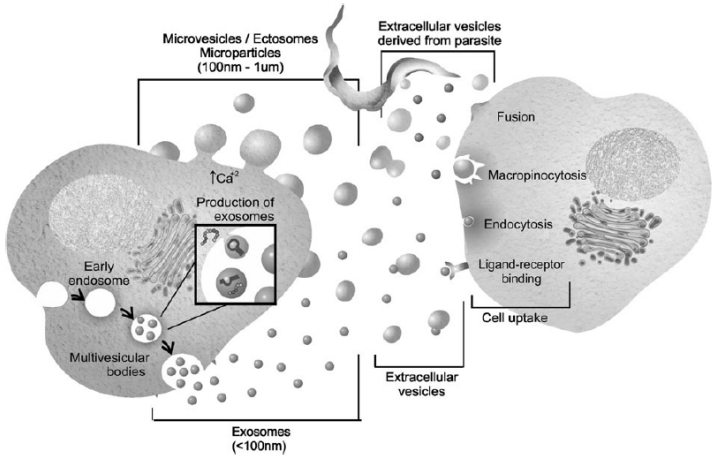

Fig. 1. Schematic representation of the dynamic flux of extracellular vesicles between host cells and parasites.

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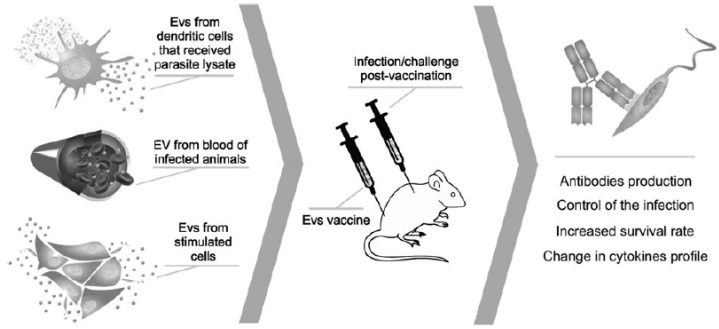

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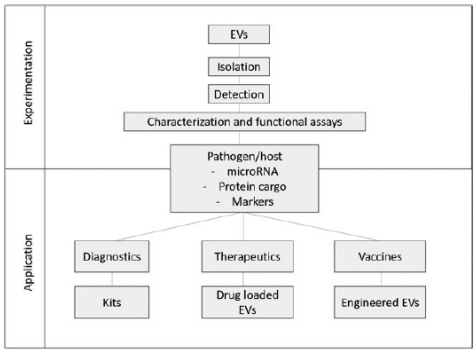

973 **Fig. 3.** Experimental steps for the Study of EVs and the possibilities of translation into clinics.