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Extracellular vesicles released by anaerobic protozoan parasites: Current situation

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Abstract

Extracellular vesicles (EVs) have emerged as a ubiquitous mechanism for transferring information between cells and organisms across all three kingdoms of life. Parasitic unicellular eukaryotes use EVs as vehicles for intercellular communication and host manipulation. Pathogenic protozoans are able to modulate the immune system of the host and establish infection by transferring a wide range of molecules contained in different types of EVs. In addition to effects on the host, EVs are able to transfer virulence factors, drug-resistance genes and differentiation factors between parasites. In this review we cover the current knowledge on EVs from anaerobic or microaerophilic extracellular protozoan parasites, including *Trichomonas vaginalis*, *Tritrichomonas foetus*, *Giardia intestinalis* and *Entamoeba histolytica*, with a focus on their potential role in the process of infection. The role of EVs in host: parasite communication adds a new level of complexity to our understanding of parasite biology, and may be a key to understand the complexity behind their mechanism of pathogenesis.

KEYWORDS

extracellular vesicles, immune response, parasites, pathogenesis, virulence

1 | INTRODUCTION

Pathogenic protozoans are responsible for a wide range of human and animal diseases globally, and cause a substantial socioeconomic burden in many developing nations. Within this group are diseases caused by protozoan parasites that reside extracellularly and in oxygen-deprived environments (anaerobic) within their hosts. These include enteric human pathogens such as *Giardia intestinalis*, *Entamoeba histolytica* and the human and bovine urogenital tract parasites, *Trichomonas vaginalis* and *Tritrichomonas foetus*, respectively. Although these parasites use diverse mechanisms for survival and persistence within their hosts, it has long been hypothesised that secretion of effector molecules might be a common feature important for pathogenesis. In this sense, extracellular vesicles (EVs) have been identified as product of secretome that contribute to the adaptive capabilities of microbial cells through delivery of proteins, lipids and nucleic acids to both adjacent and distant cells. The international society of extracellular vesicles (ISEV) endorses "extracellular vesicles" as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, that is, do not contain a functional nucleus (Thery et al., 2018). The scientific community classifies EVs in three main types; including: exosomes (50–150 nm in diameter) that arise from multivesicular bodies (MVBs), ectosomes or microvesicles (MVs) that are shed from the plasma membrane (100–1,000 nm in diameter) (van Niel, D'Angelo, & Raposo, 2018) and apoptotic bodies that are released during apoptotic cell death (bigger than 1,000 nm in size) (Caruso & Poon, 2018). There are growing numbers of studies on EV biogenesis in mammalian cells, however, full details of the molecular pathways involved are not yet resolved (Abels & Breakefield, 2016; Colombo, Raposo, & Thery, 2014). So far, it is known that various sorting machineries are involved in clustering membrane-associated proteins and lipids destined for secretion via EV. Both routes share molecular machineries for EV generation, such as "Endosomal Sorting Complex Required for Transport" (ESCRT) and tetraspanins proteins.

Both ESCRT dependent and independent mechanisms have been implicated in clustering of cargo for biogenesis of EV (Colombo et al., 2014). Differences in biogenesis of each EV population suggest that components of the cellular machinery involved in their formation may be different (Choi, Kim, Kim, & Gho, 2015). Despite apparent dissimilarities in the mechanism of formation between exosomes and MVs, it is experimentally difficult to discriminate among populations after they are secreted from cells. In recent years, the study of EVs composition has greatly advanced our understanding of the role these vesicles play in cell biology. As more vesicular proteins are identified, it has become apparent that EVs contain a specific subpopulation of proteins rather than randomly selected molecules from their cells of origin (Choi et al., 2015). In spite of this specificity, there is no clearly descriptive physical property or molecular marker that can unambiguously distinguish exosomes from MVs (Choi et al., 2015). Since current methods of vesicle isolation fail to achieve a complete separation of EVs based on their mechanism of formation, it has been suggested that they should be classified based on their size as large and small EVs (Witwer et al., 2017), or that the term exosomes should be used to name small vesicles originating both from MVBs and the plasma membrane (Pegtel & Gould, 2019). Nevertheless, we will use the terminology as defined in the original studies when discussing the results. There are different methods for enrichment of EVs that typically result in heterogeneous preparations enriched in one or more EV population(s). The traditional ones utilise the EV properties, such as size and buoyant density, namely, ultracentrifugation, microfiltration and gel filtration (Momen-Heravi, Getting, & Moschos, 2018). The methods based on the fact that EVs change their solubility and/or aggregate appeared somewhat later, namely, precipitation with polyethylene glycol, protamine and sodium acetate (Konoshenko, Lekchnov, Vlassov, & Laktionov, 2018). In addition, numerous methods for isolation of EV population based on specific interactions with molecules exposed on the EV surface or microfluidic technologies have recently appeared (Konoshenko et al., 2018). For a more thorough review of tools used to separate and analyse EVs, see (Konoshenko et al., 2018; Momen-Heravi et al., 2013; Witwer et al., 2013). Selection of a preferred method is greatly dependent on the goal to be achieved as well as individual laboratory habits. In general, researchers aim for high EV purity and yield, either at the whole population of EVs or a subclass (Momen-Heravi et al., 2018). Traditionally, the most commonly used protocol for EV isolation is differential centrifugation, which involves multiple centrifugation and ultracentrifugation steps. In general, the centrifugation protocol includes a low speed centrifugation (300–500 g for 10–15 min) to pellet cells, followed (in some cases) by filtration trough a 0.22 μm or 0.8 μm filter pore, then a centrifugation by a medium speed (10,000–20,000 g for 20–30 min) to pellet larger vesicles and a final 100,000 g ultracentrifugation step for 90–120 min to pellet small EVs. Although this technique was used in most of the studies discussed in this review (Evans-Osses et al., 2017; Moyano et al., 2019; Nievas et al., 2018; Twu et al., 2013), it is important to have in mind that protocols slightly vary between users, which may lead to differences in the recovery or degree of purification of EVs.

The study of EVs in protozoan parasites has increased in the past years and the evidence suggests, as has been described in other eukaryotes, that protozoan EVs are involved in cell communication (Szempruch, Dennison, Kieft, Harrington, & Hajduk, 2016; Wu et al., 2018). Nevertheless, our knowledge of EVs from anaerobic and microaerophilic protozoan parasites is still very limited. Here we review the literature on the main findings of EVs in anaerobic protozoan-related diseases, discuss recent advances and highlight the importance of EVs in host: cell interaction to encourage further studies that would help to understand the functions of these complex extracellular organelles in the context of parasite pathogenesis.

2 | *TRICHOMONAS VAGINALIS*

Trichomonas vaginalis is an anaerobic flagellated protozoan that causes trichomoniasis, the most common non-viral sexually transmitted infection worldwide with an estimated 156 million new cases annually (WHO, 2018). Although asymptomatic infection is common, disease manifestation varies both in symptoms and risks, including discomfort in the urogenital area as a consequence of vaginitis or urethritis and more important outcomes if acquired during pregnancy, such as preterm delivery and premature rupture of membranes (Fichorova, 2009). Furthermore, the parasite has also emerged as an important cofactor in amplifying the human immunodeficiency virus (HIV) spread (McClelland et al., 2007) and in increasing the risk of cervical and aggressive prostate cancer (Gander, Scholten, Osswald, Sutton, & van Wylick, 2009; Twu et al., 2014). Research in *T. vaginalis* has demonstrated that host: parasite interaction and cytotoxicity is a multifactorial mechanism (Mercer & Johnson, 2018), including parasites secreted vesicles as well as surface, secreted and effector proteins (Mercer & Johnson, 2018). Specifically, the analysis of *T. vaginalis* EVs has become a very exciting field in the study of its pathogenesis, and so far, the release of both exosomes and MVs have been documented (Nievas et al., 2018; Olmos-Ortiz et al., 2017; Rai & Johnson, 2019; Twu et al., 2013). Interestingly, the formation of both types of vesicles increase upon exposure of the parasites to the host cells, suggesting that they may be involved in the process of host: parasite interaction (Nievas et al., 2018; Twu et al., 2013). Using differential centrifugation followed by 200 nm filtration, Twu et al. isolated vesicles from sizes smaller than 200 nm (with a mean diameter of 95 nm) mainly enriched in exosomes (Twu et al., 2013). Similarly, MVs were enriched by differential centrifugation followed by two filtration steps: a first 800 nm pore filter to avoid cell debris and then a 200 nm pore filter to eliminate the exosome population (Nievas et al., 2018). Although a highly enriched population of vesicles with a mean diameter of 380 nm was obtained, the recovery was very poor and limited further studies. The proteomic analyses identified 215 proteins in exosomes and 592 in MVs (Figure 1). Interestingly, the proteomic data revealed that 167 out of 215 exosome proteins were found in the MVs proteomic survey. This is not surprising as the protein content of MVs and exosomes tends to overlap in many proteomic analyses. However, although many proteins may be truly shared between both

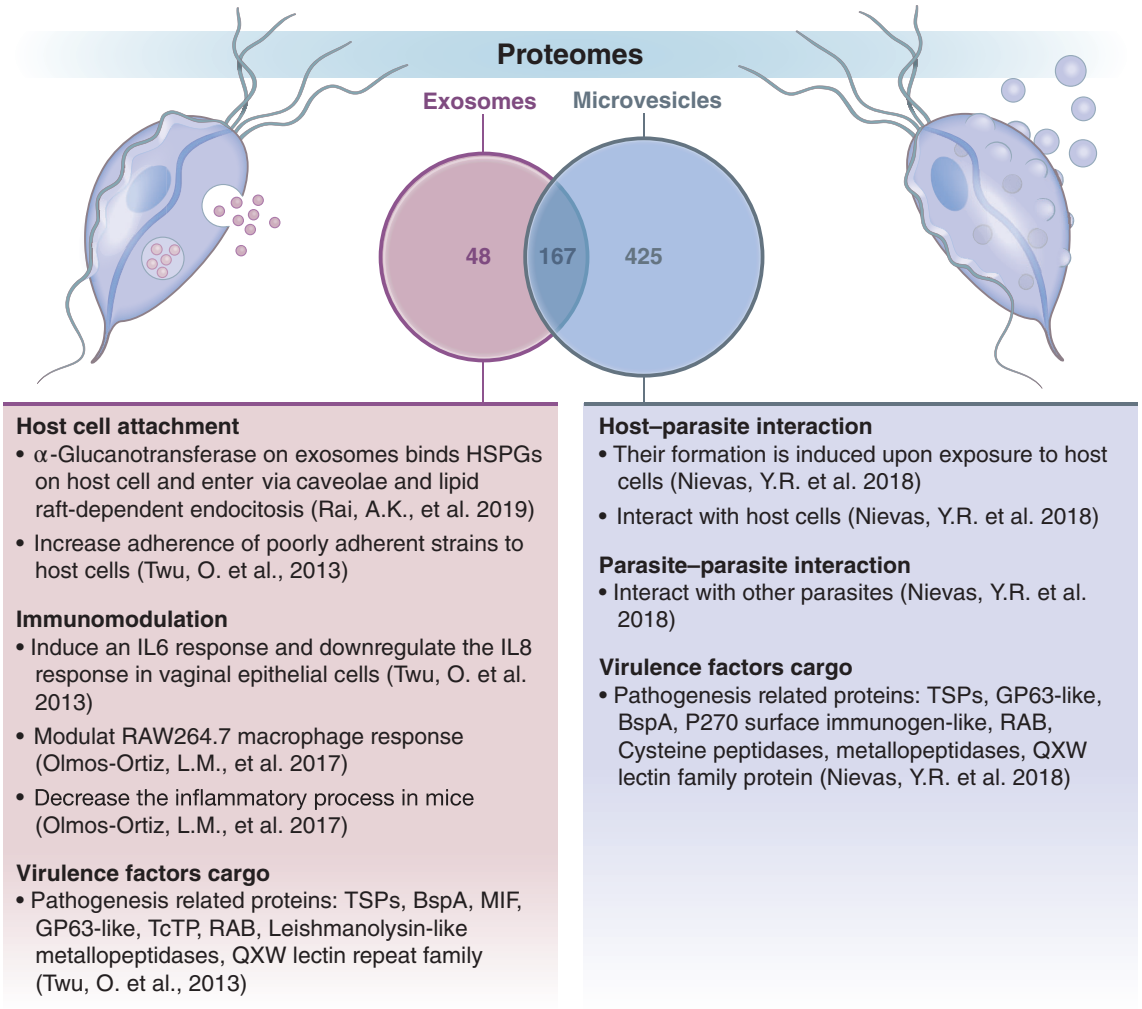

FIGURE 1 Role of extracellular vesicles in *T. vaginalis*. EVs (MVs and exosomes) mediate a wide range of effects on *T. vaginalis* including modulation of parasite adherence, delivery of virulence factor and regulation of host immune response

populations, it cannot be discarded a cross contamination due to limitations of the enrichment techniques used as both populations were isolated only relying on the sizes of them. The protein content of exosomes and MVs comprised molecules that participate in different stages of the EVs formation and/or release in other systems, such as vesicle trafficking-related proteins (Schmidt & Teis, 2012); Rab GTPases and SNARE-complex proteins (Fader, Sanchez, Mestre, & Colombo, 2009; Ostrowski et al., 2010); cytoskeletal proteins (Mathieu, Martin-Jaular, Lavieu, & Thery, 2019); metabolic enzymes and tetraspanins. For human-related EVs, tetraspanins proteins (CD9, CD63, and CD81) are often used as markers of EVs (Kowal et al., 2016). Despite extensive research on the protein composition of EVs from parasites, it is still unknown whether the same markers apply to identify EVs from all parasite species. Considering the presence of TvTSP1 and TvTSP8 in the exosome and MVs proteome, respectively (Nievas et al., 2018; Twu et al., 2013), these proteins might potentially be used as EVs marker in *T. vaginalis*. However, it is important to note that TSPs have been identified in some, but not all protozoan parasite genomes. Specifically, 17 TSPs-like sequences were found in *E. histolytica* (Tomii, Santos, & Nozaki, 2019), 1 in *Leishmania* spp., 3 in *T. cruzi* and 2 in *T. brucei*, while no TSPs-like sequences were found in *Giardia spp*., *Plasmodium spp*., *Toxoplasma gondii* and *Cryptosporidium parvum* (our HMMer search using Pfam PF00335). Therefore, TSPs proteins may not be proposed as general markers in EVs from protozoan parasites. Considering the role of TSPs in EVs biogenesis in mammalian cells (Andreu & Yanez-Mo, 2014), and the fact that EVs secretion has been demonstrated in parasites that lack TSPs within their genomes (Beauvillain, Juste, Dion, Pierre, & Dimier-Poisson, 2009; Evans-Osses et al., 2017; Martin-Jaular, Nakayasu, Ferrer, Almeida, & Del Portillo, 2011), it would be interesting to evaluate if the mechanism of EV formation differ in these parasites. Tetraspanin are transmembrane proteins that organise specialised membrane domains termed tetraspanin-enriched microdomains (TEMs) which may play a role in EVs biogenesis, cargo selection and/or binding and uptake by target cells (Andreu & Yanez-Mo, 2014). In this sense, it has been demonstrated that TvTSP1 accumulates in MVB-like structures after the parasites are exposed to vaginal epithelial cells (Twu et al., 2013). This observation could be

suggesting a role of this protein in cargo selection or biogenesis of exosomes. Interestingly, *T. vaginalis* tetraspanins have also demonstrate to have a role in the process of host parasite interaction, as the expression of both TvTSP1 and TvTSP8 are up-regulated upon exposure of the parasites to the host cell (Coceres et al., 2015). Besides TSPs, proteomics analysis showed an enrichment of other proteins that have been implicated in host: parasite interactions and immunomodulation (Nievas et al., 2018; Twu et al., 2013). Specifically, GP63-like metallopeptidases involved in the infection process (Ma et al., 2011); BspA proteins that participate in bacterial and *T. vaginalis* adherence (Handrich, Garg, Sommerville, Hirt, & Gould, 2019; Noel et al., 2010); the surface immunogen protein p270 (Alderete, 2000); TvMIF that induces inflammatory responses (Twu et al., 2014) as well as proteins that are highly expressed in adherent strains (TVAG_020780 and TVAG_239650) with unknown function (de Miguel et al., 2010; Gould et al., 2013). In order to fully understand the function of these proteins in EVs during infection, it would be interesting to analyse the protein content of the different vesicles populations in the presence and absence of host cell. Considering the presence of proteins related to pathogenesis in exosomes and MVs, it could be speculated that the parasite might use EVs to stablish infection and manipulate host defence responses. In this sense, the presence of MVs on the cell surface of *T. vaginalis* increased in presence of neighbouring parasites (Nievas et al., 2018). Additionally, it has been shown that short-term incubation of a poorly adherent *T. vaginalis* (G3) strain with exosomes isolated from a highly adherent *T. vaginalis* isolates showed that EVs can increase the adherence of G3 strain to ectocervical and prostate cells via an effect on both parasites and host cells (Twu et al., 2013). In contrast, exosomes from poorly adherent strains had no measurable effect on parasite adherence. Furthermore, exosomes from parasite strains that preferentially bind prostate cells are more effective in increasing adherence to prostate versus vaginal epithelial cells (Twu et al., 2013). Taken together, these data indicate *T. vaginalis* exosomes may package strain specific, perhaps even host cell specific, virulence factors. Based on these and the different phenotypes presented by *T. vaginalis* strains (Lustig, Ryan, Secor, & Johnson, 2013), a comparative and comprehensive proteomic analysis of the EVs content of different parasite strains may contribute to the elucidation of their involvement in parasite pathogenesis.

Trichomonas vaginalis exosomes has also been shown to modulate host immune responses (Figure 1) as were found to induce an IL6 response in vaginal epithelial cells and to downregulate the IL8 response to parasites (Twu et al., 2013). Reducing secretion of IL-8 by EVs, a key cytokine for neutrophil recruitment, may be critical in establishing infection as neutrophils are the front line of defence against this parasite. IL6 is a chief stimulator of proteins in acute inflammation and suppresses the level of other proinflammatory cytokines in an acute response (Gabay, 2006). *Trichomonas vaginalis* exosomes may lead to the regulation of IL6 and IL8 secretion, thus priming the urogenital tract for parasite colonisation (Twu et al., 2013). Additionally, they were also shown to modulate the immune response of macrophages in vitro by increasing the release of NO and inducing the release of the anti-inflammatory cytokine IL10 (Olmos-Ortiz et al., 2017). Exosomes also modulate the immune response of a BALB/c female mice in vivo, exerting a complex cytokine profile response. Mice intravaginally inoculated with exosomes prior to parasite infection increased the release of IL-10 while decrease the secretion of IL-13 and IL-17 (Olmos-Ortiz et al., 2017). Moreover, the EVs pre-treatment correlated with an increase in the parasite survival and a significant decrease in vulvar inflammation in a murine model of infection (Olmos-Ortiz et al., 2017), supporting the anti-inflammatory exosomes role that favours the parasite survival and challenging the proposal of *T. vaginalis* EVs as a vaccination method.

In 2013, Twu et al. (2013) demonstrated that *T. vaginalis* exosomes fuse and deliver their content to the host cells. Recently, Rai and Johnson (2019) evaluated the components involved in EVs internalisation and the mechanism involved in the uptake. They demonstrated that *T. vaginalis* EVs not only are able to deliver their soluble content but also transfer lipids when the membrane of the exosomes fused with membrane of the host cells (Rai & Johnson, 2019). The general mechanisms that drives EVs uptake and cargo delivery are still incompletely characterised. However, the process generally involves: the targeting of the recipient cells (that may be or not mediated by receptors), the EV internalisation that has been reported to occur through multiple routes (i.e., endocytosis, micropinocytosis) and the delivery of the content to the acceptor cell (Mathieu et al., 2019). Rai and Johnson (2019) demonstrated that *T. vaginalis* EVs interact with glycosaminoglycans on the surface of host cells and specifically bind to heparan sulfate proteoglycans (HSPGs), indicating that HSPGs on host cells act as receptors in the first step of EV internalisation (Rai & Johnson, 2019). They also identified a ligand on the surface of EVs, named 4-α-glucanotransferase (Tv4AGT), that is critical for EV uptake (Rai & Johnson, 2019). Interestingly, this protein is also present in the MVs proteome (Nievas et al., 2018), suggesting that mechanisms used to drive host: pathogen interactions could be conserved among both types of vesicles. Finally, they evaluated the EVs internalisation mechanism and identify caveolae and lipid-raft dependent endocytosis as key mediators of the EVs uptake. Future analyses of the internalisation of different types of EVs will be necessary to fully understand the mechanisms involved in EV-mediated cell: cell communication.

A diverse composition of genetic material is found in EVs (Abels & Breakefield, 2016). Overall, EVs are primarily enriched with small RNAs, with many derived from ribosomal 18S and 28S rRNAs and tRNAs (Abels & Breakefield, 2016). The presence of small RNAs in EVs led to the prediction that RNA cargo in EVs may modulate "recipient" cells gene expression. In this sense, a heterogeneous population of small RNAs ranging in size from between 25 and 200 nt were found in *T. vaginalis* exosomes (Twu et al., 2013). The small RNAs packaged inside *T. vaginalis* exosomes may have a role in modulation of parasite: parasite or parasite: host interactions. The possibility that these small RNAs could be delivered to the host cell to modulate gene activity is an appealing idea that needs to be further studied.

3 | *TRITRICHOMONAS FOETUS*

Tritrichomonas foetus is a flagellated parasitic protozoan that infects the bovine genital tract where it causes a sexually transmitted infection in bovine (Rae & Crews, 2006) and diarrhoea in domestic cats (Yao & Koster, 2015). *Tritrichomonas foetus* has a worldwide distribution and causes significant economic losses due to bovine trichomonosis (Rae & Crews, 2006). In the bull, infection normally persists for years without clinical signs. In contrast, trichomonosis in female cattle ranges from clinically almost unapparent infections to severe manifestations of disease (vaginitis, placentitis and pyometra) that result in infertility, early embryonic death, or abortion in cattle (Rae & Crews, 2006). Despite these serious health related and economic consequences, biological processes important for *T. foetus* infection are not clearly defined. The presence of EVs in this parasite has not been described so far, however, a component of the ESCRT-III complex that has been identified in the *T. vaginalis* EVs proteomes called TfVPS32 is localised in *T. foetus* intracellular vesicles (Iriarte et al., 2018). ESCRT components are responsible for generating the intraluminal vesicles of MVB through their ability to promote the bending of membranes away from the cytoplasm, culminating in either exosomal release or lysosomal degradation (Juan & Furthauer, 2018). Specifically, ESCRT-III allows the pinching off of ILVs to become exosomes (Juan & Furthauer, 2018). Additionally, recent evidence demonstrates that at least some ESCRT subunits participate in the assembly and budding of MVs (Schmidt & Teis, 2012). Considering the importance of EVs in *T. vaginalis* infection and the presence of TfVPS32 in intracellular vesicles (Iriarte et al., 2018), further investigations in the field to demonstrate the release, to elucidate the mechanisms of biogenesis and the functional role of these EVs in the process of *T. foetus* infection are encouraged.

4 | *GIARDIA INTESTINALIS*

Giardia intestinalis is a flagellated protozoan and etiological agent of giardiasis, the most common cause of diarrheal infection in children of low-income countries, producing an estimate of 280 million new infections every year (Bartelt & Sartor, 2015). Infection occurs upon ingestion of food or water contaminated with the cyst stage of the parasite. Exposure to the gastric acid triggers excystation, releasing trophozoites that adhere to the epithelial cells of the upper small intestine where they replicate (Bartelt & Sartor, 2015). Some trophozoites that get exposed to biliary fluid form cysts that are passed in the faeces, completing the transmission cycle by infecting a new host (Adam, 2001). Unfortunately, there are no vaccines available to prevent the disease and the use of drug therapies is not always successful (Leitsch, 2015). Recent reports have linked *Giardia* infections to the development of food allergies and post-infectious syndromes, such as chronic fatigue syndrome (CFS). The acquisition of the disease during early childhood can lead to impaired growth of the affected children (Fink & Singer, 2017). The long-term effects of *Giardia* infections, the prevalence of asymptomatic cases, and the establishment of chronic infections highlight the importance of finding novel clinical strategies to combat the disease.

Giardia belongs to the phylum Diplomonadida, unicellular eukaryotes that have undergone considerable reductive evolution, including the loss of mitochondria, peroxisomes, Golgi apparatus, and classical endo-lysosomal system (Adam, 2001). *Giardia* parasites contain numerous acidified peripheral vacuoles (PVs) found adjacent to the plasma membrane in both trophozoites and encysted cells which fulfil some criteria of endosomes and lysosomes (Adam, 2001; Midlej, de Souza, & Benchimol, 2019). Using high-resolution electron microscopy techniques, Midlej et al. recently reported the presence of intraluminal vesicles (ILV) of 50–100 nm in diameter inside some PVs, the number of ILVs ranging from one to seven in trophozoites and one in encysting cells (Midlej et al., 2019). These results suggested PVs are organelles with a wide range of biological characteristics and functions which could act as MVBs in certain cases (Midlej et al., 2019). In effect, Moyano et al. (2019) described the presence of two distinct populations of vesicles in the exosomal fraction of the supernatant derived from the in vitro culture of *G. lamblia* trophozoites: a group of exosome-like vesicles (ElV) with cup-like shape and a diameter of 50–100 nm and a group of smaller vesicles of a diameter of 20–25 nm (Moyano et al., 2019). Immunoblot analysis of the exosomal-enriched fraction revealed the presence classical exosome markers such as giardial 14-3-3 and tubulin, as well as proteins associated with the PV membranes, like gQa1 and the encystation-specific cysteine protease previously identified in a PV proteome (Wampfler, Tosevski, Nanni, Spycher, & Hehl, 2014), indicating that ElVs may indeed be formed in the PVs (Moyano et al., 2019). Importantly, ESCRT-associated protein Vps4a, Rab11, and ceramide were identified as important players in *Giardia* exosome formation (Moyano et al., 2019). Rab11 was implicated in the biogenesis of ILVs while ESCRT-associated protein Vps4a was found to be critical for ElVs production, supporting recent data that suggests *Giardia* reduced ESCRT machinery is functional in the parasite (Saha, Dutta, Datta, & Sarkar, 2018).

The release of small EVs was also reported in a recent work, in which two distinct populations of EVs released by *Giardia* trophozoites was described: a population of small extracellular vesicles (SEVs) with a mean vesicle diameter of 67.7 nm and a population of large extracellular vesicles (LEVs) with a mean diameter of 187.6 nm (Gavinho et al., 2020). A proteomic analysis of each EV population revealed a proteome profile that is distinct to each EV subgroup, although some proteins that are important for *Giardia* pathogenesis such as antigenic variable surface proteins (VSPs) (Adam, 2001), giardins (Weiland, Palm, Griffiths, McCaffery, & Svard, 2003), cathepsin B, arginine deiminase and ornithine carbamoyltransferase, were present in both populations (Gavinho et al., 2020). Both subtypes of EVs are internalised by the mammalian cells suggesting they could be involved in host–parasite interaction (Gavinho et al., 2020), although the final destination of each subpopulation inside the host cell remains to be determined. The authors observed that two different inhibitors of EVs release, the PAD-inhibitor Cl-amidine (Kosgodage, Trindade, Thompson, Inal, & Lange, 2017) and

cannabidiol (CBD) (Kosgodage et al., 2019), reduced the production of Giardia EVs and negatively affected parasite attachment to host cells (Gavinho et al., 2020). Surprisingly, the decrease on parasite adhesion was restored when treated parasites were incubated with Giardia LEVs, while Giardia SEVs or host cell derived EVs showed no effect (Gavinho et al., 2020), suggesting the necessary factors for parasite adhesion are probably found in the larger *Giardia* EVs. The results obtained with LEVs are consistent with a previous work by Evans-Osses et al. (2017) where they described microvesicles (MVs) released from the plasma membrane of *Giardia* trophozoites with a diameter of 150–350 nm whose formation was dependent on pH, calcium and cholesterol (Evans-Osses et al., 2017). The inhibition of MVs formation using the cholesterol depleting agent methyl-β-cyclodextrin (MβCD) (Mahammad & Parmryd, 2015) showed these MVs were essential for parasite adhesion to intestinal epithelial cells (Evans-Osses et al., 2017). Additionally, the MVs were capable of inducing a mild activation of immature dendritic cells, implicating them in parasite pathogenesis (Evans-Osses et al., 2017). Furthermore, the presence of small RNAs in trophozoite MVs was detected (Evans-Osses et al., 2017), suggesting a possible role in the modulation of host gene expression. Transcriptomic analysis of host cells incubated in the presence of parasite microvesicles could shed light on this question. The possibility that *Giardia* EVs could have a role in parasite–parasite communication also remains to be explored. Intriguingly, *Giardia* parasites transitioning to the cyst form release MVs with a protein content that is different from the MVs released by trophozoites, as determined by mass spectrometry analysis (Evans-Osses et al., 2017). In particular, many *Giardia* uncharacterised proteins of unknown function were found exclusively in MVs of the cyst stage (Evans-Osses et al., 2017). Though the particular role of cyst form MVs remains to be determined, their protein content makes them interesting targets for future studies.

Finally, a recent study sheds novel insight into the role of *Giardia* EVs in the pathophysiology of giardiasis (Siddiq, Allain, Dong, Olivier, & Buret, 2020). The authors show *Giardia* EVs have a bacteriostatic effect on commensal gut bacteria such as *E. cloacae* and *E. faecalis*. Additionally, exposure to Giardia EVs increased the motility of non-invasive commensals *E. coli* strain HB101 and *E. cloacae* (Siddiq et al., 2020). Importantly, in vitro studies using an epithelial primary cell line demonstrate that *Giardia* EVs are capable of disrupting the integrity of the host epithelial junctions (Siddiq et al., 2020), which could help explain the prevalence of post-infectious syndromes after eradication of the disease (Fink & Singer, 2017). While further studies are needed to fully understand the role of *Giardia* EVs on parasite biology and pathogenesis, the recent developments involving the study of *Giardia* EVs, in particular the detrimental effect of EVs inhibitors on parasite adherence, open up a venue for novel treatment strategies in the control of giardiasis.

5 | *ENTAMOEBA HISTOLYTICA*

Amoebiasis is a serious infectious disease that is caused by the unicellular parasite, *Entamoeba histolytica*. This parasite is an enteric protozoan parasite that infects 500 million people per year, causing disease in 50 million and killing 100,000 individuals annually (Mortimer & Chadee, 2010). The infection occurs following the ingestion of mature cysts in food and water that is contaminated with faeces. During the excystation stage, the cysts transform into the mobile trophozoites form and migrate to the colon. The life cycle is completed when the trophozoites form new cysts that are evacuated in the faces. Although in most cases the parasite lives in the large intestine without causing disease, sometimes, it invades the colon wall causing colitis, acute dysentery or diarrhoea. The infection can also spread through the blood to the liver and, rarely, to the lungs, brain or other organs (Mortimer & Chadee, 2010).

Entamoeba histolytica possesses a high content of vesicles and vesicle trafficking has been proposed as a key regulator of infection and disease. Perdomo et al. (Perdomo et al., 2015) have characterised the internal membranes from *E. histolytica* by proteomics and demonstrated that the endomembrane system contained proteins relevant to the ER, vesicle translocation, sorting, glycosylation and components of the Golgi, endosomes and MVBs (Perdomo et al., 2015). Additionally, *E. histolytica* release molecules involved in the invasion and lysis of the host tissues during the infection process. Ujang et al. (2016) identified 219 proteins excreted-secreted by this parasite into the extracellular environment and showed that such proteins were involved in the colonisation, evasion of the host immune system as well as in cyst formation process. Interestingly, many of the proteins identified do not contain *N*-terminal secretory signal sequence, suggesting that their secretion would occur by a non-classical secretion pathway (Ujang et al., 2016). In this sense, Sharma et al. (2020) recently demonstrated that amoebae secrete vesicles with a size distribution ~125 nm that have morphological features typical of EVs from other systems. The proteome of Entamoeba EVs identified 359 different proteins (Sharma et al., 2020). It is important to consider that the EVs isolation method used (PEG based) regularly produce a high total EV yield marred by a high contamination with non-EV proteins. Besides the presence of some possible contaminants, significant overlap with orthologs of the top 100 known mammalian exosome markers in Exocarta and an enrichment of proteins required for vesicle membrane production was detected in the Entamoeba EV proteome (Sharma et al., 2020) suggesting that enrichment in EVs was successful. Surprisingly, although a recent bioinformatics analysis identified at least 17 putative tetraspanin proteins in the *E. histolytica* genome (Tomii et al., 2019), the TSP family proteins were absent from the amoebic EV proteome list (Sharma et al., 2020). Similarly, none of these proteins were detected in the surface proteome (Biller et al., 2014) and localisation experiments that detected EhTSP1, EhTSP2, EhTSP8, EhTSP9, EhTSP12, EhTSP13, EhTSP15 in vesicular compartments in the cytosol, but not on the cell surface (Tomii et al., 2019). While these proteins are generally involved in EVs biogenesis, the absence of Tetraspanins in the EV and surface proteome may suggest a different mechanism of EV biogenesis in this organism.

Amoebic EVs are selectively packaged with a population of antisense small RNA population in the EVs (Sharma et al., 2020). Specifically, the 27 nt population of antisense small RNAs found in EVs is a

subset of the endogenous small RNA population identified previously in amoeba that has been shown to control gene expression and capable of strong transcriptional gene silencing in target genes (Morf, Pearson, Wang, & Singh, 2013; Zhang, Ehrenkaufer, Pompey, Hackney, & Singh, 2008). Additionally, some members of the RISC complex (Ago2-2, Tudor domain containing protein, Heat shock protein 70) has also been identified in *E. histolytica* EVs (Sharma et al., 2020). The presence of a specific small RNA population and some members of RISC complex might indicate the possibility of small RNA mediated communication through EVs in Entamoeba. Amoeba are unicellular organisms that communicate with each other to work in a concerted mechanism. In this sense, conditioned medium from *E. histolytica* growth cultures was shown to influence cell motility in amoeba demonstrating that amoeba can respond to signals released from one cell to another (Zaki, Andrew, & Insall, 2006). Consistent with a role in parasite communication, EVs derived from trophozoites or encysting parasites appear to deliver cargo that can affect the encystation efficiency of recipient amoebic cells. Specifically, EVs from encysting parasites enhanced encystation in target parasites with EVs from log-phase trophozoites decreased encystation efficiency in target parasites (Sharma et al., 2020) indicating that intercellular communication between Entamoeba parasites is mediated by EV contents. In summary, this work provide the first evidence that amoebic EVs participate in parasite: parasite communication and have important role in development; underscoring the importance of studying EVs in this parasite.

6 | CONCLUDING REMARKS AND FUTURE PERSPECTIVES

EVs are a fascinating issue with impact in cell biology, pharmacology, immunology and medicine; joining basic and applied sciences. In the last few years, studies on parasite-derived EVs have gradually gained attention (Szempruch et al., 2016; Wu et al., 2018). However, research in EVs in anaerobic parasites lag behind other protozoan parasites. As carriers of antigens and virulence factors, the biological impact of EVs in the process of parasite infection is likely to be considerable. Further investigation of EVs contents and biogenesis, their release in various environmental conditions as well as the function of EVs in host: parasite interactions, will surely reveal further examples of how these organelles, long thought to be mere artefacts, are essential to life and cell communication in these anaerobic parasites. In this sense, the identification of proteins differentially expressed in EVs might help to understand their role in parasite pathogenesis. This increased understanding can, in turn, open up new avenues for vaccine, diagnostic, and therapeutic development for different parasitic diseases. The use of EVs as diagnostic, prognostic and clinical tools offers promising new research applications. Vesicles are easily isolated from blood, urine, saliva, and other bodily fluids (Keller, Ridinger, Rupp, Janssen, & Altevogt, 2011). A number of studies have shown quantitative and qualitative differences in EVs composition between healthy individuals and those with underlying diseases. These differences, combined with their easy accessibility, make EVs excellent

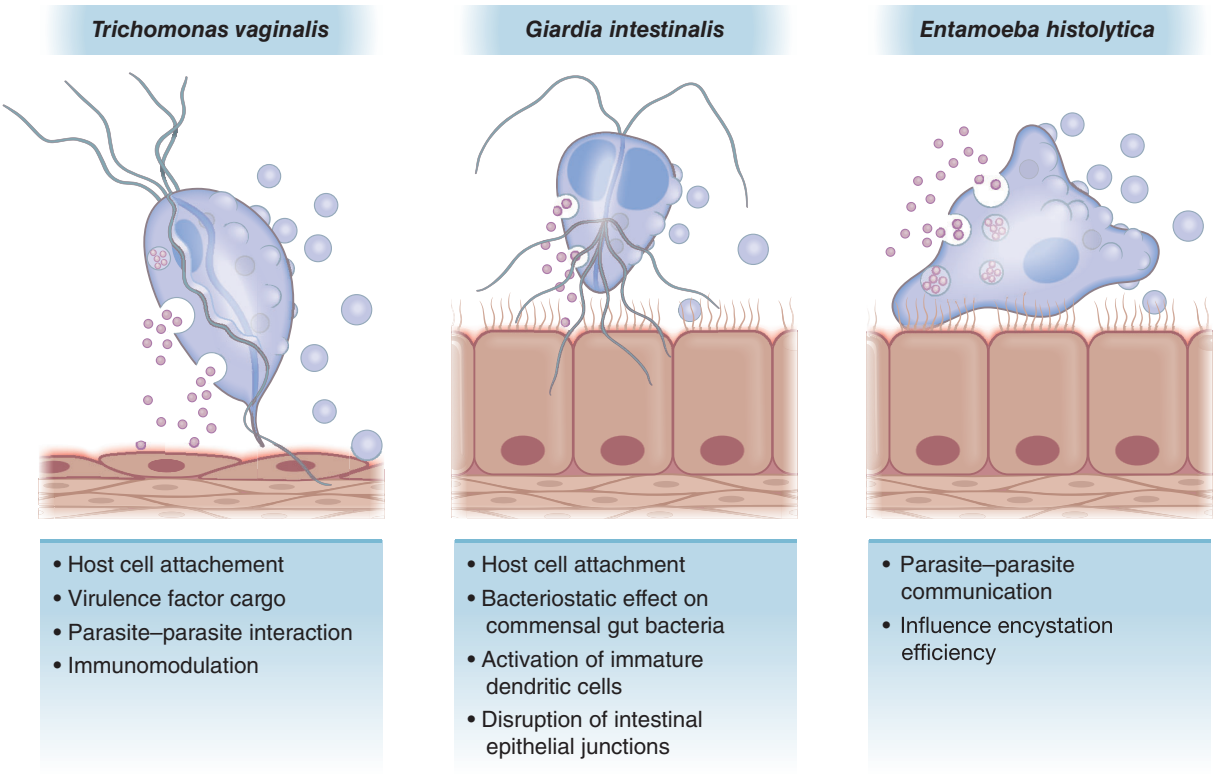

FIGURE 2 Roles of extracellular vesicles in the anaerobic protozoans *Trichomonas vaginalis*, *Giardia intestinalis* and *Entamoeba histolytica*

biomarker candidates (Momen-Heravi et al., 2018). Although the use of EVs to diagnose infectious diseases has been less studied, it shows great promise as the markers could be both host and pathogen derived. Another exciting potential use of EVs is for vaccines. The concept of using exosomes as vaccines has its origin in the cancer field (Mignot, Roux, Thery, Segura, & Zitvogel, 2006). More recently, the potential use of exosomes as vaccines against parasitic diseases has been assessed (Beauvillain et al., 2009; Martin-Jaular et al., 2011). Specifically, it has been shown that *Plasmodium yoelli* (Martin-Jaular et al., 2011) and *Toxoplasma gondii* (Beauvillain et al., 2009) parasite EVs or EVs from infected host cells can protect mice from infection. However, the results obtained in *T. vaginalis* questioned whether immunisation with EVs might be a successful vaccination method in this parasite (Olmos-Ortiz et al., 2017). There are several potential advantages to using EVs as vaccines against pathogens that include a more stable conformational conditions for the proteins; improved molecular distribution due to the ability of EVs to circulate in bodily fluids and the fact that EVs are one of the body's "natural" mechanisms for transporting antigens between cells. Thus, EVs may serve as both biomarkers for infection as well as vaccine candidates. We have only scratched the surface of understanding the functions of EVs in infection biology of anaerobic protozoan parasites (Figure 2) but the obtained results suggest that these specialised vesicles have several intriguing properties yet to be revealed.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

Natalia de Miguel conceived the study. The manuscript was drafted by Yesica Romina Nievas, Ayelen Lizarraga, Nehuen Salas, Verónica Cóceres and Natalia de Miguel. All authors reviewed, edited and approved the manuscript.

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