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The role of extracellular vesicles in *Plasmodium* and other protozoan parasites

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Summary

Protozoan parasites and other microorganisms use various pathways to communicate within their own populations and to manipulate their outside environments, with the ultimate goal of balancing the rate of growth and transmission. In higher eukaryotes, including humans, circulating extracellular vesicles are increasingly recognized as key mediators of physiological and pathological processes. Recent evidence suggests that protozoan parasites, including those responsible for major human diseases such as malaria and Chagas disease, use similar machinery. Indeed, intracellular and extracellular protozoan parasites secrete extracellular vesicles to promote growth and induce transmission, to evade the host immune system, and to manipulate the microenvironment. In this review we will discuss the general pathways of extracellular vesicle biogenesis and their functions in protozoan infections.

Introduction

Direct and indirect cellular interactions take place at many levels, both in unicellular and multicellular systems. Until recently, it was assumed that indirect cellular interactions are facilitated by secretion and transfer of soluble factors. While initially considered cellular debris, membrane-derived extracellular vesicles (EVs) have emerged as key players for the horizontal transfer of information between cells without direct cellular contact. EVs are small vesicles composed of a lipid bilayer and a size ranging from 0.1 – 1 μ m. They contain cytosol and display an extracellular plasma membrane leaflet on their outer surface. Many mammalian cell types secrete vesicles at rest or upon activation (Reviewed in (Thery *et al.*, 2002b, Raposo *et al.*, 2013)). EVs express cell type specific proteins, lipids, messenger RNA and micro RNA that are important for their function. More surprisingly, these molecules are functional and can transduce signals in recipient cells. For example, EVs from mast cells contain micro RNAs that can be transcribed into proteins in the recipient cell (Valadi *et al.*, 2007). From most studies it appears that the cargo content in EVs varies with the cell of origin suggesting a selective loading mechanism.

Different types of vesicles can be identified based on their sizes, biogenesis and cellular compartment of origin. Generally, EVs are classified into exosomes, microvesicles (MVs) and apoptotic bodies. Exosomes and MVs are small vesicles of less than 1 μ m in size, whereas apoptotic bodies are larger than 1 μ m and less structured (Raposo *et al.*, 2013). Apoptotic bodies are released during programmed cell death (Hristov *et al.*, 2004), and they carry whole organelles on their inside (Taylor *et al.*, 2008). In addition, some cancer cells release oncosomes, which are vesicles larger than the conventional MVs (Di Vizio *et al.*, 2009, Morello *et al.*, 2013). EVs are formed either at the plasma membrane or in the lumen

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of various subcellular organelles. Although their biogenesis and function may differ, it is not always possible to fully differentiate between exosomes and MVs. Therefore, in many studies the EVs analyzed may be composed of a mixture of exosomes and MVs. EVs are involved in the regulation of many processes under physiological conditions, such as immune surveillance and tissue repair. However, they are also linked to tumor formation and progression, as well as pathology and spread of viral diseases such as HIV and Epstein Barr Virus (EBV).

There is increasing evidence for the release of specific EV populations in the context of some of the major human parasitic pathogens, including those causing malaria, Leishmaniasis and Chagas disease. These diseases are all transmitted by arthropod vectors, and the parasites undergo multiple life cycle transformations, cycling between asexual and sexual replication. Such complex life cycle strategies require rapid adaptation to changing environments, and parasites have developed various strategies to respond to these changes and maintain persistence in the human host. EVs appear to play a prominent role in the parasite host interplay in order to regulate host immune responses and provide sensing mechanisms within the parasite population.

In this review we will first introduce the different types of vesicles and then discuss their biogenesis and function in protozoan parasites.

Classification and biogenesis of EVs

Exosomes

Exosomes were identified 30 years ago while studying transferrin receptor trafficking in reticulocytes. Transferrin receptor mediates intracellular iron uptake, which is required for hemoglobin synthesis. During maturation reticulocytes lose their internal compartments, and this process is accompanied by extensive membrane remodeling and the loss of transferrin receptor (Pan *et al.*, 1983). Transmission electron microscopy revealed that maturing reticulocytes contained large sacs filled with small membrane-enclosed vesicles of nearly uniform size (30 – 100 nm) within their cytoplasm. Transferrin receptor turnover was tracked using labeled transferrin, demonstrating transferrin accumulation on small vesicles within multivesicular bodies (MVB). Additional studies demonstrated that MVBs can fuse with the plasma membrane and release their internal vesicles as exosomes into the extracellular compartment (Harding *et al.*, 1983).

Generally, exosomes are formed within the endolysosomal network, a membranous compartment that sorts the various intraluminal vesicles at the late endosome (also termed multivesicular body (MVB)) for cargo degradation into lysosomes or for secretion as exosomes into the extracellular milieu (Figure 1). Exosomes carry specific protein markers of the endosomal pathway and proteins involved in vesicle formation, such as tetraspanins (multimembrane spanning proteins), chaperones such as HSP70 and members of the Rab GTPase family (Ostrowski *et al.*, 2010). The generation of exosomes is initiated in early endosomes upon endocytosis of extracellular material at the plasma membrane by intraluminal vesicle formation that results in MVB formation. Exosomes are released as MVBs fuse with the plasma membrane (Roxrud *et al.*, 2010).

The ESCRT (endosomal sorting complex required for transport) pathway consists of three complexes (ESCRT-I, ESCRT-II, ESCRT-III) and is a key mediator of MVB biogenesis, regulating membrane budding at cell surfaces and at the level of late endosomes (Raposo *et al.*, 2013). After completing MVB formation, ESCRT is separated from the MVB membrane and contributes to the transport of new cargo. ESCRT functions primarily to sort cargo proteins such as activated receptors to MVBs before their degradation in lysosomes, but

some cargo is also targeted to exosomes (Raposo *et al.*, 2013, Henne *et al.*, 2012, Henne *et al.*, 2011). Indeed, identification of the ESCRT components Tsg101 and Alix by proteomics in exosome preparations strongly suggests a role in MVB biogenesis and exosome formation (Thery *et al.*, 2002b). TSG101 (Tumor Susceptibility Gene 101) is a component of the ESCRT-I complex and Alix (Booth *et al.*, 2006) is a cytosolic protein in mammalian cells that was originally identified on the basis of its association with pro-apoptotic signaling as an ESCRT-III binding protein (Missotten *et al.*, 1999). Recent studies of syndecan sorting to exosomes have shed some light on the mechanisms underlying protein targeting to exosomes. Syntenin is a soluble protein recruited to the plasma membrane by binding to the cytosolic domain of syndecans, which are cell-surface transmembrane co-receptors for adhesion molecules and growth factors (Grootjans *et al.*, 1997). Syntenin interacts through specific motifs in its unstructured N-terminus directly with the ESCRT component Alix. Thus, the cytosolic adaptor syntenin connects syndecans to ESCRT and targets the proteins to exosomes (Baietti *et al.*, 2012). Some intracellular pathogens such as retroviruses hijack the ESCRT machinery by binding to the same motif to promote virus budding and egress (Gould *et al.*, 2003).

An alternative pathway for exosome biogenesis is ESCRT-independent and mediated by a lipid-dependent mechanism. Exosomes and lipid rafts have a very similar lipid composition and are enriched in cholesterol and sphingolipids, including a marked enrichment in ceramides compared to the plasma membrane concentration. Ceramides have a small head group and cluster into microdomains that favor budding (Matsuo *et al.*, 2004, Gulbins *et al.*, 2003). Treatment of cells with a neutral sphingomyelinase inhibitor results in a reduction of exosome release (Trajkovic *et al.*, 2008).

Microvesicles

In contrast to exosomes, MVs bud and shed directly from the plasma membrane. The terms ectosomes and microparticles are sometimes used instead to refer to MVs, particularly in the context of red blood cells. It is commonly accepted that their size is in the range of 0.1 to 1 μ m, therefore the sizes between MVs and exosomes overlap and the main difference between the two types of vesicles lies in their biogenesis. MVs form as a result of dynamic interplay between cytoskeletal protein contraction and phospholipid redistribution. Phospholipids and proteins are distributed unevenly across the plasma membrane and form microdomains similar to lipid rafts. Several enzymes of the aminophospholipid translocase type actively maintain this asymmetric distribution (Beyers *et al.*, 1999). Such “flippases” and “floppases” are translocases that transfer phospholipids from the outer leaflet to the inner leaflet of membranes and vice-versa (Leventis *et al.*, 2010). The translocation of phosphatidyl-serine to the outer membrane leaflet triggers membrane budding and therefore MV formation (van der Heyde *et al.*, 2011). Other essential regulators of MV formation have been identified more recently. For example, overexpression of GTP-binding protein ADP-ribosylation factor 6 (ARF-6) results in increased MV secretion (Figure 1). ARF-6 activation results in the contraction of cytoskeletal structures by actin-myosin interactions in a phospholipase D-dependent fashion and subsequent MV formation (Muralidharan-Chari *et al.*, 2009).

The specific interaction of the ESCRT-I component TSG101 with the plasma membrane protein ARRDC1 (Arrestin domain-containing protein 1) drives the budding of MVs. This interaction results in relocation of TSG101 from endosomes to the plasma membrane and mediates the release of MVs that contain TSG101 and ARRDC1. MV formation requires VPS4 ATPase and is enhanced by the E3 ligase WWP2, which interacts with and ubiquitinates ARRDC1 (Nabhan *et al.*, 2012).

Multiple functions for EVs in physiological and pathogenic process

The diversity of EVs and the multiple modes of information delivery are reflected in the various functions of EVs in physiological and pathogenic processes. The majority of EVs described so far are either involved in immune regulation or in tumor formation and/or progression.

Depending on the status of immune cells EVs can induce adaptive responses or inhibit inflammation. For example exosomes released by antigen-presenting dendritic cells (DCs) (Thery *et al.*, 2002a) and B cells (Raposo *et al.*, 1996) carry immune-stimulatory molecules on their surface, including MHC-I and MHC-II and the co-stimulatory molecules CD86 and CD80 (Thery *et al.*, 2002b). Exosomes released from DCs that have been pulsed by tumor peptides can trigger an antigen-specific activation of T cells and induce an anti-tumor cytotoxic T cell response *in vivo*. Tumors secrete EVs that can transfer antigens to dendritic cells to promote cross-protective anti-tumor effects and induction of cytotoxic T cell responses (Wolfers *et al.*, 2001). DC-derived exosomes can also induce activation of naïve CD4 T cells (Thery *et al.*, 2002a). Similar immunization strategies have been used in parasitic diseases (Aline *et al.*, 2004, Schnitzer *et al.*, 2010).

In some cases intracellular pathogens can change the composition and function of exosomes released from infected cells. For instance, macrophages infected with *Mycobacterium avium* release pro-inflammatory exosomes containing glycopeptidolipids (GPLs) (Bhatnagar *et al.*, 2007a). When naïve macrophages are exposed to such exosomes they secrete pro-inflammatory cytokines whereas exosomes from uninfected macrophages are not stimulatory. Similar effects are observed with *M. tuberculosis* and *Salmonella typhimurium* (Bhatnagar *et al.*, 2007b). More generally it is possible to distinguish three types of EVs in the context of infection: i) EVs secreted by free-living pathogens, ii) those secreted by mammalian cells infected with pathogens, and iii) EVs secreted by effector cells that have been stimulated directly by pathogens or through their EVs. While intracellular pathogens can take advantage of the EV machinery of the host, free-living forms must create their own vesicles. Infections by protozoan parasites can profoundly modify the composition and function of EVs (e.g., (Hassani *et al.*, 2013, Mantel *et al.*, 2013)). Several infectious conditions lead to an increased EV concentration in the body fluids of patients suggesting that EVs have a role in physiological regulation and disease outcome. Indeed EVs can mediate host-parasite and parasite-parasite interactions. Specifically they provide pathogens with the means to evade the host immune system, facilitate the spreading of virulence factors and allow cell-cell communication between parasites over long distances.

The known roles of EVs in the context of infections by protozoan parasites will be discussed in the following paragraphs.

EVs in Malaria: immunomodulation and cellular communication

Plasmodium falciparum is transmitted to the human host after a female *Anopheles* mosquito injects parasites during a blood meal into the subcutaneous tissue. Injected sporozoites migrate to the liver where they invade hepatocytes. Inside the hepatocyte each sporozoite develops into several thousand merozoites, which are released in the bloodstream upon egress from hepatocytes and invade red blood cells (RBCs). During blood stage development, ring stage parasites develop into replicative schizont forms that release multiple invasive daughter merozoites. Blood stage parasites are associated with all the pathology and complications observed with symptomatic and severe malaria (Miller *et al.*, 2002). A subset of blood stage parasites commits to the sexual cycle, developing into male

and female gametocytes in the human host before further development and sexual recombination in the mosquito.

Several human studies have demonstrated that circulating EVs from various cellular sources are elevated during infection with *Plasmodium* species, and their plasma levels are associated with severity of disease. For example, elevated levels of endothelial cell-derived EVs were measured in plasma of sick *P. falciparum*-infected children in Malawi compared to healthy controls (Combes *et al.*, 2004). In a study of *P. vivax*-infected patients in Brazil, EVs from RBCs, platelets, leucocytes and monocytes were elevated at levels similar to patients with ovarian carcinoma (a known EV-inducing condition) (Campos *et al.*, 2010). In another study levels of RBC-derived EVs were directly compared in patients infected with either *P. vivax*, *P. malariae* or *P. falciparum*, demonstrating highest levels in severe malaria patients infected with *P. falciparum* (Pankoui Mfonkeu *et al.*, 2010). Although these studies do not demonstrate causality between elevated EV levels and disease severity, *in vitro* experiments and studies in the mouse model suggest that EVs derived from platelets and endothelial cells contribute to the overall inflammatory condition (Couper *et al.*, 2010, Coltel *et al.*, 2006, Wassmer *et al.*, 2006, Grau *et al.*, 2005). Interestingly, inflammatory responses also activate receptor expression on endothelial cells and thereby increase parasite sequestration. The ABCA1 (ATP-binding-cassette transporter 1) knock out (KO) mouse model has been used to investigate the effect of EVs on severe malaria. ABCA1 promotes membrane microvesiculation and ABCA1 KO mice have a defect in EV release (Yakushi *et al.*, 2000, Hamon *et al.*, 2000). Importantly they are resistant to severe malaria induced by *P. berghei* ANKA infection probably due to significantly reduced inflammation, and they eventually die from severe anemia instead (Ling *et al.*, 2011, Combes *et al.*, 2005, Pankoui Mfonkeu *et al.*, 2010). The mouse malaria model also confirms the pro-inflammatory properties of RBC-derived EVs, as those purified from infected mice strongly induce macrophage activation in a TLR-dependent fashion *in vitro* (Couper *et al.*, 2010). We have recently shown in *P. falciparum* that EVs derived from infected RBC supernatant are rapidly internalized by macrophages and trigger strong pro- and anti-inflammatory cytokine responses (Mantel *et al.*, 2013).

Mature red blood cells are devoid of any internal membranes including machinery for exocytosis and endocytosis, and it is therefore puzzling how infected RBCs can release EVs. Ultrastructural studies first demonstrated that malaria parasites profoundly alter the host RBC environment (Luse *et al.*, 1971), suggesting that parasites create their own protein network for nutrient import and export of virulence factors (Lauer *et al.*, 1997). Indeed, more recent studies have identified hundreds of proteins that are exported across the parasite membrane and the surrounding parasitophorous vacuole membrane into the host cell (Hiller *et al.*, 2004, Marti *et al.*, 2004, Sargeant *et al.*, 2006). A subset of these exported proteins is deposited onto the RBC surface, including the parasite solute transporter Clag3 proteins and the major virulence factor PfEMP1 (Nguitragool *et al.*, 2011, Leech *et al.*, 1984, Su *et al.*, 1995). Protein trafficking to the infected RBC surface is via parasite-induced membrane platforms termed Maurer's clefts that are anchored to the host cytoskeleton (e.g., (Knuepfer *et al.*, 2005, Spycher *et al.*, 2006)). Small vesicles attached to actin filaments are trafficked between Maurer's clefts and the RBC membranes, and these structures are lost in patients with sickle cell hemoglobin mutations (Cyrklaff *et al.*, 2011), suggesting a potential mechanism of protection from severe malaria.

We have recently demonstrated release of EVs from *P. falciparum* - infected RBCs using live imaging, confirming early reports of this phenomenon by electron microscopy (Luse *et al.*, 1971). Such EVs can efficiently be isolated from *in vitro* culture and their membranous nature has been demonstrated by atomic force microscopy and electron microscopy (Regev-Rudzki *et al.*, 2013, Mantel *et al.*, 2013). Proteomic analysis of EVs derived from infected

RBCs revealed an enrichment of host lipid raft proteins such as stomatin, as well as the microvesicle markers ARF-6 and VPS4 (Mantel *et al.*, 2013), suggesting that minimal host machinery may be involved in EV biogenesis. A number of parasite proteins were also identified, in particular proteins from the Maurer's Clefts structures (Mantel *et al.*, 2013), and deletion of a specific Maurer's Clefts component inhibits EV production and uptake (Regev-Rudzki *et al.*, 2013). Quantitative and kinetic experiments of EV release revealed that infected RBCs produce about 10 times more EVs than uninfected RBCs and that the vast majority of EVs are released shortly before parasite egress (Mantel *et al.*, 2013). This time interval coincides with the timing of observed disappearance of Maurer's clefts in a recent time-lapse analysis of parasite development (Gruring *et al.*, 2011).

Surprisingly we observed that infected RBCs are also able to internalize EVs and transfer them into the parasite cytosol, pointing to their potential involvement in intercellular communication between parasites. Presence of additional membranes surrounding such internalized EVs suggests that some sort of phagocytic or endocytic process must be operational in infected RBCs (Mantel *et al.*, 2013). Considering the apparent absence of such processes in mature RBCs, such pathways are likely provided by the parasite. Importantly EV binding/internalization to infected RBCs is titratable and correlates with increased formation of malaria transmission stages (i.e., gametocytes) suggesting that EVs are involved in a cellular communication pathway with density sensing properties within the parasite population (Mantel *et al.*, 2013). EVs derived from transgenic parasites can transfer DNA encoding for a drug resistance marker between individual parasites, thereby spreading drug resistance in the parasite population (Regev-Rudzki *et al.*, 2013). Resistant parasites commit to the transmission stage pathway, again suggesting existence of a cellular communication pathway in malaria parasites. The data from these two studies confirm previous observations that parasite conditioned medium (from which EVs for the above experiments were derived) can induce gametocyte formation (Dyer *et al.*, 2003, Fivelman *et al.*, 2007), and they provide a rationale for targeted investigation of this novel cellular communication pathway. Altogether current evidence suggests that *P. falciparum* parasites have developed a unique mechanism of cellular communication via EVs to sense their population density during infection and accordingly regulate the balance between virulence (asexual growth) and transmission (production of gametocytes).

EVs in *Leishmania*: immunomodulation via host miRNA regulation

Leishmania parasites are the causal agents of visceral and cutaneous leishmaniasis, diseases transmitted to mammals by *Phlebotomus* (Old World) and *Lutzomyia* (New World) sandflies. When biting their hosts, infected sandflies regurgitate extracellular “metacyclic” promastigote forms into the skin. After phagocytosis by macrophages, the flagellated promastigotes transform into the non-motile amastigote form in the phagolysosome of the host cell. Amastigotes replicate and infect additional macrophages. Infected macrophages that are taken up by sandflies release amastigote forms into the mid gut where they transform into “procyclic” promastigote forms.

Leishmania parasites are able to secure survival and propagation within their host by altering signaling pathways involved in the ability to kill pathogens or to activate the adaptive immune system. Proteomic analysis of supernatant from infected macrophage cultures identified more than 150 secreted *Leishmania* antigens (Silverman *et al.*, 2008). Many known mammalian exosomal proteins, as well as *Leishmania* orthologs to ESCRT-III components were present in that supernatant suggesting an exosome-based secretion system (Silverman *et al.*, 2008). Indeed, ultrastructural analysis of purified material from supernatants confirmed the vesicular structure of the secreted material. It is currently not clear how amastigotes trigger exosome formation in infected macrophages and how these

exosomes cross the surrounding phagolysosome and are eventually secreted into the supernatant.

An important step in the immune evasion process is the activation of host protein tyrosine phosphatase SHP-1 (Shio *et al.*, 2012). Proteomics of exosomes released from infected macrophages identified factors involved in immune evasion such as the parasite proteins GP63 and leishmanolysin. GP63 is a zinc metalloprotease that is internalized by macrophages. Upon internalization it cleaves and activates host protein tyrosine phosphatases to prevent INF-gamma induced signaling, thereby blocking the pro-inflammatory cytokine response against *Leishmania* infection (Gomez *et al.*, 2009). In addition GP63 is targeted to hepatic cells via exosomes where it inhibits the pre-miRNA processing enzyme Dicer1 to block formation of the miRNA ribonucleoprotein complex. Indeed, miR-122 expression levels in the liver are reduced upon *Leishmania* infections, resulting in altered expression of genes involved in lipid metabolism (Ghosh *et al.*, 2011). Visceral leishmaniasis is caused by accumulation of infected macrophages in the liver and spleen and causes abnormal lipid profiles. Restoration of miR-122 and Dicer1 expression level increased serum cholesterol and reduced parasite burden (Ghosh *et al.*, 2013).

The free-living promastigote forms also release vesicles from the plasma membrane and via the MVB from the flagellar pocket (Silverman et al 2008), an invagination in the plasma membrane where the flagellum emerges and the major site of protein secretion in the parasite. Proteomic analysis suggested that the plasma membrane-derived EVs are in fact apoptotic bodies while those released from MVBs are exosomes, as demonstrated by the large overlap with orthologs from human exosome components. Purified vesicles from *L. donovani* promastigotes modulate the cytokine response of human monocytes by inducing IL-10 and down-regulating TNF-alpha. DCs exposed to such EVs are unable to induce the differentiation of naïve T cells into Th1 cells. Proteomic analysis of EVs released by a mouse macrophage cell line exposed to either stationary phase *L. mexicana* promastigote forms or to LPS revealed that most host proteins were shared between the 2 exosome populations. GP63 was the only parasite protein identified in EVs released from parasite-exposed macrophages. These EVs were able to induce phosphorylation of signaling proteins and translocation of transcription factors into the nucleus, suggesting an active transfer of GP63 into recipient cells (Hassani *et al.*, 2013). Together these data suggest that both intracellular and free-living parasite stages can release EVs as well as trigger other cells to release EVs in order to regulate host immune responses.

EVs in *Trypanosoma cruzi*: a role in chronic disease?

Trypanosomes are flagellated parasitic protozoa that include a number of medically important parasites. *Trypanosoma cruzi* infects animals and humans in the Americas and is the etiological agent of Chagas disease. After entry into the human host, *T. cruzi* trypomastigotes invade macrophages, which carry the pathogen to other sites within the body. After invasion the trypomastigotes differentiate into amastigote forms. Amastigotes replicate and differentiate back to trypomastigotes that are released in the bloodstream. These can either infect other cells or be transmitted to the vector, a *Triatoma* bug, for further differentiation into epimastigote forms.

Cardiomyopathies are complications observed during chronic Chagas disease. During this phase the inflammatory lesions are not directly related to the presence of the parasite (Rassi *et al.*, 2001) and instead assumed to be the result of an autoimmune reaction. It has also been proposed that secretion of *T. cruzi* antigens and subsequent adsorption by uninfected cells may lead to cellular damage due to the immune response directed to the parasite (Pinho *et al.*, 2002). Indeed, trypomastigote forms secrete antigens that are present in sera from *T.*

cruzi - infected animals (Dzbenksi, 1974) and from human patients in the acute and chronic phases of Chagas disease (Araujo *et al.*, 1981). As soon as trypomastigote forms disappear from the bloodstream to invade host cells, antigens are not detectable anymore in circulation. Antigens are present in vesicles at the parasite membrane and in the flagellar pocket before secretion by the trypomastigote stage (Goncalves *et al.*, 1991), suggesting release via EVs. Likewise in the mouse system antigens can be internalized into recipient cells, and those cells can be the targets of parasite-specific antibody responses (Pinho *et al.*, 2002). Injection of EVs in BALB/C mice prior to infection with *T. cruzi* leads to increased parasitemia, as well as severe heart pathology and intense inflammation, suggesting a potential role of EVs in increased virulence (Trocoli Torrecilhas *et al.*, 2009). In addition to producing their own EVs, *T. cruzi* trypomastigotes can induce production of EVs from other cells, in particular from monocytes. Such EVs can bind to trypomastigotes protecting them against lysis by the complement system (Cestari *et al.*, 2012).

A recent study has specifically compared origin and composition of EVs derived from trypomastigote forms in humans with those from epimastigotes present in the sand fly vector (Bayer-Santos *et al.*, 2013). Both forms release EVs from the plasma membrane and from MVBs localized at the flagellar pocket. Fractionation provided purified preparations of both vesicle types from each parasite stage for proteomic analysis (Bayer-Santos *et al.*, 2013). These data demonstrate presence of multiple parasite antigens as well as other protein classes including nuclear proteins in EVs, supporting the idea that *T. cruzi* modulates host cells via EVs during infection. Together with the differential density the data also suggest that plasma membrane-derived EVs are microvesicles while those released through MVBs at the flagellar pocket are exosomes. Altogether, current evidence suggests that like *Leishmania* parasites, *T. cruzi* produces exosome-like particles from the free-living and intracellular stages, as well as triggers other cells for EV production, in order to modulate the host immune response.

EVs in *Trichomonas*: regulating epithelial cell adherence

Trichomoniasis is a sexually transmitted disease caused by the flagellated protozoan parasite *Trichomonas vaginalis*. The parasite colonizes the human urogenital tract where it remains extracellular and adheres to epithelial cells (Swygard *et al.*, 2004). Person to person transmission occurs directly as the parasite does not form environmentally resistant cyst forms.

Trichomoniasis is associated with vaginitis, cervicitis, urethritis, pelvic inflammatory disease and adverse birth outcomes. Recent work has demonstrated that *T. vaginalis* secretes EVs that modulate expression of the cytokines IL-6 and IL-8 in ectocervical cells. Moreover, pre-treatment with EVs can increase cytoadherence of the parasites to epithelial cells through receptor activation (Twu *et al.*, 2013). Interestingly, EVs from highly cytoadherent strains induce strong binding, whereas exosomes from poorly adherent strains have only a minor effect on binding. These data suggest that EVs contain strain-specific factors responsible for the differential binding phenotype. Proteomics of purified EV preparations identified *T. vaginalis* surface proteins and greater than 70% overlap with mammalian exosome markers, suggesting that *T. vaginalis* EVs are in fact exosomes. Shared proteins include components of the ESCRT machinery as well as tetraspanin homologs. *T. vaginalis* exosomes also contain small RNA species of as yet unknown function.

Concluding remarks

Extracellular vesicles have been identified in multiple lineages of the eukaryotic kingdom and are also released by both Gram-negative and some Gram-positive bacteria,

demonstrating their existence as ancient mechanisms for information exchange. Presence of ESCRT components across early eukaryotic lineages (Williams *et al.*, 2007) and identification of ESCRT in exosomes released from *T. vaginalis* and *Leishmania* also supports the idea that exosome formation is a highly conserved mechanism in eukaryotes. The free-living stages of *Leishmania sp.* and *T. cruzi*, which both belong to the Kinetoplastid phylum, release exosomes from MVBs at the flagellar pocket. Likewise *T. vaginalis* exosomes are released through MVBs, again suggesting conserved mechanisms for exosome biogenesis and release in eukaryotes. Interestingly, ESCRT I and II components have been lost in the strictly intracellular protozoan parasite genera *Plasmodium* and *Toxoplasma*, which both belong to the Apicomplexan phylum. The absence of parasite or host ESCRT markers and presence of host VPS4 and ARF-6 in EVs released from *P. falciparum*-infected RBCs suggests that they are in fact microvesicles.

Recent studies have started to shed light on the diverse functions relating to exosome and microvesicle release by some of these protozoan parasites and the cells that they infect (Figure 2). Presence of parasite-derived components (protein, lipid, RNA, DNA) in EVs makes them highly immunogenic, highlighting a common EV function in immune modulation across all the parasites studied so far. Secondly, parasites can manipulate their environment by activating other cell types (indirectly or directly) in order to promote parasite phenotypes such as adherence to epithelial or endothelial cells, as shown for *T. vaginalis* and for malaria parasites, respectively. Thirdly, parasites can communicate with each other for the purpose of regulating transmission stage formation, as we have recently demonstrated in *P. falciparum*. Such communication and sensing mechanisms are likely more widespread amongst protozoa, as populations often shift between states in a synchronized fashion that is reminiscent of quorum sensing in bacterial species. The mammalian EV field has provided many tools in recent years that can now be applied to systematically investigate the fascinating biology of these vesicles in the context of host parasite interactions.

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Figure 1. Overview of EV biogenesis and functions

A. Exosome and MV biogenesis. Exosomes are derived from multivesicular bodies (MVBs). Exosome generation is initiated through inward budding of early endosomes leading to MVB formation (1). Exosomes are released when MVBs fuse with the outer cell membrane to release their cargo. ESCRT proteins, in conjunction with additional factors such as syntenin and syndecans, mediate the biogenesis of MVBs and the sorting of specific cargo to MVBs. Rab proteins regulate maturation and fusion of MVBs with the plasma membrane (2). Microvesicles (MVs) bud directly from the plasma membrane and are shed into the environment (3). Their formation also requires specific factors such as ARF-6, VPS4 and the plasma membrane protein ARRDC1. **B. EV function.** EVs are composed of a lipid bilayer, and they express specific receptors on their surface reflecting their cellular origin (i.e., tetraspanins, integrins). Exosomes can be identified by presence of specific ESCRT components such as TSG101 and Alix. EVs contain various bioactive molecules including protein, lipid, DNA and RNA (including miRNA bound to Argonaute-2 [Ago2]).

Figure 2. EVs in parasites

A. *Plasmodium* sp. *P. falciparum*-infected RBCs release EVs that contain multiple Maurer's cleft (MC) components. One MC component, PTP2, is essential for EV release and uptake by recipient cells. The EVs are phagocytosed and induce secretion of cytokines by macrophages. EVs can also be internalized by infected RBCs and trigger differentiation of the parasite in the recipient cell into gametocytes. **B. *Leishmania* sp.** During human infection, free-living promastigote forms and intracellular amastigotes secrete EVs that contain the parasite antigen GP63. GP63 induces host SHP-1 that down-regulates the immune response. EVs can also be transferred to hepatocytes where GP63 cleaves DICER1, inhibiting the maturation of the lipid regulator miR-122, promoting growth of the parasite. **C. *Trichomonas vaginalis*.** The parasite secretes EVs that can fuse with host cells and induce secretion of IL-6 and IL-8, activating receptor expression on the surface of epithelial cells and therefore inducing parasite attachment. **D. *Trypanosoma cruzi*.** Free-living trypomastigote forms secrete vesicles containing *T. cruzi* antigens. When fibroblasts and cardiomyocytes adsorb EVs they become targeted by a humoral immune response that is responsible for tissue damage. Trypomastigote forms also trigger the release of EVs from monocytes, and these monocytic EVs bind to the trypomastigotes and protect them from complement lysis by binding and neutralizing the C3 convertase.