EXTRACELLULAR VESICLES IN INFECTIOUS DISEASES

EDITED BY: Shulamit Michaeli, Neta Regev-Rudzki and
Ana Claudia Torrecilhas

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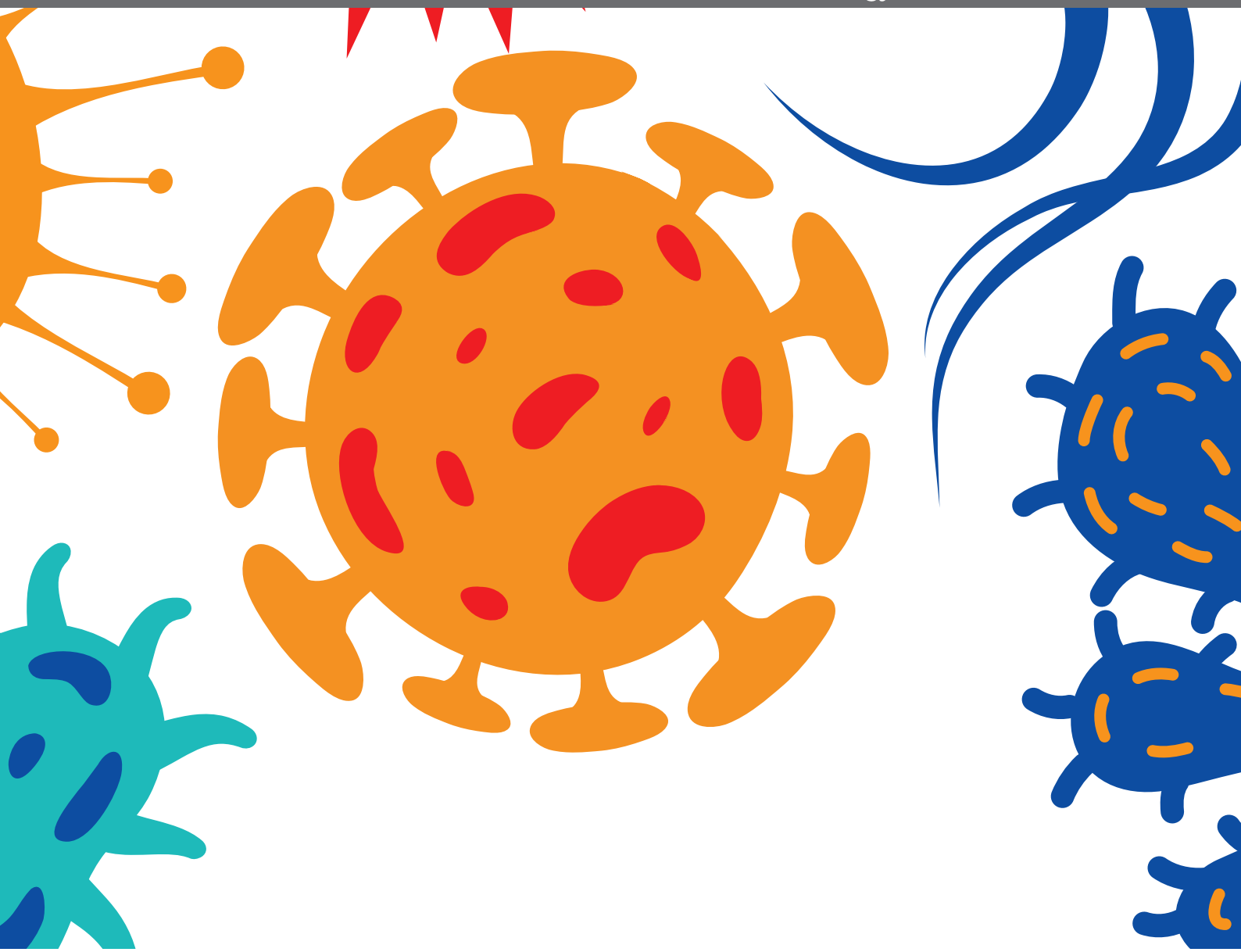

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EXTRACELLULAR VESICLES IN INFECTIOUS DISEASES

Topic Editors:
Shulamit Michaeli, Bar-Ilan University, Israel
Neta Regev-Rudzki, Weizmann Institute of Science, Israel
Ana Claudia Torrecilhas, Federal University of São Paulo, Brazil

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EDITORIAL
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Editorial: Extracellular Vesicles in Infectious Diseases

Neta Regev-Rudzki[1]*, *Shulamit Michaeli*[2]* and *Ana Claudia Torrecilhas*[3]*

[1] Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel, [2] Bar-Ilan University, Ramat Gan, Israel, [3] Department of Pharmacy, Federal University of São Paulo (UNIFESP), São Paulo, Brazil

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Editorial on the Research Topic

Extracellular Vesicles in Infectious Disease Research

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Jeroen P. J. Saeij, University of California, Davis, United States

***Correspondence:**
Ana Claudia Torrecilhas
ana.torrecilhas@unifesp.br
Neta Regev-Rudzki
neta.regev-rudzki@weizmann.ac.il
Shulamit Michaeli
Shulamit.Michaeli@biu.ac.il

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Extracellular vesicles (EVs) isolated from pathogens mediate communication between parasites and their hosts under a variety of physiological and pathological conditions. EVs deliver cell-free messages *via* a transfer of RNA, proteins, and even DNA to modulate and induce inflammation and to control the host infection process. EVs can provide valuable information on how a pathogen sends messages to other pathogens and hosts (Torrecilhas et al., 2012; Campos et al., 2015; Ofir-Birin and Regev-Rudzki, 2019; Torrecilhas et al. 2020). This Research Topic provides an overview of the mechanism of EV-mediated communication between hosts and viruses, parasites, and fungi. This Research Topic consists of 17 papers, including 8 reviews and 9 original papers. Several studies on this topic assessed the effects of EVs on the interactions between pathogens and hosts, and the mechanisms of EV-mediated communication between hosts and pathogens were also addressed.

The first original article published in this Frontiers in Cellular and Infection Microbiology Parasite and Host issue is from Duguet's group (Duguet et al.). They showed that EVs isolated from *Caenorhabditis elegans* contain microRNAs and small regulators that affect biological processes and comment on their role in host-nematode communication. Parasite-derived miRNAs regulate host immune system mRNAs.

The paper by Zhang et al. discusses how *Echinococcus granulosus* protoscoleces (PSCs) and hydatid cysts release EVs that are exosome-like based on size and morphology. The authors analyzed the miRNA, circRNA, and lncRNA profiles of the 20 most abundant miRNAs in EVs and explored their possible roles in biological processes, especially in pathways associated with pathogenicity and the host immune response. These EVs contain small RNA species, including specific miRNAs that are homologous to host miRNAs and induce immunomodulation in the host.

Another article discusses the role of *Cryptococcus deneoformans*, a fungus that causes meningoencephalitis in immunocompromised patients. In this study, the authors showed that fungal shedding of EV RNAs and cryptococcal intersectin protein (Cin1) govern a unique Cin1-Wsp1-Cdc42 endocytic pathway required for intracellular transport, virulence, and regulated particle secretion. Cin1 has clinical importance as an exRNA, and studies using cutting-edge technology in cryptococcal pathogenesis may contribute to the discovery of novel therapeutic strategies (Liu et al.).

The study by Nawaz et al. included proteomic analysis of EVs purified from the saliva of *Haemaphysalis longicornis*. The bush tick is a vector of human disease-causing agents, such as the agent causing thrombocytopenia syndrome. The authors found that EVs carry proteins that may be helpful during tick development and vesicular proteins involved in proton transport, detoxification, ECM-receptor interaction, ribosomes, RNA transport, ABC transporters, and oxidative phosphorylation.

Several studies on this topic have assessed the role of EV release in leishmaniasis. Saini and Rai showed that treatment with linoleic acid (LA) inhibited *Leishmania donovani* infection in macrophages, suppressed the parasitic load, and reduced the levels of IL-12 and *i*NOS. LA promoted a protective-type immune response in the infected host and inhibited the release of *Leishmania*-derived microvesicles. Soares' team also presented data on macrophage activation using EVs isolated from *L. infantum L. braziliensis*, and *L. amazonensis*. EVs from *L. braziliensis* and *L. infantum* parasites failed to induce a proinflammatory response. EVs from both the *L. infantum WT* and LPG-deficient mutant (LPG-KO) did not show any differences in their interactions with macrophages, suggesting that LPG alone was not sufficient for activation (Nogueira et al.). Another group investigated whether *Leishmania amazonensis* or EVs modulate B-1 cell activation and differentiation. *L. amazonensis* infection in B-1 cells decreased the production of NO and ROS. TLR-2, TLR-6 and TLR-9 had significantly higher expression in B-1 cells from mice intraperitoneally injected or stimulated with EVs (Reis et al.).

Gualdrón-López et al. discussed multiparameter flow cytometry combined with cell and EV purification techniques to determine and investigate the interactions of plasma-derived EVs from human spleen cells from *Plasmodium vivax*-infected patients (PvEVs). They used size-exclusion chromatography (SEC) to separate EVs from the bulk of the soluble plasma proteins and stained isolated EVs with fluorescent lipophilic dyes (Gualdrón-López et al.).

This Research Topic also includes reviews that discuss the role of EVs in viral infections. Dr. Soekmadji's group discussed the role of EVs in hepatic fibrosis and how understanding their biological mechanism of action might be beneficial for developing therapeutic strategies to treat chronic liver disease (Lim et al.). Another review discussed EVs containing infective viral genomes that are secreted into the extracellular space (Martins and Alves). EVs can trigger antiviral responses and cytokine secretion even in uninfected cells near the infection, and the role of EVs during viral infections is crucial for comprehending viral mechanisms and how to respond to emerging viral diseases (Martins and Alves). In this review, the symbiosis between the TV-specific endosymbiont viruses *Trichomonasvirus* and *Trichomonas vaginalis* was studied by exploiting sEVs as a vehicle for intercellular communications and by modifying their protein cargo to suppress host immune activation, including NF-κB activation and increases in IL-8 and RANTES. The virus may offer evolutionary benefits to its protozoan host, at least partially, by altering the immunomodulatory properties of EVs spreading from the site of infection to noninfected immune effector cells. EVs serve as vehicles for

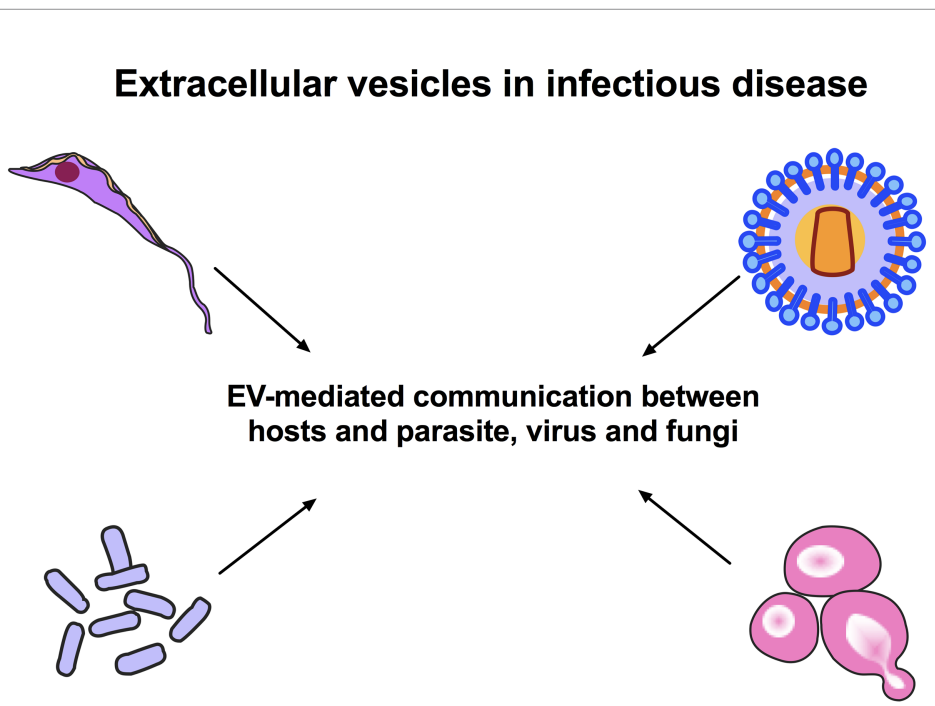

FIGURE 1 | Extracellular Vesicles in Infectious Diseases. EVs isolated from parasites, fungi and viruses are known to mediate communication under a variety of physiological and pathological conditions and modulate the host immune system. EVs are a new research area that can provide valuable information on how a pathogen sends messages to other pathogens and hosts. The fundamental point for the control of endemic parasitic diseases is an understanding of the mechanisms involved in the pathogen-host interactions.

intercellular communication and modify their protein cargo to suppress host immune activation (Govender et al.).

A review on influenza virus infection discusses the EVs released from infected cells, implying the functional relevance of EVs for influenza virus dissemination, EV-based influenza vaccines, and therapeutic strategies to combat the influenza virus (Jiang et al.).

Another review shows how cross-kingdom sRNA trafficking occurs in EVs participating in sRNA delivery in the host during infection, as well as the production of EVs by bacterial and fungal pathogens that help establish the disease. Bacterial vesicle RNA cargo plays roles in recipient host cells by regulating gene expression and modulating the immune response. In fungi, the RNA molecules present in EVs are diverse and participate in communication between the host and pathogenic fungi (Munhoz da Rocha et al.). In parallel, other authors discussed the diverse functions of EVs in fungi, from the regulation of physiological events and responses to specific environmental conditions to the mediation of highly complex interkingdom communications and the function of EVs as vehicles for the delivery of biologically active molecules (Rizzo et al.). Protozoan EV reviews showed that the EVs isolated from pathogenic protozoa are disseminated alongside their biomolecules, and there are specific immune cell responses to protozoan parasite-derived EVs. Protozoan-host interactions release EVs that are crucial for the immunomodulatory events triggered by parasites (Torrecilhas et al.). Additionally, EVs from these organisms have a role in the digestive tracts of invertebrate hosts prior to parasite transmission. This review summarizes the available data on how EVs from medically important trypanosomatids affect parasite interactions with vertebrate and invertebrate hosts (Torrecilhas et al.; Olajide and Cai). Schistosomes release EVs that modulate the host immune response and EV-harbored miRNAs to upregulate the innate immune response of the M1 pathway and downregulate differentiation towards adaptive Th2 immunity. EVs may facilitate the development of novel tools for diagnostics and deliver therapy in relation to schistosomiasis, as well as to immune-associated disorders (Avni and Avni).

Overall, this Research Topic, "Extracellular Vesicles in Infectious Diseases" bring together important original papers and reviews to understand the interactions between pathogens and hosts and deliver scientific reports on the mechanisms of EV-mediated communication between hosts and pathogens (**Figure 1**).

AUTHOR CONTRIBUTIONS

MS prepared the draft. NR-R, SM and ACT revised the draft and approved the final version of the draft. NR-R, SM and ACT assisted in submission. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Extracellular Vesicle-Contained microRNA of *C. elegans* as a Tool to Decipher the Molecular Basis of Nematode Parasitism

Thomas B. Duguet[1†], *Julien Soichot*[2†], *Rostyslav Kuzyakiv*[3,4], *Lars Malmström*[3,4,5,6] *and Lucienne Tritten*[2*]

[1] Institute of Parasitology, McGill University, Sainte-Anne-de-Bellevue, QC, Canada, [2] Institute of Parasitology, University of Zurich, Zurich, Switzerland, [3] S3IT, University of Zurich, Zurich, Switzerland, [4] SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland, [5] Institute for Computational Science, University of Zurich, Zurich, Switzerland, [6] Division of Infection Medicine, Lund University, Lund, Sweden

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Edited by:
Ana Claudia Torrecilhas, Federal University of São Paulo, Brazil

Reviewed by:
Antonio Marcilla, University of Valencia, Spain
Laurence A. Marchat, National Polytechnic Institute of Mexico, Mexico

***Correspondence:**
Lucienne Tritten
lucienne.tritten@uzh.ch

[†] *These authors have contributed equally to this work*

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Among the fundamental biological processes affected by microRNAs, small regulators of gene expression, a potential role in host-parasite communication is intriguing. We compared the miRNA complement of extracellular vesicles released by the free-living nematode *Caenorhabditis elegans* in culture to that of other adult parasitic nematodes. Expecting convergent functional roles for secreted miRNAs due to the common parasitic lifestyle of the organisms under investigation, we performed a miRNA sequence analysis as well as target search and pathway enrichment for potential mRNA targets within host immune functions. We found that the parasite miRNA seed sequences were more often identical to those of *C. elegans*, rather than to those of their hosts. However, we observed that the nematode-secreted miRNA fractions shared more often seed sequences with host miRNAs than those that are not found in the extracellular environment. Development and proliferation of immune cells was predicted to be affected several-fold by nematode miRNA release. In addition, we identified the AGE-RAGE signaling as a convergent targeted pathway by species-specific miRNAs from several parasitic species. We propose a multi-species comparative approach to differentiate those miRNAs that may have critical functions in host modulation, from those that may not. With our simple analysis, we put forward a workflow to study traits of parasitism at the miRNA level. This work will find even more resonance and significance, as an increasing amount of parasite miRNA collections are expected to be produced in the future.

Keywords: nematode, host, microRNA, secreted, extracellular vesicles, computational analysis, immunomodulation, host-parasite relationships

INTRODUCTION

MicroRNAs (miRNAs) represent a class of non-coding RNAs that function as regulators of gene expression, impacting multiple and various physiological functions within eukaryotic organisms. Approximately 20 nucleotides in length, these small molecules target messenger RNA (mRNA) by complementary base pairing and induce mRNA degradation with subsequent inhibition of translation (Bartel, 2009; Fabian et al., 2010). This phenomenon was first described in

the free-living nematode *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000), which led to the subsequent identification of so far over 2,500 human miRNAs predicted to target at least 60% of human genes (Lewis et al., 2005; Friedman et al., 2009). The past decade has seen numerous reports of the ubiquitous nature of miRNA-derived regulation of gene expression while enriching current databases with 38,589 miRNAs in the most recent miRBase release (v. 22) (Griffiths-Jones et al., 2008; Kozomara et al., 2019).

Among the fundamental biological processes affected by miRNAs, a potential role at the host-parasite interface is intriguing. Host-parasite interactions, shaped by thousands of years of co-evolution, are characterized by a highly complex molecular dialogue, including the release and transport of miRNAs. Recent studies suggest that parasites utilize their secreted miRNAome to divert and modulate responses of the host immune system to favor their own development (reviewed in Eichenberger et al., 2018b; Tritten and Geary, 2018). These interactions are at least partly mediated through the secretion and trafficking of parasite-derived extracellular vesicles (EVs), which transport a large diversity of bioactive proteins, lipids, and nucleic acids, among which miRNAs are highly represented (Valadi et al., 2007; Bernal et al., 2014; Buck et al., 2014; Eichenberger et al., 2018a; Tritten and Geary, 2018).

The significant level of identity between host and parasite miRNAs, including for *Brugia malayi* and *Fasciola hepatica* and their respective hosts, was emphasized (Buck et al., 2014; Fromm et al., 2015; Zamanian et al., 2015). This putative convergent evolution is predicted to endow the parasite with the capacity to hijack host miRNA regulatory networks. This identity extends to entire parasite-derived miRNA sequences, but may also be critically important even if restricted to the seed region that binds to cognate mRNAs.

The nematode *C. elegans* has been extensively used as a model organism for research in developmental biology. Its genome was the first to be sequenced and its miRNAome is very well-characterized, with 253 precursors and 437 mature sequences in miRBase release 22 (Kozomara et al., 2019). Based on its free-living lifestyle and our extensive knowledge of its genome and biology, comparing nematodes such as *C. elegans* with parasitic nematodes can be used to uncover the molecular basis of parasitism. *C. elegans*, too, release EVs, which play crucial roles in development and behavior (Beer and Wehman, 2017). The model has also proved valuable to improve our mechanistic understanding of EV release, and the components involved in EV formation (Wehman et al., 2011; Hyenne et al., 2015; Beer and Wehman, 2017).

To date, the secreted miRNAomes of many nematode species have been sequenced and characterized, providing a basis for a computational and experimental effort to identify the targets of key immunomodulatory parasite-derived miRNAs. Here, we have characterized the miRNA complement of EVs released by *C. elegans* in culture. We compared our data to the excretory/secretory (E/S) miRNAs released by other adult parasitic nematodes *in vitro*. Expecting convergent functional roles for secreted miRNAs due to the common parasitic lifestyle of the organisms under investigation, we performed target search and pathway analysis for potential mRNA targets within host immune functions.

MATERIALS AND METHODS

C. elegans Cultures and Media Processing

The hermaphrodite strain N2 (Bristol) was obtained from the *Caenorhabditis* research center, University of Minnesota. They were fed with *Escherichia coli* OP50, maintained, and synchronized as described (Stiernagle, 2006). Synchronized young adult cultures were obtained 3 days after seeding ∼10,000 eggs per nematode growth medium plate. Young adults were thoroughly washed before incubations for 5 or 24 h in M9 buffer (Stiernagle, 2006). Between 35,500 and 158,000 worms were used for each experiment (listed in **Supplementary Table S1**). Worms were removed from media by sedimentation and supernatants were processed by sequential centrifugations at 4°C: 10 min at 1,500 × g and 45 min at 12,000 × g. Ultracentrifugations were performed in a Beckman ultracentrifuge (Optima L-80 XP), using a SW40Ti rotor and Ultra-Clear thinwall tubes (14 × 95 mm, Beckman 344060) as follows: 2 h at 120,000 × g, 4°C; the pellet was washed with cold sterile PBS (Gibco), and centrifuged again for 2 h at 120,000 × g, 4°C, as described (Théry et al., 2006; Sotillo et al., 2016). The resulting pellet was lyzed in Trizol reagent (Ambion) and stored at −20°C until further processing. Total RNA was isolated from all 4 samples in parallel, using a modified phenol/chloroform extraction protocol designed to increase the yield and quality of small RNA species (Zununi Vahed et al., 2016). Briefly, following the Trizol reagent manufacturer's instructions and after the usual phase separation, 3M potassium acetate pH 5.2 was added at 1:10 ratio to the collected aqueous phase, incubated for 30 min at −20°C, and spun at 12,000 × g for 12 min at 4°C. Subsequently, the supernatant was mixed with 1 equal volume of 2.5 M LiCl and 2 equal volumes of pre-chilled ethanol. After 2 h at −80°C, the mixture was spun at 16,000 × g for 20 min at 4°C. Pellets were air-dried, solubilized in nuclease-free dH_2O, and stored at −80°C. RNA from two 5 and 24 h incubations each, respectively, were pooled and shipped on dry ice to LC Sciences for miRNA sequencing.

miRNA Sequencing and Analysis

RNA quality was checked using an Agilent Technologies 2100 Bioanalyzer (**Supplementary File S1**). The library was prepared following Illumina's TruSeq small-RNA-sample preparation protocols. Single-end sequencing (50 bp) was performed on Illumina's HiSeq 2500 sequencing system. Raw reads were subjected to LC Sciences proprietary program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to analyze the data. Adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats were removed (**Supplementary File S1**). Unique sequences with 18∼26 nucleotides in length were mapped to *C. elegans* precursors in other nematode miRNA precursors in miRBase v. 22 to identify known miRNAs and novel 3p- and 5p-derived miRNAs. The unmapped sequences were BLASTed against the *C. elegans* genome, and hairpin structures were

predicted from the flanking 80 nt sequences using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Normalization of sequence counts in each sample was achieved by dividing the counts by a library size parameter of the corresponding sample, as in Tritten et al. (2016; GEO accession GSE144289). Mature sequences were re-annotated using MirGeneDB 2.0, a database containing recently curated miRNA information (Fromm et al., 2020).

Mass Spectrometry Analysis

EV-enriched samples from two independent incubations of 5 and 24 h each were submitted for mass spectrometric analysis at the Functional Genomics Center Zurich. Briefly, samples were precipitated using trichloroacetic acid. Protein pellets were washed twice with cold acetone, dried and resuspended in 45 µl 10 mM Tris, 2 mM $CaCl_2$, pH = 8.2, and 5 µl trypsin 100 ng/µl in 10 mM HCl). A microwave assisted digestion of 30 min at 60°C was observed, after which samples were dried and dissolved in 10 µl ddH_20 + 0.1% formic acid. Protein concentrations were measured by Nanodrop. For the LC-MS/MS analysis, 105 and 195 ng (5 h samples), and 212 and 420 ng (24 h samples) digested protein were injected, respectively, on a nanoAcquity UPLC coupled to a Q-Exactive mass spectrometer (Thermo Scientific). Database searches were performed on a Mascot search engine Perkins et al. (1999) against the *Escherichia coli* and *C. elegans* databases (downloaded from Uniprot on 19 November 2019 and from Wormbase on 18 November 2019, respectively), independently and sequentially, in order to maximize number of hits. Further analysis was performed in Scaffold v. 4 (Proteome Software) using stringent settings: 1% protein FDR, a minimum of 2 peptides per protein, and 0.1% peptide FDR. In Scaffold, a quantitative analysis was performed based on normalized total spectra (default parameters) whereby the sum of all the spectra associated with a specific protein within a sample is used, which includes also those spectra that are shared with other proteins and is referred to as the total spectrum count. Proteins were further analyzed by gene ontology using Blast2GO (Götz et al., 2008).

Transmission Electron Microscopy and Nanoparticle Tracking Analysis

Representative *C. elegans* supernatants processed as above (twice independently) were used to verify the presence of extracellular vesicles by transmission electron microscopy at the center for microscopy and imaging analysis (University of Zurich). For negative staining, samples were fixed by adding 4% formic acid in PBS at a 1:1 ratio with sample. Samples were glow-discharged for 20 min on the grid, washed with PBS, and 1% glutaraldehyde was added for 5 min. Samples were washed 5 × 2 min in dH_2O. A contrast enhancement step of 5 min with uranyl acetate 1% was performed, followed by 10 min incubation on ice with uranyl acetate/methylcellulose (900 µl methylcellulose 2%/100 µl 3% uranyl acetate). Samples were dried and imaged by transmission electron microscopy (TEM). Nanoparticle tracking analysis (NTA; ZetaView, Particle Metrix, Germany) was performed on a fresh 24 h incubation sample, diluted 1:250 in commercial PBS (Gibco) confirmed to be particle-free by NTA. Measurements of particle concentration and size were based on 11 frames.

Comparative Analysis Across Several Nematode Species

Experimental E/S miRNA collections from *in vitro* studies on adult gastrointestinal nematodes were retrieved from the literature, for which sequence data was available. These comprised miRNAs from *Ascaris suum* (n = 29; Hansen et al., 2019), *Haemonchus contortus* (n = 40; Gu et al., 2017), and *Trichuris muris* (n = 56; Eichenberger et al., 2018c). miRNA family annotations were obtained from MiRGeneDB 2.0 (Fromm et al., 2020), based on homology with *C. elegans* and *A. suum* mature miRNA collections and/or miRBase v. 22 (Kozomara et al., 2019). High-confidence E/S miRNA data from *H. polygyrus*, present in 10 copies or more in the worm secretions (although not confined to EVs) and registered in miRBase v. 22, were included in the seed sequence identity analysis (n = 74; Buck et al., 2014).

UpSetR package was used to plot the extent of common and unique seed sequences (nucleotides 2–8) across experimentally validated E/S parasite miRNA datasets (Lex et al., 2014).

miRNAs from the corresponding mammalian host species were also extracted from miRBase (v. 22): *Sus scrofa* (n = 457), *Mus musculus* (n = 1,978), *Ovis aries* (n = 153), as well as those from *C. elegans* (n = 437). In order to evaluate seed sequence conservation toward host miRNAs vs. nematode miRNAs, we assessed the proportion of identical seed sequences in parasite vs. host, and in parasite vs. *C. elegans* in a similarity matrix (Hamming distance), as proposed previously (Kehl et al., 2017). Briefly, the matrices consisted of pairwise comparisons between (i) all parasite and host seeds for each host-parasite association, and (ii) all parasite vs. *C. elegans* seeds (using 437 *C. elegans* mature miRNAs from miRBase). Approximate string distances were calculated for each nematode/host miRNA pair with the function "stringdist" package (van der Loo, 2014) in R v. 3.5.1 (method = hamming). The number of nucleotides matching in position between two sequences was used to create graphs expressed as a percentage of the total number of comparisons. Whether the proportions of 100% matching seeds between parasite vs. host and parasite vs. *C. elegans* are equal was tested in two-proportions z-tests (two-sample test for equality of proportions with continuity correction) in R v. 3.5.1. Briefly, we tested whether the observed proportion of identical nematode seeds against host miRNAs is greater than the observed proportion of nematode seeds against *C. elegans* miRNAs.

miRNA target prediction was performed using the TargetScan software package v. 7 (http://www.targetscan.org), implemented as a standalone workflow under iPortal (Kunszt et al., 2015) and openBIS (Bauch et al., 2011), using default parameters (Agarwal et al., 2015). The 84 species alignment allows mapping by homology. All host genes with a weighted context ++ score ≤ -1.0 and targeted at least twice were used in enrichment analyses, as described (Benna et al., 2019). The R version of the

program Enrichr was employed, using the "KEGG_2019_Mouse," and "Reactome_2016" databases (Kuleshov et al., 2016).

RESULTS

EVs Found in the Secretions of *C. elegans* Contain Numerous Proteins and miRNAs

Imaging by transmission electron microscopy (TEM) revealed a heterogeneous EV population in our samples, as shown in **Supplementary Figure S1**. Some EVs show the typical deflated ball structure as well as the size of exosomes (about 100 nm Ø). Further smaller (possibly) vesicular structures of ∼20 nm, which may represent *E. coli* EVs. Nanoparticle tracking analysis (NTA) of a representative sample (24 h incubation) revealed a median particle size of 107.3 nm Ø (**Supplementary Figure S2**) and a concentration of 1.5×10^{10} particles per ml.

Protein species associated with the EV-enriched fractions of culture supernatants were identified by mass spectrometry. The large majority of identified proteins originated from *E. coli*, with 753 proteins (**Supplementary Table S2**). A total of 170 unique proteins (based on total unique peptide counts, all samples confounded) were identified as *C. elegans* proteins; 128 were observed in the secretions after both 24 and 5 h incubations, 62 were only seen after 24 h incubation and 37 were only detected after 5 h of incubation (**Supplementary Table S2**). Based on our stringent search criteria, one sample (5 h_2) displayed a different and less diverse protein profile (only 51 proteins) compared to the three others, which were in agreement regarding the ranking in abundance, for unexplained reasons. In each of these three samples, eight proteins were common to the top 10 with slight variations in the exact ranking. These included two IRG-7 proteins (Q19853 and A0A131MBU3), two members of the neprilysin metallopeptidase family (B6VQ96 and Q22763), two carboxypeptidases (Q94269 and K8ESM2), two uncharacterized proteins with a C-type lectin domain (G5ECR0), and regulated by DAF-16 (H2L0Q1). The outlying 5 h sample showed one uncharacterized common protein with the other samples' top 10 (H2L0Q1), two actins (P10986 and O45815), two lysozymes (O62415 and Q20964), as well as four proteins with protease/peptidase activity (P34528, Q94271, Q22972, and O01530). Actin was detected in both 5 h samples but not after 24 h. Highly represented in the *C. elegans* EV-enriched secretions were proteins with protease/(carboxy)peptidase activity, and carbohydrate binding properties. In line with the experimental design, 80/127 (63%) proteins assigned to a gene ontology cellular component (74% of all proteins) were associated with membrane (GO:0016020, GO:0016021, GO:0005886, GO:0045121), or the extracellular space (GO:0005615, GO:0005576). This is in agreement with a previous report (Russell et al., 2018).

miRNA sequencing revealed 100 high confidence miRNA species in our samples in total. These encompassed miRNAs from groups 1, and 2a, present in 10 copies or more (see **Supplementary Table S3**). This represents ∼20% of the known *C. elegans* mature miRNAs registered in miRBase. We found 94 and 69 high confidence miRNA species in samples resulting from 5 and 24 h incubation, respectively. **Table 1** shows the most abundant high confidence miRNAs found in each sample, based on normalized read counts. The agreement between the two samples was good: 11/15 most abundant miRNAs at 5 h were also among the top 15 after 24 h incubation.

C. elegans vs. Other Nematode miRNAs

Experimental EV-contained miRNA collections from *in vitro* studies on adult gastrointestinal nematodes were retrieved from the literature, for which sequence data was available.

TABLE 1 | Top 15 most abundant high confidence mature miRNAs in the supernatants of *C. elegans* after 5 h and 24 h in liquid culture.

Abundance rank	*C. elegans* 5 h	*C. elegans* 24 h
1	**cel-bantam-P2_3p (cel-miR-80-3p)**	**cel-let-7-P5_5p (cel-let-7-5p_1ss12GA)**
2	**cel-miR-54-P3_3p (cel-miR-56-3p)**	**cel-miR-54-P3_3p (cel-miR-56-3p)**
3	**cel-miR-36-P1_3p (cel-miR-35-3p)**	**cel-bantam-P2_3p (cel-miR-80-3p)**
4	**cel-let-7-P5_5p (cel-let-7-5p_1ss12GA)**	**cel-miR-36-P1_3p (cel-miR-35-3p)**
5	**cel-miR-52-P1_5p (cel-miR-52-5p)**	**cel-miR-10-P2l_5p (cel-miR-51-5p)**
6	**cel-miR-10-P2l_5p (cel-miR-51-5p)**	**cel-bantam-P4_3p (cel-miR-82-3p)**
7	**cel-miR-36-P3_3p (cel-miR-37-3p)**	**cel-miR-54-P2_3p (cel-miR-55-3p)**
8	**cel-miR-10-P3e_5p (cel-lin-4-5p)**	**cel-miR-36-P3_3p (cel-miR-37-3p)**
9	**cel-miR-96-P3_5p (cel-miR-228-5p)**	**cel-miR-52-P1_5p (cel-miR-52-5p)**
10	**cel-miR-36-P6_3p (cel-miR-40-3p)**	cel-miR-90_3p (cel-miR-90-3p)
11	**cel-miR-54-P2_3p (cel-miR-55-3p)**	**cel-miR-36-P6_3p (cel-miR-40-3p)**
12	cel-miR-10-P3f_5p (cel-miR-237-5p)	cel-miR-10_3p (cel-miR-238-3p)
13	cel-miR-29-P2_3p (cel-miR-49-3p)	cel-miR-1_3p (ppc-miR-1_R+2)
14	**cel-bantam-P4_3p (cel-miR-82-3p)**	**cel-miR-96-P3_5p (cel-miR-228-5p)**
15	cel-miR-31_5p (cel-miR-72-5p_R-2)	**cel-miR-10-P3e_5p (cel-lin-4-5p)**

cel, C. elegans; ppc, Pristionchus pacificus. Both annotations provided by MirGeneDB and miRBase (in brackets) are shown. Deviations from sequences registered in miRBase are indicated as follows: ss, substitutions; R, addition or deletion of nucleotides on the 3′ end of the mature sequence. Names indicated in bold show miRNA candidates common to the top 15 of both incubation periods. Please note that all sequences listed in this table are present in material from both 5 and 24 h.

These comprised miRNAs from *A. suum, H. contortus*, and *T. muris*. Based on MirGeneDB family annotations, the LET-7, MIR-10, MIR-34, and MIR-8 (miR-236 in miRBase) families were consistently identified across all datasets, including among the high-confidence *C. elegans* miRNAs (**Figure 1** and **Supplementary Table S4**). The MIR-216 family (hco-miR-259-5p, asu-miR-5362-5p, asu-miR-5342-3p according to miRBase) was only represented in parasitic species, in the three species examined. The MIR-71 and MIR-54 families were detected in the EVs from all species except *T. muris*. Similarly, the BANTAM family was represented in EVs from all except *H. contortus*. The MIR-2, MIR-36, MIR-29, MIR-279, and MIR-87 families were not described in EVs from *A. suum*.

miRNA seed sequences mediate most of the interaction with and confers specificity to the target mRNA. Unique seed sequences (i.e., a given seed appears only once in the list, even if common to several miRNAs) were compared across datasets, this time also including *H. polygyrus* miRNAs (E/S miRNAs not restricted to EVs; **Figure 2**). Most seed sequences appeared to be unique to each miRNA collection; three seed sequences were shared by all five species among secreted datasets and using miRBase annotations [ACCCGUA, as in miR-100 (or miR-51-56 in *C. elegans*); GAGAUCA, as in miR-81, and GAGGUAG, as in let-7]. One sequence was common to the four parasites, but absent from *C. elegans* (AAGCUCG; as in miR-993). A given seed sequence may not stem from the same miRNA, but may target the same genes. Based on the limited number of studies and species examined here, there is *a priori* no clade-clustering with our small study sample, besides three seeds shared by clade V organisms (*H. contortus*, *H. polygyrus*, *C. elegans*; GAUAUGU, as in miR-50; UCAUCAG, as in miR-5899, and AUGACAC, as in miR-60,−63, -64).

The mRNA targeting properties of miRNAs is directly related to the seed sequence. Here, we focused on the nucleotides in position 2–8 on the mature sequence. Considering the possibility of a potential evolutionary advantage for the parasite to secrete miRNAs sharing identical seeds with its host's and so, subvert to existing host regulatory pathways, we hypothesized that a higher proportion of seed sequence identity (7/7 matching nucleotides) would be observed compared to host miRNAs than to *C. elegans* miRNAs. To test this hypothesis, nematode parasite E/S miRNA seeds (nucleotides 2–8) were aligned to all host miRNAs, creating similarity matrices. The same was done with non-E/S miRNA helminth seeds, aligned to host or *C. elegans* miRNA seeds. Systematic pairwise comparisons between all parasite and host or *C. elegans* miRNAs resulted in between 5,814 and 816,501 comparisons. This is due to the varying numbers of miRNAs available from miRBase for each host species, and to the number of nematode miRNA entries. Applying a two-sample test for equality of proportions with continuity correction, the hypothesis was refuted for every nematode-host association (**Table 2**). On the contrary, nematode E/S miRNAs were more conserved (i.e., more often identical) toward the phylogenetically closer organism, since the proportion of identical seeds was higher in alignments to *C. elegans* than to host miRNA seeds ($p < 0.05$). However, nematode E/S miRNA seeds were found to be more often identical to host miRNAs than non-E/S (somatic) miRNAs ($p < 0.001$). This

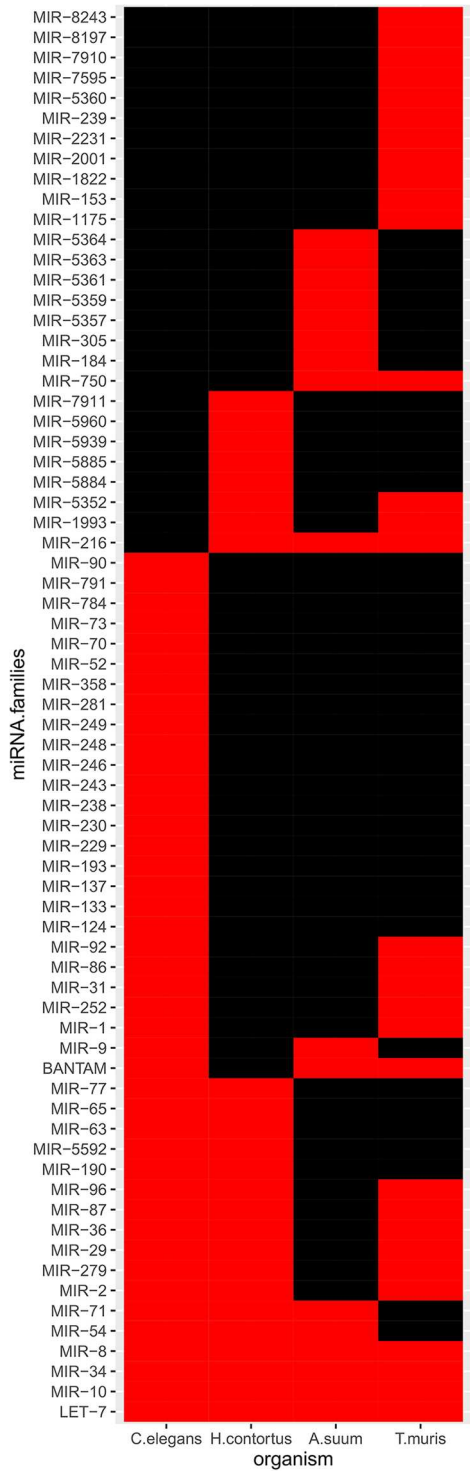

FIGURE 1 | miRNA families in available EV-contained datasets. Only high-confidence *C. elegans* miRNAs were considered. Black, absence; red, presence.

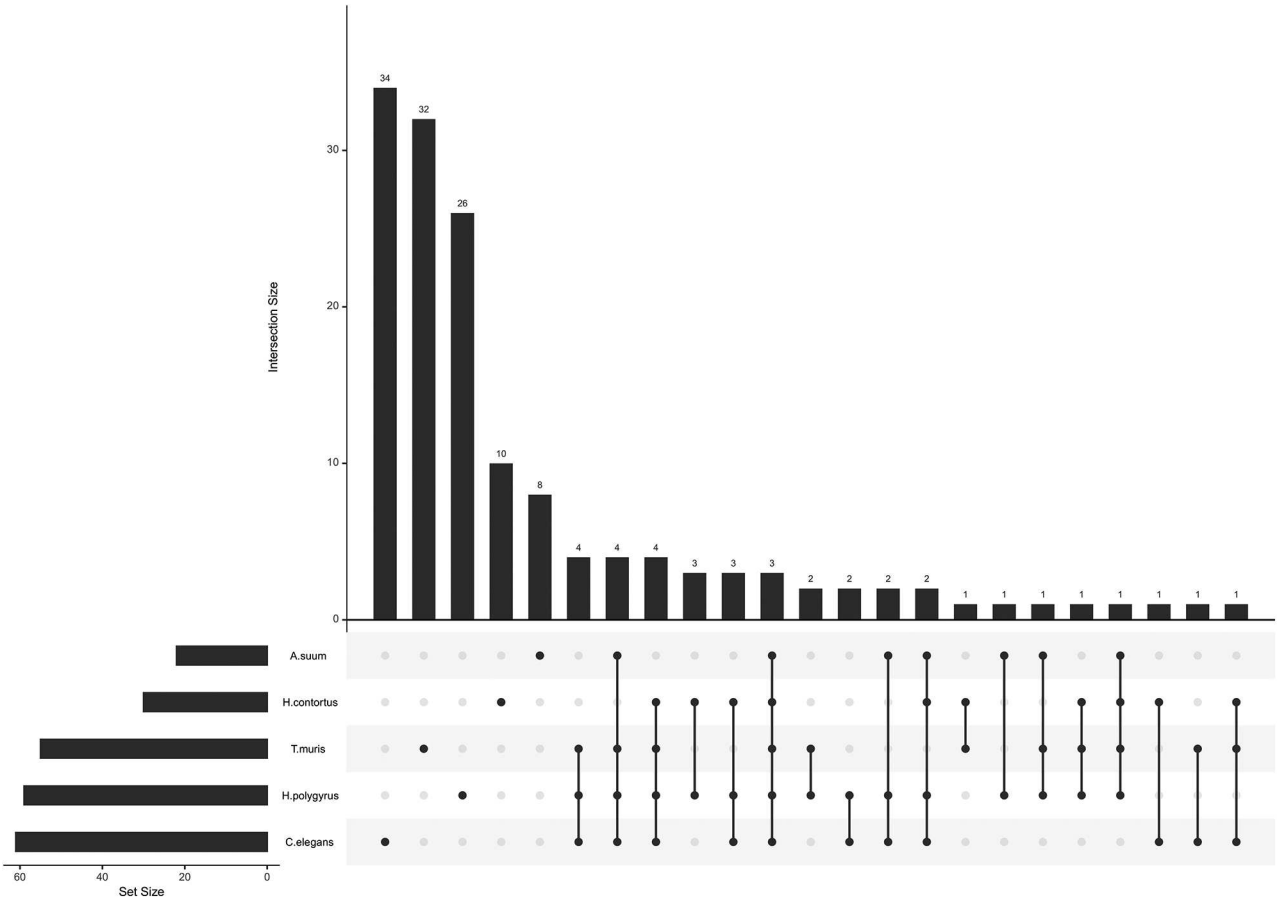

FIGURE 2 | miRNA seed sequences across secreted nematode datasets. The plot was generated with all unique seed sequences across each dataset. The intersection size represents the number of unique seed sequences; (connected) dots under the bar chart indicate the distribution across datasets and show sequences shared by multiple datasets.

was also the case for *C. elegans* artificially aligned to mouse miRNAs. Similarly, parasite E/S miRNA seeds were also found to be more often identical to *C. elegans* miRNAs than non-E/S miRNA seeds ($p < 0.001$). This suggests that miRNA species that tend to be secreted may be more conserved across nematodes, than somatic miRNAs.

Predicted Targets in Host Genomes

Host genes predicted to be targeted by helminth miRNAs were used in pathway enrichment analyses. The number of genes resulting from target predictions from each helminth-host association and used for downstream analysis was as follows: *A. suum*/*S. scrofa* ($n = 3{,}594$), *H. contortus*/*O. aries* ($n = 3{,}833$), *T. muris*/*M. musculus* ($n = 13{,}952$), *H. polygyrus*/*M. musculus* ($n = 14{,}525$), *C. elegans*/*M. musculus* ($n = 15{,}111$), *C. elegans*/*S. scrofa* ($n = 9{,}896$), *C. elegans*/*O. aries* ($n = 8{,}510$). Interrogating the KEGG and Reactome databases via the tool Enrichr returned hundreds of overrepresented pathways. Significantly overrepresented pathways (adjusted $p < 0.05$) were compared across helminth-host associations including *C. elegans* and the various mammalian hosts used in this study (**Table 3** and **Supplementary Table S5**). While no KEGG term was uniquely associated with parasitism (i.e., present in all parasitic species, but absent in the *C. elegans* vs. hosts predictions), 34 terms were common to all seven associations. These included T cell receptor signaling pathway, as well the interconnected Ras, Rap1, FoxO, and ErbB signaling pathways. Similarly, the putative miRNA targets of helminth miRNAs were enriched in genes belonging to 18 Reactome pathways (adj. $p < 0.05$) common to all helminth-host associations. Among those, 12 pathways (with extensive redundancy) are related to the PI3K/Akt signaling (not shown). Again, this qualitative analysis did not allow the clustering of pathways associated with parasitic lifestyle. Pathway analysis further revealed some features of interest among host-parasite associations (without *C. elegans*), worthy of attention. For instance, mucin synthesis is predicted to be affected in the pig by *A. suum* miRNAs, which was not observed in the other host-parasite pairs. Notch signaling pathway is targeted by *A. suum* and *H. polygyrus* miRNAs, while TGF-β signaling counted among targeted pathways by *H. contortus*, *T. muris*,

TABLE 2 | Seed identity across miRNAs from nematodes, hosts, and *C. elegans*.

	Parasite vs. host (in % alignments)		Parasite vs. *C. elegans* (in % alignments)		Are nematode E/S miRNA seeds more often identical to their host's miRNAs than to those of *C. elegans*?	Are nematode E/S miRNA seeds more often identical to their host's miRNAs than non-E/S miRNAs?
	E/S	Non-E/S	E/S	Non-E/S		
Proportion of identical seeds across repeated pairwise alignments						
*C. elegans**	0.09	0.02	NA	NA	NA	Yes ($p < 0.001$)
H. contortus	0.34	0.08	0.61	0.14	No ($p = 0.99$)	Yes ($p < 0.001$)
H. polygyrus	0.07	0.01	0.14	0.09	No ($p = 1$)	Yes ($p < 0.001$)
A. suum	0.36	0.06	0.59	0.28	No ($p = 0.99$)	Yes ($p < 0.001$)
T. muris	0.04	NA	0.24	NA	No ($p = 1$)	NA

*Each nematode miRNA seed set (nt 2–8, E/S, and non-E/S) was aligned pairwise to each host or C. elegans total miRNA list from miRBase. This resulted in thousands of alignments, of which a proportion showed 100% match (7/7 nucleotides). *C. elegans miRNAs were aligned to mouse miRNAs, which is the largest host miRNA dataset.*

TABLE 3 | Host biological pathways targeted by helminth miRNAs according to predictions.

Pathways	Helminth-host association
Common to all helminth-host associations	
T cell receptor signaling, Ras signaling, Rap1 signaling, FoxO signaling, ErbB signaling	Asu/Ssc, Hco/Oar, Hpo/Mmu, Tmu/Mmu, Cel/Mmu, Cel/Ssc, Cel/Oar
Other pathways of interest in associations involving parasitic species	
Mucin type O-glycan biosynthesis	Asu/Ssc
Notch signaling pathway	Asu/Ssc, Hpo/Mmu
TGF-beta signaling pathway	Hco/Oar, Tmu/Mmu, Hpo/Tmu
mTOR signaling pathway	Asu/Ssc, Hco/Oar, Tmu/Mmu, Hpo/Mmu
Th17 cell differentiation	Asu/Ssc
Pathways of interest targeted by species-specific miRNA seeds	
AGE-RAGE signaling pathway	Asu/Ssc, Hco/Oar, Hpo/Tmu

Selected significantly enriched host pathways are listed, according to adjusted p-value ($p < 0.05$), focusing on elements of the host immune system. Asu, A. suum; Hco, H. contortus; Hpo, H. polygyrus; Tmu, T. muris; Cel, C. elegans; Ssc, S. scrofa; Oar, O. aries; Mmu, M. musculus.

and *H. polygyrus* miRNAs in their respective hosts. The mTOR signaling pathway might be altered by all four parasites, while the capacity of Th17 cell differentiation may be modulated by *A. suum* miRNAs.

We further stratified the results, focusing on the targets of species-unique miRNAs, based on seed sequences. Varying numbers of unique, species-specific miRNAs [*A. suum* = 8; *H. contortus* = 10; *H. polygyrus* = 26; *T. muris* = 32; *C. elegans* (target search vs. mouse genes) = 36] targeted varying numbers of KEGG pathways under the significance level (adj. $p < 0.05$) [*A. suum* = 42; *H. contortus* = 3; *H. polygyrus* = 25; *T. muris* = 54; *C. elegans* (target search vs. mouse genes) = 80]. All targeted the FoxO signaling pathway, via different genes or sites on the same gene, illustrating miRNA pleiotropy. Here too, pathways such as the MAPK, and TGF-β signaling pathways were enriched in most host-parasite associations (**Supplementary Table S6**). Interestingly, all parasites but *H. contortus* and *C. elegans,* targeted AGE-RAGE signaling, a pro-inflammatory pathway.

DISCUSSION

Despite methodological differences, our miRNA sequencing results are in agreement with those of Russell et al. (2018). Similarly, the proteomic data parallel those from the prior report at the functional level, and a large proportion of proteins are associated with membranous structures, which we expected from EV-rich preparations; both studies report many c-type lectins. On EV surfaces, lectins may mediate docking to recipient cells via proteoglycans (French et al., 2017). Interestingly, lectins were not observed on the surface of *F. hepatica* derived EVs, while molecules with peptidase activity, also abundant in our dataset, were highly represented (de la Torre-Escudero et al., 2019). Helminth EVs were reported to typically transport cytoskeletal proteins (e.g., actin), stress-related proteins (e.g., heat-shock proteins), along with Rab proteins and others (Mekonnen et al., 2018), which we also identified in the present work.

miRNA sequencing allowed the identification of 100 miRNAs from EV-enriched culture media. Whether this list results from sorting of specific miRNA species for export via EVs, showing higher proportions of 3′ uridylated isoforms, as shown in mammalian B cells and derived EVs (Koppers-Lalic et al., 2014), remains to be defined. The miRNA profile we obtained from EV-enriched processed culture media is fairly different from those from parasitic nematodes characterized so far, where the usually abundant miR-100 isoforms (miR-10-P2 reads according to MirGeneDB), bantam, mir-71, and mir-279, typically occupy the top ranks in abundance. A previous comparative study revealed that miR-100, miR-92, miR-279, and miR-137 (miRBase IDs) are hallmarks of parasitic nematode species, as they could not be identified in *C. elegans* (Wang et al., 2015b), suggesting their importance in parasitic processes. These miRNAs are also missing from our high-confidence list. *Let-7* strongly accumulates in the L4 stage and is required for the transition between larval to adult stages (Reinhart et al., 2000). miR-80 (cel-bantam-P2_3p) is a major metabolic regulator, broadly expressed in tissues of well-fed animals and down-regulated in the absence of food (Vora et al., 2013). The abundance of miR-80 in the EV-enriched fraction suggests that no starvation response had been activated during the short incubations. The MIR-216 family, comprising miRNAs with highly heterogenous

miRBase annotations, appeared to be represented in parasitic species only; the literature mentioning miRNAs assigned to this family is very scarce. MIR-71 and MIR-54 were absent in the only clade I parasite and may reflect an evolutionary distance. BANTAM was missing in *H. contortus*; mir-5885 isoforms were, however, found to be homologous to *Drosophila* bantam (Marks et al., 2019). Similarly, five further miRNA families were found in all nematodes except *A. suum*. Although these differences may reflect phylogeny, life-cycle requirements, and/or host specificity, they might as well be the result of varying dataset sizes and completeness.

The first experimental confirmation of host manipulation by parasitic nematode miRNAs was obtained with the murine pathogen *H. polygyrus*, which releases EVs enriched with Argonaute proteins and miRNAs (Buck et al., 2014; Coakley et al., 2017). The nematode's miRNAs were shown to downregulate host *Il33r* and *Dusp1 in vitro*, leading to the suppression of the Th2 innate immune response to an allergen *in vivo* (Buck et al., 2014). This pioneer work was followed by several further reports on other helminths (Wang et al., 2015a; Zamanian et al., 2015; Fromm et al., 2017; Zheng et al., 2017; Eichenberger et al., 2018a,c; non-exhaustive list).

miRNAs are highly conserved through the animal kingdom. miRNA function is characterized by redundancy and pleiotropy, whereby the expression level of one transcript may be regulated by several miRNAs, and one miRNA binds to many targets, establishing a complex regulatory network. Differences in the miRNA profiles of *C. elegans* and parasites had no overt functional consequences resulting from the target search analysis. Narrowing down the search to targets of the top 20 most abundant miRNAs did not lead to substantial differences in targeted pathways. This is best explained by the fact that identical seed sequences are found in different miRNAs from different families: for instance, if the miR-100 isoforms are absent in *C. elegans*, the corresponding seed is found in miR-51 and others, abundantly represented in the nematode secretions. There is currently no consensus on the amounts of miRNAs required to observe a phenotype in the interaction between a host and a parasite, or in other words, whether a threshold for biological significance may exist. The overall low exosomal miRNA concentrations were even proposed to be biologically insignificant in natural settings (Chevillet et al., 2014).

The miRNA species, number, families, and seed sequences appeared to vary substantially across the examined reports and our current data. There are a number of elements that require caution while comparing miRNA datasets, currently. It is expected that not all organisms release equal amounts of EVs; it was shown previously that some nematode stages are more productive than others (Zamanian et al., 2015). The general experimental designs implemented for EV collection may not reflect the natural *in vivo* situation, inducing stress on nematodes, which may have consequences on EV release and their cargo. Here, we attempted a comparison between miRNA species from juvenile non-parasitic hermaphrodites and adult dioecious parasites; however, it is known that nematode stages release different miRNA profiles (Tritten et al., 2016; Hansen et al., 2019). Finally, the miRNA library preparation and sequencing strategies are known to impact on the data, and there are no recommendations for standardized reporting to date.

Comparing free-living with parasitic species can be used as an approach to uncover the molecular basis of nematode parasitism. The underlying rationale is that parasitic nematodes have evolved from free-living ancestors, and therefore, parasites will have adapted existing ancestral traits and evolved new ones, which together underlie the parasitic lifestyle (Viney, 2018). By doing so, the most relevant comparisons are those taking into account the multiple evolutions of nematode parasitism, reflected in the nematode phylogeny (Blaxter et al., 1998). Ideally, the most appropriate and powerful phylogenomic comparisons are those that compare taxa within the clades, rather than between clades (Viney, 2018). The limited number of studies providing a characterization of EVs and sufficient miRNA information including sequence data does not allow such a design currently. In our current study, involving by chance only gastrointestinal pathogens, only *H. contortus*, *H. polygyrus*, and *C. elegans* belong to the same clade (V). They shared three seed sequences *a priori* absent in the other organisms.

Taking all reported high confidence miRNAs into account, we noted that, regardless of the size of the dataset, a large proportion of seed sequences was unique to each species (or dataset). We assessed whether nematode miRNA seed identity to host miRNAs may constitute an advantageous trait associated with parasitism, bringing the capacity to subvert to existing host regulatory pathways. The proportion of identical seed sequences was not higher in parasite-host comparisons than in those between parasites and *C. elegans*. We observed that the parasite-secreted miRNA fractions shared more often seed sequences with host miRNAs than those that are not found in the extracellular milieu. Overall, this suggests that miRNA release by parasites could constitute an evolved mechanism of host modulation, in an extent that we have barely started to recognize. Regulating conserved host endogenous sites could be effective for two reasons: (i) the context of these sites already permits functional repression, and (ii) these sites cannot mutate to prevent pathogen regulation without modifying/compromising the host physiology (Claycomb et al., 2017). The likely mechanisms and implications of co-evolution between host mRNA and parasite miRNA have been addressed by Claycomb et al. (2017).

Target search and pathway analysis based on whole miRNA repertoires did not allow clustering across lifestyles or clades. *C. elegans* miRNA targets among mammalian genes revealed the same most highly enriched pathways within broad immune functions. This is likely due to the redundant and pleiotropic properties of miRNA-driven gene regulation. According to predictions, aspects in T cell differentiation and proliferation were repeatedly among impaired pathways. Similarly, the PI3K/Akt signaling is central in T cell development (among other cells; Juntilla and Koretzky, 2008). In lymphocytes, Notch is essential for specifying T cell fate and for promoting early T cell differentiation (Laky and Fowlkes, 2008). The importance of mTOR has been shown in DC-mediated T helper cell differentiation (Salmond and Zamoyska, 2011; Hussaarts et al., 2013). Interestingly, *A. suum* miRNAs were predicted to affect the mucin type O-glycan biosynthesis pathway. Increased

mucus production represents a key protective mechanism against gastrointestinal nematodes, to facilitate expulsion of the pathogens (Sharpe et al., 2018). Defective mucin type O-glycan biosynthesis leads to disruption of gut homeostasis and to the re-shaping of the whole gut microbial community (Bergstrom and Xia, 2013). In *T. muris* infections, transforming-growth factor beta (TGF-β) seems to be pivotal in regulating responses to the worm: when CD4+ T cells have reduced ability to respond to TGF-β (due to the expression of truncated version of TGF-β receptor II), mice harbored higher worm burdens and down-regulated levels of Th2 cytokines (Veldhoen et al., 2008; Klementowicz et al., 2012). The AGE-RAGE signaling pathway was the only convergent enrichment result common to species-specific miRNA seeds from three parasitic species, but not *C. elegans*. The influence of dietary advanced glycation end-products (AGE) and its receptor RAGE in an immune context is known in other infectious diseases (Traoré et al., 2016). RAGE is a pattern recognition receptor expressed by B and T cells, as well as dendritic cells. Its expression and response to released RAGE ligands have been observed in human eosinophils Curran and Bertics (2011), a hallmark of immune responses to helminth infections. Upon activation, it is involved in immediate inflammatory response, and a shift toward lymphocyte Th1 differentiation (Kierdorf and Fritz, 2013; Traoré et al., 2016).

Due to inherent bias in the length, conservation, and nucleotide composition of 3′UTR gene portions, target prediction tools suffer some imperfection that can result in prediction inaccuracy. Therefore, computational target predictions require experimental validation to provide more conclusive answers. Our efforts were restricted to seed sequence length of 7 nucleotides for practical reasons; in reality, these may range from 6 to 8 nucleotides.

There is as yet no general mechanistic framework describing how RNA trafficking is programmed; whether and how EV cargo is targeted, what needs to be co-delivered, and how a given miRNA may be integrated into a functional pathway in a recipient cell remain unknown (Claycomb et al., 2017). Cross-species and cross-kingdom communication relying on RNA is a young research field that holds promise for the design of novel diagnostic and disease control strategies. Based on presence or absence of miRNA candidates in each dataset, this approach does not address the quantitative dynamics of sequences with immunomodulatory properties. Here, we propose a simplistic multi-species comparative approach to identify clustering patterns among miRNA sequence-based regulatory networks across organisms. With increasing parasite miRNA collections with improved data depth and quality, this work will certainly find even more resonance and significance in a near future.

DATA AVAILABILITY STATEMENT

The miRNA sequencing data have been deposited to GEO (NCBI) with the dataset GSE144289. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset PXD017352. The analysis codes are available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

TD and LT designed the study and wrote the manuscript. TD, JS, and LT carried out the experiments and analyzed the data. RK and LM assisted in processing the data. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00217/full#supplementary-material

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Transcriptomic Analysis of Extracellular RNA Governed by the Endocytic Adaptor Protein Cin1 of *Cryptococcus deneoformans*

*Muxing Liu[1], Zhengguang Zhang[1], Chen Ding[2], Tuo Wang[3], Ben Kelly[4] and Ping Wang[4,5]**

[1] Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Nanjing, China, [2] College of Life and Health Sciences, Northeastern University, Liaoning, China, [3] Department of Chemistry, Louisiana State University, Baton Rouge, LA, United States, [4] Department of Microbiology, Immunology, and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA, United States, [5] Department of Pediatrics, Louisiana State University Health Sciences Center, New Orleans, LA, United States

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***Correspondence:**
Ping Wang
pwang@lsuhsc.edu

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Membrane vesicles are considered virulence cargoes as they carry capsular and melanin components whose secretory transport is critical for the virulence of the human fungal pathogen *Cryptococcus* species. However, other components of the vesicles and their function in the growth and virulence of the fungus remain unclear. We have previously found that the cryptococcal intersectin protein Cin1 governs a unique Cin1-Wsp1-Cdc42 endocytic pathway required for intracellular transport and virulence. Using RNA sequencing, we compared the profiles of extracellular RNA (exRNA), including microRNA (miRNA), small interference RNA (siRNA), long noncoding RNA (lncRNA), and messenger RNA (mRNA) between the wild-type (WT), and derived Δ*cin1* mutant strains of *Cryptococcus deneoformans*. Seven hundred twelve miRNAs and 88 siRNAs were identified from WT, whereas 799 miRNAs and 66 siRNAs were found in Δ*cin1*. Also, 572 lncRNAs and 7,721 mRNAs were identified from WT and 584 lncRNAs and 7,703 mRNAs from Δ*cin1*. Differential expression analysis revealed that the disruption of *CIN1* results in many important cellular changes, including those in exRNA expression, transport, and function. First, for miRNA target genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that cellular processes, components, and macromolecular functions are the most affected pathways. A higher number of genes were involved in the intracellular transport of endocytosis. Second, the results of GO term and KEGG analysis of differentially expressed lncRNA target genes and mRNA genes were consistent with those of miRNA targets. In particular, protein export is the topmost affected pathway among lncRNA target genes and one of the affected pathways among mRNA genes. The result of quantitative real-time reverse transcription PCR (qRT-PCR) from 12 mRNAs tested is largely agreeable with that of RNA-Seq. Taken together, our studies provide a comprehensive reference that *Cryptococcus* secretes abundant RNAs and that Cin1

plays a critical role in regulating their secretion. Given the growing clinical importance of exRNAs, our studies illuminate the significance of exploring this cutting-edge technology in studies of cryptococcal pathogenesis for the discovery of novel therapeutic strategies.

Keywords: extracellular RNA, endocytic protein, fungal intersectin, intracellular trafficking, fungal pathogenesis

INTRODUCTION

Cryptococcus spp. are encapsulated basidiomycetous fungi that infect both the healthy people and immunocompromised individuals, causing meningoencephalitis (Perfect, 1989). Virulence of *Cryptococcus* is multifaceted, with the production of the polysaccharide capsule, melanin pigment, and extracellular proteinases characterized as the common virulence factors (Kozel, 1995; Buchanan and Murphy, 1998; Lengeler et al., 2000). The elaboration of these virulence factors depends on intact intracellular transport, including exocytosis and endocytosis, which is a highly conserved and essential cellular process. Endocytosis is a process in which living cells uptake foreign materials through the invagination of the plasma membrane (PM) to form vesicles, whereas exocytosis is the release of vesicle contents to the cell exterior through vesicle fusion with PM (Oka and Krieger, 2005). Normal intracellular transport is required for the prolific cellular growth and differentiation, as well as for the pathogenicity of infectious microbes. For example, the uptake of transferrin provides iron necessary for the parasitism of *Plasmodium falciparum*, a unicellular protozoan parasite (Rodriguez and Jungery, 1986). The secretory transport of glucuronoxylomannan (GXM) and aspartic proteinases (SAPs) is considered to be important for capsule formation, a virulence factor of *Cryptococcus* spp. and the virulence of *Candida albicans*, respectively (Schaller et al., 2005).

Intracellular transport is also highly organized and complex involving the concordant function of many protein partners, particularly, the endocytic adaptor proteins, including human intersectin 1 (ITSN1), and cryptococcal Cin1 [reviewed in Wang and Shen (2011)]. These proteins contain multiple domains that couple endocytic uptake with secretion, interconnect transport with actin cytoskeleton regulation, and interact with signaling events mediated by Rho/Rac/Cdc42 family GTPases (Bourne et al., 1990; Jenna et al., 2002; Huang and Cai, 2007; Kaksonen, 2008). Cryptococcal Cin1 is a multi-domain adaptor protein that plays a pleiotropic function in the growth, transport, and production of virulence factors of the fungus (Shen et al., 2010). Previous studies also demonstrated that Cin1 functions upstream of Wsp1, a homolog of human GTPase-binding domain (GBD)-containing Wiskott–Aldrich syndrome protein (WASp), and Cdc42 to regulate actin polymerization, and dynamics (Shen et al., 2011, 2012). However, other functions of Cin1 remain unknown.

Extracellular vesicles (EVs), including microvesicles and exosomes, are spheroid lipid membrane structures containing cytoplasmic and membrane proteins, phospholipids, metabolites, and nucleic acids. Microvesicles (50–1,000 nm in diameter) are formed by the outward budding of the surface PM, whereas the smaller exosomes (40–120 nm in diameter) are intraluminal vesicles primarily formed by the fusion of the multivesicular membrane or the outward budding of the surface PM [reviewed in Ibrahim and Marban (2016), Quesenberry et al. (2015)]. Recent studies also characterized apoptotic bodies (1–5 μm in diameter), the by-products of cell disassembly during apoptosis, as EVs (Poon et al., 2014). EVs and vesicular RNAs are recognized to be important in diseases, including cancer, neurodegenerative disorders, and infectious diseases, as they carry signals that not only identify themselves but also are capable of altering the function of targeted cells (El Andaloussi et al., 2013; Barile and Vassalli, 2017). Previous studies identified at least 1,244 and ∼2,000 vesicular extracellular RNAs (exRNAs) from *Cryptococcus deneoformans* and *C. albicans*, respectively (Jiang et al., 2012; Peres da Silva et al., 2015). We have also identified ∼3.3 million small exRNAs from two clinical strains of *Rhizopus delemar* in a previous study (Liu et al., 2018).

To further characterize Cin1 function in secretory transport relevant to the virulence of *Cryptococcus*, we performed next-generation sequencing of exRNAs from *C. deneoformans* wild-type (WT) and Δ*cin1* mutant strains. We showed that *Cryptococcus* secretes abundant RNAs, including microRNA (miRNA), small interference RNAs (siRNA), long noncoding RNA (lncRNA), and messenger RNA (mRNA) and that Cin1 plays a regulatory role including that in secretion. Based on the emerging importance of exRNA as a determinant of various biological processes, including pathogenesis, further exploration of differential exRNA expression and target characterization are highly warranted for *Cryptococcus* species.

RESULTS

Small Extracellular RNA Characterization, MicroRNA Identification, and Target Prediction

EVs are heterogeneous nanoparticles naturally released from cells during growth, and they carry cargoes containing nucleic acids, proteins, lipids, and other metabolites. EVs were isolated from *Cryptococcus deneoformans* WT (JEC21) (Kwon-Chung et al., 1992) and the Δ*cin1* strains (Shen et al., 2010) grown in liquid yeast peptone dextrose (YPD) for 3 days at 30°C with shaking (225 rpm), and RNA extraction and size fractionation were all similar to those described previously (Liu et al., 2018). RNA quality assessment, cDNA synthesis, library construction, and RNA sequencing were performed by the Beijing Genome Institute (BGI, Shenzhen, China).

A BGISEQ500 platform was used for RNA-Seq of secretory small RNA (sRNA). Approximately 27.4 and 27.1 million sRNA clean reads were obtained from WT and Δ*cin1*, respectively. The clean reads accounted for ∼93–94% of the raw reads, with

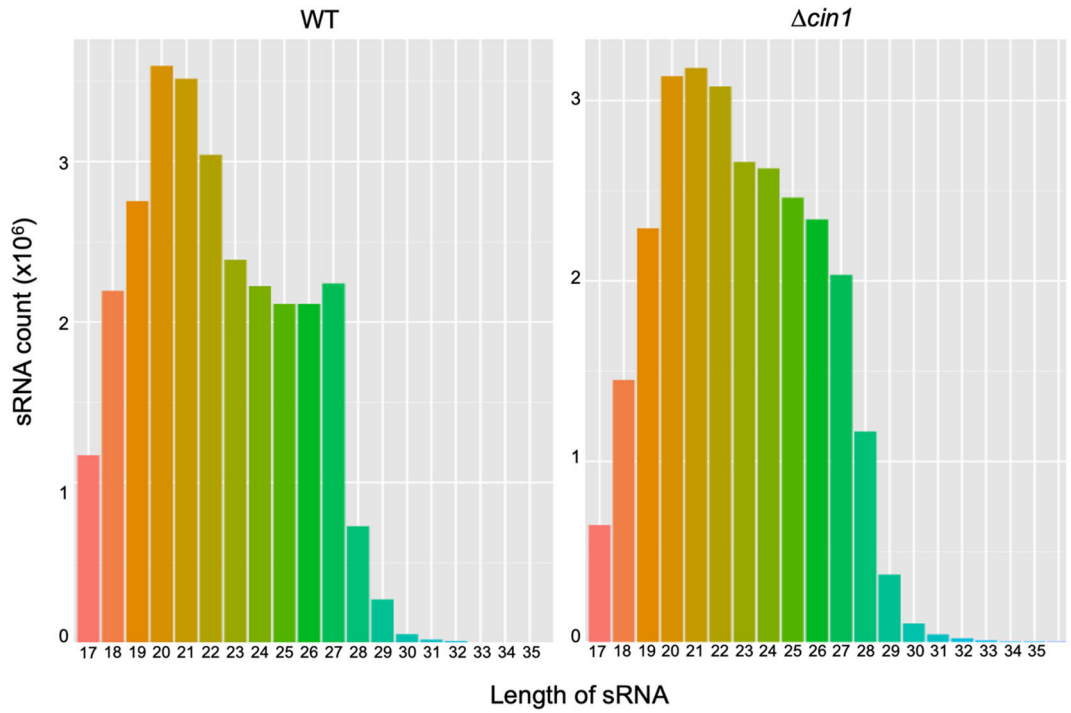

FIGURE 1 | A comparison of extracellular small RNA (sRNA) length distribution between the *Cryptococcus deneoformans* WT (JEC21) and Δ*cin1* mutant strains. The majority of sRNA is within the range of 19- and 22-nt in length, with 20 nt as the major size group in JEC21 (left panel) and 21 nt in *cin1* (right panel). The *x*-axis indicates tag lengths, and the *y*-axis indicates tag read numbers.

∼96% mapped to the genome of *C. deneoformans*, suggesting a reasonable sequencing depth and accuracy. The median lengths of sRNAs were 20 nucleotides (nt) in WT but were shifted to 21 nt in Δ*cin1*, with a minimum length of 17 nt and a maximum length of 30 nt (**Figure 1**). Approximately 90–92% of sRNAs were mapped to intergenic regions, whereas 7.5–9.4% were mapped to exons and 0.5–0.6% to introns.

The clean reads were mapped to the sRNA reference database to identify 690 and 787 known miRNAs from WT and Δ*cin1*, respectively (**Tables S3**, **S4**). Both strains also contain 12 previously unknown novel miRNAs (**Table S5**). In addition to miRNAs, 100 siRNAs were identified (88 from WT and 66 from Δ*cin1*, respectively) (**Table S6**). None of the siRNAs were previously described. Similar to miRNAs, siRNAs are also short duplex RNA molecules that exert gene silencing effect at the post-transcriptional level by targeting mRNA. However, the major difference between siRNAs and miRNAs is that the former are highly specific with only one mRNA target, whereas the latter have multiple targets (Lam et al., 2015). Differential expression screening revealed that WT has more upregulated miRNAs (by 82) than Δ*cin1* but less downregulated (by 61) than Δ*cin1* (**Figure 2**, left graph). The opposite was true for siRNA: more downregulated (by 29) in Δ*cin1* than WT (**Figure 2**, right graph). The functional significance of such differential expression is not yet known; however, studies in mammalian systems suggested that miRNA is required for tissue homeostasis as the expression levels of many tissue-restricted miRNAs are usually downregulated owing to illness (Hammond, 2015).

Functional Annotation of Extracellular MicroRNA Targets

To characterize possible functions of secretory miRNAs, TargetFinder (Fahlgren and Carrington, 2010; Kielbasa et al., 2010) was used to identify ∼5,732 miRNA targets out of ∼10, 527 known miRNA counts. To categorize the putative functions of miRNA targets, Gene Ontology (GO) term enrichment analysis was performed. In all, 21 GO functional categories belonging to three main categories (biological process, cellular component, and molecular function) were identified. For biological process, the top three functional categories were "cellular process" (16), "single-organism process" (12), and "metabolic process" (12). For cellular component, the top three were "cell" (14), "cell part" (14), and "organelle" (10). For molecular function, the top three were "binding" (10), "catalytic activity" (10), and "transporter activity" (2) (**Figure 3**).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed, and both the bar graph and the scatter plot were generated to demonstrate functional classification and pathway assignment of miRNA targets. The top two KEGG pathways were "global and overview maps" (12, metabolism) and "transport and catabolism" (10, cellular process), whereas the "carbohydrate metabolism" (5, metabolism), "folding, sorting, and degradation" (5,

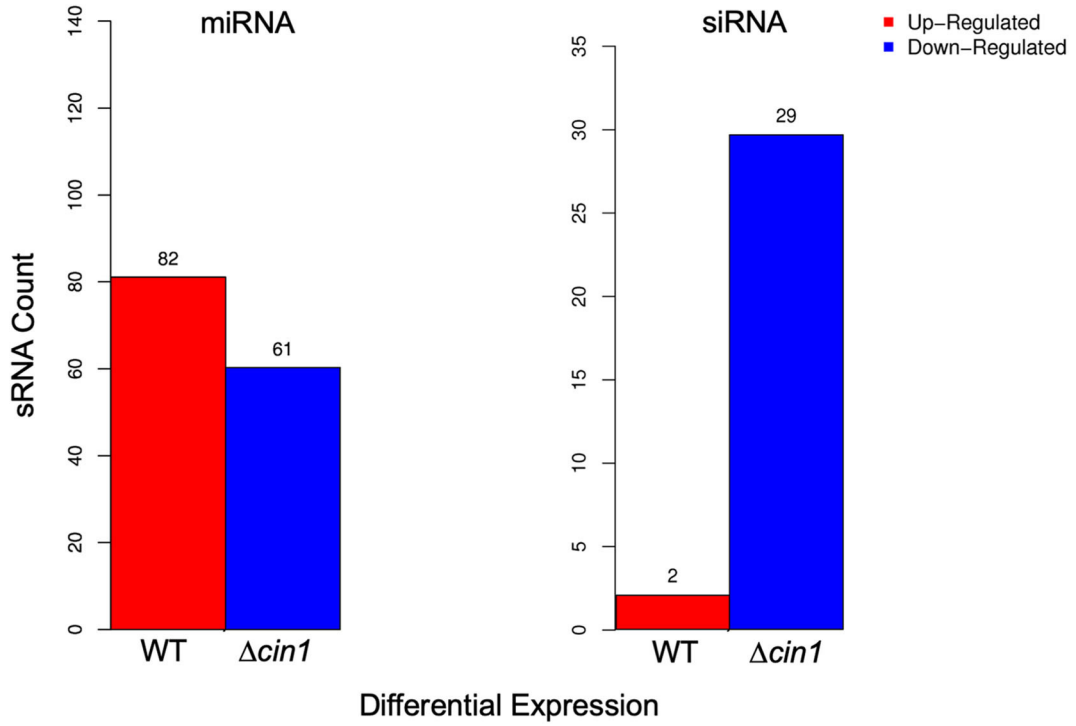

FIGURE 2 | Differential expression of extracellular small RNA (sRNA). Differentially expressed microRNA (miRNA) (left graph) and small interference RNA (siRNA) (right graph) between the wild-type (WT) and Δ*cin1* strains. Red color represents upregulation, and blue color represents downregulation. The expression level is calculated using TPM (transcripts per kilobase million; 't Hoen et al., 2008).

genetic information processing), and "translation" (5, genetic information processing) pathways were all ranked in the third place (**Figure 4**, left panel). A scatter plot of the top 20 pathways indicated that the topmost enriched pathways were "basal transcription factors," "citric acid cycle (TCA)," and "starch and sucrose metabolism" (**Figure 4**, right panel). "RNA transport" and "protein export" pathways were also enriched, and more genes were involved in the "endocytosis" and "biosynthesis of secondary metabolites" pathways (**Figure 4**, right panel). These results are consistent with that Cin1 plays a pleiotropic regulatory function, including membrane transport (Shen et al., 2010).

Identification of Extracellular Long Noncoding RNA

In comparison with extracellular sRNAs sequenced by BGISEQ500, exRNAs of >50 nt were sequenced using an Illumina HiSeq4000 platform by BGI. Approximately 60.5 and 59.7 million clean reads were obtained from WT and Δ*cin1*, with ∼61 and ∼67% mapping to the *C. deneoformans* genome, respectively. LncRNA and mRNA were distinguished by predicting the coding ability of the transcripts using CPC, txCdsPredict, and CNCI against the Pfam database (Kong et al., 2007; Nawrocki et al., 2009; Sun et al., 2013; Finn et al., 2016; El-Gebali et al., 2019) (**Table S7**). A separation was made if it satisfies three out of the four prediction methods, as illustrated in the Venn diagrams (**Figure 5**).

LncRNAs are RNAs of longer than 200 bp in length; and in contrast to mRNA, they lack the potential for coding proteins. Recently, there has been accumulating evidence that indicates that lncRNA participates in a broad range of cellular processes [reviewed in Meng et al. (2017)]. A total of 690 extracellular lncRNAs were identified from *C. deneoformans* (**Table S8**). Among them, 572 were from WT and 584 from Δ*cin1*. Also, 600 lncRNAs were found to be differentially expressed using the PossionDis method to detect gene expression (Audic and Claverie, 1997) (**Figure 6A**). The lncRNAs were also grouped into 10 known lncRNA families when compared against the Rfam dataset using INFERNAL software (Nawrocki et al., 2009; Kalvari et al., 2018) (**Figure 6B**). Consistent with lncRNA characteristics, cryptococcal lncRNA has a shorter coding sequence (**Figure 6C**) with fewer exons (**Figure 6D**), and most contain a single transcript (**Figure 6E**).

Functional Annotation of Extracellular Long Noncoding RNA Targets

The regulatory role of lncRNAs in gene expression lies within their functions as either miRNA precursors or miRNA sponge through lncRNA–miRNA interactions (Jarroux et al., 2017; Ulitsky, 2018). Differentially expressed lncRNAs were functionally annotated against GO and KEGG databases. GO

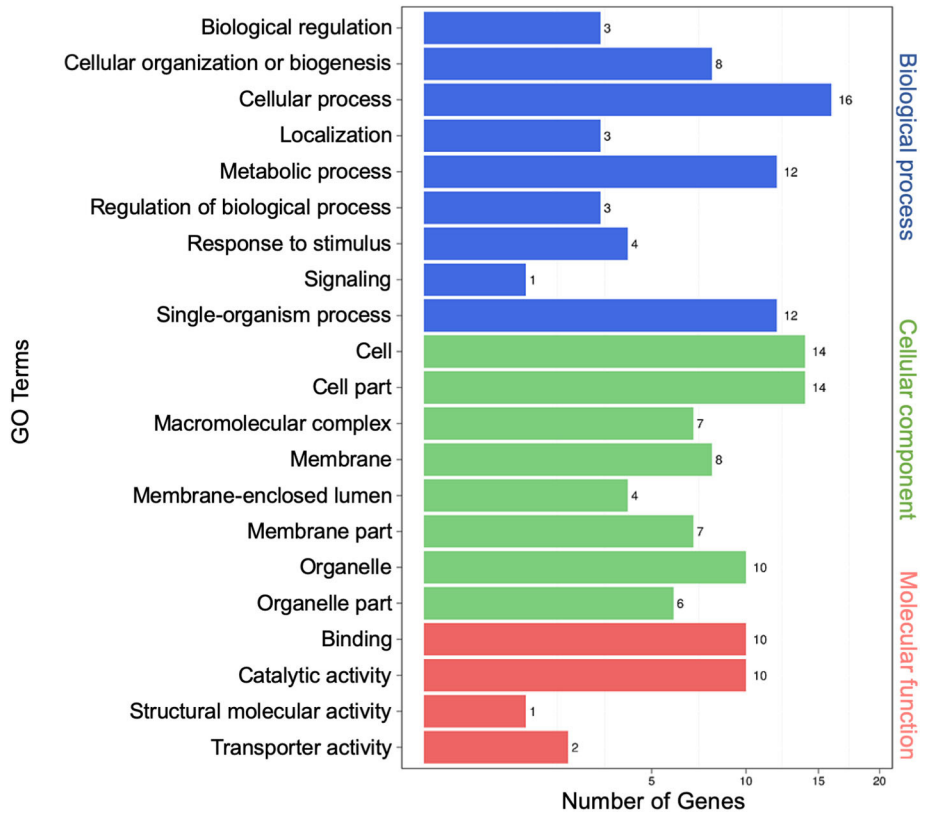

FIGURE 3 | Gene Ontology (GO) analysis of differentially expressed extracellular microRNA (miRNA) target genes. The left vertical coordinates refer to GO terms, the bottom horizontal coordinates are the numbers of differentially expressed genes, and the right vertical coordinates indicate the class of GO terms.

terms representing 33 functional categories were identified. The top five categories for biological process are "cellular process" (97), "metabolic process" (91), "single-organism process" (79), "localization" (35), and "biological regulation" (32). The top five categories for cellular components are "cell" (84), "cell part" (84), "membrane" (78), "membrane part" (74), and "organelle" (70). The top three categories for molecular function are "binding" (77), "catalytic activity" (67), and "transporter activity" (20) (**Figure 7**, left panel). This profile was similar to that of miRNAs (**Figure 3**). The KEGG pathway analysis showed the top 20 most enriched pathways that were largely similar to miRNA annotation (**Figure 4**, right panel). Significantly, the scatter plot showed that the topmost enriched pathway involving a relatively high number of genes was "protein export" (**Figure 7**, right panel).

Identification and Annotation of Extracellular Messenger RNA

mRNAs are abundant single-stranded RNA molecules directing protein synthesis, and fragmented mRNAs are also proposed to play a role in regulating stability, localization, and translational activity of mRNAs through RNA binding (Batagov and Kurochkin, 2013). In all, 6,899 known extracellular mRNAs were identified (**Table S9**), with 5,626 and 5,608 mRNAs identified from WT and Δ*cin1*, respectively. In addition, 2,095 novel mRNAs were identified from both strains (**Table S10**). PossionDis detection for gene expression showed that 5,789 known and 2,096 novel mRNAs were differentially expressed between WT and Δ*cin1* (**Figure 6A**).

Differentially expressed known mRNAs were functionally annotated against GO and KEGG databases. GO terms representing 41 functional categories were identified. The top five categories for biological process are "cellular process" (642), "metabolic process" (615), "single-organism process" (478), "localization" (208), and "biological regulation" (206). The top five categories for cellular components are "cell" (527), "cell part" (525), "organelle" (437), "membrane" (426), and "membrane part" (390). The top five categories for molecular function are "binding" (486), "catalytic activity" (461), "transporter activity" (95), "nucleic acid binding transcription factor activity" (60), and "structural molecular activity" (41) (**Figure 8**, left panel). This profile was similar to that of extracellular miRNAs (**Figure 3**) and lncRNAs (**Figure 7**). The result of the top 20 KEGG pathway analysis was also largely similar to that of lncRNA annotation (**Figure 7**), with the scatter plot showing that the top-ranked enriched pathways were involved in fatty acid, pentose, and glucuronate interconversions, and peroxisome regulation (**Figure 8**, right panel). "Protein export" remained as one of the

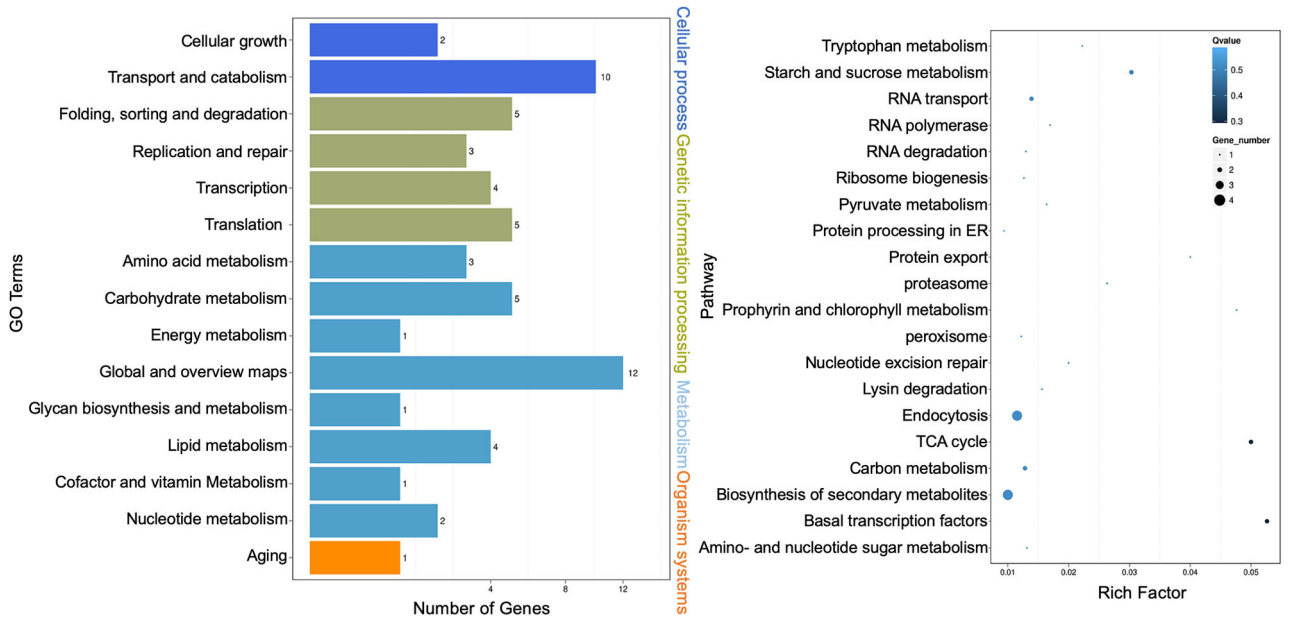

FIGURE 4 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of extracellular microRNA (miRNA) target genes. Bar graph: the left vertical coordinates are 15 KEGG terms of differentially expressed target genes, the bottom horizontal coordinates are the numbers of genes, and the right vertical coordinates indicate the class of Gene Ontology (GO) terms (left panel). Scatter plot: the left vertical coordinates indicate 20 pathways, while the horizontal numbers refer to rich factors (right panel). The rich factor means that the ratio of differentially expressed gene numbers and the number of genes annotated in the pathway. The greater the rich factor, the greater the degree of enrichment. A *Q*-value is the corrected *p*-value ranging from 0 to 1, and a lower value indicates greater pathway enrichment.

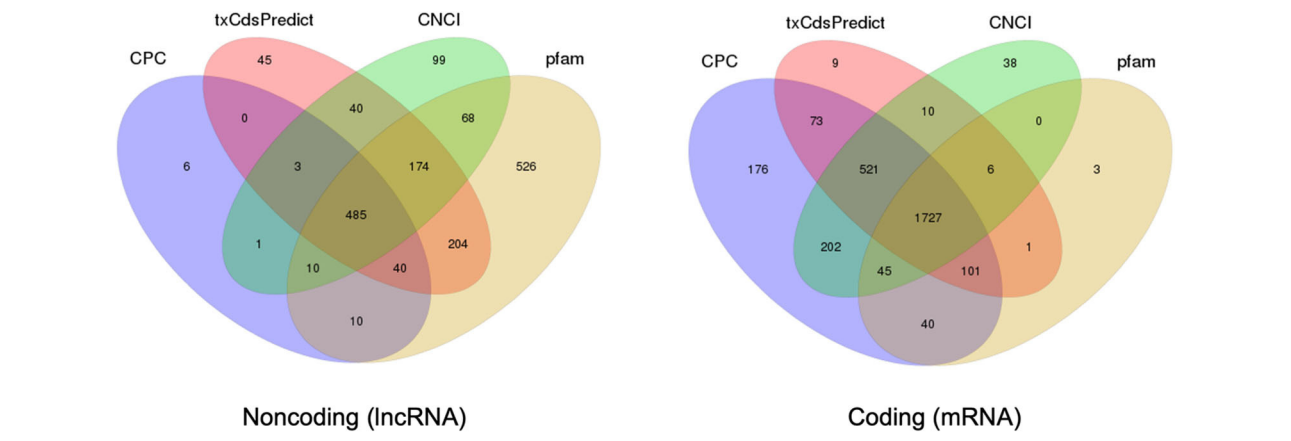

FIGURE 5 | Prediction of long noncoding RNA (lncRNA) and messenger RNA (mRNA) based on the coding ability. Venn diagrams depicting the predication of lncRNA (left diagram) and mRNA (right diagram) by three out of four prediction methods including CPC, txCdsPredict, CNCI, and the Pfam database.

most enriched pathways, albeit less than that of the lncRNA targets (**Figure 8**, right panel).

Quantitative RT-PCR Validation

To validate the accuracy of results from RNA-Seq, we examined the expression of selected mRNAs using quantitative real-time reverse transcription PCR (qRT-PCR). For upregulated mRNAs, we selected XM_568032.1 (3254810, conserved HNG-box protein), XM_569986.1 (3256653, BET1 membrane protein), XM_572299.1 (3259265, lipid particle protein), XM_568399.1 (3255266, tartrate transporter), and XM_568399.1 (3255266, lysophospholipase). For downregulated mRNAs, we chose XM_572526.1 (3254145, a membrane protein), XM_567889.1 (3254501, alcohol dehydrogenase), XM_571004.1 (3257991, hexose transport-related protein), XM_570958.1 (3257778, succinate:fumarate antiporter), and XM_568389.1 (3255146, peptide alpha-*N*-acetyltransferase). These 10 mRNAs exhibited the most apparent differential expression, both up and down.

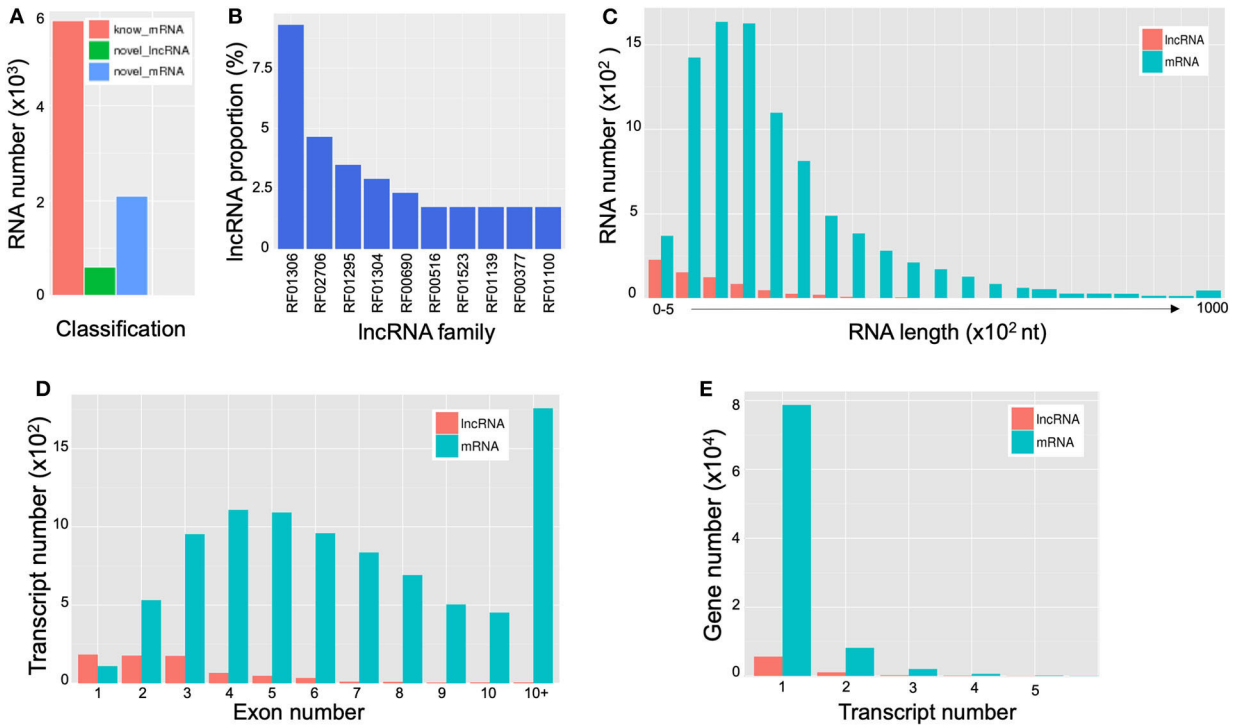

FIGURE 6 | Characterization of extracellular long noncoding RNAs (lncRNAs) and messenger RNAs (mRNAs) of *Cryptococcus deneoformans* wild-type (WT) and Δ*cin1* strains. **(A)** Differential expression; red color represents known mRNA, green color represents lncRNA, and blue color represents novel mRNA. **(B)** Distribution of lncRNA families. The vertical coordinates refer to the percentage of lncRNA, and the horizontal coordinates indicate lncRNA families. **(C)** The RNA length distribution of lncRNA and mRNA. The vertical coordinates refer to RNA numbers, and the horizontal coordinates indicate transcript lengths. **(D)** Distribution of lncRNA and mRNA exon numbers. The vertical coordinates refer to transcript numbers, and the horizontal coordinates indicate exon numbers. **(E)** The distribution of lncRNA and mRNA transcript numbers. The vertical coordinates refer to gene numbers, and the horizontal coordinates refer to transcript numbers.

We also selected two mRNAs for hypothetical proteins (XM_571287.1, #3258287 and XM_573013.1, #3259540). qRT-PCR revealed that nine had a similar expression profile (three up and six down) whereas three did not (#3254810, HMG-box protein; #3259717, tartrate transporter; and #3255146, peptide alpha-acetyltransferase) (**Figure 9** and **Table S1**). The reasons for inconsistency remain unknown but could be attributed to variations in sample processing (usage of total cellular RNA instead of vesicular RNA) or errors. While most of these proteins remain functionally uncharacterized, their predicted identities of being membrane and lipid proteins, as well as transport-related proteins, suggest that they are likely involved in intracellular transport. Thereby, their altered expression, either up or down, owing to *CIN1* gene disruption is in accordance with that Cin1 playing an important role in intracellular trafficking.

DISCUSSION

Cryptococcus spp. are unique pathogenic fungi that are characterized by their propensity for the human central nervous system, causing fungal meningitis. Previous studies have provided strong evidence that *Cryptococcus* produces vesicles containing GXM, a capsule precursor, and melanin pigment, and that it secretes laccase, metalloprotein urease, and phospholipases involved in melanin synthesis, stress resistance, and others (Cox et al., 2000, 2001; Olszewski et al., 2004; Rodrigues et al., 2007; Nosanchuk et al., 2008; Vu et al., 2014). Additional studies showed that attenuated expression of genes encoding vesicular proteins, including the small GTPase Sec4/Sav1 protein, the exocyst complex component protein Sec6, and the phospholipid transfer protein Sec14, individually or collectively, affected the secretion of GXM, laccases, ureases, and phospholipases and, thereby, virulence (Yoneda and Doering, 2006; Panepinto et al., 2009; Chayakulkeeree et al., 2011). The composition and architecture of cryptococcal cell walls have also been suggested as the critical factors for the anchoring of melanin pigments, and structural investigation using high-resolution techniques such as solid-state NMR spectroscopy could enhance the understanding of pathogenesis mechanisms (Kang et al., 2018; Chrissian et al., 2020).

Intracellular transport is a complex and highly organized process involving concordant functions of many proteins, in particular, the endocytic adaptor proteins [reviewed in Wang and Shen (2011)]. In contrast to most Sec proteins that are either small GTPases determining transport specificity or vesicle constituents, endocytic proteins function as chaperones interconnecting, and modulating many steps of transport: endocytosis, exocytosis, actin cytoskeleton dynamics, and signal

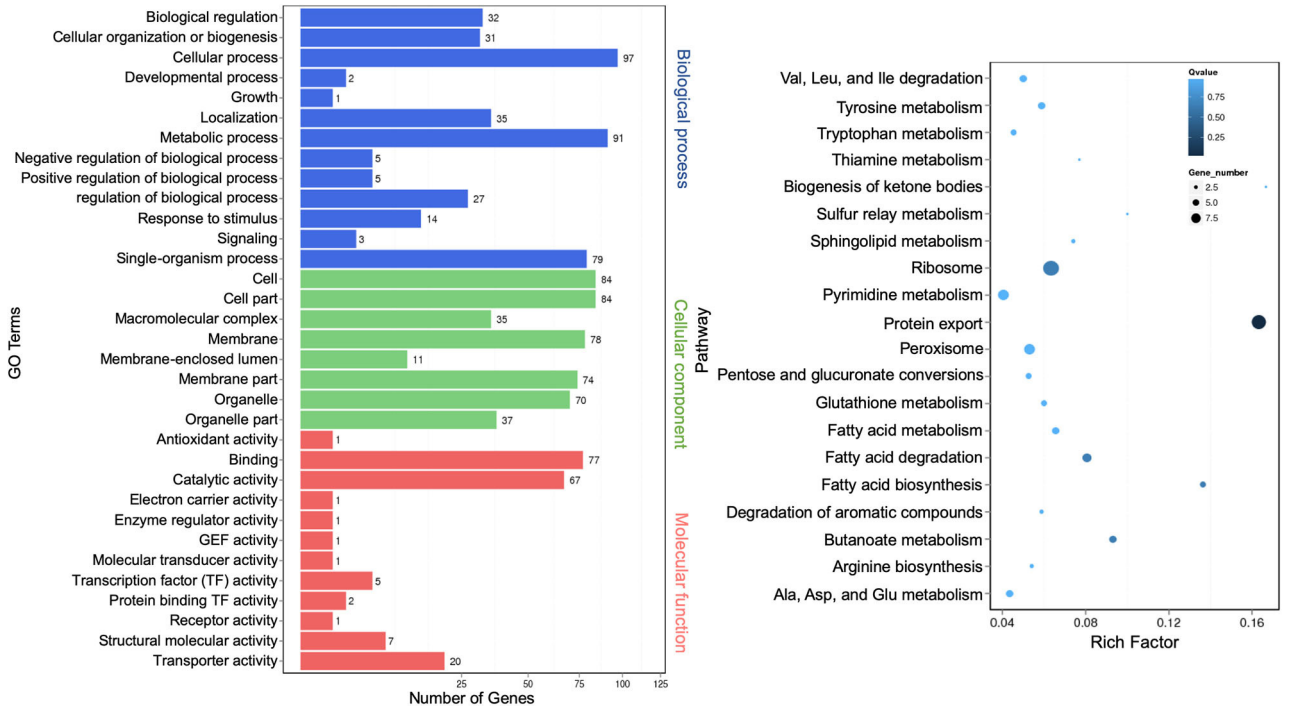

FIGURE 7 | Gene Ontology analysis of differentially expressed extracellular long noncoding RNAs (lncRNA) target genes. A graph plot of Gene Ontology (GO) term enrichment analysis (left panel). The left vertical coordinates refer to 33 GO terms, the bottom horizontal coordinates are the numbers of differentially expressed genes, and the right vertical coordinates are GO classes. A scatter plot of a 20-pathway enrichment analysis (right panel). The left vertical coordinates are pathways, and the horizontal coordinates refer to rich factors.

transduction (Bourne et al., 1990; Jenna et al., 2002; Huang and Cai, 2007). The cryptococcal Cin1 protein contains multiple domains, including one Eps15-containing domain, a coiled-coil region, an actin monomer-binding WH2 domain, two SH3 motifs, and a RhoGEF-PH domain (Shen et al., 2010). Cin1 has pleiotropic functions in growth, transport, and the production of virulence factors. Similar to ITSN1, Cin1 functions upstream of the human WASp homolog Wsp1 and Cdc42 GTPase to regulate actin polymerization and organization (Shen et al., 2011, 2012). Collectively, our previous published findings support that the Cin1-Wsp1-Cdc42 regulatory pathway, not described from any other pathogenic fungi, may also contribute to the unique pathogenesis mechanism of *Cryptococcus*.

sRNAs, including miRNAs, siRNAs, and PIWI-interacting RNAs (piRNAs) play a critical role in the regulation of cellular growth and development, as they modulate the expression of target genes via RNA cleavage or transcriptional silencing (Hwang and Mendell, 2006; Osada and Takahashi, 2007). MiRNAs regulate gene expression by targeting the 3′-untranslated region (UTR) of their target mRNAs, whereas siRNAs interfere with gene expression by complementary base pairing to trigger mRNA degradation [reviewed in Bartel (2004), Fabian et al. (2010)]. In a proof-of-principle study, Jiang et al. identified two miRNAs, miR1, and miR2, from a collection of 200 cellular sRNAs of *Cryptococcus deneoformans*. miR1 and miR2 were found to interfere with the expression of *URA5* and *CLC1* genes when inserted in the 3′-UTRs, respectively (Jiang et al., 2012).

In comparison with sRNAs, lncRNAs are RNAs longer than 200 bp in length but are noncoding. There is emerging evidence suggesting that lncRNA also has a broad range of regulatory functions, either as a miRNA precursor or a target/sponge (Paraskevopoulou and Hatzigeorgiou, 2016). However, the existing knowledge regarding lncRNA function is still far less than complete than that for miRNAs. mRNAs are a large family of coding molecules specifying protein sequence information in eukaryotic cells. Previous studies have found that EVs contain a substantial number of mRNAs from their parent cells (Wei et al., 2017). These mRNA molecules are protected from RNase degradation, and those polyadenylated may be capable of encoding polypeptides (Valadi et al., 2007; Lai et al., 2015). In addition, fragmented mRNA resided within EVs could potentially regulate protein functions (Batagov and Kurochkin, 2013).

ExRNAs were previously identified in several pathogenic and non-pathogenic fungi. Peres da Silva et al. identified 344, 423, 145, and 532 miRNAs from *Cryptococcus neoformans* (var. *grubii*), *Candida albicans*, *Paracoccidioides brasiliensis*, and *Saccharomyces cerevisiae*, respectively (Peres da Silva et al., 2015). The identification of multiple miRNAs, lncRNAs, and mRNAs

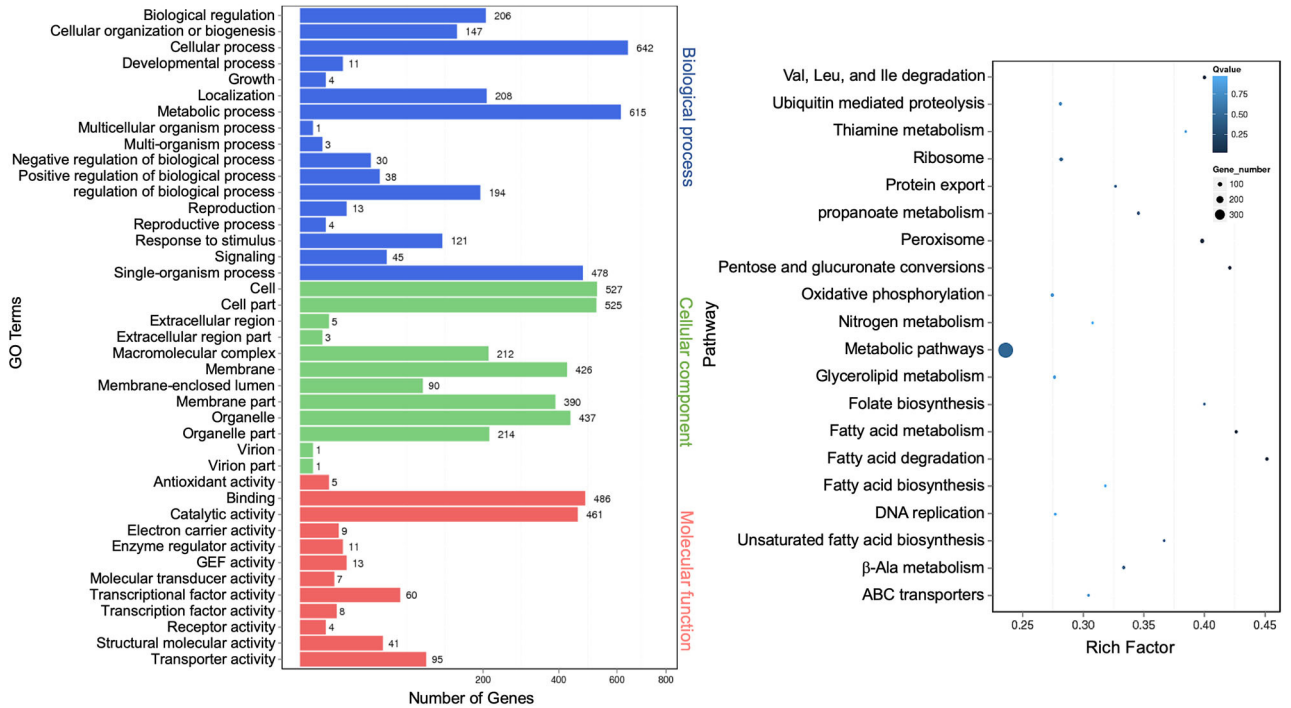

FIGURE 8 | Gene Ontology analysis of differentially expressed extracellular messenger RNAs (mRNAs). A graph plot of Gene Ontology (GO) term enrichment analysis (left panel). The left vertical coordinates refer to 41 GO terms, the bottom horizontal coordinates are the numbers of differentially expressed genes, and the right vertical coordinates are GO classes. A scatter plot of a 20-pathway enrichment analysis (right panel). The left vertical coordinates are pathways, and the horizontal coordinates refer to rich factors.

hypothesized to be involved in vesicle-mediated transport and metabolic pathways led da Salvia et al. to propose that RNA-containing vesicles may be a key determinant for various biological processes, including cell–cell communication and pathogenesis (Peres da Silva et al., 2015). A study by Bielska et al. showed that EVs derived from *Cryptococcus gattii*, a different but related species, could mediate virulence transfer between strains and the characteristics depending on both proteins and RNAs (Bielska et al., 2018). More recently, an RNA sequencing study has also described abundant exRNAs produced by two strains of the mucoralean fungus *Rhizopus delemar* (Bruni et al., 2019). In agreement with these findings, we here identified significant amounts of extracellular miRNAs and siRNAs from *C. deneoformans*. We have also identified a significant amount of extracellular lncRNAs and mRNAs produced by *C. deneoformans*.

Finally, Cin1 was previously characterized to play a pleiotropic function required for the growth, transport, and the production of virulence factors of the fungus (Shen et al., 2010). The Δ*cin1* mutant strain does not produce melanin or capsule, and it also exhibits defects in cytokinesis and growth (Shen et al., 2010). Additional studies suggested that Cin1 functions through a unique Cin1-Wsp1 (Wiskott–Aldrich syndrome protein homolog)-Cdc42 endocytic pathway to regulate growth and virulence, as well as actin dynamics and transport (Shen et al., 2011, 2012). The findings of our current studies are consistent with this conclusion. Analysis of differentially expressed gene targets of miRNA and lncRNA and mRNAs all indicated that Cin1 plays a wide array of functions in cellular processes, composition, and function. All three types of RNAs have roles in RNA and protein export and endocytosis. Further detailed analysis of differential expression of each class of RNAs and their pathways will be required to gain further insight into the regulatory function of Cin1.

MATERIALS AND METHODS

Extracellular Vesicle Isolation

Cryptococcus deneoformans (previously *Cryptococcus neoformans* var. *neoformans*) WT JEC21 and Δ*cin1* mutant strains were grown in YPD broth at 30°C for 3 days with 225-rpm rotation (Shen et al., 2010). Yeast cells were precipitated by centrifugation at 4,000 × g for 15 min, and supernatants were recovered. Smaller debris or particles were removed by second centrifugation at 15,000 × g for 15 min at 4°C. The supernatant was then filtered through an ultrafiltration filter with a molecular weight cutoff of 100 kDa (Amicon), and the remaining liquid was precipitated by ultracentrifugation at 100,000 × g for 1 h at 4°C. The precipitated fraction containing membrane fractions was re-suspended in phosphate-buffered saline (PBS), washed

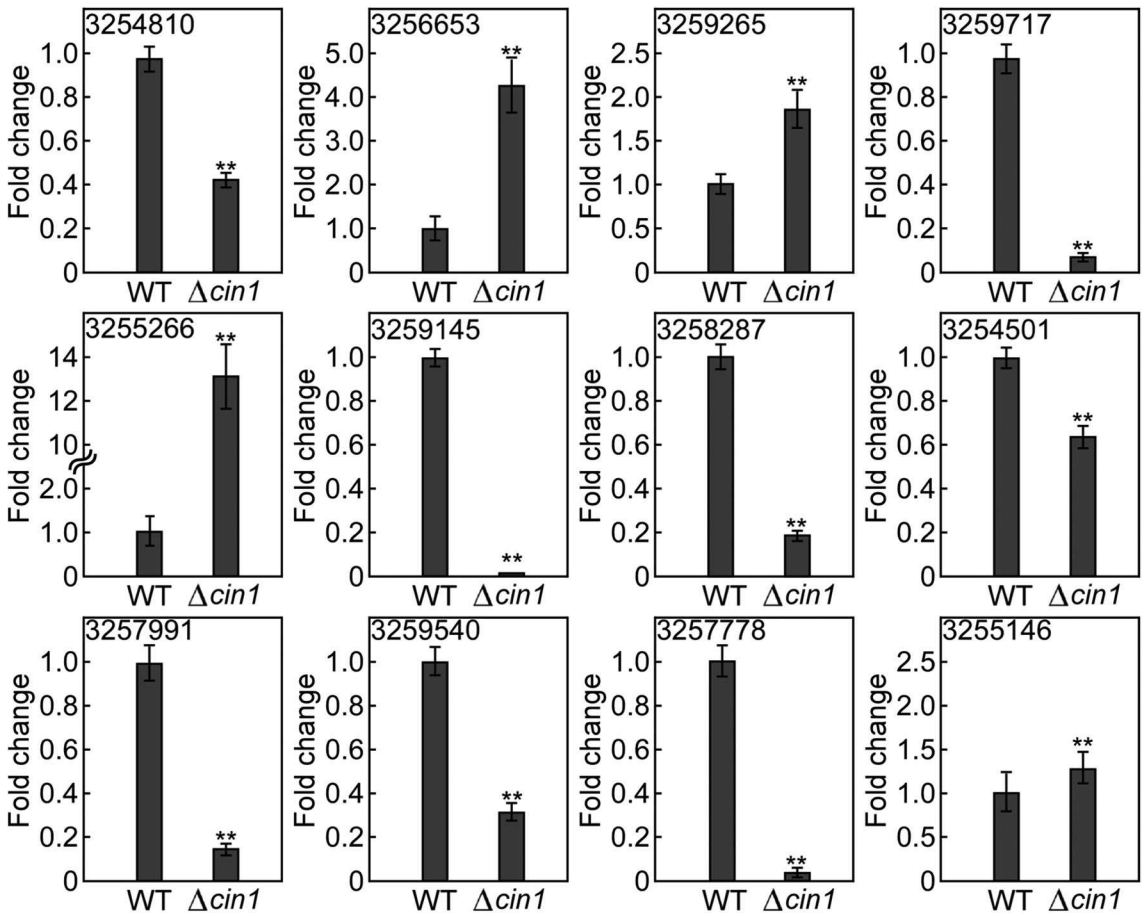

FIGURE 9 | Expression validation by quantitative real-time PCR. The expression of 12 extracellular messenger RNAs (mRNAs) was quantified by qRT-PCR in reference to the expression of the constitutively active *ACT1* gene in *Cryptococcus*. Error bars show standard deviations ($n = 3$), whereas asterisks indicate statistically significant correlations ($p < 0.05$). Gene ID 3254810 (XM_568032.1, a conserved HNG-box protein), 3256653 (XM_569986.1, a BET1-like membrane protein), 3259265 (XM_572299.1, a lipid particle protein), 3255266 (XM_568399.1, a tartrate transporter), 3255266 (XM_568399.1, lysophospholipase), 3254145 (XM_572526.1, a membrane protein), 3254501 (XM_567889.1, alcohol dehydrogenase), 3257991 (XM_571004.1, a hexose transport-related protein), 3257778 (XM_570958.1, a succinate:fumarate antiporter), and 3255146 (XM_568389.1, peptide alpha-*N*-acetyltransferase). 3258287 (XM_571287.1) and 3259540 (XM_573013.1) potentially encode hypothetical proteins.

twice with PBS, and lyophilized prior to RNA extraction. Owing to the reduced growth of Δ*cin1* in comparison with WT, a larger volume (2×) of YPD was used for its growth. Cultures were grown in duplicated flasks (2×), and vesicle preparations were pooled.

Extracellular RNA Extraction, Library Construction, and Sequencing

Briefly, vesicular RNAs were extracted with TRIzol (Sigma-Aldrich) and separated by polyacrylamide gel electrophoresis (PAGE), and sRNA bands of ∼18–30 nt in size were recovered. 5′ and 3′ adaptors were then added to sRNA prior to cDNA synthesis. The resultant products were purified and amplified by PCR. The PCR yield was quantified and subjected to single-strand circularization (ssDNA circle) for final library construction. According to the BGI protocol, DNA nanoballs (DNBs) were generated with the ssDNA circle by rolling circle replication (RCR) to intensify the fluorescent signals during the sequencing process. The DNBs were then loaded into the patterned nanoarrays, and paired-end reads of 100 bp were read through on a DNBseq™ platform (BGISEQ-500, BGI).

For lncRNA and mRNA characterization, extracted exRNA was first mixed with a biotin-labeled specific probe (Ribo-ZeroTM rRNA Removal Kit) to remove ribosomal RNA (rRNA) and then fragmented. cDNA first strand was synthesized using a TruSeq® Stranded kit (Illumina) and second strand with DNA polymerase I and RNaseH. Double-stranded cDNA was then ligated with an "A" base and a linker and amplified, and the cDNA library generated the following purification. Sequencing was carried out in an Illumina HiSeq4000 platform (BGI). Description and comparison of BGISEQ-500 and Illumina HiSeq for RNA-Seq were previously described by (Zhu et al., 2018).

MicroRNA Identification, Differential Expression, and Target Prediction

For small exRNA, the sequence tags were subjected to data cleaning analysis to remove transfer RNA (tRNA), rRNA, and other impurities. The clean tags (reads) were then mapped to the *C. deneoformans* genome. Known miRNAs were identified by searching against the miRbase and Rfam reference sRNA database using AASRA software, and novel miRNAs were predicted if they were mapped to the intergenic regions, introns, the reverse repeat sequence of a coding sequence, but not to any other RNAs (Nawrocki et al., 2009; Kozomara and Griffiths-Jones, 2014; Chong et al., 2017; Kalvari et al., 2018). The expression levels of sRNA were calculated using TPM (transcripts per kilobase million; 't Hoen et al., 2008), and differentially expressed sRNAs were screened using ExpDiff (Yang et al., 2017). The false discovery rate (FDR) control method was used to determine the threshold of *p*-value, and the ratio of TPM was used to calculate the fold change in expression. An FDR of < 0.001 and an absolute value of log2-ratio ≥ 1 was set as the threshold for determining the significance of gene expression difference (Kim and van de Wiel, 2008). Once miRNA results were obtained, their target prediction was performed using TargetFinder (Fahlgren and Carrington, 2010; Kielbasa et al., 2010).

Long Noncoding RNA and Messenger RNA Identification, Coding Ability Prediction, and Differential Expression

The large exRNA clean reads were compared with the *C. deneoformans* genome at National Center for Biotechnology Information (NCBI) using HISAT (Kim et al., 2015) and assembled with StringTie (Pertea et al., 2015). All of the transcript sequences were compared with known lncRNA and mRNA with Cuffcompare (Trapnell et al., 2010). LncRNA and mRNA were distinguished by predicting the coding ability of the transcripts using CPC, txCdsPredict, and CNCI and against the Pfam database (Kong et al., 2007; Nawrocki et al., 2009; Sun et al., 2013; Finn et al., 2016).

Gene Ontology Term and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis

The differentially expressed miRNA and lncRNA target genes were subjected to GO term and KEGG pathway enrichment analysis. GO provides a common descriptive framework and functional annotation and classification for analyzing gene set data (www.geneontology.org; Ashburner et al., 2000; The Gene Ontology Consortium, 2019), whereas the KEGG pathway database is a recognized and comprehensive database including all known biochemical pathways (www.annotation.jp/KEGG; Kanehisa and Goto, 2000; Kanehisa et al., 2016). GO term and KEGG pathway analyses for mRNA genes were the same as for miRNA and lncRNA target genes.

Quantitative RT-PCR Verification

As a verification step for RNA sequencing, selected mRNAs were examined for expression by qRT-PCR. One microgram of total RNA was reverse transcribed into first-strand cDNA using the oligo(dT) or random primers and M-MLV Reverse Transcriptase (Invitrogen) following previously described methods (Chong et al., 2017; Liu et al., 2018). The expression of the constitutively expressed actin gene *ACT1* was used as an internal control. Primers used for qRT-PCR are listed in **Table S2**. qRT-PCR was performed with an ABI 7500 Fast Real-Time System, and transcripts were analyzed by 7500 System SDS software. To compare the relative abundance of target gene transcripts in different samples, the average threshold cycle (Ct) was normalized to *ACT1* for each sample as $2^{-\Delta Ct}$ [$-\Delta Ct =$ (Ct, target gene-Ct, *ACT1*)]. Fold changes between different samples were calculated as $2^{-\Delta\Delta Ct}$ [$-\Delta\Delta Ct =$ (Ct, experimental-Ct, *ACT1*) – (Ct, control-Ct, *ACT1*)].

DATA AVAILABILITY STATEMENT

This data can be found here: https://www.ncbi.nlm.nih.gov; BioSample accessions SAMN14775367, SAMN14775368, SAMN14775369.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00256/full#supplementary-material

Table S1 | Differential expression of secreted mRNA between *C. deneoformans* JEC21 and *cin1* strains revealed by RNA-Seq.

Table S2 | Primers used in this study.

Table S3 | miRNAs identified from *C. denewformans* JEC21 strain.

Table S4 | miRNAs identified from *C. denewformans cin1* mutant strain.

Table S5 | Novel miRNAs identified from *C. denewformans*.

Table S6 | Novel siRNAs identified from *C. denewformans*.

Table S7 | Coding and noncoding RNAs identified from *C. denewformans*.

Table S8 | Novel lncRNAs identified from *C. denewformans*.

Table S9 | Known mRNAs identified from *C. denewformans*.

Table S10 | Novel mRNAs identified from *C. denewformans*.

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REVIEW
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Extracellular Vesicles in Fungi: Past, Present, and Future Perspectives

Juliana Rizzo[1]*, *Marcio L. Rodrigues*[2,3] *and Guilhem Janbon*[1]

[1] *Unité Biologie des ARN des Pathogènes Fongiques, Département de Mycologie, Institut Pasteur, Paris, France,* [2] *Instituto Carlos Chagas, Fundação Oswaldo Cruz, Curitiba, Brazil,* [3] *Instituto de Microbiologia Paulo de Góes, Universidade Federal Do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil*

Extracellular vesicles (EVs) have garnered much interest in the cell biology and biomedical research fields. Many studies have reported the existence of EVs in all types of living cells, including in fifteen different fungal genera. EVs play diverse biological roles, from the regulation of physiological events and response to specific environmental conditions to the mediation of highly complex interkingdom communications. This review will provide a historical perspective on EVs produced by fungi and an overview of the recent discoveries in the field. We will also review the current knowledge about EV biogenesis and cargo, their role in cell-to-cell interactions, and methods of EV analysis. Finally, we will discuss the perspectives of EVs as vehicles for the delivery of biologically active molecules.

Keywords: extracellular vesicles, fungal infections, fungal physiology, intercellular communication, pathogenesis

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Marilyn Anderson,
La Trobe University, Australia

***Correspondence:**
Juliana Rizzo
juliana.rizzob@gmail.com;
jrizzoba@pasteur.fr

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INTRODUCTION

Extracellular vesicles (EVs) is a general term used to describe cell-derived double-layer phospholipid membrane particles that reach the extracellular environment (van Niel et al., 2018). These membranous particles are released by cells in all the three domains of life (Gill et al., 2019), and many studies have highlighted their relevance in diverse biological contexts (Maas et al., 2017; van Niel et al., 2018; Bielska and May, 2019; Rybak and Robatzek, 2019; Palacios et al., 2020). EVs are heterogeneous in biogenesis pathways, size, cargo, membrane composition, and biological functions. They participate in many cellular physiological events, including disease development in humans and animals (Shah et al., 2018; Xu et al., 2018). The recent advances in this still emerging field have contributed to the construction of solid knowledge that is rapidly evolving to the formulation of applied tools, including drug delivery systems and vaccine prototypes.

Based on their biosynthetic pathways, mammalian EVs are usually categorized in two broad classes: microvesicles (also called ectosomes or microparticles) and exosomes (van Niel et al., 2018; Latifkar et al., 2019; Mathieu et al., 2019). Microvesicles are generally larger vesicles, from 50 nm to 2,000 nm in diameter, that are formed by the direct outward budding of the plasma membrane. Exosomes are smaller EVs ranging from 30 to 150 nm in diameter that are produced by the endosomal pathway. The biogenesis of exosomes starts when endosomes maturate to form multivesicular bodies (MVBs). These structures fuse with the plasma membrane and release their luminal vesicles to the outer space (van Niel et al., 2018; Latifkar et al., 2019). The exosome population is apparently more complex than initially thought. Recently, by employing an asymmetric-flow field-flow fractionation analysis of melanoma-derived exosomes, two exosome subpopulations were identified, including a large subset ranging from 90 to 120 nm, and a small subset from 60 to 80 nm (Zhang et al., 2018). These subsets of nanoparticles were shown to be packed with different cargo, highlighting the diversity of particles secreted by living cells and opening new questions on their biogenesis and functions (Zhang et al., 2018; Mathieu et al., 2019).

In fungi, since their first description in 2007 (Rodrigues et al., 2007), EVs have been identified in twenty different species, comprising yeast and filamentous fungi. Despite the increasing number of studies on EVs, we still have limited information on their structural properties, biogenesis, and functional outcomes. In this review, we will discuss early reports suggesting the existence of fungal EVs. We will then move our discussion to recent insights, technical hurdles, and the relevance of EVs for fungal biology and intercellular communication during interaction with different host cells.

EXTRACELLULAR VESICLES IN FUNGI: HISTORICAL ASPECTS AND OVERVIEW OF RECENT DISCOVERIES

Studies in the early 1970s suggested the existence of fungal EVs in different models. In 1972, Gibson & Peberdy analyzed the ultrastructure of *Aspergillus nidulans* protoplasts and described a "region of protoplast plasmalemma exhibiting outpushing," which lead to the production of outer membranous particles, once called "subprotoplasts" (Gibson and Peberdy, 1972). Another example of microscopical evidence of the existence of fungal EVs was provided in 1973 by Takeo and collaborators (Takeo et al., 1973). They reported the presence of "spherical invaginations which secrete the vesicles outside the cell membrane" in *Cryptococcus neoformans* (Takeo et al., 1973). In 1977, "extracellular vesicles" was used for the first time in the fungal literature by Chigaleichik and colleagues during the analysis of extracellular lipid structures of *Candida tropicalis* cultivated in the presence of n-alkanes (Chigaleichik et al., 1977).

In 1990, "membrane-bound vesicles which traverse the wall through specialized pimple structures" were reported in *Candida albicans* (Anderson et al., 1990). Eight years later, studies on the cell wall dynamics of *Schizosaccharomyces pombe* demonstrated that protoplasts under cell wall regeneration manifested an increased number of secretory vesicles, including vesicle-like particles in the outer space (Osumi, 1998). In the same study, particles at the *C. albicans* cell surface, at that time called "warty projections," were also reported (Osumi, 1998). In 2000, membrane formations across the periplasmic space, linking the plasma membrane to the inner face of the cell wall, were reported in *C. neoformans*, suggesting the occurrence of vesicular traffic across the fungal cell wall (Rodrigues et al., 2000). Noteworthy, it is very likely that other reports similarly suggested vesicle-like particles in the outer space of fungi, but to our knowledge, these studies compose the first set of experimental evidence suggesting the existence of fungal EVs.

The first study directly focusing on fungal EVs was published in 2007 in *C. neoformans*. Fungal EVs were proposed to be the vehicles for polysaccharide export across the fungal cell wall (Rodrigues et al., 2007). Many subsequent studies demonstrated EV production in yeast forms of *C. gattii, Histoplasma capsulatum*, *C. albicans*, *C. parapsilosis*, *Sporothrix schenckii*, *S. brasiliensis*, *Paracoccidioides brasiliensis*, *P. lutzii*, *Malassezia sympodialis*, *Saccharomyces cerevisiae*, *Pichia fermentans,* and *Exophiala dermatitidis* (Albuquerque et al., 2008; Gehrmann et al., 2011; Vallejo et al., 2011; Vargas et al., 2015; Leone et al., 2017; Bielska et al., 2018; Ikeda et al., 2018; Peres Da Silva et al., 2019; Lavrin et al., 2020).

Compared to yeasts, little is known about EVs in filamentous fungi. However, their presence has been described in different species, including *Trichoderma reesei,* a fungus involved in lignocellulosic degradation (de Paula et al., 2019), in the phytopathogens *Alternaria infectoria* (Silva et al., 2014) and *Fusarium oxysporum* f. sp. *vasinfectum* (Bleackley et al., 2019b), and in the dermatophyte *Trichophyton interdigitale* (Bitencourt et al., 2018). In human filamentous pathogens, EVs have been described in the emerging pathogen *Rhizopus delemar* (Liu et al., 2018) and in the major common causative agents of invasive aspergillosis, *A. fumigatus and A. flavus* (Souza et al., 2019; Brauer et al., 2020; Rizzo et al., 2020). A timeline pointing point out the historical aspects and the recent discoveries of fungal EVs is presented in **Figure 1**.

BIOGENESIS, SELECTION OF CARGO AND RELEASE OF EVs IN FUNGI

The processes regulating fungal EV biogenesis and the specificity of cargo remain unresolved, and most of our hypotheses come from mammalian studies. Fungal EVs are carriers of proteins, lipids, nucleic acids, polysaccharides, toxins, allergens, pigments, and even prions, as recently reviewed (Bleackley et al., 2019a; De Toledo Martins et al., 2019). Many of these molecules are associated with fungal physiological aspects, such as metabolism and cell wall biogenesis, but also with stress responses, antifungal resistance and pathogenesis (Rodrigues et al., 2007, 2008; Albuquerque et al., 2008; Eisenman et al., 2009; Vallejo et al., 2012a,b; Gil-Bona et al., 2015; Kabani and Melki, 2015; Peres Da Silva et al., 2015b; Vargas et al., 2015; Zarnowski et al., 2018; Alves et al., 2019; Zhao et al., 2019).

By analogy with metazoan counterparts, it has been suggested that the release of fungal EVs and selection of cargo can require diverse secretory routes, including regulators of conventional and unconventional secretory pathways (Oliveira et al., 2013; Bielska and May, 2019; Silva et al., 2019). Among the conventional secretory regulators, the Sec6 protein, involved in the exocytosis of post-Golgi secretory vesicles to the plasma membrane, was reported to be associated with EV release in *C. neoformans*. A reduction of EV release level in a sec6 mutant strain was associated with the impaired secretion of virulence-associated molecules (Panepinto et al., 2009). Additionally, mutation of *SEC4*, which encodes a Rab family GTPase; essential for vesicle-mediated exocytic secretion and autophagy, altered EV composition and the kinetics of extracellular release in *S. cerevisiae* (Oliveira et al., 2010b). The Sec1 protein, which is involved in the fusion of Golgi-derived exocytic vesicles with the plasma membrane, also participated in EV composition, but *SEC1* deletion did not affect EV release (Oliveira et al., 2010b). These studies suggest a key role for the Golgi-derived secretory pathway in the vesicular trans-cell wall traffic.

Among the regulators of unconventional secretion, Oliveira and collaborators suggested that the Golgi reassembly stacking

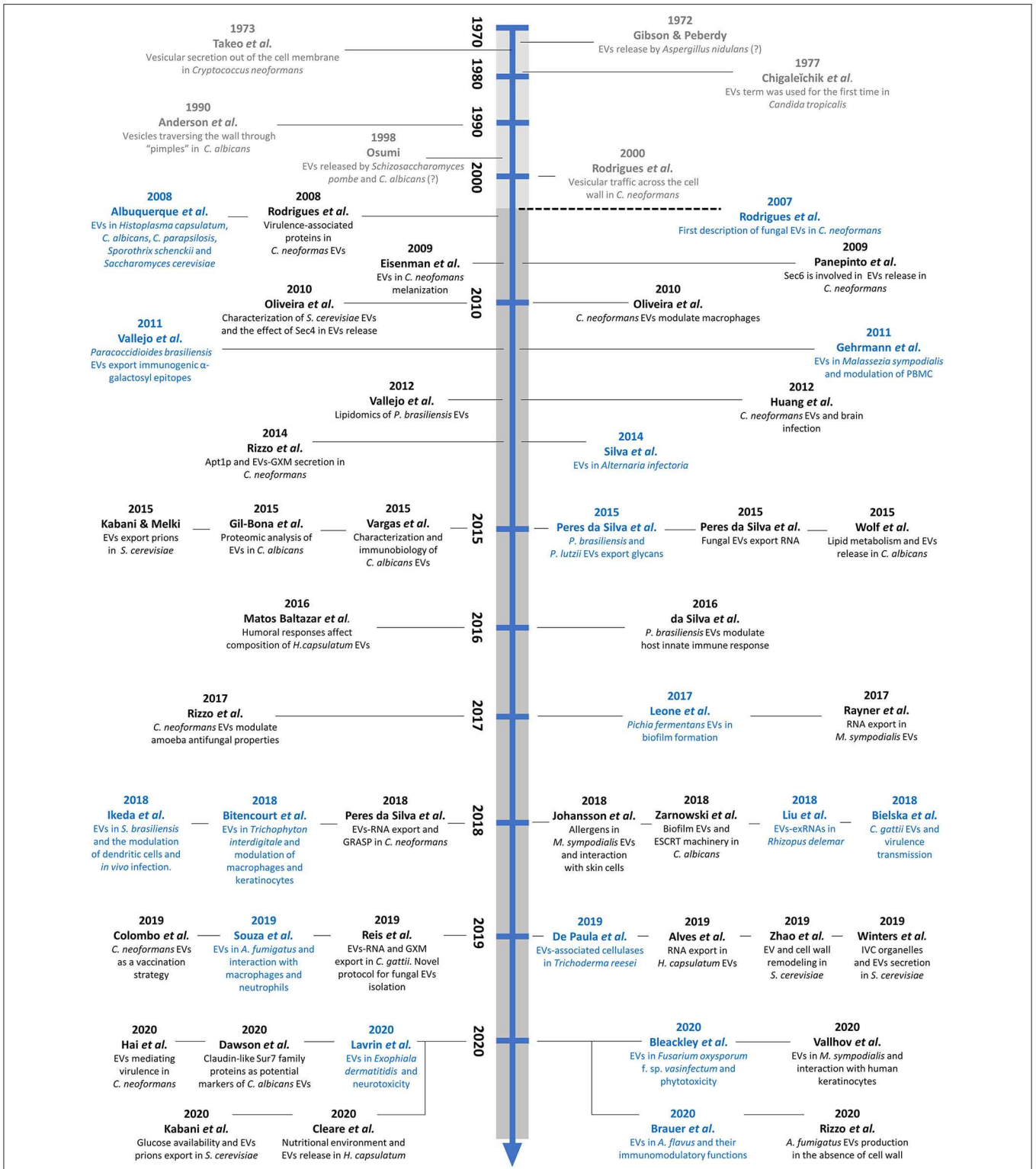

FIGURE 1 | Timeline showing early evidence and recent discoveries in the field of fungal EVs. The studies in gray represent early suggestions of fungal EVs. The dashed line represents the first direct description of fungal EVs, in the *C. neoformans* model. The studies in blue illustrate the initial characterization of EVs in the twenty different fungal species, and those in black represent compositional, methodological, or functional discoveries regarding fungal EVs.

protein (GRASP) was involved in EV release in *S. cerevisiae* (Oliveira et al., 2010b). GRASP was recently shown to participate in EV-mediated export of mRNA in *C. neoformans* (Peres Da Silva et al., 2018) and is an important regulator of *C. neoformans* virulence (Kmetzsch et al., 2011). In addition to GRASP, members of the Endosomal Sorting Complex Required for Transport (ESCRT) machinery involved in the formation and functionality of MVBs, such as the Snf7 and Vps23 proteins, affected EV protein profile in *S. cerevisiae* (Oliveira et al., 2010b).

It was also demonstrated by Zhao and colleagues in *S. cerevisiae,* that mutation of the *VPS2*, *VPS23,* and *VPS36* genes, which encode components of the ESCRT machinery, affected EV proteomic profile and EV abundance (Zhao et al., 2019). EVs produced by ESCRT mutants showed enrichment in cell wall remodeling enzymes, including the glucan synthase subunit Fks1 and the chitin synthase Chs1 (Zhao et al., 2019). Similar results were obtained in *C. albicans*, in which mutations in the subunits of the ESCRT machinery resulted in decreased EV production, in comparison with wild-type strains (Zarnowski et al., 2018). It was recently shown that the lack of another protein of ESCRT complex, Vps27, resulted in the accumulation of MVB structures and release of enlarged EVs in *C. neoformans.* This phenotype was associated with an impaired laccase trafficking to the cell wall (Park et al., 2020). Since EVs were already reported to contain laccase (Rodrigues et al., 2008), this result provides further insights into laccase-associated EV transport and virulence in *C. neoformans.*

Additional mutations have been shown to affect EVs production or cargo composition, although it remains to be known whether these phenotypes are their direct or indirect consequences of these mutations. For instance, the deletion of the lipid flippase encoding gene *APT1* has been reported to alter EV size, EV-mediated secretion of the major capsular polysaccharide, glucuronoxylomannan (GXM), as well as virulence in *C. neoformans* (Rizzo et al., 2014). In *C. gattii*, the deletion of *AIM25* encoding a putative lipid scramblase was reported to result in the production of larger EVs and with altered RNA content, despite the normal EV-associated GXM content (Reis et al., 2019). These data suggest that lipid transporters, and perhaps other regulators of lipid metabolism, could play an important role in biogenesis and cargo selection of fungal EVs. Indeed, lipid biosynthetic genes such as the phosphatidylserine decarboxylase encoding genes (*PSD1* and *PSD2*) in *C. albicans* were reported to influence EV size and protein composition, suggesting an association between lipid metabolism and composition of EVs (Wolf et al., 2015).

It has also been suggested that other regulators of fungal cell physiology could play a role in the release of EVs. For instance, a *C. neoformans* mutant lacking a putative G_1/S cyclin gene had increased production of EVs (Garcia-Rodas et al., 2014). Chitin synthase genes (CHS) were also suggested to play a role in vesicular release, as inferred from the observation that the deletion of chitin synthases genes in *C. neoformans* resulted in a significantly reduced release of EVs (Rodrigues et al., 2018). Recently, it was also shown that *S. cerevisiae* cytoplasmic organelles, called intracellular vesicle clusters (IVCs), serve as sites for the synthesis and selection of EV-associated proteins tagged for secretion (Winters et al., 2020).

Although the studies above provided relevant information on potential mechanisms of EV production in fungi, the key genetics and transcriptional networks, and eventually the post-translational processes underlying fungal EV production and selection of cargo, are unknown. EV release is influenced by fungal lifestyle and modulated by environmental or host-posed conditions (Eisenman et al., 2009; Matos Baltazar et al., 2016; Baltazar et al., 2018; Zarnowski et al., 2018). Additionally, externally added compounds such as EDTA, molecules produced by mammalian cells, such as Galectin-3 (Gal-3) or serum albumin, were reported to reduce vesicular release, or lead to EV disruption (Robertson et al., 2012; Wolf et al., 2012; Almeida et al., 2017). These studies highlight the complex mechanisms associated with EV formation and release, including their extracellular stability.

EVs AND CELL WALL CROSSING

The presence of a thick cell wall was historically considered as a barrier for the outward transition and release of membrane-derived vesicles, as recently discussed (Coelho and Casadevall, 2019). This view contrasts with early studies demonstrating that the fungal cell wall contains several major lipids, which could be components of transitory membrane structures associated with trans-cell traffic (Kanetsuna et al., 1969; Domer, 1971; Cox and Best, 1972). The idea of the cell wall as a friendly environment for lipids was consolidated by numerous reports on membranous structures in association with the cell wall and in culture supernatants. Consequently, several hypotheses have been proposed to understand how the cell wall structure is compatible with the transit of lipid membranes. There are three non-mutually exclusive hypotheses that related to this point. First, vesicles could move across the cell wall through a guide channel. Second, cell wall remodeling enzymes could generate areas facilitating EV transit. Finally, turgor pressure could force vesicles to pass through cell wall pores (Wolf and Casadevall, 2014; Brown et al., 2015).

In *C. neoformans*, Wolf and collaborators used electron microscopy techniques to identify single and multiple vesicle-like particles directly in the cell wall, without any apparent trans-cell wall channel or changes in vesicle surroundings, which argues against the presence of channels for vesicle release in fungi (Wolf et al., 2014). It was also demonstrated that liposomes containing amphotericin B (AmBisome) ranging from 60 to 80 nm in diameter crossed the fungal cell wall from outside to the intracellular space and reached the plasma membrane in their intact form, even though the predicted porosity of the cell wall was too small (pore size around 5.8 nm) to allow their transit (Walker et al., 2018). After these observations, Walker and colleagues described yeast cell walls as viscoelastic structures, permeable to membranous particles.

Recently, it was also shown that EVs from *S. cerevisiae* contain cell wall-related proteins, including enzymes that participate in the degradation and reorganization of polysaccharides,

suggesting a role in cell wall remodeling (Zhao et al., 2019). Additionally, it was recently shown that wall-less *A. fumigatus* cells export plasma membrane-derived EVs containing a complex combination of proteins and glycans. EVs produced by germinating conidial protoplasts increased in number and differed in cargo when the cells were incubated under cell wall regeneration conditions (Rizzo et al., 2020). Finally, another recent study demonstrated that polymorphonuclear granulocytes produce EVs that associate with the cell wall of *A. fumigatus* and even enter fungal hyphae, resulting in alterations in the morphology of the fungal cell wall (Shopova et al., 2020). These studies highlight the cell wall as a dynamic structure with flexible viscoelastic properties and provide new insights into how vesicles can cross the cell wall to reach the extracellular space. Clearly, these studies also open questions on how EVs can play a role in cell wall biosynthetic processes and inter-kingdom communication as signaling entities.

CELL-TO-CELL COMMUNICATION MEDIATED BY EVs

The process of cell wall crossing by EVs has functional consequences in recipient cells (Regente et al., 2017; Bielska et al., 2018; Cai et al., 2018; Rodrigues and Casadevall, 2018). Therefore, here we will explore the literature on EV-mediated communication between fungal cells and in the bidirectional cross-talk between fungi and other organisms.

The relevance of fungal EVs during cellular interaction at the community level, in different stages of fungal lifecycle or even transferring virulence-associated molecules from one strain to another, has been suggested. For instance, it was recently described that EVs produced in *C. albicans* biofilms are different from those produced by free-living planktonic cells, and the release of EVs is an important feature for the proper biofilm formation and drug resistance (Zarnowski et al., 2018). Previously, in the dimorphic yeast *P. fermentans* it was also suggested that EVs could play an active role during the dimorphic transition in response to the growth conditions, including biofilm formation (Leone et al., 2017). Both studies suggest that EVs could participate in intercellular communication during biofilm formation, stimulating studies on the relationship of other microbial biofilms with fungal EVs.

Other examples of cell-to-cell communication mediated by fungal EVs came from studies on *Cryptococcus.* It was previously described that the Vancouver Island outbreak lineage of *C. deuterogattii* display an increased ability to proliferate inside host macrophages through a mechanism called "division of labour" (Voelz et al., 2014). During this process, cells coordinate their behavior to increase the intracellular proliferation of the population as a whole (Voelz et al., 2014). This process has been recently shown to be regulated by EVs (Bielska et al., 2018). EVs obtained from this outbreak lineage were internalized by macrophages pre-infected with cells from a non-outbreak lineage and trafficked to the phagosome, inducing a rapid intracellular proliferation of the non-outbreak fungal cells. This process seems to be restricted to intra-species communication since EVs purified from a virulent strain of *C. neoformans* did not result in the same outcome, even at the highest concentrations of EVs (Bielska et al., 2018).

Very recently, Hai et al. reported that sterile culture filtrates from highly virulent VNIa-5 strains of *C. neoformans* isolated from immunocompetent patients, but not from HIV patients, promoted an increase in the pathogenic potential of less virulent VNIa-5 isolates. This process probably required EV-associated proteins (Hai et al., 2020). These results open new avenues on how EVs can act in virulence transfer in many different contexts, such as in fungal co-infections.

The complexity of the relationship of EV cargo and their functions in intercellular communication was reinforced by the discovery that fungal EVs contain prions and cell wall remodeling enzymes (Kabani and Melki, 2015; Zhao et al., 2019). In *S. cerevisiae*, it was demonstrated that the fungal prion Sup35p was exported via EVs both in its soluble and aggregated infectious states (Kabani and Melki, 2015). Considering that prions are transmitted vertically to the progeny or horizontally during mating, it is reasonable to suggest that EVs could mediate vertical and horizontal transfer of prions-like protein in fungi (Kabani and Melki, 2016; Zhao et al., 2019). Still in *S. cerevisiae,* it was recently shown that EVs can be taken up by fungal cells and play a critical role in cell wall remodeling. Fungal EVs containing cell wall associated enzymes, such as glucan and chitin synthases, were able to enhance yeast cell viability upon cell wall stress, induced by the presence of the 1,3-β-glucan synthase inhibitor antifungal drug, caspofungin (Zhao et al., 2019). This latter data raises the question if the drug resistance could also happen in a community level basis and bolster fungal infection processes, as previously discussed for drug resistance in *C. albicans* biofilms (Zarnowski et al., 2018).

In addition to what is known about communication between fungal cells, several studies on the relevance of fungal EVs in cellular communication with mammalian cells are available in the literature, as previously reviewed (Zamith-Miranda et al., 2018; Bielska and May, 2019; Freitas et al., 2019; Silva et al., 2019). In all morphological stages of many pathogens, fungal EVs were shown to be internalized by mammalian cells in processes that culminated with the modulation of antimicrobial activities and diverse immunogenic responses, including the activation of pro-inflammatory and anti-inflammatory cytokines. In these experiments, host cells interacting with EVs comprise murine macrophages (including bone marrow-derived macrophages), dendritic cells, and neutrophils, in addition to human peripheral blood mononuclear cells, keratinocytes, monocytes, macrophages, and brain microvascular endothelial cells (Oliveira et al., 2010a; Gehrmann et al., 2011; Vallejo et al., 2011; Huang et al., 2012; Peres Da Silva et al., 2015a; Vargas et al., 2015; Da Silva et al., 2016; Bielska et al., 2018; Bitencourt et al., 2018; Ikeda et al., 2018; Johansson et al., 2018; Souza et al., 2019; Vallhov et al., 2020).

Recently, it was shown that melanized-EVs obtained from the extremophilic fungus *Exophiala dermatitidis* were able to strongly affect the viability of human neuroblastoma cells, while non-melanized EVs were considerably less neurotoxic (Lavrin et al., 2020). These data demonstrated that EV cargo is relevant

for their biological effect on host cells, and strengthen the notion that fungal EVs enclose diverse virulence-associated molecules, such as melanin, as previously reported for *C. neoformans* (Rodrigues et al., 2008; Eisenman et al., 2009).

Several elements can interfere in EV-mediated interactions between fungi and mammalian cells. Baltazar and collaborators showed that the binding of monoclonal antibodies to *H. capsulatum* modulated vesicle composition at both quantitative and qualitative levels, leading to diverse immune effector mechanisms (Matos Baltazar et al., 2016; Baltazar et al., 2018). Reales-Calderon and colleagues also showed that macrophage-derived EVs change their size and protein composition in response to *C. albicans* infection, suggesting a role of host cells EVs in fungi-macrophage communication (Reales-Calderon et al., 2017). Recently, it was also shown *A. fumigatus* cells triggered EV release by human neutrophils. EVs released by neutrophils exposed to conidia had antifungal properties and inhibited the growth of *A. fumigatus* hyphae. The same outcome was not observed for EVs released by uninfected neutrophils (Shopova et al., 2020). These data suggest that EV release by mammalian cells represent a still unexplored mechanism of antifungal defense during host-pathogen interactions.

Besides regulating the responses of mammalian cells, fungal EVs were also described to modulate the physiology of environmental predators, including *Acanthamoeba castellanii* (Rizzo et al., 2017). During the fungi-amoebae interaction, EVs released by *C. neoformans* were internalized by *A. castellanii* with no impact to the predator's viability. EVs modulated amoebal antifungal properties by inducing enhanced yeast intracellular survival. The same effect did not occur when amoebae were treated with the capsular polysaccharide GXM (Rizzo et al., 2017). Although *A. castellannii* was also shown to release EVs (Goncalves et al., 2018), the impact of amoeba EVs on fungal biology remains to be elucidated.

EVs also participate in plant-fungi communication (Cai et al., 2019). In 2011, based on the ultrastructural characterization of the haustorium-forming phytopathogen *Golovinomyces orontii*, it was observed MVB-like structures fusing with (or budding off from) the fungal plasma membrane and also membrane-bound vesicles in the extra-haustorial matrix, suggesting the occurrence of vesicular release outside of the haustorial cell wall during plant-fungi interactions (Micali et al., 2011). Later on, EVs from sunflowers were shown to be internalized by the fungus *Sclerotinia sclerotiorum*, a phytopathogen able to infect numerous host plants and cause severe rot (Regente et al., 2017). Plant-derived EVs were enriched in cell wall remodeling enzymes and defense proteins. Once in contact with fungal cells, plant EVs caused morphological changes, impairment of growth and cell death, which led to the hypothesis that EVs could function as vehicles for the delivery of components involved in plant defense mechanisms against fungal infections (Regente et al., 2017).

Cai and collaborators demonstrated that *Arabidopsis* cells release EVs containing siRNAs that were efficiently taken-up by the fungus *Botrytis cinerea*, which resulted in the silencing of virulence-associated fungal genes (Cai et al., 2018). The mechanism behind this observation involved the silencing of fungal virulence-associated genes through mRNA cleavage (Cai et al., 2018). *Arabidopsis* EVs were also proposed to deliver siRNAs into the plant pathogen *Phytophthora capsici*, possibly contributing to host-induced gene silencing during natural infection (Hou et al., 2019).

EVs were also involved in arbuscular mycorrhizal symbioses (Roth et al., 2019), as concluded from the accumulation of EVs in the contact area of fungi with plant cells. It is not known if these EVs are originated from plant and/or fungal cells, but their detection in the interaction interface suggests that these membranous particles could be mediating the cell-to-cell communication between the two symbionts. Although these studies highlight the relevance of EVs in mediating molecule exchange from plant to fungi, an unanswered question is whether fungi use EVs to deliver effector molecules to plants during mutualistic or parasitic relationships. Accordingly, in EVs from the cotton pathogen *Fusarium oxysporum* f. sp. *vasinfectum* were described to induce a phytotoxic response in plants (Bleackley et al., 2019b), which reinforce this hypothesis. This subject has been discussed in detail in recent reviews (Huang et al., 2019; Kwon et al., 2020).

METHODS OF PURIFICATION AND TECHNICAL HURDLES OF EVs CHARACTERIZATION

The difficulties posed by EV isolation methods, quantification, imaging, and functional analysis are technical hurdles that challenge the field of EV research (Margolis and Sadovsky, 2019). The debate concerning the principles for EV isolation, characterization of cargo and biological functions has motivated numerous consortiums in the field of mammalian EVs to establish collaborative projects directed to the development of robust methods for isolation, analysis, interpretation and reproducibility of experiments (Consortium et al., 2017; Thery et al., 2018; Das et al., 2019).

Despite substantial progress, research on fungal EVs is still in its infancy (Bielska and May, 2019). In this context, it is essential to point out the limitations and advances of the investigation of fungal EVs, including the experimental models of EV analysis. The regular protocols that have been used in the past decade to isolate fungal EVs are time-consuming and rely on handling liters of culture supernatant using low-speed centrifugation, followed by filtration, volume concentration, and collection of EV-rich fractions by ultracentrifugation (Rodrigues et al., 2016). This general protocol has been used since the first description of EVs in *C. neoformans (Rodrigues et al., 2007),* but there are serious limitations, including low yield, possible co-isolation of non-vesicular extracellular molecules, and the isolation of mixed EV populations.

The recent use of an asymmetric flow field-flow fractionation analysis revealed a previously unknown population of particles smaller than 50 nm (around 35 nm), named exomeres. These structures lack the external membrane and, therefore, are considered as non-vesicular nanoparticles that can be co-isolated with exosomes (Zhang et al., 2018; Mathieu et al., 2019). Exomeres have not been documented in fungi so far. Therefore,

one can speculate that their presence in fungal secretomes could also represent a contamination of EV fractions. The hurdles of isolation and characterization of homogeneous subgroups of EVs have been a matter of debate in the EV field as a whole, and recent calls for protocol improvements and accurate analysis have been published (Thery et al., 2018; Raposo and Stahl, 2019).

Different approaches have been suggested to increase the purity, yield, and functional potential of mammalian cells EV purification protocols (Mateescu et al., 2017; Thery et al., 2018; Takov et al., 2019). In fungi, density gradient ultracentrifugation with sucrose or iodixanol was successfully used for EV fractionation (Rodrigues et al., 2007; Oliveira et al., 2009; Kabani and Melki, 2015; Rayner et al., 2017; Bleackley et al., 2019b). Also, size-exclusion chromatography after clarification and ultracentrifugation of culture supernatants was used to isolate EVs from *C. albicans* biofilms (Zarnowski et al., 2018). However, the main disadvantage of applying additional purification steps during EV isolation is the decrease in the purification yield (Chutkan et al., 2013).

An alternative approach for the isolation and analysis of fungal EVs has been recently described. Reis and collaborators have demonstrated that isolating EVs from cells growing on solid media had many advantages in comparison to the previously used protocol in liquid medium (Reis et al., 2019). The optimized protocol was shown to be faster, with higher yields, and applicable to the biological evaluation of EVs (Reis et al., 2019). Nonetheless, all the protocols used so far end up in the generation of EV-rich centrifugation pellets, which consistently comprise heterogeneous populations, with varying sizes and physical-chemical properties. These latter aspects make EVs subpopulations indistinguishable, thus lessening the accuracy of their characterization, which can culminate in misleading interpretations of functional roles (Raposo and Stahl, 2019).

The analysis of the dimension of fungal EVs has been predominantly based on three different technical approaches: dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and electron microscopy (EM) (Bielska and May, 2019; Palacios et al., 2020). A combination of these methods and others certainly improves accuracy. In this sense, NTA proved to be a useful method to evaluate the distribution of EV subtypes on different fungal species, in association with particle quantification (Reis et al., 2019). DLS provides similar results, with the limitation of not being quantitative. However, considering that DLS and NTA techniques are based on particle sizes, with limitations for analyzing particles smaller than 100 nm, the combination of two different and complementary techniques, such as single-particle analyzers (NTA, DLS, high-resolution flow cytometry) with EM-based analysis is highly recommended in order to better characterize fungal EVs (Thery et al., 2018; Margolis and Sadovsky, 2019).

Methods of dehydration or chemical fixation for conventional EM pose many questions regarding artifacts and the possibility of altered morphology, size, and membrane stability of EVs (Noble et al., 2020). In this sense, the use of cryo-electron microscopy seems to be an appropriate method to visualize a broad spectrum of sizes and morphologies of EVs (Emelyanov et al., 2020; Noble et al., 2020). The use of asymmetric-flow field-flow fractionation for EV analysis is promising for the identification of larger and smaller vesicles (Zhang et al., 2018), but this remains to be confirmed in the analysis of fungal EVs. It is also important to state that linking size information to other biophysical and biochemical EV properties is of high relevance in order to define the vesicle subtypes better (Thery et al., 2018; Margolis and Sadovsky, 2019).

Clearly, there are still many open questions regarding the biological relevance of EV size diversity (Margolis and Sadovsky, 2019). Yet, novel approaches to optimize EV purification and size-based fractionation are needed. If resolved, this experimental limitation would facilitate the analysis of the impact of external factors on EV production, including growth conditions, cell cycle stage, growth phase, and cellular density, among others. In association with the optimized EV purification protocols, improved methods of data interpretation could positively impact the field.

For instance, comparative analyses of the enrichment of selected sets of molecules in EVs with their global cellular levels could reveal the existence of specific sorting mechanisms of molecular loading, in addition to selective delivery of EVs to different cell targets. Accordingly, it was recently shown that the claudin-like Sur7 family proteins Sur7 and Evp1 were enriched in *C. albicans* EVs, compared to whole cell lysates. The authors suggested these proteins as putative *C. albicans* EV positive markers, based on their potential topological similarity to tetraspanins, markers used for mammalian EVs (Dawson et al., 2020).

The analysis of cargo and the functional diversity of EVs is highly dependent on the purification methods and the nutritional availability (Tkach et al., 2018; Cleare et al., 2020). It was also demonstrated that EV-mediated prion export is regulated by glucose availability in *S. cerevisiae* (Kabani et al., 2020). Additionally, recent data showed that different nutrition environments play an essential role in EV formation and cargo loading in *H. capsulatum* (Cleare et al., 2020). Variable nutrient availability impacted the released EVs in size, protein, lipid, and carbohydrate metabolites profiles, which reinforce the plasticity of EV composition and its possible impact on fungal virulence (Cleare et al., 2020). Therefore, improvement of existing purification protocols and standardization of data analysis are highly desirable, as previously discussed (Coumans et al., 2017; Mateescu et al., 2017; Thery et al., 2018; Srinivasan et al., 2019; Thane et al., 2019; Turchinovich et al., 2019). The main challenges faced by the fungal EVs community, based on the technical hurdles and conceptual gaps described in this manuscript, are summarized in **Figure 2**.

EVs AS VEHICLES FOR THE DELIVERY OF BIOLOGICALLY ACTIVE MOLECULES

There is an urgent need for new strategies to prevent and combat fungal infections, which affect over a billion people worldwide and kill more than 1.5 million, annually (GAFFI; http://www.gaffi.org/). Moreover, it is important to emphasize that the emergence of new fungal pathogens and resistance to

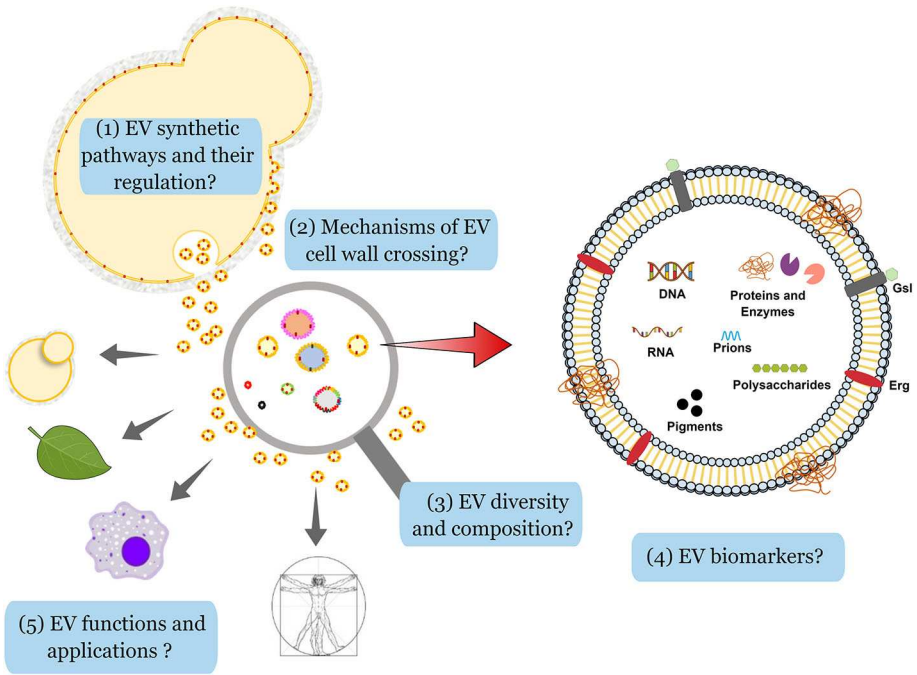

FIGURE 2 | Challenges in the analysis of fungal EVs. By analogy with the metazoans, and based on the knowledge on secretion and regulation of vesicle production in eukaryotes, one can anticipate the existence of a least two pathways for the production of EVs in fungi. Although some elements implicated in these pathways (ESCRT proteins, for instance) have been already identified in fungi, little is known about the differential regulation in the formation of fungal EVs (1). In fungi, the presence of a thick cell wall was initially considered as a physical barrier for EV crossing, but recent evidence has shown that the cell wall is a viscoelastic structure and different hypotheses have been proposed to explore how it is compatible with the transit of lipid membranes (2). On the experimental side, a large diversity of EV morphology, size, and content has been observed, but different technical limitations have impaired the analysis. For instance, NTA or flow cytometry analyses do not detect particles smaller than 100 nm in diameter in a reliable fashion. Moreover, the current protocols used to purify EVs do not prevent contamination with aggregates potentially containing proteins, nucleic acids and polysaccharides, thus limiting the knowledge on EV structure and cargo. Differential EV purification according to their size and/or density from the contaminating aggregates should shed light on their intrinsic diversity (3). Despite their complex composition, including nucleic acids, glycans, pigments, proteins, prions, and different lipids (ergosterol, Erg; glycosphingolipids, Gsl), specific markers of fungal EVs have not been identified so far (4). Finally, very little is known on the role fungal EVs in cell-to-cell communication between different hosts and fungi, but also between fungal cells. One can anticipate that EV diversity could be associated with a diversity of functions and responses of the recipient cells in the presence of different types of vesicles. EV components may also have applicability in biomedical fields, including immunomodulation tools, drug-delivery vehicles, and vaccine candidates (5).

antifungal drugs are health security threats around the world (Fisher et al., 2018; Rhodes and Fisher, 2019). Despite this public health burden, fungal infections have been widely neglected in terms of research funding compared to other infectious diseases (Rodrigues and Albuquerque, 2018; Rodrigues and Nosanchuk, 2020). In this sense, investments in combating fungal pathogens, including EVs-based approaches, can be beneficial for the development of new mechanisms of fighting fungal diseases.

Despite the already mentioned open questions (Margolis and Sadovsky, 2019; Raposo and Stahl, 2019), substantial progress has been made to elevate EVs to the position of important mediators of intercellular and interkingdom communication processes, given their ability to transfer bioactive components (Maas et al., 2017; van Niel et al., 2018; Cai et al., 2019; Mathieu et al., 2019; Correa et al., 2020) and surmount biological barriers, including the blood-brain barrier (BBB) (Alvarez-Erviti et al., 2011).

Potential EV-based applied tools are increasing in number and they are expected to positively affect diagnosis and therapy in a number of diseases (Lane et al., 2018; Shah et al., 2018). Their use as disease biomarkers, drug delivery, or even bioengineered for vehicles in therapeutics has also been proposed (Shah et al., 2018; Xu et al., 2018; Wiklander et al., 2019). In a sense, bacterial, fungal and parasite RNA sequences were identified in human body fluids (Beatty et al., 2014), suggesting that RNA-associated EVs could represent biomarkers of infectious diseases (Beatty et al., 2014; Hoy et al., 2014). EV-based strategies for the control of infectious diseases have also been suggested, including the use of artificial vesicle-protected RNA antifungal strategies, and other RNA-based techniques for host-induced gene silencing (Cai et al., 2019).

EVs have also been suggested to have important functions as adjuvants, vaccine or immunotherapy platforms for fighting infectious diseases, including fungal infections (Schorey and Harding, 2016; Fuhrmann et al., 2017; Kuipers et al., 2018; Freitas et al., 2019). In bacteria, for instance, *Staphylococcus aureus* EVs have been recently suggested to be used as a vaccine platform, since mice immunized with native *S. aureus* EVs produced a robust T-cell response and were protected against lung infection (Choi et al., 2015; Wang et al., 2018). Vaccine candidates for parasitic helminths are also being discussed since

no effective vaccines are available to control the transmission of these pathogens (Mekonnen et al., 2018). Similarly, there are no licensed antifungal vaccines (Nami et al., 2019), which highlights the need for novel strategies to develop vaccines to prevent fungal infections.

Previous studies using EVs released by *C. neoformans* and *C. albicans* revealed their ability to delay the mortality of *Galleria mellonella* after challenge with yeast cells (Vargas et al., 2015; Colombo et al., 2019), probably due to an innate mechanism of infection control (Freitas et al., 2019). Considering that fungal EVs are efficient immunomodulators (Freitas et al., 2019), it is reasonable to suggest that native or engineered EVs could be promising structures for the development of vaccine platforms.

CONCLUDING REMARKS

Fungal EVs have been the focus of many studies over the past few years and have emerged as important signaling particles, therefore opening new avenues for investigation of their use in different pathogenic models. Despite substantial advances in the field, important challenges and unanswered questions about the structure and functions of EVs remain active. There is a clear need to strengthen our knowledge of the genetic, biochemical, and physical aspects of fungal EVs in order to clarify the mechanisms regulating their production, composition, and diversity toward a better comprehension of their biological function.

AUTHOR CONTRIBUTIONS

JR contributed to the conception and design of the review. MR and GJ contributed equally to the manuscript text and revision. MR was currently on leave from his position of Associate Professor in the Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), Brazil. All authors have made substantial intellectual contributions to the work, have read, and approved the submitted version.

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REVIEW
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Role of Extracellular Vesicles in Influenza Virus Infection

Yuan Jiang[1,2], *Xiaowen Cai*[1], *Jiwen Yao*[1], *Huanhuan Guo*[1], *Liangjun Yin*[3], *Wingnang Leung*[4]* *and Chuanshan Xu*[1]*

[1] *Key Laboratory of Molecular Target and Clinical Pharmacology, State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences and Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou, China,* [2] *Department of Rehabilitation Medicine, The First Affiliated Hospital of Chengdu Medical College, Chengdu, China,* [3] *Department of Orthopedic Surgery, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China,* [4] *Asia-Pacific Institute of Aging Studies, Lingnan University, Tuen Mun, China*

Influenza virus infection is a major health care concern associated with significant morbidity and mortality worldwide, and cause annual seasonal epidemics and pandemics at irregular intervals. Recent research has highlighted that viral components can be found on the extracellular vesicles (EVs) released from infected cells, implying a functional relevance of EVs with influenza virus dissemination. Therefore, exploring the role of EVs in influenza virus infection has been attracting significant attention. In this review, we will briefly introduce the biogenesis of EVs, and focus on the role of EVs in influenza virus infection, and then discuss the EVs-based influenza vaccines and the limitations of EVs studies, to further enrich and boost the development of preventative and therapeutic strategies to combat influenza virus.

Keywords: influenza virus, extracellular vesicles, exosomes, immune response, vaccine

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Neta Regev-Rudzki,
Weizmann Institute of Science, Israel

Reviewed by:
Teneema Kuriakose,
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Asuka Nanbo,
Nagasaki University, Japan

***Correspondence:**
Wingnang Leung
awnleung@gmail.com
Chuanshan Xu
xcshan@163.com

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INTRODUCTION

Influenza virus, a single-stranded negative-sense RNA virus, belongs to *Orthomyxoviridae* family, and can infect the nasal and tracheal airways, and then spread throughout the upper and lower respiratory tract (Tumpey and Belser, 2009). Influenza virus infection usually causes various syndromes such as fever, cough, headache, body ache, runny nose and even severe pneumonia depending largely on the health status and immunologic function of the patient and virus pathogenicity. Seasonal influenza and pandemic influenza are manifestations of influenza virus infection in human beings, moreover, avian-origin influenza virus occasionally jumped species and spread to human successfully, leading to limited, non-sustained human-to-human avian influenza virus transmission, and the mortality is high (Malik et al., 2009). During the influenza season, according to statistics, 5–15% of the human population could be infected, causing 250,000–500,000 deaths a year worldwide (Ginsberg et al., 2009; Goeijenbier et al., 2014). Since 2010, 140,000–710,000 inpatients of the United States were infected with influenza viruses, and 12,000–56,000 deaths each year (McGowan et al., 2019). Most seasonal influenza virus infections are self-limiting, however, they can cause serious or fatal pulmonary dysfunction and even acute respiratory distress syndrome (ARDS) (Khatri et al., 2018). Meanwhile, the oversecretion of inflammatory cytokines (known as "cytokine storm") induced by the immune or inflammatory response, aggravate the

damage degree of ARDS, even induce many organs failures and finally raise the death risk (Tisoncik et al., 2012). Moreover, during or shortly after influenza virus infection, patients usually face increased risk of thrombosis-related cardiovascular events, such as myocardial infarction and stroke (Warren-Gash et al., 2009; Antoniak et al., 2016). Dreadfully, a new strain or variant of influenza virus might result in a pandemic with millions of fatalities.

According to the different antigenicity of the nucleoprotein (NP) and matrix protein (M), influenza viruses are usually classified into three different serotypes: influenza A virus (IAV), influenza B virus (IBV), and influenza C virus (ICV) (Zheng et al., 2020). IAV has genetically distinct subtypes based on 18 hemagglutinin (HA) and 11 neuraminidase (NA) surface glycoproteins. Some subtypes of IAV have established stable transmission profiles among birds, swine, and humans (Huo et al., 2019). In particular H5N1 and H7N9 are highly pathogenic subtypes of avian origin, and directly transmitted from birds to humans with highly contagious and widespread outbreak patterns (Li et al., 2015, 2017). H1N1 is the earliest emerging subtype with available genomic sequences, has caused several pandemics and seasonal epidemics, resulting in millions of deaths and enormous economic losses (Yin et al., 2018). Once influenza virus settle in the respiratory tract of humans, the virus can break the mucus barriers and rapidly infect the primary host cells (i.e., epithelial cells) to release the genome segments into the cell to start virus transcription and replication. First of all, influenza viruses bind to sialic acid receptor on the surface membrane of the epithelial cells using HA, and then enters into host cells via viropexis and/or receptor-mediated endocytosis process. Viropexis is the predominant metabolical activity-independent mechanism of influenza viruses attachment envelopment and the subsequent formation of virus-containing intracellular vacuoles for entry into the host cell (Patterson et al., 1979). The virus particles are encapsulated into endosomes, and transported to locations near the cell nucleus. After the viral membranes fused with endosomes, viral ribonucleoprotein complex (vRNPs) as the templates are released into the cell nucleus to synthesize messenger RNA (mRNA) and complementary RNA (cRNA) for viral translation and replication. Newly synthesized vRNPs are assembled and exported from the cell nucleus and then directed to the plasma membrane. Finally, vRNPs are incorporated into budding virions and released into extracellular environment (Keshavarz et al., 2018), and then spread to uninfected cells or local immune cells such as macrophages (MΦ), which are the principal effector cells of the innate immune system (Cypryk et al., 2016). The intercellular transfer of viral materials released from infected cells to neighboring and distant recipient cells affect virus spread and pathogenesis. Importantly, this kind of cell- cell communication can directly activate host response to influenza virus infection, and modulate the host homeostasis (Assil et al., 2015).

It was widely believed that cell-cell communication is critical for maintaining homeostasis in body under many different physiological and pathologic conditions, not just viral infection, and can be mediated by direct cell-cell contact via transferring some soluble factors including hormones, cytokines, and inflammatory mediators (Schorey and Harding, 2016). Extracellular vesicles (EVs), a heterogeneous group of natural membrane vesicle released from various cells, are found in the plasma and other body fluids, such as saliva, sputum, urine, semen, and breast milk. EVs are initially regarded as cellular waste (Kalamvoki and Deschamps, 2016; Pleet et al., 2018), however, recent research has highlighted that EVs can also as an important mediator play autocrine/paracrine role in intercellular communication (Fujita et al., 2018). EVs attach to the surface of recipient cells by means of adhesion molecules, and then release their contents (e.g., proteins, lipids, and RNAs) into cytosol of recipient cells by phagocytosis, endocytosis, and macropinocytosis or direct fusing with cell membrane (Villarroya-Beltri et al., 2014; Tkach and Théry, 2016). Currently, EVs are called by different names in many articles according to their size and biogenesis, but only three main classes of EVs have been indentified, namely exosomes, microvesicles/ectosomes and apoptotic bodies (Hessvik and Llorente, 2018; Vidal, 2019). Exosomes, <150 nm in diameter, are released into the extracellular environment after fusion of multivescular bodies (MVBs) with the plasma membrane. Nevertheless, microvesicles and apoptotic bodies, both larger than 100 nm in diameter, are directly formed by plasma membrane outward budding and then released from living and dying cells, respectively (Palmulli and van Niel, 2018).

Many *in vivo* infection studies have shown that an elevated concentration of blood EVs was along with infection, and viral components (e.g., proteins and genome) were found on the EVs released from infected cells, demonstrating a functional relevance of these vesicles with virus dissemination in the body during an infection (Schorey and Harding, 2016). Recently, the relationship of EVs and viral infection has also been extensively investigated, including human immunodeficiency virus (HIV), hepatitis B virus (HBV), Hepatitis C virus (HCV), human papilloma virus (HPV), Epstein-Barr virus (EBV), human T-lymphotropic virus (HTLV), and members of the herpesvirus family (Meckes, 2015; Cone et al., 2019). Studies confirmed that EVs encapsulating viral materials could stimulate host response in the absence of direct infection of cells. For example, HIV-1 accessory protein Nef targets the MAL, a tetraspanning membrane protein, and then exploits MAL-dependent passway of exosome biogenesis for hijacking exosome release into Jurkat T cells to educate quiescent T cells permissive to HIV-1 replication (Ventimiglia et al., 2015). Moreover, EVs released from virus infected cells could also interact with non-immune cells such as hepatocytes for persistent infection (Devhare et al., 2017). Based on the above, not surprisingly, viruses have evolved to hijack the biosynthetic machinery of EVs as an astute survival strategy. The sorting and release of virion or viral components have to use or require the endosomal-sorting complexes required for transport (ESCRT) pathway (Schorey and Harding, 2016), as well as exosome and other EVs, implying that there exist some similarities between viral assembly and biogenesis of EVs in host cells. The EVs carrying viral proteins and genetic material, play a significant role in viral infection (Nolte-'t et al., 2016).

In this review, we will briefly introduce the biogenesis of EVs, and focus on the role of EVs in influenza virus infection,

and then discuss the EVs-based influenza vaccine and the limitations of EVs studies, to further enrich and boost the development of preventative and therapeutic strategies to combat influenza virus.

SIMILARITY IN EVs BIOGENESIS AND RELEASE OF INFLUENZA VIRUS PARTICLES

Exosomes are the smallest one of EVs with round double membrane structure in appearance (Hessvik and Llorente, 2018). As the best-characterized subclass of EVs, exosomes have been arouseing general concern and widely researched. Besides mature red blood cells, nearly all eukaryotic cells can secrete exosomes depending on their endocytic capacities (Vidal, 2019). Exosomes are the only EVs formed from internal membrane, and the biogenesis of them starts from early endosomes mature into late endosomes, and budding into intraluminal vesicles (ILVs) after encapsulating selected cargo composed of proteins, nucleic acids, and other bioactive molecules (Anderson et al., 2016). ILVs are a direct result from parts of the limiting membrane bud into the lumen of the endosome, and encapsulated into mulitivesicular bodies (MVBs) (Schöneberg et al., 2017). MVBs are fused with autophagosomes for delivering them to lysosomes, or directly fused with lysosomes where their contents can undergo degradation, or directly transported to fuse with the plasma membrane for ILVs release to extracellular environment as exosomes (Gruenberg, 2020; Kalluri and LeBleu, 2020). The above process can be regulated through ESCRT-dependent pathway or ESCRT-independent pathways (e.g., Rab GTPases, tetraspanin complexes and ceremide pathway; Tkach et al., 2018). The ESCRT machinery consists of approximately thirty proteins, and those proteins assemble into core four complexes (e.g., ESCRT-0, -I, -II, and -III) and some associated proteins (e.g., VPS4, ALIX, and VTA1), especially the ESCRT-II subunits can bind to RNA resulting in the cytoplasmic mRNA and miRNA to sort into exosomes during the biogenesis process (Kouwaki et al., 2017).

Microvesicles are also named as ectosomes or microparticles, and less known in their biogenesis process compared to exosomes. In general, microvesicles are formed by direct budding of the outward plasma membrane via ARF6 and RHOA-dependent rearrangement of the actin cytoskeleton (Li et al., 2012), and Ca^{2+}-activated scramblases (Cocucci and Meldolesi, 2015). The formation of microvesicles seems to be affected by some other mechanisms in different cells. In cancer cells, Rab GTPases Rab5 locating at the plasma membrane of cells, might play vital roles in microvesicles biogenesis (Das et al., 2018). Rab22a and hypoxia-inducible factors (HIFs) can also regulate microvesicles formation in the cancer cells under hypoxia condition (Wang et al., 2014). The ESCRT-III Pathway participates in microvesicles formation in cardiomyocytes. Like exosomes, microvesicles can also transfer mRNA and miRNAs to recipient cells, however, the mechanisms underlying the sorting of RNAs in microvesicles biogenesis remain unclear.

Apoptotic bodies are the only EVs formed during programmed cell death (PCD) (Ståhl et al., 2019). Apoptotic bodies biogenesis starts from budding of the outward plasma membrane through apoptotic pathway. After the critical morphological changes of apoptotic cells (e.g., membrane blebbing and membrane protrusion), apoptotic bodies encapsulateing chromatin, low molecular weight RNA, glycosylated proteins, nuclear fragments and even intact mitochondria, and are ultimately released into extracellular environment as a product of apoptotic cell disassembly. Moreover, apoptotic bodies not only modulate the response of immune system, but also transfer bioactive molecules to recipient cells that represent certain signaling pathway in cell-cell communication (Hristov et al., 2004).

As described above, ESCRT machinery might contribute to the release of other types of EVs besides exosomes, because it is involved in remodeling of plasma membrance (Assil et al., 2015). To date, many studies have reported that viral assembly and EVs biogenesis share some similarities, implying that the "hijacking" behavior of viruses in host cells could be in charge of sorting the viral proteins and RNAs into EVs. HIV is a typical example, it can assemble and release from infected cells via ESCRT machinery (Schorey et al., 2015). Influenza viruses bud from the plasma membrane of host cells similar to microvesicles (Lakdawala, 2019), and the similarities of protein profile between influenza virions and microvesicles secreted by uninfected cells have been confirmed by Hutchinson and colleagues, suggesting that influenza viruses can manipulate certain pathway normally used for microvesicles formation (Hutchinson et al., 2014). However, current evidences have suggested that no core ESCRT component involved in IAV particles assembly and budding, implying influenza virus may utilize the ESCRT-independent pathways to facilitate transportation of virus particles to the plasma membrane for release (Bruce et al., 2009; Watanabe and Lamb, 2010; Alenquer and Amorim, 2015). Bruce E. A. et al. (2010) found that Rab 11 involved in the formation of virus particles of IAV by budding from the apical plasma membrane. Morphologically, the virus particles of IAV can take the shape of pleomorphic spheres or vastly elongated filaments. In the study of Bruce et al., Rab11 depletion caused defective budding, low formation of filamentous virions, and virus particles failed to pinch off from the plasma membrane, leading to virus particles apparently stalled in the process of budding. Those results suggested that Rab11 pathway was important in directing vesicular traffic, and influenza virus utilizes the Rab11 pathway for budding. Thereafter, other scholars suggested that Rab 11 was a critical host factor with an essential contribution to transport of vRNPs cargo to the plasma membrane in IAV-infected cells, revealing the potential mechanism of influenza virus genome delivery via a Rab11-dependent vesicular transport pathway (Amorim et al., 2011; Eisfeld et al., 2011). Given Rab family proteins are essential regulators of intracellular vesicle transport between different compartments and vesicle budding, especially RAB11 was the first Rab reported to be involved in exosome secretion (Colombo et al., 2014). Existing studies have shown that the Rab 11 vesicle trafficking is one of the similarities in EVs biogenesis and release of influenza virus particles, implying

that influenza virus RNPs may access a Rab11-dependent vesicular transport pathway in recycling endosome (RE) that contributes to the budding of influenza virus particles via EVs secretion.

ROLE OF EVs IN HOST IMMUNE RESPONESE FOR INFLUENZA VIRUS INFECTION

During the viral infection, host innate and acquired immune responses can be activated by virus-derived products, accompanied by the recruitment and activation of eukocytes and other cells by cytokines, chemokines, and inflammatory mediators released from infected or resident immune cells, eliciting antiviral action (Schorey et al., 2015). Innate immunity provides the first line of defense and triggers pro-inflammatory responses, while adaptive immunity eliminates the viruses during the later stages of infection (Chen et al., 2018). The recognition and respones to viruses are very important for innate immune system. The viral components such as proteins, lipids, carbohydrates and genetic material, are generally defined as pathogen associated molecular patterns (PAMPs). PAMPs can bind with pattern recognition receptors (PRRs) on the plasma membrane of host cells or dendritic cells (DCs) and MΦ to induce cell-signaling cascades, culminating in the activation of innate immune response against the viruses (Kouwaki et al., 2017). PRRs include Toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors. Interestingly, viruses have evolved special strategies to evade and/or inhibit host immune responses to promote their virulence and evade immune surveillance. Influenza viruses evolved multiple ways to escape from the host immunity, for instance, some multi-functional proteins such as non-structural protein-1 (NS1) protein and PB1-F2 protein were produced to suppress innate immune signaling pathways (Chen et al., 2018). Moreover, EVs may evoke a "Trojan horse" ploy to help influenza viruses avoid the immune surveillance and favor viral entry into the recipient cells, due to their lower immunogenicity and better biocompatibility. Notably, EVs provide shelter for host and viral proteins and genome by protecting them from DNase, RNase and proteinases in extracellular environment. Some host miRNAs with function of promoting virus replication were found in EVs during influenza virus infection (Keshavarz et al., 2018). For example, miR-17-5p highly expressed in EVs derived from IAV-infected lung epithelial cells and patients' bronchoalveolar lavage fluid (BALF) can result in decreased expression of the antiviral factor Mx1 and significantly enhanced IAV replication (Scheller et al., 2019). Therefore, some scholars speculated that those miRNAs within EVs could also promote influenza virus replication in recipient cells (Keshavarz et al., 2018; Zheng et al., 2020).

In contrast, EVs also are found as an antiviral mediator participating in antiviral immunity. Virus-infected cells can release EVs with functional viral RNAs (e.g., mRNA and miRNA) to DCs and MΦ, and then the viral RNAs are internalized and recognized by PRRs to produce a large quantity of type I interferon and pro-inflammatory cytokines. For example, IAV-RNA can be recognized by TLR7 in plasmacytoid DCs (pDCs), triggering the signal to induce antiviral innate immune responses (Diebold et al., 2004). Recent studies have revealed that infected epithelial cells release EVs that specifically regulate responses of neighboring epithelial cells and immune cells to limit the virus's transmission. Liu et al. (2019) observed that hsa-miR-1975, a Y5 RNA-derived small RNA, was activated in the apoptosis process of influenza virus-infected human lung adenocarcinoma epithelial A549 cells. Subsequently, hsa-miR-1975 was delivered into neighbor cells by exosomes, and fused with other antiviral proteins or nucleotides to produce interferon, and thereby inhibited influenza virus replication when viruses invaded the recipient cells. Besides the infected cells, respiratory epithelial cells not only produce cytokines and chemokines that communicate with immune cells to activate and regulate antiviral responses (Miura, 2019), but also secrete EVs to induce the antiviral responses. Kesimer et al. (2009) firstly observed that human tracheobronchial epithelial (HTBE) cells derived exosome-like vesicles with characteristic exosomal size (30–100 nm) and morphology (cup-shaped), and multivesicular and late endosomal membrane markers Tsg101 and CD63, can neutralized influenza viruses by mean of α-2,6 linked sialic acid on the their surface which can preferentially be bound to influenza viruses, implying an antiviral role for exosomes in mucosal innate defense. Thereafter, Maemura et al. (2018) firstly examined the presence of exosomes in BALF of influenza virus-infected mice, and found the quantity of exosomes enriched miR-483-3p was increased. Those miR-483-3p-containing exosomes might mainly derived form MΦ, but not lung tissues. After exosomal miR-483-3p transfection in lung epithelial cells, the expressions of type I interferon and proinflammatory cytokine were increased by miR-483-3p targeting negative regulators of the RIG-I signaling pathway. Besides BALF, furthermore, high levels of exosomal miR-483-3p was also found in serum of influenza virus-infected mice, and high inflammatory cytokines in vascular endothelial cells (Maemura et al., 2020). Meanwhile, microparticles were found in the BALF of relatively normal subjects who underwent bronchoscopy and bronchoalveolar lavage in the study by Suptawiwat et al. (2017). Those vesicles were originated from bronchial epithelial cells and alveolar epithelial cells, and might exert their anti-influenza activity by trapping influenza virions using their surface sialic acid. The further study have shown that transformed bronchial epithelial BEAS-2B cells enriched both a-2,6- and a-2,3-linked sialic acids, and their microparticles could combat both H1N1 and H5N1, while human lung alveolar epithelial A549 cells only enriched a-2,6-linked sialic acid and their microparticles could only combat H1N1 virus (Jantaratrirat et al., 2018). Additionally, EVs from immune cells are also involved in inflammatory responses to viral infection. Huo et al. demonstrated that mast cells may support the productive replication of influenza virus such as H1N1, H5N1, and H7N2 in their previous studies, however, they observed that exosomes were preferentially secreted from H1N1 or H7N2-infected mouse mastocytoma cell in follow-up study, and speculated that those exosomes

were potentially pivotal in innate immunity to fight IAV infection via triggering the robust innate immunity of cells (Huo et al., 2019).

The above findings demonstrate that EVs containing viral components are two sides of the same coin. EVs not only offer convenience to influenza virus replication and immune evasion, and also act as antigen carriers to promoting an innate and acquired immune response to control infection. This enigmatic dual roles of EVs may occur simultaneously and in dynamic balance, that seems to depend on the origin cells, recipient cells and likely many other as well as environmental factors yet undefined factors.

EVs AS AN EMERGING VACCINE CARRIERS FOR INFLUENZA VIRUS INFECTION

The occurrence of new influenza viral strains by continually antigenic variation of influenza viruses usually make them resistant to currently used antiviral drugs. Vaccination is the best prophylactic measures for combating influenza virus infection (Okamoto et al., 2018), and recommended for all residents age 6 months and older regardless of the state of their health in United States. During the 2018–2019 influenza season, in United States, the overall adjusted vaccine effectiveness (VE) against all influenza virus infection associated with medically attended ARI was 47%, and VE was 61% in children aged 6 months−17years. VE was estimated to be 74% against illness caused by H1N1, and 26% against H3N2 (Doyle et al., 2019). It is estimated that vaccination could prevent 300–4,000 deaths annually in the United States alone, however, vaccination rates remain low, only 37% of employed adults were vaccinated in 2016 (Mossad, 2018). The currently available influenza vaccines, including inactivated viral particles, M2e-based vaccine, live attenuated influenza vaccine (LAIV) and virus like particle (VLP), are effective against influenza pandemic (Keshavarz et al., 2019). LAIV and VLP vaccines can stimulate both humoral and cellular immune responses, while inactivated vaccines can only induce systemic humoral responses. With the emergence of new influenza viral strains each year, these above vaccines just can provide limited protection. Exosome-based vaccines have been regarded as a new platform as influenza vaccines which have many advantages over traditional vaccines produced in cell culture or eggs (Jungbauer, 2018). For example, avoiding glycosylation directly affect recombinant proteins immunogenicity in avian eggs or endogenous viruses in avian-derived cell lines interfere with the structure of the introduced exogenous virus, causing an allergic reaction after the vaccination. Anticoli et al. (2018) employed an exosome-based vaccine platform to elicite a cytotoxic T lymphocyte (CTL) immunity against influenza virus. Murine muscle cells were transfected with DNA vectors expressing the Nef mutant/Influenza virus A-NP (Nef^{mut}/Flu-NP) in this subject, and the murine muscle cell-derived exosomes were purified and injected in mice can lead to a well detectable antigen-specific $CD8^+$ T cell response associating with a cytotoxic activity potent enough to kill peptide-loaded and/ or antigen-expressing syngeneic cells, suggesting that this kind of vaccine platform was applicable for further pre-clinical and clinical investigations or applications. Schorey et al. have enumerated the potential advantages to using exosomes as vaccines in their review (Schorey et al., 2015), (1) exosomes are capable of providing a more stable conformational conditions for the proteins. (2) the ability of exosomes to circulate and reach distal organs can improve molecular biodistribution in body fluids. Thirdly, the expression of adhesion molecules on exosomes can provid a more efficient presentation to the antigen-presenting cells. (3) exosomes act one of the body's "natural" mechanisms to transpor antigens between cells, and may play a role in cross-priming. Interestingly, EVs from Gram-negative bacteria, what are now referred to as outer membrane vesicles (OMVs) (Coelho and Casadevall, 2019), have received increased attention as emerging and feasible vaccine carriers (Acevedo et al., 2014; Wang et al., 2018; Yu et al., 2018; Kis et al., 2019). Numerous studies have shown that OMV-derived vaccines could induce protective immunity against influenza viruses (Rappazzo et al., 2016; Lee et al., 2017; Watkins et al., 2017).

SUMMARY AND OUTLOOK

Overall, current evidences suggest EVs play an important role in influenza virus infection. During the process of influenza virus infection, EVs can deliver virus particles that serve as regulators of host defense and mediators of immune evasion, or that serve as antigens of innate immune receptors to stimulate host defense and immunity. Moreover, Exosomes could potentially be used as cell-free vaccines to help people prevent influenza. Many scholars suggested that EVs can not only be a source of diagnostic markers in influenza virus infection, and may also be used as a kind of cell-free therapy, for instance, isolated EVs from swine bone marrow-derived mesenchymal stem cells (MSCs) could inhibit the HA activity of influenza viruses and suppress the replication of influenza viruses (Khatri et al., 2018).

So far, however, some limitation would still affect the EVs studies. For example, nomenclature of EVs have been originated in many literature in accordance with their size, origin and functions, such as microvesicles, microparticles, exosomes, ectosomes, oncosomes, and so on (Colombo et al., 2014). Jurj and colleagues had a detailed introduces of the classification of EVs and their main characteristics (e.g., size, appearance, marker, release process, pathways, etc.) in their recently published review (Jurj et al., 2020). On the basis of the recently updated guidelines of ISEV, "EVs" was defined as the "generic term for particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate," but not all EVs researchers agree with the nomenclature of EVs (Théry et al., 2018; Witwer and Théry, 2019). Hence, the exact meaning of EVs cannot be discerned immediately from the names' constituent parts without further explanation. Heterogeneitythe is an inevitable challenge associated with the studies of EVs, which may explain to the confusing and sometimes even

conflicting results between laboratories (Suptawiwat et al., 2017; Maemura et al., 2018). Exosome and other EVs usually have various compositions and size will vary depending on their origin, donor cell culture conditions (e.g., glucose levels, fetal bovine serum, antibiotics, mycoplasma and other microbes.) and even physiological or pathological environment of donor cells or tissues including hypoxia, hyperthermia, infections, circadian rhythms, hormones, and stage of cell cycle, etc. (Burger et al., 2017; Németh et al., 2017; Gaurivaud et al., 2018; Ludwig et al., 2019; Pegtel and Gould, 2019; Kalluri and LeBleu, 2020; Zubair et al., 2020). Moreover, the existing isolation and purification technologies (e.g., ultracentrifugation, nanoscale flow cytometry, immunoprecipitation/affinity capture, Exosome Isolation Reagent, etc.) do not meet the special requirements in isolate large quantities of pure and specific EVs from mixtures of different vesicle types in cell culture medium or body fluids (McNamara and Dittmer, 2019). Notably, influenza virus particles and EVs share similarities in density, diameter, etc (McNamara and Dittmer, 2019). Thus, it is sometimes difficult to discriminate the roles of exosomes and other EVs in influenza virus infection and completely explain the specific role of EVs' subtype.

More importantly, some questions such as double-sided actions of EVs in influenza virus infection, and their role in cytokine storm frequently accompanied by ARDS, and even the stability and safety of exosome-based vaccines with foreign antigens on the recipient remain clarified. Therefore, further studies are still needed to investigate EVs composition and function during the pathogenesis process of influenza virus infection.

AUTHOR CONTRIBUTIONS

YJ, CX, and WL contributed to the conception and design of the study. YJ wrote the first draft of the manuscript. LY, HG, and JY wrote sections of the manuscript. CX proofread the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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Comprehensive Analysis of Non-coding RNA Profiles of Exosome-Like Vesicles From the Protoscoleces and Hydatid Cyst Fluid of *Echinococcus granulosus*

Xiaofan Zhang[1,2,3,4,5], *Wenci Gong*[1,2,3,4,5], *Shengkui Cao*[1,2,3,4,5], *Jianhai Yin*[1,2,3,4,5], *Jing Zhang*[1,2,3,4,5], *Jianping Cao*[1,2,3,4,5]* *and Yujuan Shen*[1,2,3,4,5]*

[1] Chinese Center for Disease Control and Prevention, National Institute of Parasitic Diseases, Shanghai, China, [2] Chinese Center for Tropical Diseases Research, Shanghai, China, [3] Key Laboratory of Parasite and Vector Biology, Ministry of Health, Shanghai, China, [4] WHO Collaborating Centre for Tropical Diseases, Shanghai, China, [5] National Center for International Research on Tropical Diseases, Ministry of Science and Technology, Shanghai, China

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***Correspondence:**
Yujuan Shen
shenyj@nipd.chinacdc.cn
Jianping Cao
caojp@yahoo.com

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Cystic echinococcosis is a worldwide chronic zoonotic disease that threatens human health and animal husbandry. Exosome-like vesicles (ELVs) have emerged recently as mediators in the parasite–parasite intercommunication and parasite–host interactions. Exosome-like vesicles from parasites can transfer non-coding RNAs (ncRNAs) into host cells to regulate their gene expression; however, the ncRNAs profiles of the ELVs from *Echinococcus granulosus* remain unknown. Here, we isolated protoscolece (PSC)–ELVs and hydatid fluid (HF)–ELVs from the culture medium for *E. granulosus* PSCs *in vitro* and the HF of fertile sheep cysts, respectively. The microRNA (miRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA) profiles of the two types of ELVs were analyzed using high-throughput sequencing, and their functions were predicted using Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes pathway analysis. In PSC–ELVs and HF–ELVs, 118 and 58 miRNAs were identified, respectively, among which 53 miRNAs were present in both ELVs, whereas 65 and 5 miRNAs were unique to PSC–ELVs and HF–ELVs, respectively; 2,361 and 1,254 lncRNAs were identified in PSC–ELVs and HF–ELVs, respectively, among which 1,004 lncRNAs were present in both ELVs, whereas 1,357 and 250 lncRNAs were unique to PSC–ELVs and HF–ELVs, respectively. Intriguingly, the spilled PSCs from cysts excrete ELVs with higher numbers of and higher expression levels of miRNAs and circRNAs than HF–ELVs. The miRNA sequencing data were validated by quantitative reverse transcription–polymerase chain reaction. Furthermore, the target lncRNAs and mRNAs regulated by the 20 most abundant miRNAs were screened, and a ceRNA regulatory network containing 5 miRNAs, 41 lncRNAs, and 23 mRNAs was constructed, which provided new ideas and the molecular basis for further clarification of the function and mechanism of *E. granulosus* ELVs ncRNAs in the parasite–host interactions. Egr-miR-125-5p and egr-miR-10a-5p, sharing identical seed sites with host miRNAs, were predicted to mediate inflammatory response, collagen catabolic process, and mitogen-activated

protein kinase cascade during parasite infections. In conclusion, for the first time, we identified the ncRNAs profiles in PSC–ELVs and HF–ELVs that might be involved in host immunity and pathogenesis, and enriched the ncRNAs data of *E. granulosus.* These results provided valuable resources for further analysis of the regulatory potential of ncRNAs, especially miRNAs, in both types of ELVs at the parasite–host interface.

Keywords: *Echinococcus granulosus*, exosome-like vesicles, miRNA, non-coding RNAs, parasite–host interactions

INTRODUCTION

Cystic echinococcosis is a worldwide zoonotic disease that is caused by the metacestodes (larval stages) of the tapeworm *Echinococcus granulosus*. Humans may be infected by the accidental ingestion of *E. granulosus* eggs (McManus et al., 2012). *Echinococcus granulosus* infection is characterized by unilocular fluid-filled hydatid cysts within the internal organs (mainly the liver and lungs) of humans and other livestock intermediate hosts (Wen et al., 2019). It is endemic in countries and regions with extensive animal husbandry, causing huge economic losses and disease burden (Budke et al., 2006).

Hydatid cysts comprise cyst walls and cyst contents, such as the brood capsule, protoscoleces (PSCs), hydatid fluid (HF), and daughter cyst. The cyst walls consist of an inner germinal layer (GL) supported externally by a tough, elastic, acellular laminated layer, surrounded by a host-produced fibrous adventitial layer. The GL undergoes asexual multiplication to produce the brood capsule and large numbers of PSCs. In addition to its proliferative activity, the GL is involved in secretory activity, secreting several proteins into the HF that could play a role in host evasion (Monteiro et al., 2010). A proteomic analysis of the exosome-like vesicles (ELVs) from the HF of *E. granulosus*–infected fertile sheep identified a number of host proteins and parasite-derived proteins, among which antigen B and antigen five could reach the host and elicit a strong specific antibody response (Siles-Lucas et al., 2017). For *E. granulosus* PSCs, the excretory/secretory products can directly regulate the differentiation of B10, B17, and Th17 cells, and stimulated B10 cells produce interleukin (IL)10 via Toll-like receptor 2 (TLR2) signaling (Pan et al., 2017, 2018). A recent study isolated and analyzed the proteome of PSC–ELVs, which can be internalized by dendritic cells and induce their maturation (Nicolao et al., 2019). These studies mainly focused on the proteins in the two types of ELVs.

Extracellular vesicles (EVs) are small membranous vesicles that are generally classified in three major types, exosomes, microvesicles, and apoptotic bodies, based on their size, biogenesis, and composition. These EVs can carry a wealth of bioactive molecules such as proteins, carbohydrates, lipids, and nucleic acids, which are mainly found in specific vesicles known as exosomes. Exosomes are endocytic vesicles of ∼30–150 nm in size that are released after the fusion of multivesicular bodies with the plasma membrane. Therefore, exosomes contain specific membrane markers such as ALIX, enolase, 14-3-3, CD63, and CD9 (Ying et al., 2017). Exosome-like vesicles have been identified in a number of parasite species, including several protozoa, trematodes, and nematodes, and act in intercellular communication and parasite–host interactions (Marcilla et al., 2014; Coakley et al., 2015). Exosome-like vesicles contain specific repertoires of non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), which regulate gene expression at the levels of transcription, RNA processing, and translation (Cech and Steitz, 2014; Lambertz et al., 2015; Fan et al., 2018). MicroRNAs are small single-stranded ncRNAs molecules with a length of ∼22 nt. As key posttranscriptional regulators, miRNAs are capable of regulating gene expression by degrading or suppressing the target mRNA, mostly through specifically binding to the 3′ untranslated region (3′ UTR) (Shukla et al., 2011). Long ncRNAs are more than 200 nucleotides in length and have limited protein-coding potential, which are also involved in posttranscriptional regulation by interacting with miRNAs, mRNAs, or proteins (Guttman et al., 2013; Ulitsky and Bartel, 2013). Recent evidence has shown that parasite-derived ELVs ncRNAs are involved in the regulation of host gene expression upon internalization of ELVs (Garcia-Silva et al., 2014a) and have been implicated in the host immune response and pathogenesis of a variety of parasite infections (Buck et al., 2014; Garcia-Silva et al., 2014b).

Helminth miRNAs encapsulated into ELVs and internalized by host immune cells induced regulation and expansion of regulatory T cells, resulting in the control of inflammation (Siles-Lucas et al., 2015). The miR-10 family and miR-277 were the most highly enriched miRNAs in ELVs of both *Dicrocoelium dendriticum* and *Fasciola hepatica* and seem to have potential immune-regulatory functions (Fromm et al., 2017). *Schistosoma japonicum*–derived ELVs contained miR-bantam, which may be involved in the hepatic pathogenesis of schistosomiasis (Zhu et al., 2016a). Parasite ELVs cargoes have been characterized mainly at the protein level, although other molecules, such as miRNAs, have been identified inside. However, there has been no study to identify and characterize miRNAs and other ncRNAs in *E. granulosus* PSC–ELVs and HF–ELVs.

In the present study, we showed that *E. granulosus* PSCs secrete EVs *in vitro*, and hydatid cysts produce EVs, whose size and morphology are consistent with exosomes. High-throughput sequencing was conducted to analyze the ncRNAs (miRNAs, circRNAs, and lncRNAs) profiles in the two types of ELVs, and bioinformatics analysis of the 20 most abundant miRNAs in the two types of ELVs was performed to explore the possible biological processes and pathways associated with pathogenicity and the host immune response. These ELVs

contain small RNA species, including specific miRNAs that are homologous to host miRNAs with known immunomodulatory roles (Zhang et al., 2016; Vaher et al., 2019). The results demonstrated that the most abundant miRNAs in both types of ELVs might be new candidates for the mechanism of parasite–parasite communication and parasite–host interactions of ELVs in parasitic diseases.

MATERIALS AND METHODS

Parasite Material

Liver hydatid cysts were collected aseptically from naturally infected sheep slaughtered at abattoirs located in Xinjiang Uygur Autonomous Region, China. The HF and PSCs were obtained from the fertile cysts under aseptic conditions and were placed in 50 mL falcon tubes. The HF was centrifuged at 2,000 × *g* for 5 min to remove the PSCs and other solid material. PSC-free HF (300 mL) from more than 10 fertile cysts was pooled and stored at −80°C until use. The PSCs were washed 5–8 times using 0.9% NaCl containing 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Frederick, MD, USA) and maintained in sterile conditions until *in vitro* culture.

In vitro Culture of PSCs

In vitro culture of PSCs ($n = 20{,}000/75\ cm^2$) and viability assays were carried out as previously described, with modifications (Cumino et al., 2010). Briefly, PSCs were cultured in RPMI (Roswell Park Memorial Institute) 1,640 medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with antibiotics (200 U/mL penicillin and 200 μg/mL streptomycin) and glucose (4 mg/mL) in 75 cm^2 cell culture flasks at 37°C under 5% CO_2. Viability was determined using a trypan blue dye exclusion test. The parasite culture medium was harvested and changed every 12 h. Briefly, 20,000 PSCs were maintained in serum-free media (300 mL) for 7 days. The medium collected from different time points was pooled and stored at −80°C until use.

Purification of Exosome-Like Vesicles

Protoscolece–ELVs and HF–ELVs were isolated from the PSCs culture medium and the pooled HF, respectively, and enriched by differential centrifugation. The PSCs culture medium and pooled HF were centrifuged at 300 × *g* for 10 min, at 2,000 × *g* for 20 min, and finally at 10,000 × *g* for 40 min to remove large dead cells and cell debris. The supernatant was filtered using low-protein binding 0.22 μm pore filters (Millipore, Bedford, MA, USA). The filtered PSCs culture medium and HF were subjected to ultracentrifugation at 110,000 × *g* for 90 min at 4°C to pellet the vesicles using a Beckman Coulter Optima L-100 XP ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA) with an SW 41 Ti rotor. The final pellets were washed with phosphate-buffered saline (PBS) and subjected to further ultracentrifugation at the same high speed. Protoscolece–ELVs and HF–ELVs were resuspended separately in 100 μL of PBS and stored at −80°C until use.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was conducted using a NanoSight LM10 (NanoSight, Malvern Panalytical Ltd., Malvern, UK) to determine the size and frequency distribution of PSC–ELVs and HF–ELVs preparations, performed in triplicate. NanoSight analysis allows a better statistical resolution of vesicle size and concentration by measuring the Brownian motion of particles in solution related to particle size (Oosthuyzen et al., 2013).

Transmission Electron Microscopy

Purified PSC–ELVs and HF–ELVs (both 10 μL) resuspended in 100 μL of PBS were added to 200-mesh formvar-coated grids (Agar Scientific Ltd., Stanstead, UK) for 1 min, and the remaining liquid was wiped off using filter paper. The grids were negatively stained with 3% phosphotungstic acid for 1 min and dried at room temperature. The grids were loaded onto the sample holder of the transmission electron microscope (TEM) (HITACHI, Tokyo, Japan) and exposed at 80 kV for image capture.

Western Blotting

The ELV pellet (20 μL) was mixed with 10 μL of a solution of 5 × sodium dodecyl sulfate (SDS) and 20 μL of 1 × PBS and then boiled at 100°C for 10 min. Thirty micrograms of each protein sample was separated on 10% SDS–polyacrylamide gel electrophoresis, and the proteins were then transferred to polyvinylidene fluoride membranes (Millipore) at 15 W for 30 min. After a 1 h blocking step in Tris-buffered saline (TBS) 0.05% - Tween 20 (TBST) containing 5% bovine serum albumin, the membranes were incubated at 4°C overnight with the following primary antibodies: anti−14-3-3 zeta/delta (1/1,000), antienolase (1/1,000), and anti-CD9 (1/1,000) antibodies (Cell Signaling Technology, Danvers, MA, USA). For all secondary antibody incubations, horseradish peroxidase–conjugated or goat anti–rabbit antibodies (Cell Signaling Technology) were used at a 1:5,000 dilution. The membranes were visualized using an ECL Western blotting Detection System (Clinx Science Instruments Co., Ltd., Shanghai, China).

Small RNA Library Construction, Sequence Analysis, and Identification of miRNAs

Total RNA containing small RNAs was extracted from PSC–ELVs and HF–ELVs using the mirVanaTM miRNA Isolation Kit (Ambion, Austin, TX, USA), following the manufacturer's protocol. The small RNA library construction and sequencing were conducted by OE Biotech Co., Ltd. (Shanghai, China). The quality, quantity, and integrity of the total RNAs were assessed using a NanoDrop 2,000 instrument (Thermo Fisher Scientific, Waltham, MA, USA), a Qubit 2.0 Fluorometer (Life Technologies), and an Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The small RNA libraries were constructed using TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Subsequently, RNA-Seq libraries were sequenced on the Illumina sequencing platform (HiSeq™ X Ten).

The basic reads were converted into raw data by base calling. Low-quality reads without a 3′ adapter and insert tag were filtered, and the reads with 5′ primer contaminants and poly (A) were removed. Clean reads with high quality and the specified length distribution (15–41 nt) in the reference genome were determined and then used for further analysis. These RNAs were aligned and then subjected to BLAST searching against the Rfam v.12.0 (Nawrocki et al., 2015) and GenBank databases. The ncRNA sequences annotated as rRNAs, tRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) were removed. The remaining reads were matched against the miRBase v.21 database (http://www.mirbase.org/), and the known miRNAs were identified (Griffiths-Jones et al., 2008). The expression patterns of the known miRNAs in different samples were analyzed. Unannotated small RNAs were analyzed using mirdeep2 (Friedlander et al., 2012) to predict novel miRNAs. Based on the hairpin structure of a pre-miRNA and the miRBase database, the corresponding miRNA star sequences were also identified. The threshold set for upregulated or downregulated miRNAs was fold change $\geq$2.0 and $P < 0.05$.

Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)

Total small RNAs from 100 μL of ELVs of both types were extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Boston, MA, USA) according to the manufacturer's instructions. During the RNA purification step the same amount of cel-miR-39 (*Caenorhabditis elegans* miRNA; Qiagen) spike in control was added to each sample to monitor the efficiency of miRNA extraction and allowed for normalization of sample-to-sample variation. Three hundred nanograms of total RNA were used for reverse transcription. cDNA was synthesized using the miScript II RT Kit (Qiagen) according to the manufacturer's protocols. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed to use a miScript SYBR Green PCR Kit (Qiagen) on a Bio-Rad CFX96 system. The primers used for the qRT-PCR are shown in **Supplementary Table 1**. The miRNA expression levels were quantified based on the threshold cycle (Ct) values. Cel-miR-39 served as the external control for the expression analysis of ELVs-derived miRNAs (Sohn et al., 2015; Lovett et al., 2018). The relative gene expression values for ELVs miRNAs were normalized to cel-miR-39 and calculated using the comparative Ct [2(−ΔΔCt)] method. Triplicate independent experiments were performed for each type of ELVs.

Sequencing and Identification of lncRNAs and circRNAs

After extracting total RNA from the samples and removing the ribosomal RNA using a Ribo-Zero Gold rRNA Removal Kit (Illumina), the RNA was fragmented using fragment reagent. First-strand cDNA was synthesized using 8 μL of First Strand Synthesis Act D Mix and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols. Second-strand cDNA was synthesized using 5 μL of End Repair Control and 20 μL of Second Strand Marking Master Mix. The double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter; cat. no. A63881). After repairing the ends and ligating the adenylated 3′ ends and sequence adapters, the DNA fragments were enriched via PCR. Finally, the products were purified (Agencourt AMPure XP; Beckman Coulter), and the library quality was assessed on an Agilent Bioanalyzer 2,100 system (Agilent Technologies). An Illumina sequencing platform (HiSeq™ X Ten) was used for sequencing, and 150-base-pair (bp) paired-end reads were generated.

Raw reads in the fastq format were subjected to quality preprocessing using Trimmomatic (0.36). After removing the adapter and low-quality reads, the clean reads were obtained. Hisat2 (2.2.1.0) was used to align the clean reads with the *E. granulosus* reference genome. For the mapped reads, transcripts were reconstructed based on the probability model and the comparison results of each sample using StringTie (1.3.3b). Finally, the candidate lncRNAs were identified using the software CPC (0.9-r2), CNCI (1.0), PFAM (v30), and PLEK (1.2), which were used to predict the coding capacity of the transcripts. The clean reads were analyzed by CIRI software for circRNAs prediction. The abundance of lncRNAs and circRNAs was calculated using FPKM (fragments per kilobase of transcript per million mapped reads), and RPM (reads per million reads), respectively. The differentially expressed lncRNAs and circRNAs were detected using a negative binomial distribution test based on the DESeq package (1.18.0). The threshold set to identify differentially expressed lncRNAs and circRNAs was fold change $\geq$2.0 and $P < 0.05$.

Target Prediction and Functional Annotation of the 20 Most Abundant miRNAs

The candidate host target genes of the 20 most abundant miRNAs in PSC–ELVs were predicted using the software miRanda v3.3a (Betel et al., 2010) with the following parameters: S $\geq$ 150; $\Delta G \leq -30$ kcal/mol; and demanding strict 5′ seed pairing. To analyze the biological functions of ELVs miRNAs, the predicted miRNA target genes were annotated using Gene Ontology (GO) enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Gene Ontology analysis labels these target genes with a function, such as biological process, molecular function, or cellular component. Kyoto Encyclopedia of Genes and Genomes analysis provides the information of signal transduction and disease pathways for target genes, thus providing a basis for research into the function and involved pathways of ELVs miRNAs. The GO enrichment and KEGG pathway enrichment analysis of the 20 most abundant miRNA-target genes were both performed using R software (v3.6; https://www.r-project.org/) based on the hypergeometric distribution.

Construction of the ceRNA Regulatory Network

Regulatory relationships between ELVs miRNAs and host mRNAs and between ELVs miRNAs and lncRNAs were predicted by Shanghai OE biotech Co., Ltd., using miRanda v3.3a.

Based on the ELVs miRNA–mRNA interaction analysis and the miRNA–ELVs lncRNA interaction analysis, an lncRNA–miRNA–mRNA regulatory network was constructed and visualized using Cytoscape (v3.6.1; https://cytoscape.org) software.

RESULTS

Identification of Exosome-Like Vesicles From HF and PSCs

Transmission electron microscopy and NTA were applied to evaluate the morphology and size distribution of the isolated PSC–ELVs and HF–ELVs. Spherical vesicles in the 30–150 nm range were observed under TEM (**Figures 1A,B**). Nanoparticle tracking analysis also showed that the majority of purified vesicles derived from PSCs and HF were between 40–70 nm and 60–90 nm in diameter, respectively (**Figures 1C,D**), which were compatible with the size of exosomes (Gillan et al., 2019). Western blotting analysis confirmed the presence of exosomal marker proteins, including 14-3-3, enolase, and CD9 (**Figure 1E**).

Overview of Small RNAs Sequencing

Illumina Hiseq X Ten sequencing resulted in 23–27 million raw reads obtained per ELV. After trimming of adaptors and length filtering, high-quality (quality score, $Q \geq 20$) clean reads were obtained from the two types of ELVs, with no significant differences in length distribution of small RNAs (21–23 nt) (**Supplementary Figure 1**), which were used for further analysis. BLASTN was used to search against the RFAM and GenBank databases using the pooled data for the two types of ELVs. Approximately 0.02–0.43% of the clean reads were annotated as rRNA, snRNA, snoRNA, or tRNA (**Supplementary Table 2**). The clean reads were compared with *E. granulosus* genome, and the results showed that 58.08–58.24% of the clean reads could map to *E. granulosus* (**Supplementary Table 2**).

Identification of Conserved miRNAs in PSC–ELVs and HF–ELVs

The clean reads from the two types of ELVs were aligned to the *E. granulosus* database in miRBase for characterization. According to the database results, 118 miRNAs and 58 miRNAs were identified in PSC–ELVs and HF–ELVs, respectively. Among them, 72 and 48 miRNAs were known miRNAs belonging to *E. granulosus*, and the remaining 46 and 10 miRNAs were putative novel miRNAs identified by MiRDeep2. Among the 118 miRNAs in PSC–ELVs, 12 were very abundant (>10,000 reads) in the PSC–ELVs including egr-miR-4989-3p, egr-miR-125-5p, egr-let-7-5p, egr-miR-71-5p, egr-miR-61-3p, egr-miR-277a-3p, egr-miR-10a-5p, egr-bantam-3p, egr-miR-2162-3p, egr-miR-2a-3p, egr-let-7-3p, and egr-miR-96-5p (**Supplementary Table 3**). Intriguingly, among the 58 miRNAs in HF–ELVs, the most abundant miRNAs (counts >10,000 reads) were the same as those in the PSC–ELVs.

However, the expression levels of egr-miR-3479a-5p, egr-miR-1992-3p, egr-miR-3479b-5p, egr-miR-124a-5p, and some other miRNAs were rather low in PSC–ELVs. The expression levels of egr-miR-3479a-5p, egr-miR-8-3p, egr-miR-133-5p, egr-miR-7-3p, and some other miRNAs were rather low in HF–ELVs (**Supplementary Table 3**).

The 20 most abundant and known miRNAs identified from the sRNA library with read counts of more than 3,000 in PSC–ELVs are listed in **Table 1**. Egr-miR-4989-3p was the most abundant in the two types of ELVs, followed by egr-miR-125-5p and egr-let-7-5p in PSC–ELVs, and egr-let-7-5p and egr-miR-125-5p in HF–ELVs. Other miRNAs commonly found in ELVs derived from helminthes, such as miR-1, miR-9, and miR-10, were also found in the vesicles.

Differentially Expressed miRNAs in PSC–ELVs and HF–ELVs

The differentially expressed miRNAs, including known and novel miRNAs between PSC–ELVs and HF–ELVs, were analyzed using high-throughput sequencing. Comparing the miRNAs numbers and expression levels of HF–ELVs with those of PSC–ELVs identified 53 miRNAs that were present in both types of ELVs, whereas 65 and 5 miRNAs uniquely existed in PSC–ELVs and HF–ELVs, respectively (**Figure 2A**). Eighty-five miRNAs were differentially expressed, among which 73 were upregulated and 12 were downregulated in PSC–ELVs (**Supplementary Table 4**). The differentially expressed miRNAs were defined as having a fold change $\geq$2.0 and $P < 0.05$. The expression levels of the 20 most abundant miRNAs in PSC–ELVs were higher than those of the corresponding miRNAs in HF–ELVs (**Figure 2B**). These results indicated that the miRNAs exhibited a wide range of expression levels in the two types of ELVs.

qRT-PCR Validation

To verify the results of miRNAs sequencing, we used qRT-PCR to detect the expression of 10 abundant miRNAs selected from the both PSC–ELVs and HF–ELVs. The qRT-PCR results were consistent with the high-throughput sequencing, and these miRNAs were likely to play roles in response to the biological functions of ELVs in parasite development and infections (**Figure 3**).

Identification of lncRNAs and circRNAs in PSC–ELVs and HF–ELVs

According to the database results, 2,361 and 1,254 lncRNAs were identified in the two types of ELVs, respectively, among which 1,357 and 250 lncRNAs uniquely existed in PSC–ELVs and HF–ELVs, respectively, and 1,004 lncRNAs were present in both types of ELVs (**Figure 4A**). Among the 2,361 identified lncRNAs in PSC–ELVs, nine were the most abundant lncRNAs (counts >10,000) in the PSC–ELVs, including TCONS_00010188, TCONS_00043757, TCONS_00019710, TCONS_00038979, TCONS_00035186, TCONS_00003209, TCONS_00012812, TCONS_00006741, and TCONS_00003723 (**Supplementary Table 5**). Among the lncRNAs, 42 were differentially expressed, among which 19 were upregulated, and 23 were downregulated in PSC–ELVs. Intriguingly, among the 1,254 identified lncRNAs in HF–ELVs, the most abundant

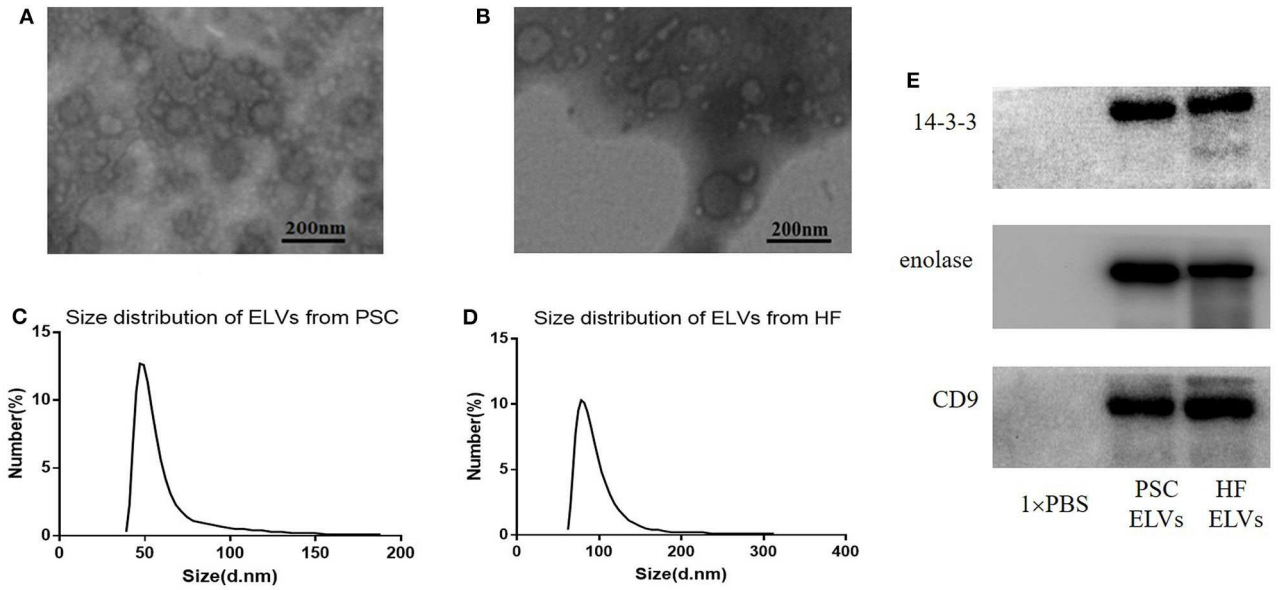

FIGURE 1 | Identification ELVs from protoscoleces **(A,C)** and hydatid fluid **(B,D)**. **(A,B)** Images of the rounded or cup-shaped vesicles obtained using negative staining under TEM. Scale bars: 200 nm. **(C,D)** The diameter distribution analysis of the purified ELVs assessed using nanoparticle tracking analysis. **(E)** Exosome-associated markers 14-3-3, enolase, and CD9 were measured using Western blotting analysis.

TABLE 1 | Twenty known miRNAs abundant in PSC-ELVs and HF-ELVs.

miRNA	HF-ELVs (read count)	PSC-ELVs (read count)	Sequence	Length
egr-miR-4989-3p	731785	1218418	AAAATGCACCAACTATCTGAGA	22
egr-miR-125-5p	98624	214209	TCCCTGAGACCCTAGAGTTGTC	22
egr-let-7-5p	122110	186487	TGAGGTAGTGTTTCGAATGTCT	22
egr-miR-71-5p	78485	141374	TGAAAGACGATGGTAGTGAGA	21
egr-miR-61-3p	20891	102383	TGACTAGAAAGAGCACTCACATCC	24
egr-miR-277a-3p	14315	57284	TAAATGCATTTTCTGGCCCGTA	22
egr-miR-10a-5p	53478	45031	CACCCTGTAGACCCGAGTTTGA	22
egr-bantam-3p	27505	43375	TGAGATCGCGATTACAGCTGAT	22
egr-miR-2162-3p	12562	40877	TATTATGCAACTTTTCACTCC	21
egr-miR-2a-3p	19234	28392	AATCACAGCCCTGCTTGGAACC	22
egr-let-7-3p	10762	16581	ACATCCGTTTCACTATCTGCATA	23
egr-miR-96-5p	11001	14662	ATTGGCACTTTTGGAATTGTC	21
egr-miR-4991	9587	8628	GATCCTGGAATCCAACCTCATT	22
egr-miR-184-3p	7063	7938	GGGACGGAAGTCTGAAAGGTTT	22
egr-miR-7-5p	3843	7024	TGGAAGACTGGTGATATGTTGT	22
egr-miR-7b-5p	5685	6572	TGGAAGACTTGTGATTAGATTGTT	24
egr-miR-124b-3p	4012	6328	TAAGGCACGCGGTGAATACC	20
egr-miR-281-3p	3064	4389	TGTCATGGAGTTGCTCTCTATA	22
egr-miR-9-5p	1646	4173	TCTTTGGTTATCTAGCTGTGTG	22
egr-miR-3479a-3p	1395	3541	TATTGCACGTTCTTTCGCCATC	22

lncRNAs (counts >10,000) were almost the same as those in the PSC–ELVs.

However, the expression levels of TCONS_00006556, TCONS_00050975, TCONS_00050974, and some other lncRNAs were rather low in PSC–ELVs, whereas the expression levels of TCONS_00052899, TCONS_00018540, TCONS_00053812, and some other lncRNAs were rather low in HF–ELVs. Intriguingly, the expression levels of the 20 most abundant lncRNAs in

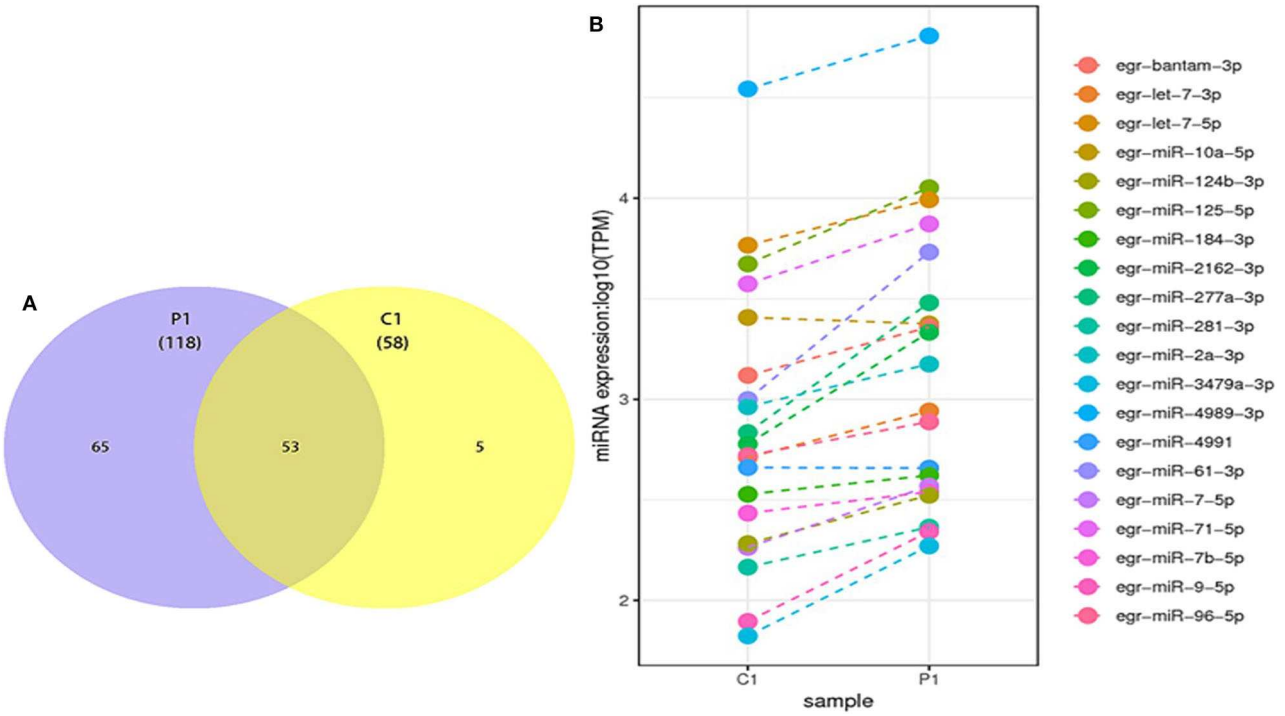

FIGURE 2 | Discovery and profiling of miRNAs in PSC-ELVs and HF-ELVs. **(A)** Comparative numbers of miRNAs in PSC-ELVs and HF-ELVs. **(B)** Comparative expression analysis of the 20 most abundant miRNAs identified in PSC-ELVs and HF-ELVs. P1: PSC-ELVs. C1: HF-ELVs.

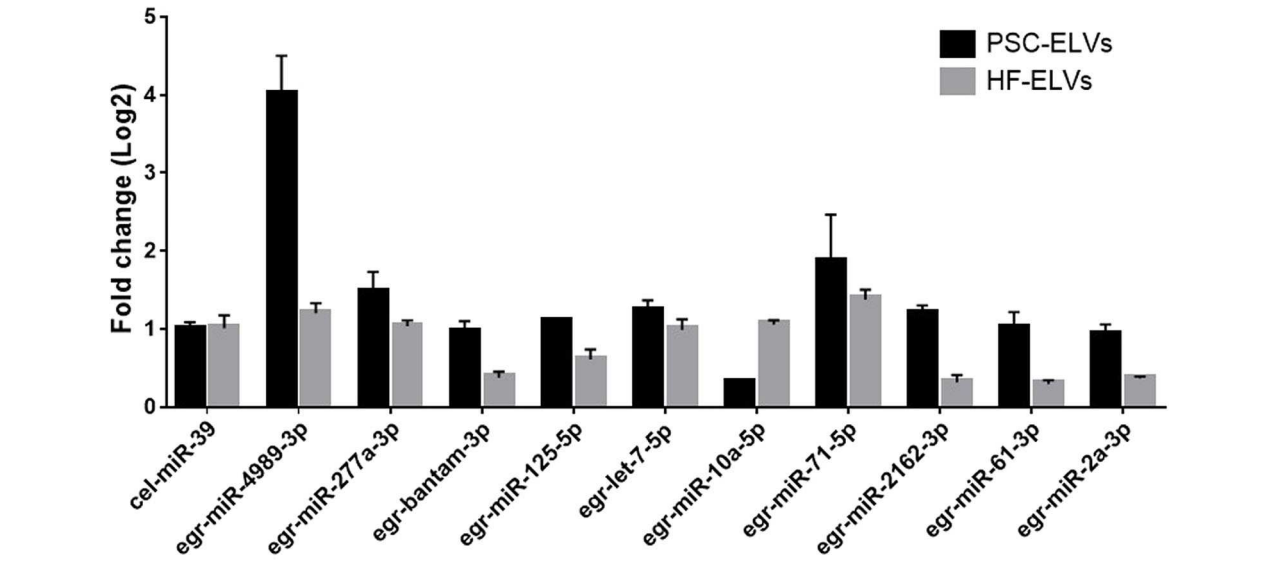

FIGURE 3 | Identification of abundant miRNAs in PSC-ELVs and HF-ELVs using qPCR. Cel-miR-39 acted as external control to confirm normalization of sample-to-sample variation. Results are presented as log2-fold changes in expression ± standard error.

PSC–ELVs were mostly lower than those of the corresponding lncRNAs in HF–ELVs (**Figure 4B**).

The clean reads were analyzed using the CIRI software for circRNAs prediction, which identified 1,277 and 512 circRNAs in PSC–ELVs and HF–ELVs, respectively. Among them, 1,094 and 329 known circRNAs existed uniquely in PSC–ELVs and HF–ELVs, respectively, and 183 circRNAs were present in both types of ELVs (**Supplementary Figure 2A**). Among the 1,277 identified

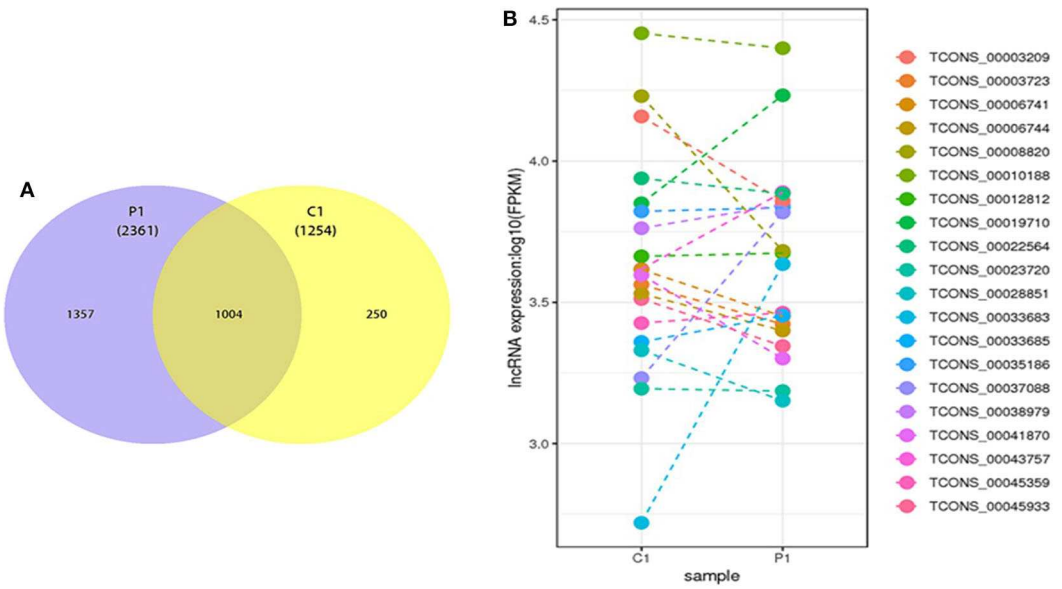

FIGURE 4 | Discovery and profiling of lncRNAs in PSC-ELVs and HF-ELVs. **(A)** Comparative numbers of lncRNAs in PSC-ELVs and HF-ELVs. **(B)** Comparative expression analysis of the 20 most abundant lncRNAs identified in PSC-ELVs and HF-ELVs. P1: PSC-ELVs. C1: HF-ELVs.

circRNAs in PSC–ELVs, circRNA_1446, circRNA_0954, and circRNA_1451 were the most abundant circRNAs, whereas circRNA_1446, circRNA_0954, and circRNA_0977 were the most abundant circRNAs (counts >1,000 reads) in the HF–ELVs (**Supplementary Table 6**).

However, the expression levels of circRNA_0082, circRNA_0646, circRNA_1161, and some other circRNAs were rather low in PSC–ELVs, whereas circRNA_0082, circRNA_0646, circRNA_1162, and some other circRNAs were rather low in HF–ELVs (**Supplementary Table 6**). Meanwhile, the expression levels of the 20 most abundant circRNAs in PSC–ELVs were higher than those of the corresponding circRNAs in HF–ELVs, similar to the miRNAs (**Supplementary Figure 2B**).

miRNA Target Prediction

Most studies focus on the mRNA targets of miRNAs, which can reduce protein expression either by inhibiting translation or by promoting target mRNA degradation. Furthermore, miRNAs play an important role in cross-species communication (Liang et al., 2013). The host target genes of the 20 most abundant miRNAs in PSC–ELVs were predicted using the miRanda software. In total, 895 target mRNAs were predicted against the 20 most abundant miRNAs (**Supplementary Table 7**). Among them, 13 miRNAs could bind to more than three genes, two miRNAs could bind to a single human mRNA, and five miRNAs had no predicted host target genes.

To gain insights into the functions of the 20 most abundant miRNAs in the PSC–ELVs, the host target genes were analyzed using the GO and KEGG databases. In the GO analysis, the host target genes of the 20 most abundant miRNAs in PSC–ELVs were mostly enriched in regulation of transcription, DNA-templated, and actomyosin structure organization in biological processes; cytoplasm, plasma membrane, and nucleus in cellular component; and metal ion binding, DNA binding, and RNA binding in molecular functions (**Figure 5**).

The KEGG pathway analysis indicated 179 enriched pathways of human genes. The most significant enriched pathways were protein processing in endoplasmic reticulum, shigellosis, and RIG-I-like receptor signaling pathway for the PSC–ELVs miRNAs. The main KEGG pathway classification included signal transduction, folding, sorting, and degradation, endocrine system, and infectious diseases (**Figure 6A**). The miRNAs in parasite ELVs can be transferred to host cells to modulate host gene expression and the immune response. These most abundant miRNAs might also participate in inflammatory signaling pathways, such as the tumor necrosis factor (TNF) signaling pathway, the TLR signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, and the nuclear factor kappa B (NF-κB) signaling pathway. Additionally, KEGG analysis showed some pathways related to parasite infections, such as Th1 and Th2 cell differentiation pathway, Th17 cell differentiation pathway, and the IL-17 signaling pathway (**Figure 6B**). Therefore, these most abundant miRNAs in PSC–ELVs might regulate *E. granulosus* infectious and inflammatory processes.

Interactions Among lncRNA-miRNA-mRNA

Many reports have indicated that RNA transcripts, such as mRNAs, lncRNAs, pseudogenes, and circRNAs, can act as competing endogenous RNAs (ceRNAs) or natural miRNA sponges, which communicate with and coregulate each other by competitive binding to shared miRNAs (Tay et al., 2014). Understanding the ceRNA interactions and crosstalk

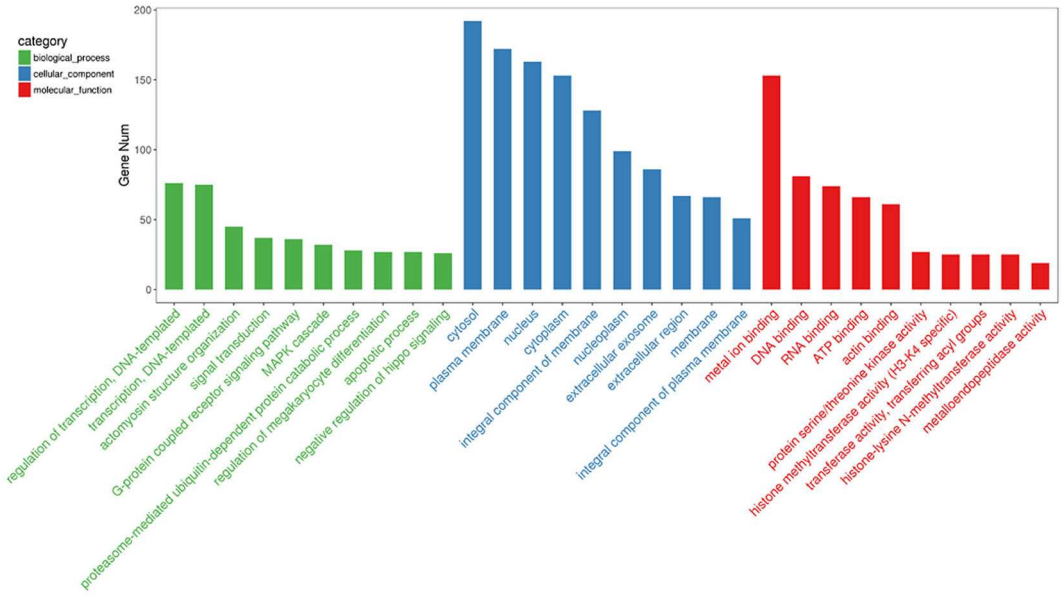

FIGURE 5 | Top 30 GO terms from the genes enrichment analysis among the 20 most abundant miRNAs in the PSC-ELVs.

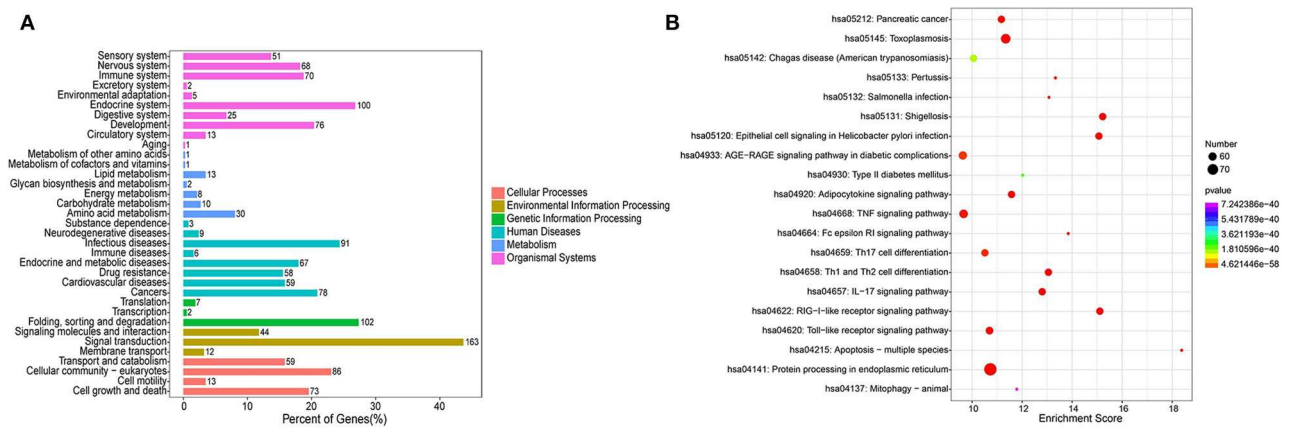

FIGURE 6 | KEGG enrichment analysis of the predicted targets of the 20 most abundant miRNAs in the PSC-ELVs. **(A)** KEGG pathway classification. **(B)** Top 20 significant KEGG pathways.

in intertwined networks will provide significant insights into gene regulation and will have implications in human disease. Therefore, the interaction between PSC–ELVs miRNAs and human mRNAs were analyzed using miRanda according to the miRNA–mRNA binding sites. Significantly, there were 23 miRNA–mRNA target pairs comprising 5 miRNAs and 23 mRNAs, which might play crucial roles in the host immune response and pathogenesis of *E. granulosus* infections. Three significant miRNAs, egr-miR-125-5p (degree = 11), egr-miR-10a-5p (degree = 7), and egr-miR-4991 (degree = 3) had the most target mRNAs. The host target genes of these miRNAs might be involved in the MAPK cascade (*NRG2*, *PSME2*, *MARK3*, and *PSMD6* genes), inflammatory response (*DDTL* and *ADORA2A* genes), collagen fibril organization and catabolic process (*COL5A2*, *COLGALT1*, *COL4A6*, and *ADAMTS14* genes), and apoptotic process (*NSMAF*, *UBE2D3*, *CRYAA2*, *TNFSF14*, *C19orf12*, *ITSN1*, *CRYAA*, and *ADORA2A* gene). Furthermore, egr-miR-125-5p and egr-miR-10a-5p share seed sequence (nucleotides 2–8) identity with *Homo sapiens* mature miRNAs (**Supplementary Figure 3**).

In addition, the lncRNAs regulated by miRNAs were analyzed using the miRanda algorithm. In total, 44 miRNA-lncRNA regulatory pairs were identified including 5 miRNAs and 41 lncRNAs. In the miRNA-lncRNA network, egr-miR-71-5p (degree = 17), egr-miR-125-5p (degree = 8), egr-miR-10a-5p (degree = 6), and egr-miR-61-3p (degree = 6) had the most target lncRNAs. Based on the regulatory pairs of miRNA–mRNA and miRNA-lncRNA, a ceRNA network consisting of 5 miRNAs,

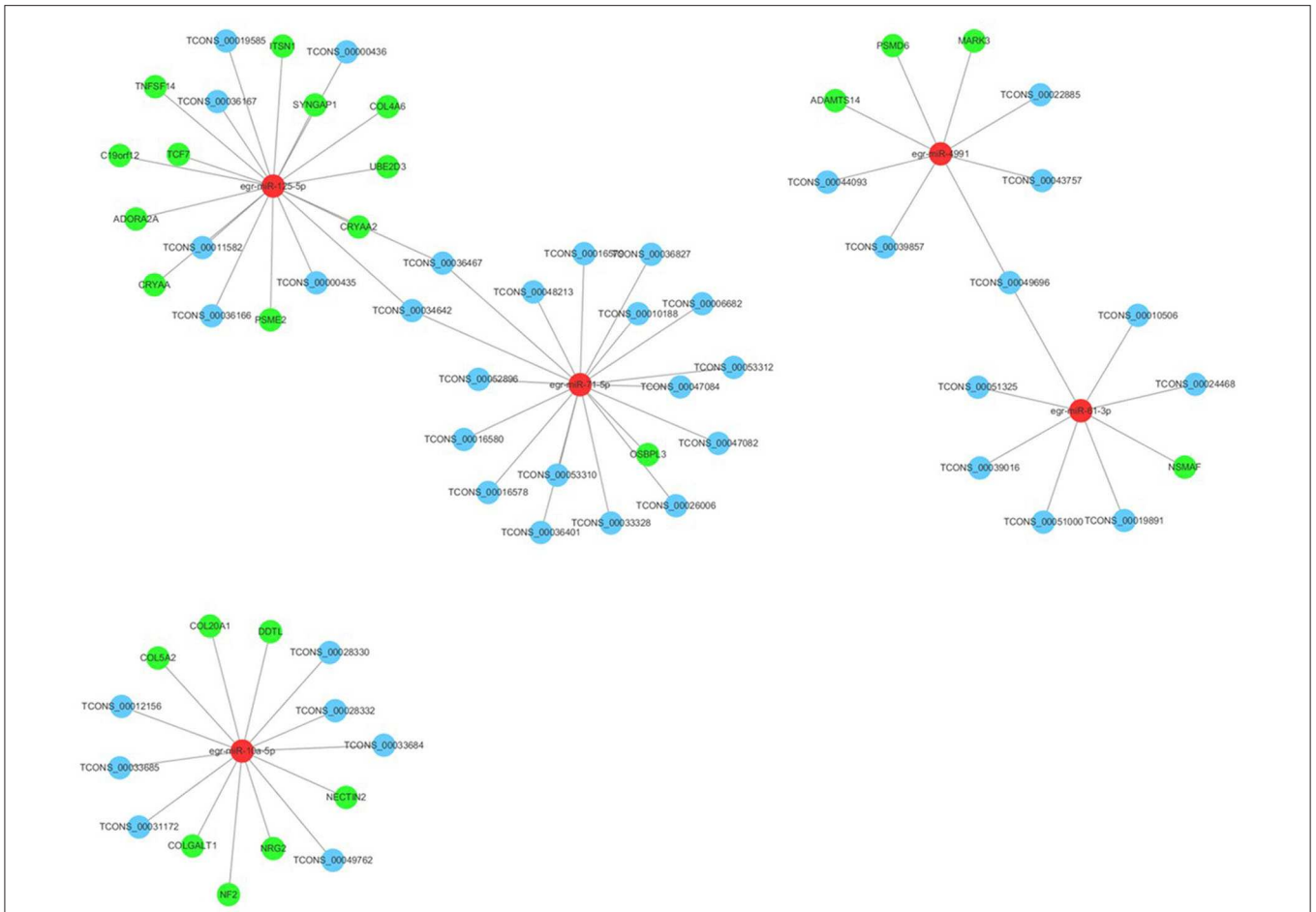

FIGURE 7 | Long ncRNA–miRNA–mRNA interaction networks in PSC-ELVs. Red, light blue, and green circles represent miRNAs, lncRNAs, and mRNAs, respectively.

41 lncRNAs, and 23 mRNAs was constructed (**Figure 7**). Each mRNA or lncRNA could be regulated by one or more miRNAs and *vice versa*. The constructed ceRNA network was conducive to depicting the role of miRNAs during *E. granulosus* infection.

DISCUSSION

A true exchange of biological macromolecules across of the laminated layer occurs between the parasite and the host, with constant vesicular trafficking through the tegument (Diaz et al., 2011; Teichmann et al., 2015). *Echinococcus granulosus* ELVs were observed in the metacestode culture medium, possibly because extracellular InsP6 (Inositol hexaphosphoric acid) binds to proteins on *E. granulosus* ELVs, acting as a "dynamic anchorage" that promotes their passage across the laminated layer (Nicolao et al., 2019). Recent studies also confirmed that parasite-derived ELVs participate in intercellular communication, immune responses, and pathology, in which a number of ncRNAs in ELVs play a crucial role during these processes (Buck et al., 2014; Hansen et al., 2019). Integration analysis of ncRNAs in parasite-derived ELVs would help understand the parasite–host interactions. Nevertheless, it is unknown whether *E. granulosus*–derived ELVs contain ncRNAs and whether ELVs miRNAs could target host genes to modulate the immune responses. In the present study, we isolated and characterized the ELVs produced from *in vitro* cultures of *E. granulosus* PSCs and HF obtained from naturally infected sheep. The isolated PSC–ELVs and HF–ELVs were similar in size and morphology to the ELVs characterized in previous reports that referred to *E. granulosus* PSCs, metacestodes, fertile sheep hydatid cysts, and other helminths (*Echinostoma caproni*, *F. hepatica*, *S. japonicum*, *Brugia malayi*, and *Heligmosomoides polygyrus*) (Marcilla et al., 2012; Buck et al., 2014; Zamanian et al., 2015; Zhu et al., 2016a).

Larval PSCs are an important transition of the life cycle stage, capable of developing either into a secondary hydatid cyst in the intermediate host or an adult worm in the final host; therefore, we compared the numbers and expression levels of ncRNAs in PSC–ELVs with those in HF–ELVs to identify the miRNAs and lncRNAs that might be crucial in the host immune response. There were 53 miRNAs and 1,130 lncRNAs that were present in both types of ELVs. In addition, there were more

miRNAs and lncRNAs in the PSC–ELVs than in the HF–ELVs; however, the 20 most abundant miRNAs were almost the same in both types of ELVs. Intriguingly, the expression levels of the 20 most abundant miRNAs and circRNAs in PSC–ELVs were higher than those of the corresponding miRNAs and circRNAs in HF–ELVs, whereas the expression levels of the 20 most abundant lncRNAs in PSC–ELVs were mostly lower than those of corresponding lncRNAs in HF–ELVs. *Echinococcus granulosus* cysts (in particular PSCs and the GL) produce and release HF–ELVs; therefore, HF–ELVs and PSC–ELVs have certain common biological components such as ncRNAs; however, their numbers and expression levels are different. Given that PSCs spilled from the cysts can develop into secondary cysts, which is the most frequent reason for relapse, we propose that PSCs spilled from the cysts are more active in excreting ELVs containing ncRNAs. Cucher et al. (2011) identified 38 miRNAs from *E. granulosus* and found that miR-2, miR-71, miR-9, miR-10, let-7, and miR-277 were enriched in PSCs and cyst walls of secondary hydatid cysts, in which their expression in different life cycle stages might be related to parasite development. These miRNAs were also identified in PSC–ELVs and HF–ELVs, which indicated that these miRNAs of PSCs and hydatid cysts could be encapsulated into their excretory ELVs. Moreover, another study compared the expression levels of miRNAs in adult worms or the cyst membrane with those in the PSCs and identified miR-125, miR-277, and miR-4989 upregulated in adult worms but downregulated in the cyst membrane (Bai et al., 2014). Let-7 has been shown to be positively correlated with its putative target gene (vitamin D receptor), which may promote the PSCs to develop into adult worms (Bai et al., 2014). In our study, compared with the ncRNAs in HF–ELVs, there were 85 miRNAs and 42 lncRNAs that were differentially expressed, among which 73 miRNAs and 19 lncRNAs were upregulated, and 12 miRNAs and 23 lncRNAs were downregulated in PSC–ELVs. miR-125-5p, miR-277a-3p, and miR-4989-5p were upregulated in PSC–ELVs, but downregulated in HF–ELVs. miR-4989 is the most abundant miRNA in both ELVs and belongs to the miR-277 family. Recently, miR-4989 and miR-277 have been reported to regulate the transcriptional landscape during juvenile to adult transition in *Schistosoma mansoni* (Protasio et al., 2017). miR-4989 was confirmed to be encapsulated into *Echinococcus multilocularis*–derived ELVs and was demonstrated to be capable of modulating nitric oxide production, the expression of TNF-α, and the key components in the LPS/TLR4 signaling pathway in RAW264.7 macrophages (Ding et al., 2019). Furthermore, *E. multilocularis* miR-4989-3p and miR-277 were detected in the sera of infected mice (Guo and Zheng, 2017). These results simply that the differential expression levels of ncRNAs in the two types of ELVs from different life stages might be involved in parasite development, and some miRNAs might moderate the host immune response.

Parasite-derived miRNAs could be involved in host immunity and pathogenesis via cross-species regulation of host mRNAs (Hu et al., 2019; He et al., 2020; Wang et al., 2020). Previous reports have shown that *S. japonicum*–derived miR-2162 can directly promote host hepatic fibrosis through cross-species regulation of host transforming growth factor β receptor III (He et al., 2020), and this miRNA was present in *S. japonicum* egg ELVs (Zhu et al., 2016b). For further analysis of the biological functions of *E. granulosus* ELVs in the parasite–host interaction, the 20 most abundant miRNAs in PSC–ELVs were selected to predict their host target genes. The GO enrichment analysis showed that these target mRNAs, which were inversely correlated with miRNAs, were involved in signal transduction, biological regulation, immune system processes, and other cellular processes. KEGG pathway analysis further demonstrated that the mRNAs targeted by the miRNAs were mainly involved in the regulation of signal transduction, infectious diseases, and the immune system, which have been widely researched and demonstrated to be associated with the functions of exosomes (Coakley et al., 2015). The predicted host target genes are involved in the inflammatory responses, including encoding members of the TNF, TLR, MAPK, and NF-κB signaling pathways, which have previously been documented to modulate cytokines during chronic *E. granulosus* infection (Refik et al., 2005; Tuxun et al., 2015; Labsi et al., 2018; Zhang et al., 2019). Additionally, the most abundant miRNAs were predicted to participate in Th1 and Th2 cell differentiation, Th17 cell differentiation, and IL-17 signaling pathway, and these immune responses occur during *E. granulosus* infections (Tuxun et al., 2012; Labsi et al., 2018). These results indicated that these 20 most abundant miRNAs likely participate in parasite–host interactions and might regulate the host immune response. A better understanding of the immune response induced by *E. granulosus* will help to further clarify the parasite–host interaction and provide a scientific basis for its treatment.

To further study the roles of PSC–ELVs miRNAs in immunity and pathogenicity, the specific miRNA sequences were compared with the corresponding host miRNAs, and an lncRNA–miRNA–mRNA regulatory network was constructed to investigate their correlations. The integrative analysis of the ceRNA interaction network demonstrated the regulatory functions of miRNAs and the specific interplay with other RNAs via the lncRNA–miRNA–mRNA regulatory axis during the parasite–host interactions. In the ceRNA network, five core miRNAs (egr-miR-125-5p, egr-miR-71-5p, egr-miR-4991, egr-miR-61-3p, and egr-miR-10a-5p) and 41 core lncRNAs were identified according to the literature and the regulatory relationship between lncRNAs and miRNAs. Intriguingly, three core lncRNAs (TCONS_00010188, TCONS_00043757, and TCONS_00033685) were also among the 20 most abundant lncRNAs in PSC–ELVs, which indicated that there may be some correlations between the most abundant miRNAs and lncRNAs in the PSC–ELVs. For the miRNAs, miR-61 showed significantly higher expression in PSCs under exposure to long-term or high albendazole sulfoxide drug levels, indicating that miR-61 might be a potential new biomarker to assess the response to chemotherapy (Mortezaei et al., 2019). In the present study, egr-miR-125-5p was predicted to target the *CRYAA*, *ADORA2A*, *COL4A6*, and *PSME2* genes, which were involved in apoptosis, inflammatory response, collagen catabolic process, and MAPK cascade, respectively (Sund et al., 2005; Billing et al., 2011; Li et al., 2017; Kobold et al., 2019). miR-125b has been shown to regulate inflammatory responses by desensitizing TLR activation after recognition

of pathogens (Gracias and Katsikis, 2011). In addition, miR-125 promoted apoptosis by reducing the expression of *VEGF* (Wu et al., 2018), which is possibly associated with the neovascularization required by the hydatid cyst metabolism in patients with cystic echinococcosis (Matera et al., 2018). Egr-miR-10a-5p was predicted to target the *NECTIN2*, *COLGALT1*, *COL5A2*, and *DDTL* genes, which were involved in the positive regulation of natural killer cell–mediated cytotoxicity and T-cell receptor signaling pathway, collagen fibril organization, collagen catabolic process, tumor necrosis factor production, and ERK1 and ERK2 cascades, respectively (Stanietsky et al., 2013; Park et al., 2017; Miyatake et al., 2018). It has been reported that hsa-miR-10a-5p had a role in regulating the proliferation and invasiveness of cancer cells and inflammatory responses in endothelial cells (Vaher et al., 2019). miR-10a-5p inhibited *MAP3K7* and β-transducin repeat-containing gene expression, thereby reducing NF-κB activation and displaying anti-inflammatory effects in the athero-susceptible endothelium (Fang et al., 2010). Further study showed that PSC–ELVs could be internalized by dendritic cells and induce their maturation (Nicolao et al., 2019). These abundant miRNAs were detected in dendritic cells upon internalization of PSC–ELVs and induced dendritic cells to secrete certain inflammatory cytokines (unpublished data). The ceRNAs predicted in the present work provided a theoretical basis for further study of how *E. granulosus* ELVs miRNAs moderate the host immune response. More importantly, egr-miR-125-5p and egr-miR-10a-5p shared seed site sequence identity with *H. sapiens* mature miRNAs and might alter the corresponding host miRNA expression, representing a potential advantage for parasite invasion. Therefore, it was reasonable to propose that these miRNAs in PSC–ELVs participate in parasitic infections, immune responses, and pathogenesis by acting in concert with their correlated mRNAs and lncRNAs.

CONCLUSIONS

Exosome-like vesicles are critical for intercellular communication, modulation of immune responses, and pathology. This is the first study to analyze the ncRNAs (miRNAs, circRNAs, and lncRNAs) profiles of the PSC–ELVs and HF–ELVs. In addition, a lncRNA–miRNA–mRNA regulatory network was constructed according to the regulatory mechanisms of miRNAs, which provided new ideas and a theoretical basis for further clarification of the function and mechanism of *E. granulosus* ELVs miRNAs in the parasite–host interaction. Egr-miR-125-5p and egr-miR-10a-5p, sharing identical seed sites with host miRNAs, were predicted to mediate the immune response during parasite infections. Although these abundant miRNAs might be involved in the host immune response and pathogenesis, the sensitivity and specificity of functional miRNAs as potential biomarkers or novel treatment strategies for cystic echinococcosis should be further investigated. Understanding the underlying mechanisms and functions of the ncRNAs in ELVs will pave the way for new parasite vaccine strategies, diagnostic markers, and treatments.

DATA AVAILABILITY STATEMENT

The miRNA and lncRNA datasets generated for this study can be found in the BioProject under accession numbers PRJNA573515 and PRJNA592079, respectively. (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA573515, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA592079).

AUTHOR CONTRIBUTIONS

YS and JC conceived and designed the study. XZ, WG, SC, JY, and JZ performed the experiments and data analysis. YS and JC contributed reagents and materials. XZ wrote the manuscript. YS and JC revised the manuscript. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00316/full#supplementary-material

Supplementary Figure 1 | Length distribution of small RNAs in ELVs derived from PSCs and HF. P1: PSC-ELVs. C1: HF-ELVs.

Supplementary Figure 2 | Discovery and profiling of circRNAs in PSC-ELVs and HF-ELVs. (JPG 337 kb). **(A)** Comparative numbers of circRNAs in PSC-ELVs and HF-ELVs. **(B)** Comparative expression analysis of the 20 most abundant circRNAs identified in PSC-ELVs and HF-ELVs. P1: PSC-ELVs. C1: HF-ELVs.

Supplementary Figure 3 | PSC-ELVs and HF-ELVs miRNA sequence homology to Homo sapiens miRNAs. (JPG 432 kb). miRNAs from PSC-ELVs, HF-ELVs, and *Homo sapiens* were grouped by seed site sequence identity (nucleotides 2–8) for the sequence alignments.

Supplementary Table 1 | Primers used for qPCR detection of selected miRNAs. (XLSX 19 kb).

Supplementary Table 2 | Distribution of small RNAs among different categories in two samples. (DOCX 15 kb).

Supplementary Table 3 | The numbers and expression levels of miRNAs in PSC-ELVs and HF-ELVs. (XLSX 40 kb).

Supplementary Table 4 | Differential expression profiles of miRNAs. (XLSX 34 kb).

Supplementary Table 5 | The numbers and expression levels of lncRNAs in PSC-ELVs and HF-ELVs. (XLSX 676 kb).

Supplementary Table 6 | The numbers and expression levels of circRNAs in PSC-ELVs and HF-ELVs. (XLSX 303 kb).

Supplementary Table 7 | The host target genes of the 20 most abundant miRNAs in PSC-ELVs. (XLSX 280 kb).

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Immunomodulatory Properties of *Leishmania* Extracellular Vesicles During Host-Parasite Interaction: Differential Activation of TLRs and NF-κB Translocation by Dermotropic and Viscerotropic Species

*Paula Monalisa Nogueira[1], Armando de Menezes-Neto[1], Valéria M. Borges[2], Albert Descoteaux[3], Ana Claudia Torrecilhas[4], Patrícia Xander[4], Or-Yam Revach[5], Neta Regev-Rudzki[5] and Rodrigo Pedro Soares[1]**

[1] Instituto René Rachou, Fundação Oswaldo Cruz - FIOCRUZ, Belo Horizonte, Brazil, [2] Instituto Gonçalo Moniz, Fundação Oswaldo Cruz - FIOCRUZ, Salvador, Brazil, [3] INRS - Centre Armand-Frappier Santé Biotechnologie, Université du Québec, Laval, QC, Canada, [4] Departamento de Ciências Farmacêuticas, UNIFESP, Diadema, Brazil, [5] Department of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel

Leishmania infection causes considerable human morbidity and may develop into a deadly visceral form in endemic regions. The parasite infects macrophages where they can replicate intracellularly. Furthermore, they modulate host immune responses by using virulence factors (lipophosphoglycan, glycoprotein-63, and others) that promote survival inside the cells. Extracellular vesicles (EVs) released by parasites are important for cell-cell communication in the proinflammatory milieu modulating the establishment of infection. However, information on the ability of EVs from different *Leishmania* species to modulate inflammatory responses is scarce, especially from those species causing different clinical manifestations (visceral vs. cutaneous). The purpose of this study was to compare macrophage activation using EVs from three *Leishmania* species from New World including *L. infantum*, *L. braziliensis,* and *L. amazonensis*. EVs were released from promastigote forms, purified by ultracentrifugation and quantitated by Nanoparticle Tracking Analysis (NTA) prior to murine macrophage exposure. NTA analysis did not show any differences in the EV sizes among the strains. EVs from *L. braziliensis* and *L. infantum* failed to induce a pro-inflammatory response. EVs from both *L. infantum* WT and LPG-deficient mutant (LPG-KO) did not show any differences in their interaction with macrophages, suggesting that LPG solely was not determinant for activation. On the other hand, EVs from *L. amazonensis* were immunomodulatory inducing NO, TNF-α, IL-6, and IL-10 via TLR4 and TLR2. To determine whether such activation was related to NF-κB p65 translocation, THP-1 macrophage cells were exposed to EVs. In the same

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***Correspondence:**
Rodrigo Pedro Soares
rodrigosoares28@hotmail.com

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way, only EVs from *L. amazonensis* exhibited a highly percentage of cells positive for NF-κB. Our results suggest an important role of EVs in determining the pattern of immune response depending on the parasite species. For *L. infantum*, LPG was not determinant for the activation.

Keywords: extracellular vesicles, *Leishmania*, host-parasite interaction, innate immunity, lipophosphoglycan (LPG)

INTRODUCTION

Leishmaniasis is a spectrum of human diseases caused by the protozoan parasite *Leishmania* and transmitted by sandflies. Annually, an estimated 1.3 million new cases and around 30,000 deaths are associated with these diseases (WHO, 2016). There is currently no effective and well-tolerated vaccine and treatments are difficult and toxic. Clinical manifestations associated with Leishmaniasis range from self-healing ulcers to lethal visceral form (VL), and depend on parasite species and the effective host immune response (Desjeux, 2004). In Latin America, especially in Brazil, VL is caused by *Leishmania infantum*, the potentially fatal infection is due to the systemic spread of the parasite and whose symptoms are characterized by progressive chronic fever, hepatosplenomegaly and pancytopenia. *Leishmania braziliensis* is the major cause of the most common form of cutaneous leishmaniasis (CL), and less frequent the mucosal leishmaniasis (MCL). Additionally, *Leishmania amazonensis* may cause different cutaneous forms ranging from regular CL to multiple non-ulcerative lesions, known as diffuse cutaneous leismaniasis (DCL) often resistant to antimonial chemotherapy (Herwaldt, 1999; Silveira et al., 2009). More importantly, most mechanisms underlying the events responsible for those different clinical forms are unknown but may be related to specific virulence factors from the parasites.

Leishmania has developed strategies to evade or subvert macrophage microbicidal effector mechanisms. Inhibition of macrophage activities may be induced by a range of *Leishmania*-derived virulence factors. In the New World, Lipophosphoglycan (LPG) is a highly abundant surface molecule and it has been shown to interfere with macrophage signaling through generation of nitric oxide (NO), cytokines, and MAPKs (Ibraim et al., 2013; Nogueira et al., 2016). Moreover, LPG-defective *L. infantum* promastigotes (Δ*lpg1*) displayed reduce ability to replicate inside murine macrophages and induced a robust *iNOS* expression compared to parental WT, supporting the status of LPG as a virulence factor (Lázaro-Souza et al., 2018). Interspecies polymorphisms in the LPG structure are important during host immune responses, and may explain, at least in part, the differences in the immunopathology. In murine macrophages, *L. braziliensis*/*L. amazonensis* LPGs were more pro-inflammatory than that *L. infantum* via TLR4 and TLR2 (Ibraim et al., 2013; Nogueira et al., 2016; Vieira et al., 2019). Together with GP63, LPG is also a virulence factor found in the EVs from *Leishmania* (Barbosa et al., 2018). *Leishmania* parasites and/or EVs can modulate macrophage transcription factors in an LPG and GP63-dependent manner (Silverman et al., 2010b; Hassani et al., 2014; Atayde et al., 2015). However, a wide range of molecules found in EVs structures are crucial for orchestrating the interaction between a given pathogen with its host (Szempruch et al., 2016).

Most studies using *Leishmania* EVs involved Old World *Leishmania* such as VL species *Leishmania donovani*. An interesting feature of *Leishmania* EVs was the presence of the zinc metalloprotease GP63. Functionally, *L. donovani* EVs were found to be anti-inflammatory modulating cytokines expression at the site of infection (Silverman et al., 2010a,b). This modulation could be due to IL-17 production and IL-1β decrease impairing NLRP3 inflammasomes (Atayde et al., 2015).

Recently, EVs isolate from *L. amazonensis* were shown to modulate immune responses in B-1 cells by inducing the production of IL-6 and TNF-α and by inhibiting IL-10 (Barbosa et al., 2018). However, how those mechanisms contribute to the severity of the disease in New World species of *Leishmania* is still unknown. Recognizing the importance of glycoconjugates and EVs in pathogenesis of Leishmaniasis, the purpose of this study was to compare their effect during macrophage activation in the innate immune compartment.

EXPERIMENTAL PROCEDURES

Ethics Statement

All animals were handled in strict accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais (MG), Brazil (protocol P-17/14-2). This protocol followed the guidelines of CONCEA/MCT. Knock-out mice handling protocol was approved by the National Commission on Biosafety (CTNBio) (protocol no. #01200.006193/2001-16).

Parasites

Promastigotes *L. infantum* (MCAN/BR/89/BA262), *L. braziliensis* (MHOM/BR/01/BA788), and *L. amazonensis* (MHOM/BR/87/BA125) species were cultured in M199 medium supplemented with 10% fetal bovine serum (FBS) 26°C. The *L. infantum* BA262 LPG-deficient mutant (Δ*lpg1*) (LPG-KO) was cultured in the same medium, supplemented with Hygromycin (50 μg/mL) and G418 (70 μg/mL) (Lázaro-Souza et al., 2018). After the 6th day, stationary phase parasites were washed in PBS (1,000 *g*, 15 min) and Hanks' balanced salt solution (HBSS) and incubated for 2 h in M199 without FBS under agitation (cell density of 1×10^8 cells/mL) (37°C, 5% CO_2) for EVs release.

The viability of parasites was assessed by Trypan blue 0.4% exclusion (Gibco). Cultures were centrifuged (1,000 g for 15 min) and parasites from the pellet were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for scanning electron microscopy

(SEM). Fixed parasites were post fixed with 1% osmium tetroxide, treated with tannic acid (0.1%), and dehydrated with ethanol. Samples were observed in a Field Emission FEI Quanta 250 FEG scanning electron microscope (FEI, OR, USA), as described (Nogueira et al., 2015).

For functional assays, supernatants were filtered (0.22 μm sterile vacuum filter) to remove residual cells and debris, 3 mL of PBS was added and the solution ultracentrifuged (100,000 *g* 4°C, 2 h) for EVs isolation. EVs were quantitated by NTA and resuspended in RPMI medium without FBS to a final concentration of 1×10^8 particles/mL. Protein concentration was determined using the Micro BCA proteins assay kit (Thermo Scientific Waltham, MA).

Nanoparticle Tracking Analysis (NTA)

All the batches collected were pooled. Size, distribution and concentration of isolated particles were measured in a Nanosight NS300 instrument (Malvern Instruments Ltd, Malvern, UK) equipped with a 405-nm laser and coupled to a CCD camera (the laser emitting a 60-mW beam at 405-nm wavelength), and data were analyzed using the NTA software (version 2.3 build 0017). The detection threshold was set to 10. Blur, Min track Length, and Min Expected Particle Size were set to auto. To perform the measurements, each sample diluted (1:100) in PBS was analyzed in triplicate; and loaded into the instrument for 30 s at 20 frames per second with the camera level set to 14 and manual monitoring of temperature (20°C).

Purification of Murine Peritoneal Macrophages

Thioglycollate-elicited peritoneal macrophages were isolated from C57BL/6 and C57BL/6 (TLR2–/– and TLR4–/– knockouts) by peritoneal washing with ice cold serum-free RPMI and enriched by plastic adherence for 1 h at 37°C/5% CO_2. Cells (3×10^5 cells/well) were washed with fresh RPMI then cultured in RPMI, 2 mM glutamine, 50 U/ml of penicillin and 50 μg/mL streptomycin supplemented with 10% FBS in 96-well culture plates (37°C/5% CO_2). Cells were primed with Interferon-γ (IFN-γ) (0.5 ng/mL) for 18 h prior to incubation with EVs (1×10^8 particles/mL) from *L. infantum* (WT and LPG-KO), *L. braziliensis,* and *L. amazonensis*. A positive control included lipopolysaccharide from *Escherichia coli* (LPS–TLR4+) (100 ng/mL) and extract of *Staphylococcus aureus* (Sa–TLR2+) (100 ng/mL). Negative control included medium with IFN-γ.

Cytokines and Nitrite Measurements

The cytokines TNF-α, IL-6, and IL-10 were determined using BD CBA Mouse Cytokine Assay kits according to the manufacturer's specification (BD Biosciences, CA, USA). Flow cytometry measurements were performed on a FACSCalibur flow cytometry (BD Bioscience, CA, USA) collected using the Cell-QuestTM software package provide by the manufacturer (1200 events). FlowJO software 7.6.4 (Tree Star, Inc, Ashland, OR) was used for data analysis. Results are representative of two experiments in triplicate. Nitrite concentration (NO) was determinate by Griess Reaction as described (Nogueira et al., 2015).

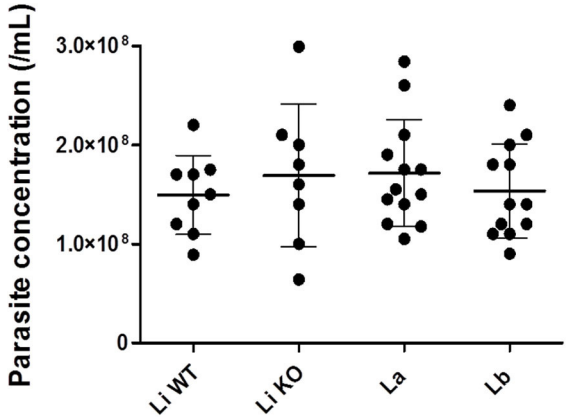

FIGURE 1 | *Leishmania* promastigotes concentrations for EVs release. Statistical analysis was performed based on Mann-Whitney test ($P > 0.05$).

NF-κB Activation Assay

THP-1 (ATCC® TIB-202™) monocyte cells were differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) for 3 days (Cells were tested for *Mycoplasma* contamination by using the polymerase chain reaction (PCR) methodology (Uphoff et al., 2002). Cells were cultured on gelatin coated glass bottom plates (Cellvis) (1×10^6 cells/well) and incubated for 1 h, following incubation with EVs (1×10^8 particles/mL) from different *Leishmania* species for another 1 and 6 h. Cells were fixed with 4% Paraformaldehyde (PFA), blocked with 5% bovine serum albumin (BSA) and stained with p65 antibody (IgG monoclonal–Santa Cruz) and Hoechst (H33342 Molecular Probes Ltd.—1 ng/mL). Images were acquired using the DeltaVision microtiter system (Applied Precision, Inc.), using a 40×/1.3 oil objective (Olympus). Image analysis was performed using ImageJ software (rsbweb.nih.gov/ij). In each sample more than 100–200 cells were quantified for cytoplasmic or nuclear localization of p65 (Sisquella et al., 2017; Ofir-Birin et al., 2018).

Statistical Analyses

For nitrite and cytokine measurements the Shapiro Wilk test was conducted to test the null hypothesis that data were sampled from a Gaussian distribution. In this case, student's *t*-test was performed. For the non-parametric distribution, it was performed the Mann-Whitney test. Data were analyzed using GraphPad Prism 5.0 software (Graph Prism Inc., CA, USA). $P < 0.05$ was considered significant.

RESULTS

Characterization of EVs Isolated From Different *Leishmania* Species

Parasites were grown in a simple serum-free media to ensure that EVs were from *Leishmania* origin. EVs were released by $1–2 \times 10^8$ parasites/mL (**Figure 1**) and viability was above 90% (data not shown) after 2 h in incubation. Those parasites were fixed and subjected to SEM. All promastigote forms exhibited the expected

shapes and microscopic images off all strains/species revealed shedding of EVs from cellular body and flagellum of the parasite (**Figure 2**).

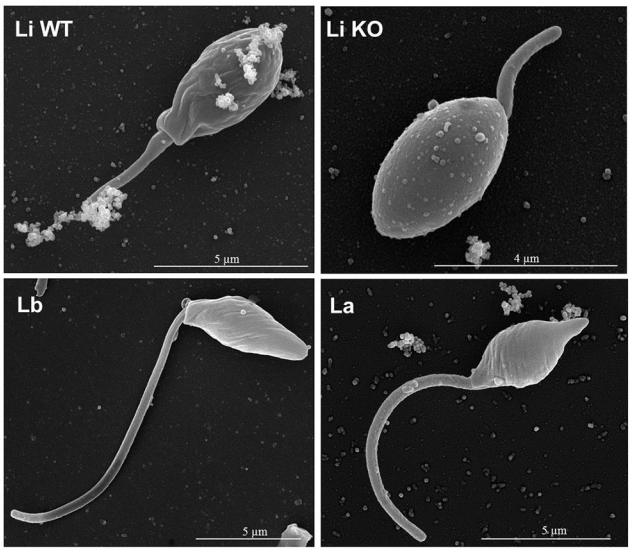

FIGURE 2 | Representative SEM images of *Leishmania* structure and release EV from entire parasite surface. Each panel shows promastigotes pre-incubated in RPMI and attached to glass coverslips containing poly-L-lysine obtained from the *L. infantum* (WT and KO), *L. braziliensis*, and *L. amazonensis* fixed and processed for SEM. The bar sizes are indicated in each image. Li WT = EVs from *L. infantum*; Li KO = EVs from *L. infantum* LPG-KO; Lb = EVs from *L. braziliensis*; La = EVs from *L. amazonensis*.

NTA analysis is a very important way to quantitate EVs from several cell types. The EVs exhibited mean diameter sizes of 123.5 ± 3.5 and 128.5 ± 3.4 nm for *L. infantum* (WT and LPG-KO), 140.0 ± 1.5 nm for *L. braziliensis*, and 129.0 ± 4.0 nm for *L. amazonensis* (**Figures 3A–D**). Statistical analysis did not show differences any in the average sizes according to species and strains ($P > 0.05$, **Figure 3E**). As expected, no particles were found in the medium alone (negative control, data not shown).

Leishmania EVs Differentially Activate TLRs

Toll-like receptors are very important molecules involved in the recognition of different pathogen-associated molecular patterns (PAMPs). It is already reported that EVs from *T. cruzi* carry important PAMPs involved in the activation of TLR2 (Nogueira et al., 2015). Since LPGs from *Leishmania* species are polymorphic and differentially modulate the immune system (Ibraim et al., 2013; Nogueira et al., 2016), the ability of their respective EVs to induce NO and TNF-α, IL-6, and IL-10 cytokines in murine macrophages was investigated. We have used knock-out mice lacking TLR2 and TLR4 receptors in order to ascertains the type of recognition by EVs. As expected, the controls represented by LPS (TLR4) and *S. aureus* (TLR2) activated TLR2KO and TLR4KO macrophages, respectively. In the wild type C57BL/6, those agonists activated both TLRs. *Leishmania infantum* and *L. braziliensis* EVs induced lower amounts of NO, TNF-α, IL-6, and IL-10 than those from *L. amazonensis* ($p < 0.05$) (**Figures 4A–D**). No differences

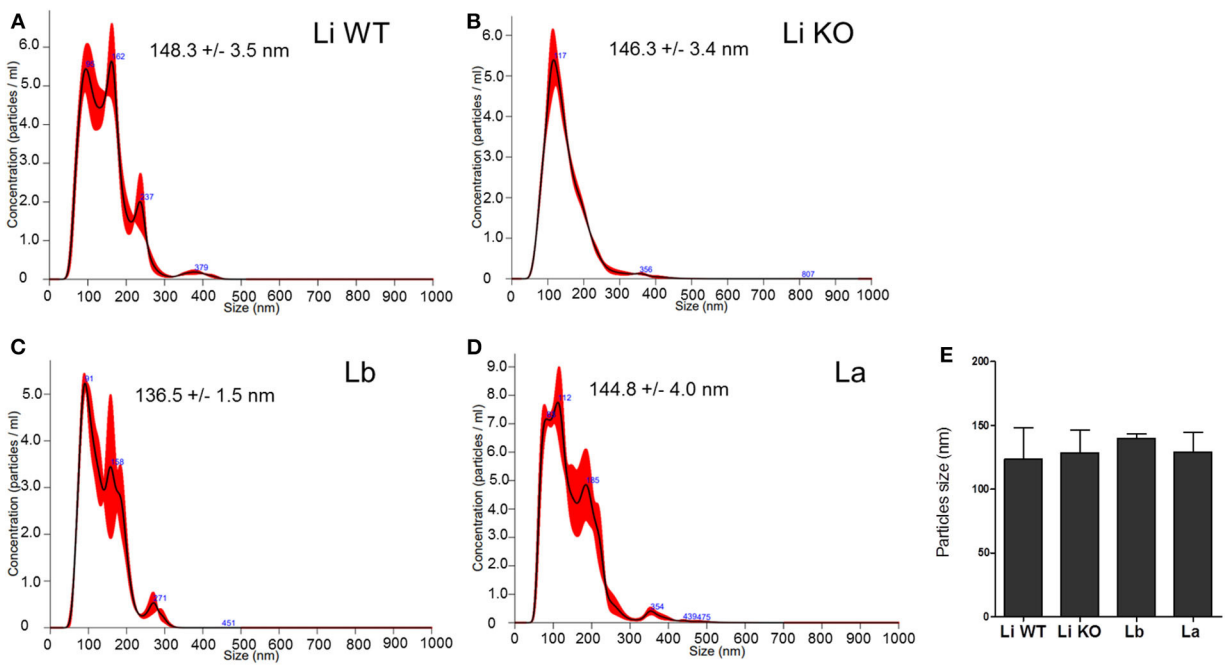

FIGURE 3 | EVs size (nm) determined by NTA analysis. Sample size distributions were calibrated in a liquid suspension by the analysis of Brownian motion via light scattering. Nanosight provides single particle size and concentration measurements. Readings were taken in triplicates. Averaged Finite Track Length Adjustment (FTLA) concentration/size for experiment. The red line is error bars indicate ±1 standard error of the mean. **(A)** Li WT = EVs from *L. infantum*; **(B)** Li KO = EVs from *L. infantum* LPG-KO; **(C)** Lb = EVs from *L. braziliensis*, and **(D)** La = EVs from *L. amazonensis*. **(E)** This figure represents a typical analysis of average EV sizes from at least two independent experiments. Statistical analysis was performed based on Mann-Whitney test ($P > 0.05$).

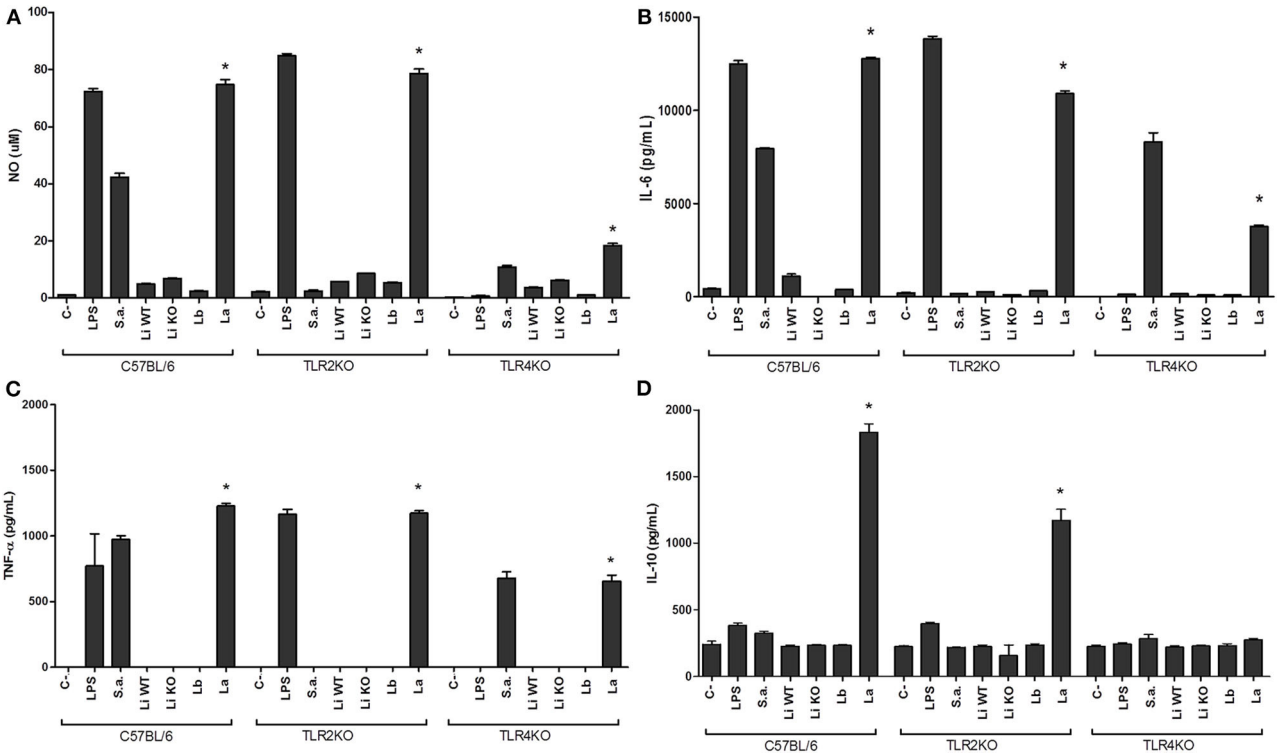

FIGURE 4 | NO and cytokines production by IFN-γ primed murine macrophages stimulated with EVs from different *Leishmania* species. Supernatants were used for NO **(A)** and cytokines IL-6 **(B)**, TNF-α **(C)**, and IL-10 **(D)** measurements were collected 48 h latter. Nitrite concentration was measured by Griess reaction. TNF-α, IL-6, and IL-10 levels were determined by CBA analysis. Li WT = EVs from *L. infantum*; Li KO = EVs from *L. infantum* LPG-KO; Lb = EVs from *L. braziliensis*; La = EVs from *L. amazonensis*. Statistical analysis was performed based on student's *t*-test ($P > 0.05$). *Asterisks indicate statistical differences.

were observed between EVs from *L. infantum* WT and LPG-KO ($p > 0.05$). Activation of macrophage immunomodulatory responses was markedly increased by EVs from *L. amazonensis* and this was primarily mediated by TLR4 and secondarily via TLR2 (**Figures 4A–D**).

To confirm whether EV-induced cytokine production was associated with NF-κB p65 nuclear translocation, THP-1 macrophages cells were exposed to EVs from all *Leishmania* species/strains for 1 and 6 h and the percentage of positive cells was evaluated. This nuclear factor is important for inducing cytokine production in the innate immune compartment. This evaluation is based on the green fluorescence detected inside the nucleus. With exception to LPS (positive control), no significant nuclear translocation of the NF-κB p65 for all *Leishmania* species was detected after 1 h (**Figures 5A,C**). As expected, after 6 h a decrease in nuclear NF-κB was observed for LPS (positive control). Different from *L. braziliensis* and *L. infantum* (WT and LPG KO), only *L. amazonensis* EVs were able to induce the nuclear translocation of NF-κB among *Leishmania* species after 6 h of exposure (**Figures 5B,D**). Those results confirmed the higher pro-inflammatory ability of *L. amazonensis* EVs in the NO and cytokine measurements (**Figures 4A–D**).

DISCUSSION

Extracellular vesicles are cell-derived particles released from a variety of cell types, including pathogens and infected cells. These structures are recognized as important mediators of cell-cell communication, immune regulation and dissemination of pathogenic material (Campos et al., 2015). EVs possess a cargo of several biomolecules including proteins, lipids, glycoconjugates, metabolites, DNA, mRNA, microRNA, and other non-coding RNA species (Mathieu et al., 2019). Secretion of EVs has been reported in several pathogens such as *Leishmania*, *Trypanosoma*, *Toxoplasma*, *Plasmodium*, *Trichomonas*, and *Mycobacterium*. Thus, the importance of those structures during the interaction with the immune system has been extensively explored (Regev-Rudzki et al., 2013; Szempruch et al., 2016).

Several research groups have focused on the study of extracellular vesicles in *Leishmania* especially in their role during cell-cell communication and innate immune responses (Atayde et al., 2016; Dong et al., 2019). Recently, EVs were shown to be important for LRV1 release in *Leishmania guyanensis* reinforce their role as potential in increasing severity in cutaneous leishmaniasis (Atayde et al., 2019). The recovered *Leishmania* EVs from New World species did not differ in size, were below

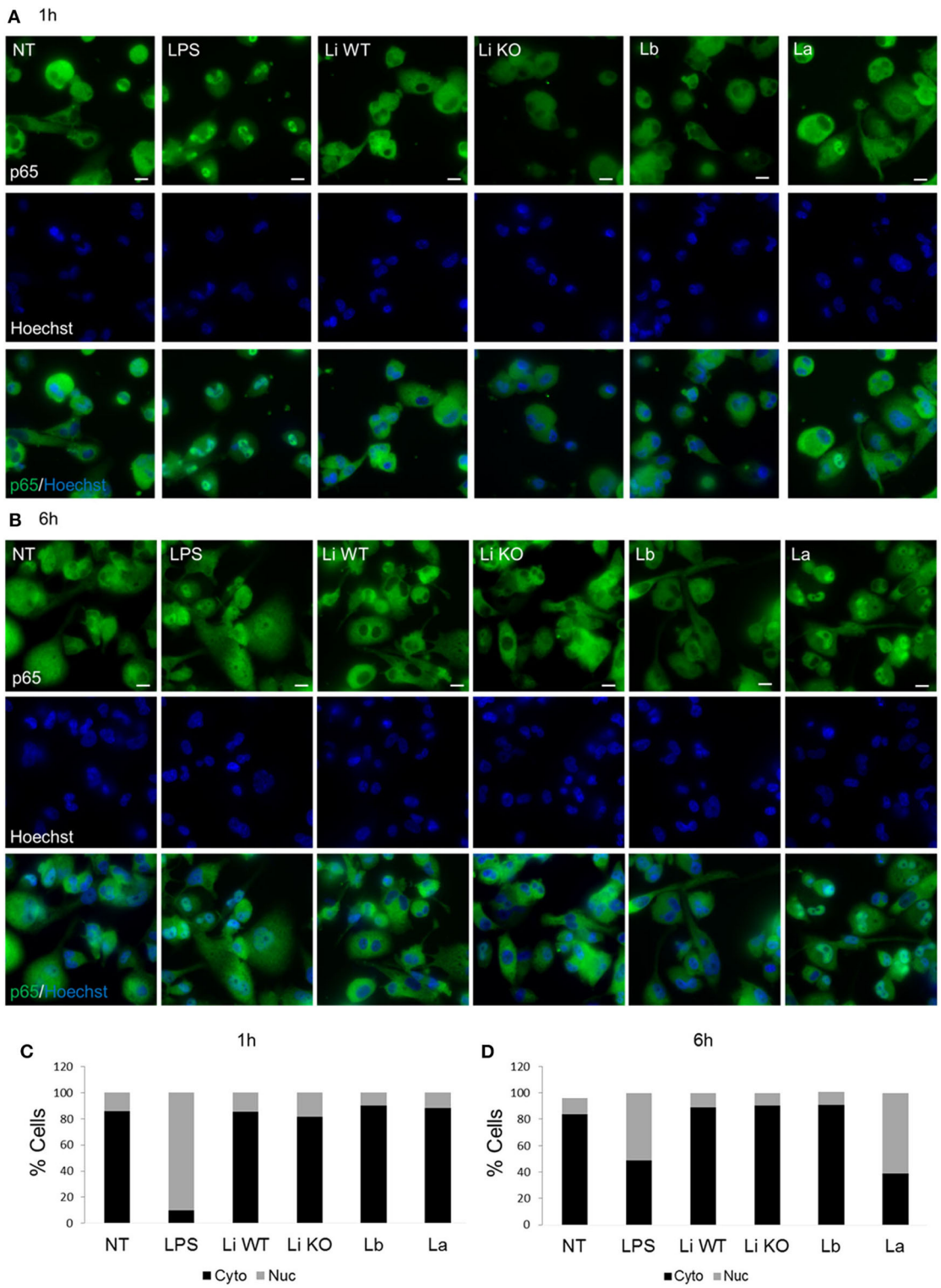

FIGURE 5 | THP-1 macrophages were treated for 1 h **(A)** and 6 h **(B)** with EVs from different *Leishmania* species to evaluate the nuclear translocation of NF-κB p65. Cells were stained with p65 (green) and Hoechst (blue) and an overlay of the two images are presented. Quantification of percentage of cells with cytoplasmic or nuclear localization of p65 for1 h **(C)** and 6 h **(D)**. NF-κB p65 fluorescence was evaluated by intranucleus intensity (the sum of the background-subtracted pixel values within the masked area of the image) and the max pixel (the largest value of the background subtracted pixel) as previously reported (Sisquella et al., 2017).

1,000 nm and viability was higher than 90%, indicating they were free of apoptotic bodies (Silverman et al., 2010a; Atayde et al., 2016; Barbosa et al., 2018). Also, those data were confirmed by SEM showing EVs being released from entire parasite surface in concentrations detected by NTA, consistent with the size and morphology of exosomes released by others parasites.

Healing of Leishmaniasis requires an effective immune response capable of eliminating the parasites with minimal damage of tissues. As part of sand fly inoculum, EVs influence the early events in the establishment of the parasite infection, play a role in pathogenesis by modulating cytokines/chemokines at the site of infection (Silverman and Reiner, 2011; Atayde et al., 2015). Cytokines such as IFN-γ and TNF-α are important activators of macrophage parasite killing whereas IL-10 participates in the control of immunopathology (Gazzinelli and Denkers, 2006). Functionally, early studies have shown that viscerotropic *L. donovani* EVs are anti-inflammatory (Silverman et al., 2010b). Here, with another viscerotropic species (*L. infantum*), no activation was detected upon macrophage stimulation by its EVs. It has been reported that those immunomodulatory and signaling-inducing activities are due to the presence of parasitic virulence factors like GP63 (Silverman et al., 2010b; Hassani et al., 2014; Marshall et al., 2018). Exosomes from WT *L. major* differentially modulated the induction of transcription factors compared to an isogenic *L. major* gp63 KO (Hassani et al., 2014). Interestingly, in our experiment, the lack of LPG in EVs from *L. infantum* did not affect macrophage activation.

Recently, EVs from *L. infantum* induced IL-10 and reduced the production of IL-18, suggesting their role in creating a permissive environment for parasite establishment (Castelli et al., 2019). Also, injection of *L. infantum* EVs increased the pro-inflammatory burden resulting in higher parasite loads (Pérez-Cabezas et al., 2018). Under our experimental conditions, EVs from *L. infantum* were also able to induce IL-10 confirming previous findings (Castelli et al., 2019). However, the presence/absence of LPG from *L. infantum* EVs did not affect their ability to induce NO and cytokine production, suggesting that other molecules could explain at least in part this lack of activation. Several EVs molecules in *L. infantum* EVs have been described (Santarém et al., 2013). However, glycoconjugates have been neglected in such approaches. The presence of other potent anti-inflammatory molecules, like glycoinositolphospholipids (GIPLs) (Assis et al., 2012) could have contributed for this lack of activation. Consistent with these observations, extracellular products (EVs or EV-depleted) were shown to act together for immunomodulating host-parasite interaction in *L. infantum* both *in vitro* and *in vivo* (Pérez-Cabezas et al., 2018). However, this activation by LPG may be determinant for *L. amazonensis*. This glycoconjugate paradoxically was very pro-inflammatory/immunosuppressive via TLR4 (Nogueira et al., 2016). Interestingly, *L. braziliensis* LPG has been shown to be very pro-inflammatory (Ibraim et al., 2013; Vieira et al., 2019) via TLR2/TLR4. This feature was not observed in its EVs, which exhibited a similar pattern than those from *L. infantum*. Overall, it is very clear that interspecies polymorphisms in EVs cargo results in differential stimulation of macrophages.

Here, a remarkable activation of macrophage immunomodulatory response by *L. amazonensis* EVs was noticed compared to the other species/strains. This species is very pro-inflammatory causing ulcerated and non-ulcerated lesions. Also, it is able to disseminate causing lesions in different parts of the body associated with a lack of cellular immune response (anergy) and therapeutic failure (Silveira et al., 2009). The first studies with *L. amazonensis* showed that EVs from macrophage infected with this species were important for induction of IL-12, IL-1β, and TNF-α (Cronemberger-Andrade et al., 2014). Further, *L. amazonensis* EVs could be involved not only in the severe immunopathology of this species but also could favor dissemination of the parasites throughout the host (Barbosa et al., 2018). In this paper, co-injection with *L. amazonensis* EVs and promastigotes increased the pro-inflammatory milieu and promote a higher parasite load in the footpad. More recently, we have demonstrated that co-injection of EVs derived from B1 cells infected with *L. amazonensis* and parasites modulated iNOS and cytokine production in mouse footpads (Toledo et al., 2020). Although an increase in the parasite load and pro-inflammatory infiltrate was detected, no protective effect on lesion size was observed. Consistent with these observations, pre-treatment with *T. cruzi* EVs increased parasite invasion and spreading to larger areas in heart leading to higher mortality in mice (Trocoli Torrecilhas et al., 2009). A distinguishing feature of EVs from this species compared to *L. infantum* and *L. braziliensis*, was their ability to highly activate TLR4/TLR2 and to induce the nuclear translocation of NF-κB p65 by THP-1 monocytes. Human monocytes (THP-1 and RAW-ELAN) cells have been successfully used upon stimulation with EVs from *Plasmodium falciparum*. Alike *L. amazonensis*, EVs from the malaria parasite were also very pro-inflammatory inducing several transcriptional factors (NF-kB and IRF3) and cytokines/chemochines via TLRs (Sampaio et al., 2017; Sisquella et al., 2017; Ofir-Birin et al., 2018). Since EVs are involved in cell-cell communication, this higher ability to induce inflammation could be more exacerbated in dermotropic species. Consistent with this idea, not only EVs from *L. infantum* from our work, but also from *L. donovani*, both viscerotropic species, exhibited a more immunosuppressive profile.

In conclusion, this study shows that EVs from different *Leishmania* species were differentially recognized by murine macrophages. In contrast to GP63, LPG did not functionally affect macrophage stimulation. However, this effect was observed for an immunosuppressive viscerotropic species. Since EVs can contribute in the immunopathology, further studies are necessary for understanding the mechanism of infection, especially during its dissemination by *L. amazonensis*. EVs are remarkable structures to understand parasite-host interactions and may serve as potential novel entities for vaccine therapy and immunodiagnostics of Leishmaniasis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All animals were handled in strict accordance with animal practice as defined by the Internal Ethics Committee in Animal Experimentation (CEUA) of Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Minas Gerais (MG), Brazil (protocol P-17/14-2). This protocol followed the guidelines of CONCEA/MCT. Knock-out mice handling protocol was approved by the National Commission on Biosafety (CTNBio) (protocol no. #01200.006193/2001-16).

AUTHOR CONTRIBUTIONS

RS, AT, PN, and AM-N: designed and performed experiments. AD and VB: development of *L. infantum* mutants. NR-R and O-YR: NF-κB translocation experiments. PX, AT, and PN: performed macrophage and CBA experiments. All authors have contributed for data analysis and writing of the manuscript.

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
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Linoleic Acid Inhibits the Release of *Leishmania donovani* Derived Microvesicles and Decreases Its Survival in Macrophages

*Sheetal Saini and Ambak Kumar Rai**

Department of Biotechnology, Motilal Nehru National Institute of Technology Allahabad, Prayagraj, India

Visceral leishmaniasis is a neglected tropical disease caused by *Leishmania (L.) donovani* parasite in the Indian subcontinent. Macrophages (mϕ) are the harboring cells for parasite and their interactions dictate the pathogenesis of this disease. Polyunsaturated fatty acids are an integral part of the mϕ cell membrane and are derived from linoleic acid (LA), which is a principal essential fatty acid. Here, we have investigated the effect of the simultaneous presence of LA during *L. donovani* infection in mϕ. Treatment with LA suppresses the parasitic load in mϕ (kDNA expression) and promotes the Th-1 type immune response (IL-12, iNOS). However, no significant change in kDNA expressions was observed when *L. donovani* promastigotes were treated with LA. Intrigued by this observation, we explored mechanism(s) by which LA promoted the protective type immune response in infected mϕ. Interestingly, LA decreased the release of *L. donovani* derived extracellular vesicle later characterized as microvesicles. Moreover, these microvesicles were suppressive concerning their bias toward the Th-2 type of immune responses (IL-10, Arginase) in mϕ. We suggest that LA plays a protective role in the immune response against *L. donovani* infection by inhibiting the release to *Leishmania* derived microvesicles and thus promoting Th-1 type immune response in mϕ.

Keywords: visceral leishmaniasis, *Leishmania donovani*, linoleic acid, *Leishmania* derived microvesicles, immune-modulation

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****Correspondence:***
Ambak Kumar Rai
ambakrai@mnnit.ac.in

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INTRODUCTION

Leishmania (L.) donovani is the causative agent of disease visceral leishmaniasis (VL) in the Indian subcontinent. The disease affects primarily underprivileged people in Bihar, Jharkhand, West Bengal and eastern part of Uttar Pradesh. The global incidences of VL are between 50,000 and 90,000 (WHO, 2019). There is no human vaccine available against VL in the market and chemotherapy has its own drawbacks especially evolving drug resistance (Mishra et al., 2007). The emergence of post kala-azar dermal leishmaniasis (PKDL) cases in the past few years (NVBDCP, 2019), HIV-VL co-infection (Sinha et al., 2005), the emergence of drug resistance (Mishra et al., 2007) and unavailability of a human vaccine against VL suggest a need for more effective control measures. Advancement in controlling the disease requires an improved understanding of the host-parasite interaction in VL infection.

The pathogenesis of VL depends significantly upon macrophage (mϕ)-*Leishmania* interactions and further their encounter with T cells. As major components of the cellular membrane, polyunsaturated fatty acids (PUFA) play a pivotal role in maintaining the membrane fluidity and increase the innate immune threshold in mϕ, which is essential for appropriate antigen presentation to T cells (Sen et al., 2001). It is evident that mϕ enriched with PUFAs specially arachidonate showed ∼50% enhancement of phagocytic and adhesion capacity (Calder et al., 1990). Linoleic acid (LA) is an essential polyunsaturated fatty acid (PUFA) and the precursor of long-chain PUFAs in the mammals. Various groups including ours have reported the role of prostaglandins and leukotrienes in *Leishmania* infection (Morato et al., 2014; Saha et al., 2014; Chaves et al., 2016; Saini et al., 2019). However, supplementation with their dietary precursor i.e., LA is a more viable and feasible approach in the treatment of human VL, but such studies are lacking.

In the present study, we have analyzed the effect of the simultaneous presence of LA during *L. donovani* infection in mϕ. LA suppressed parasitic load (kDNA gene expression) in infected mϕ and promoted Th-1 type protective immune response. However, it did not show any direct leishmanicidal activity *in vitro* on *L. donovani* promastigotes. Further exploration showed that LA reduces the release of extracellular vesicles from *L. donovani* parasite (promastigote form), which were later characterized as microvesicles (Muralidharan-Chari et al., 2010; Raposo and Stoorvoel, 2013; Marcilla et al., 2014). The immunomodulatory properties of these *L. donovani* derived microvesicles (*Ld*Mv) were also analyzed in *vitro* and were found to be immunosuppressive. Taken together, our data indicate that LA modulates the release of *L. donovani* derived microvesicles (*Ld*Mv) that restraint the parasitic load and promote pro-inflammatory type immune response.

MATERIALS AND METHODS

Reagents

RPMI-1640 media (Cat. No. 31800-022, Gibco Life Technologies, USA), FBS (Cat. No. 10270106, Gibco Life Technologies, USA), antibiotic cocktail (Penicillin + Amphotericin B + streptomycin; only for maintenance media; Cat. No. E485, Amresco Inc., USA), Kanamycin (only for infection media; Cat. No. Kanamac-750, Macleod Pharmaceuticals Ltd., India), $NaHCO_3$ (Cat. No. 27765, Fischer Scientific Pvt. Ltd., India), HEPES (Cat. No. MB016, Himedia Laboratories Pvt. Ltd., India), and L-Glutamine (Cat. No. G0063, TCI Chemicals Pvt. Ltd., India) were procured for cell culture purpose. Pure Linoleic Acid (LA; Cat. No. sc-200788A) was purchased from Santa Cruz Biotechnology, USA. Details of other reagents are given in their respective method sections.

Parasite

Promastigote form of *L. donovani* (dd8; MHOM/IN/80/Dd8; WHO reference strain of Indian origin) was maintained in M199 medium with 10% FBS at 25–27°C in anaerobic condition (Tiwari et al., 2016). The parasite strain was obtained from the Central Drug Research Institute, Lucknow, India. These parasites were being routinely maintained in our laboratory (MNNIT Allahabad) and were tested for expressions of *L. donovani* specific actin/tubulin gene regularly. For experiments, cells were fixed with formaldehyde (4%) and counted using a Neubauer chamber.

Culture of Macrophage Cell Line and *in vitro* Assays

The mouse mϕ cell line (J774A.1) was obtained from Central Drug Research Institute, Lucknow, India and maintained in RPMI-1640 media (with 10% FBS) at 37°C in 5% CO_2 incubator (Model No. ESCO CelSafe, Esco Micro Pvt. Ltd., Singapore) at MNNIT Allahabad (Saini et al., 2019). For experiments, 2×10^6 mϕ cells were counted with the help of the Neubauer chamber and seeded in the six well plates. After 3–4 hours (h), non-adherent cells were removed and the assay was performed with adhered cells. To establish the infection of *L. donovani* to mϕ, adherent J774A.1 mϕ cells were incubated with the 20×10^6 *L. donovani* parasites (MOI; 1:10). After 12 h of incubation, the non-infecting parasites were removed by washing with sterile PBS/incomplete RPMI (thrice). As per the experimental plan, cells were treated with 500 nM LA in culture (on the basis of literature) along with the parasite infection. After completion of assays, cells were processed for RNA/DNA isolation.

Gene Expression Analysis Using Real-Time PCR (qPCR)

Total RNA was isolated from mϕ (uninfected as well as infected) and processed further as described by Saini et al. (2020a). All the primers used in the present study (**Supplementary Table 1**) were commercially synthesized from Eurofins genomics, India. Expression analysis of gene was performed using qPCR (Cat. No. TCR0096, PikoReal real-time PCR System). The housekeeping gene i.e., HGPRT (cell line)/α-tubulin (parasite) was used to normalized cT values in control and experimental tubes. The data is represented as fold change in gene expression ($2^{-\Delta\Delta cT}$ value) considering untreated mϕ as reference/control (fold change = 1) (Schmittgen and Livak, 2008; Sindhu et al., 2018).

Measurement of Parasite Load Using kDNA

A modified Fan and Gulley method was used for the isolation of DNA from infected mϕ cells (Fan and Gulley, 2001). Briefly, the parasitic load in infected mϕ was quantified by measuring the copy number of *L. donovani* specific kDNA in isolated DNA using qPCR (Verma et al., 2019).

Fluorimetry Analysis

The culture supernatant of *L. donovani* parasite was incubated with 10 μM Diphenylhexatriene (DPH; Cat. No. 66525, SRL Pvt. Ltd., India) for 60 min at room temperature. After completion of incubation, the analysis was performed in a fluorescence spectrophotometer (LS-45, Perkin Elmer Inc., USA) in the Center for Interdisciplinary Research (CIR), MNNIT Allahabad. Scan between 385 and 650 nm was performed and maxima was observed at 430 nm. Hanks' balanced salt solution (HBSS) (Tiwari et al., 2016) alone was taken as background and was deducted to get normalized fluorescence intensity. Experiments

were performed three times independently and every time in triplicate.

GC-HRMS Analysis

To assess the fatty acid profile of culture supernatant of *Leishmania donovani*, GC-HRMS was used. Fatty acids from culture supernatant were extracted using Bligh and Dyer method (chloroform/methanol method) (Bligh and Dyer, 1959). Briefly, 3.75 ml chloroform and methanol (1:2) were added to 1 ml sample (supernatant). Heptadecanoic acid (5 mM/L) (Cat. No. H3500, Sigma Aldrich, USA) was added as the internal standard. Tubes were vortexed well and 1.25 ml Chloroform was added. After mixing, an equal amount (1.25 ml) of double distilled water was added. Then, the tubes were centrifuged at 1,000 rpm for 5 min at room temperature. The lower organic phase was recovered and the solvent was dried. The lipid extract obtained was dissolved in 2 ml BF_3-Methanol (14%). Sealed tubes were kept at 55°C for 1.5 h with vigorous shaking after every 20 min. After 1.5 h, 2 ml saturated $NaHCO_3$ solution and 2 ml Hexane was added. Tubes were centrifuged at 1,000 rpm for 5 min and the organic phase was collected for analysis. Final samples were transferred to screw cap glass tubes and were transported to SAIF- IIT Bombay for GC-HRMS analysis under appropriate conditions.

Characterization of Extracellular Vesicles Released by *L. donovani (Ld)* Promastigotes

5×10^6/ml parasites were cultured for 6 h with and without LA (significant uptake of LA was observed by promastigotes form of parasite after 6 h). After 6 h, the supernatants were harvested after centrifugation at 1,200 rpm for 10 min to remove parasites. These stationary phase *L. donovani* promastigotes were washed with PBS and fixed in 2.5% glutaraldehyde overnight (25°C). Fixed parasites were washed, dissolved in sterile water, placed on glass coverslips and kept at 37°C for complete drying. The supernatant was further centrifuged twice at 10,000 rpm for 30 min to harvest secreted microvesicles (Mv) (Greening et al., 2015). These Mv and dried parasite samples were transported to All India Institute of Medical Sciences (AIIMS) New Delhi for further analysis. Samples were analyzed under the Scanning Electron Microscope (EVO18 Zeiss, Oberkochen, Germany) in the sophisticated analytical instrumentation facility of AIIMS New Delhi. Besides this, the isolated Mv was finally dissolved in PBS and analyzed in particle size analyzer (Nanotrac Wave, Microtrac, USA) in CIR, MNNIT Allahabad.

Statistical Analysis

Experiments were performed independently in three sets. Each reaction was carried out in duplicates. The data are represented as Mean ± S. D. To compare the differences between two groups, student's *t*-test (paired and unpaired) was used. A $p < 0.05$ was regarded as significant and shown with the graph. The analysis was done using SPSS 15.0 and Graph Pad Prism-5.0. All the graphs and figures were made using Graph Pad Prism-5.0.

RESULTS

LA Decreases the Parasitic Load in mϕ and Promotes Pro-inflammatory Response

Presence of LA at the time of *L. donovani* infection to mϕ suppresses the parasite load in infected mϕ (**Figure 1A**; Mean ± S. D., 1.092 ± 0.3801) as compared to infected control (infected mϕ without any treatment) (Mean ± S. D., 9.018 ± 1.243) (**Figure 1A**). However, we didn't observe any significant change in kDNA of *L. donovani* promastigotes upon treatment with LA (without mϕ; **Figure 1B**). Findings showed increased expressions of immune markers of Th-1 type upon LA treatment in *L. donovani* infected mϕ [IL-12 (**Figure 1C**; Mean ± S. D., 3.917 ± 0.833) and iNOS (**Figure 1D**; Mean ± S. D., 4.167 ± 0.917)] as compared to control (IL-12; Mean ± S. D., 2.497 ± 0.209 and iNOS; Mean ± S. D., 1.945 ± 0.497). Treatment of LA also showed a simultaneous decrease in the Th-2 type immune markers in *L. donovani* infected mϕ [IL-10 (**Figure 1E**; Mean ± S. D., 0.7025 ± 0.0638) and Arginase-I (**Figure 1F**; Mean ± S. D., 0.816 ± 0.070)] as compared to infected but untreated mϕ (IL-10; Mean ± S. D., 3.243 ± 0.942 and Arginase-I; Mean ± S. D., 2.807 ± 0.195). For calculation of relative fold change in expressions, uninfected mϕ were taken as control (fold change = 1, shown as a dashed line in figures). The significant difference between two groups was analyzed using paired *t*-test.

LA Reduced the Release of Membranous Bodies in the Supernatant of *L. donovani* Culture

Supernatants of *L. donovani* promastigote cultures (LA treated and untreated) were analyzed for the presence of membrane bodies using diphenylhexatriene (DPH) dye (10 μM) (**Figures 2A,B**). DPH dye binds to cell membranes and demonstrates strong fluorescence when intercalated into lipid membranes. We have observed high fluorescence intensity of DPH in the supernatant of untreated culture (Mean ± S. D., 0.223 ± 0.020) as compared LA treated culture (Mean ± S. D., 0.121 ± 0.019) (**Figure 2B**), which suggests a low number of membranous bodies in treated samples.

The presence of fatty acid (FA) in the culture supernatant of promastigote culture was analyzed by GC-HRMS, as it also indicates the presence of membranous bodies (Chromatograph-**Supplementary Figure 1**). Internal standard (Heptadecanoic acid) was identified at 15.4 min. GC-HRMS results revealed that the concentrations of Palmitic acid (PA, 16:0; 14.3 min) and Stearic acid (SA, 18:0; 16.5 min) were decreased in LA treated *L. donovani* culture supernatant (Mean ± S. D., 442,600 ± 182,800 and 20,800 ± 153,200, respectively) as compared to untreated samples (**Figures 2C,D**; Mean ± S. D., 1,056,000 ± 141,600 and 691,600 ± 205,000, respectively). Hence, our GC-HRMS data also points toward the presence of membranous bodies in the *L. donovani* promastigote culture supernatant, which are decreased upon LA treatment.

Our SEM data showed the release of membranous bodies/extracellular vesicles by *L. donovani* promastigotes under appropriate culture conditions, which was characterized

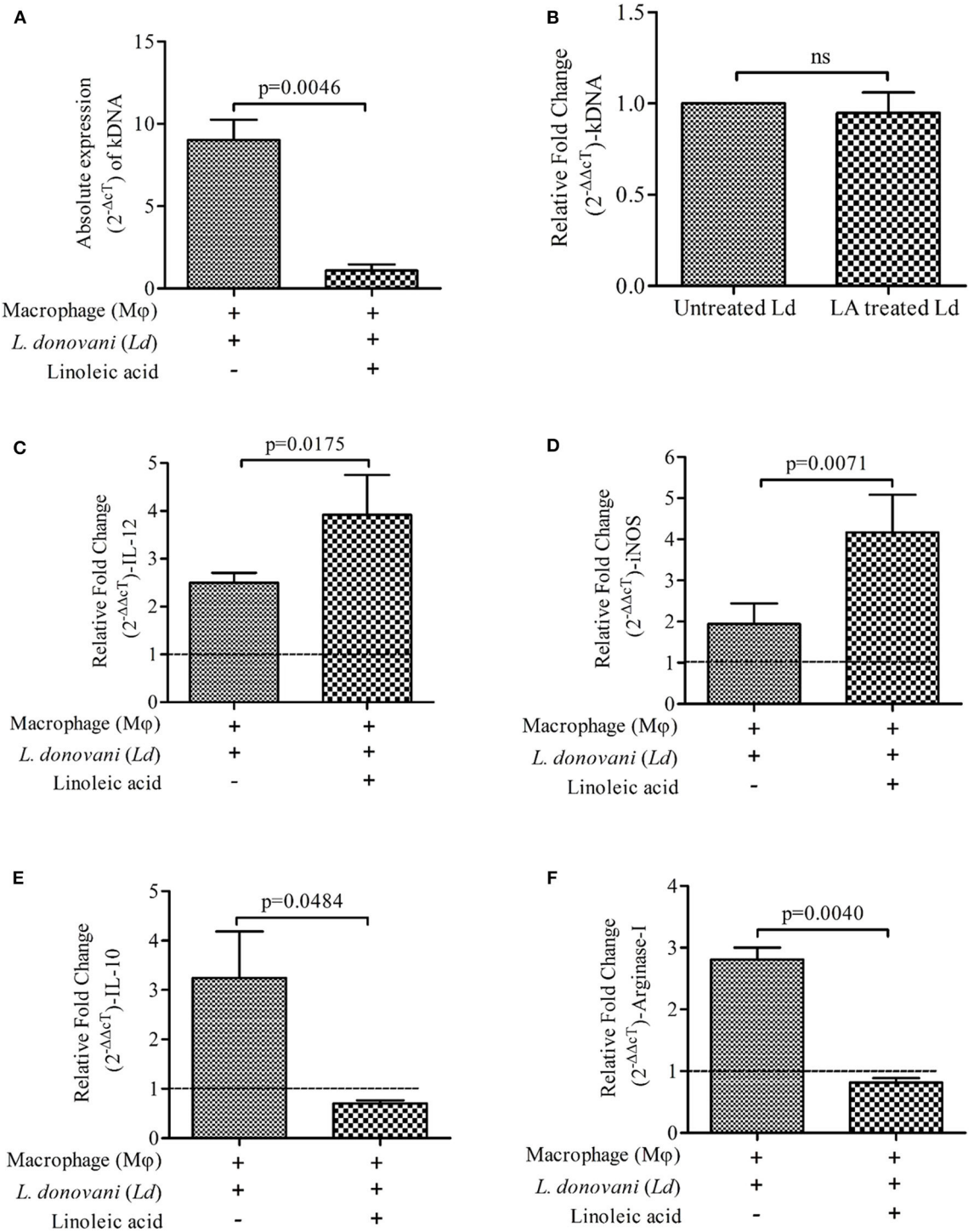

FIGURE 1 | Presence of Linoleic acid (LA) at the time of *Leishmania donovani (Ld)* infection to macrophages (mϕ) decreases the parasite load and modulates the immune response. **(A)** The absolute expression ($2^{-\Delta cT}$) of kDNA i.e., parasite load, is shown in *Ld* infected mϕ with and without supplementation of LA. The treatment of LA has been given simultaneously at the time of infection. **(B)** The bar diagram shows the relative expressions ($2^{-\Delta\Delta cT}$) of kDNA in *Ld* promastigotes upon treatment with LA. Relative fold change ($2^{-\Delta\Delta cT}$) in mRNA expressions of IL-12 **(C)**, iNOS **(D)**, IL-10 **(E)**, and Arginase-I **(F)** being shown under three different conditions i.e., mϕ (J774A.1) alone (uninfected control; horizontal dotted line, fold change = 1), *Ld* infected mϕ and *Ld* infected mϕ + LA. The results are representative of three independent experiments and each experiment was performed in triplicate. Data are expressed as Mean ± S. D. and significant differences are shown as the *p*-value on the graph.

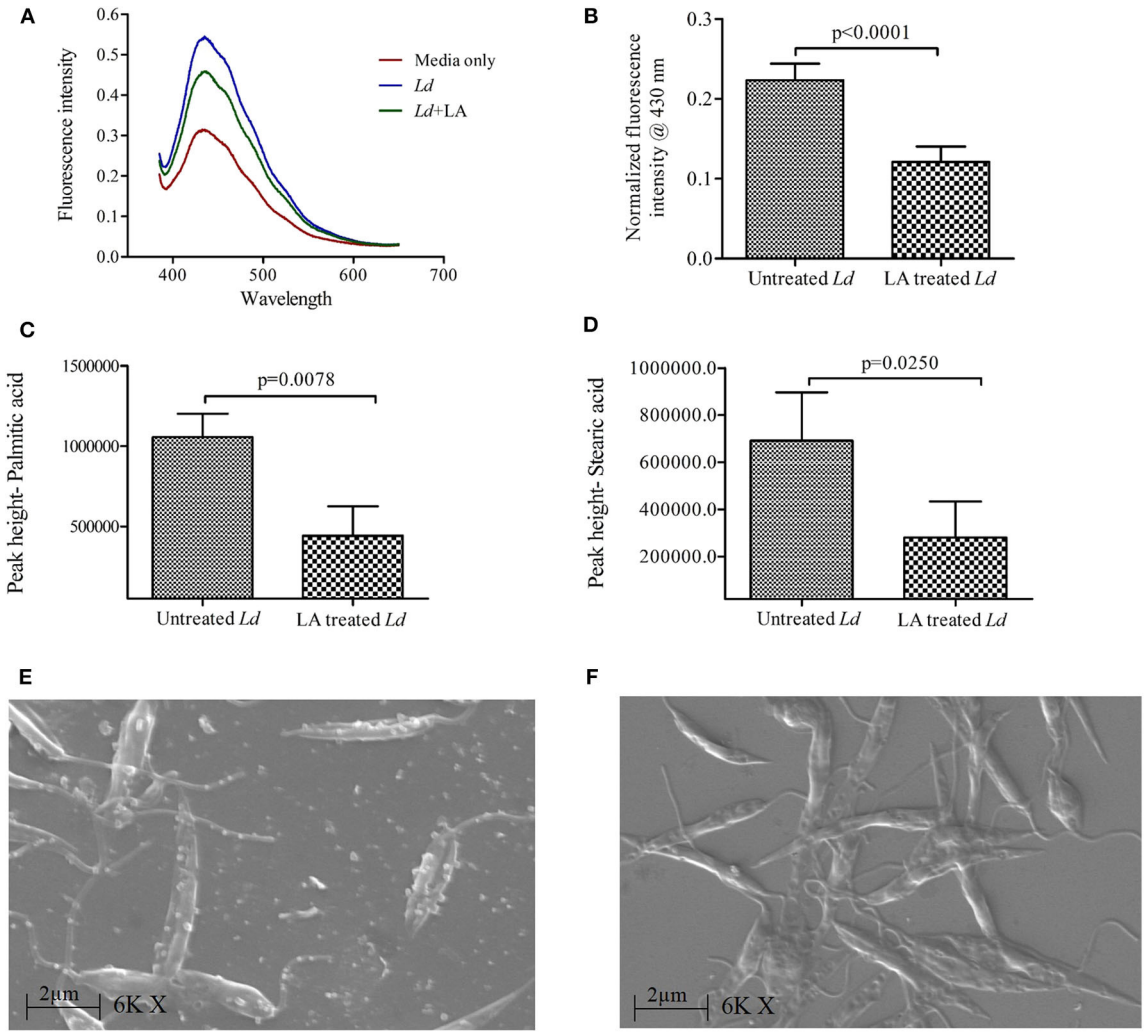

FIGURE 2 | Release of extracellular membranous bodies in the culture supernatant of *Leishmania donovani* (*Ld*) promastigote. **(A)** Fluorescence intensity spectra of culture supernatants of *Ld* promastigotes are shown using diphenylhexatriene dye (DPH, 10 µM) under three conditions i.e., HBSS media only, *Ld* culture and *Ld* culture treated with linoleic acid (LA). **(B)** The cumulative findings of fluorescence intensity (at 430 nm) are shown where culture supernatants of *Ld* (LA treated and untreated) were incubated with DPH dye. The intensities in experimental conditions were first normalized using that of HBSS media only. Bar diagram shows the differences in levels (peak height) of **(C)** palmitic acid and **(D)** stearic acid in culture supernatants of untreated and LA treated *Ld* promastigotes using GC-HRMS. **(E,F)** The scanning electron micrographs of *Ld* promastigotes are shown in two different conditions i.e., untreated and LA treated, respectively. Magnification is 6,000× with 2 µm scale and EHT 20.00 kV. Experiments were performed three times independently in triplicates and significant differences (*paired t-test*) are shown as the *p*-value on the graph.

by vesicular blebbing of the cell membrane (**Figure 2E**). The same was not observed upon treatment with LA (**Figure 2F**). To characterize whether these membranous bodies are exosomes (30–100 nm) or/and microvesicles (0.1–1 µm) (Marcilla et al., 2014), excreted membranous bodies were characterized using SEM and particle size analysis (PSA). The findings of SEM showed the presence of vesicles of sizes ranging from 0.5 to 1 µm in size (**Figures 3A,B**; Mean ± S. D., 0.819 ± 0.200 µm) which characterize these vesicles as microvesicles (Muralidharan-Chari et al., 2010; Raposo and Stoorvoel, 2013; Marcilla et al., 2014). Results obtained from PSA also suggested the same and the average size of vesicles was also ranging between 0.5 and 1 µm (**Figure 3C**; Mean ± S. D., 0.729 ± 0.269 µm).

Immuno-Modulatory Properties of *L. donovani* Derived Microvesicles (*Ld*Mv)

Intrigued by our observation, we were interested to understand the immunomodulatory properties of *Leishmania donovani* derived microvesicles (*Ld*Mv) and its effects on parasite load within mϕ. *Ld*Mv were first generated from 5 × 10^6/ml of parasite culture and used in *in vitro* experiments as per the plan. In the presence of *Ld*Mv, *Ld* infected mϕ showed decreased expressions of pro-inflammatory immune markers [IL-12 (**Figure 4A**; Mean ± S. D., 0.834 ± 0.242) and iNOS (**Figure 4B**; Mean ± S. D., 0.622 ± 0.198)] as compared to *Ld* infected mϕ without treatment (infected mϕ only) (IL-12: Mean ± S. D., 2.095 ± 0.305 and iNOS:

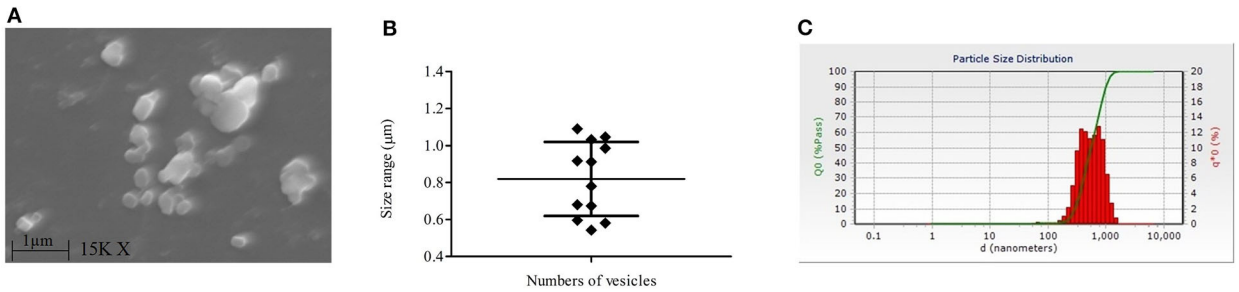

FIGURE 3 | Characterization of extracellular vesicles released by *L. donovani (Ld)* promastigotes. **(A)** Image of scanning electron microscopy (SEM) shows the presence of extracellular vesicles in the culture supernatant of the *Ld* parasite. **(B)** Sizes of these vesicles in SEM analysis are shown and it ranges from 0.5 to 1 μm in size (Mean ± S. D., 0.819 ± 0.200). **(C)** Findings of particle size analysis are shown where culture supernatants of *Ld* culture were checked for particles' sizes. Experiments were performed three times independently in triplicates.

Mean ± S. D., 1.458 ± 0.404). Simultaneously, expressions of anti-inflammatory markers (IL-10 and Arginase-I) were increased after *Ld*Mv treatment [IL-10 (**Figure 4C**; Mean ± S. D., 5.470 ± 1.011) and Arginase-I (**Figure 4D**; Mean ± S. D., 3.236 ± 0.405)] as compared to infected control (IL-10: Mean ± S. D., 2.036 ± 0.700 and Arginase-I: Mean ± S. D., 1.886 ± 0.370). Uninfected mϕ (i.e., without *Ld* infection) were taken as control (fold change = 1, *dashed line*). Interestingly, we observed an increase in parasite load (absolute gene expression; $2^{-\Delta cT}$) when mϕ were infected with *Ld* + *Ld*Mv (**Figure 4E**; Mean ± S. D., 8.696 ± 1.254) as compared to only infected mϕ as control (Mean ± S. D., 106.2 ± 8.921). This should also be noted that culture supernatant without *Ld*Mv (from LA treated culture supernatant) has no effect on immune response against *Ld* infection (**Supplementary Figure 2**).

DISCUSSION

At the cellular level, the deficiency of LA i.e., ω-6 PUFA, an essential fatty acid, impairs cell-to-cell interaction by modifying cell adhesion (Jianga et al., 2000) and possibly leading to the poor synapse formation and thus compromised activation and antigen presentation (Courtney et al., 2003). LA is the dietary precursor of arachidonic acid (AA) and precursor of the long-chain PUFAs in the mammals. It is evident that mϕ enriched with AA showed ∼50% enhancement of phagocytic and adhesion capacity (Calder et al., 1990). AA gives rise to various bioactive molecules e.g., prostaglandins (PGs) and leukotrienes (LTs). Various groups have emphasized on roles of PGs and LTs in the immune response against *Leishmania* infection (Morato et al., 2014; Saha et al., 2014; Chaves et al., 2016; Saini et al., 2019). However, the possibilities of their therapeutic applications are limited, as these molecules are transient in nature as well as not cost-effective. Instead of them, using its dietary precursor i.e., LA may have a beneficial role in the containment of the disease. Our previous observation showed that the preventive, as well as therapeutic usage of LA, significantly contains the parasite load in infected mϕ (Saini et al., 2020b). Before taking these leads further, we were interested to observe the establishment of *Ld* infection in mϕ under LA sufficient condition.

In the present study, LA enriched macrophages successfully eliminated *Ld* infection. We measured the expressions of pro- and anti-inflammatory markers in LA treated and *Ld* infected mϕ. Our finding clearly showed the strengthening of pro-inflammatory and weakening of anti-inflammatory markers, when *Ld* infection was being established in presence of LA (**Figure 1**). We conclusively demonstrated that the parasite load (i.e., kDNA expression) was also decreased under the same condition, suggesting the poor establishment of infection of *L. donovani* in an LA sufficient condition. However, we failed to observe the killing of *Ld* promastigotes when treated with LA. Summarily, there is no direct leishmanicidal activity of LA and the observed decrease in parasite load in LA treated infected mϕ is possibly due to protective switching from anti- to pro-inflammatory type of immune response. Similar results were obtained when LA was given to either prior to *Ld* infection (preventive) or after *Ld* infection (therapeutic) (Saini et al., 2020b).

Our findings suggest the release of membranous bodies in the *Ld* culture, which is clearly visible on the surface of the parasite in SEM i.e., vesicular blebbing. These bodies which are released under normal culture conditions gets inhibited in the presence of LA and these categorically belong to microvesicles (0.5–1.0 μm) (Muralidharan-Chari et al., 2010; Raposo and Stoorvoel, 2013; Marcilla et al., 2014). The presence of palmitic and stearic acid in the culture supernatant of parasite suggest the same and these fatty acids contribute to ∼98% of phospholipids (constituents of the cell membrane) in *L. donovani* promastigotes (Messaoud et al., 2017). Szempruch et al. (2016) summarizes the immunomodulatory activities of membranous bodies by protozoan parasites. Previous studies show the release of exosomal membranous bodies by amastigotes form of *Leishmania* parasite and their immunomodulatory properties are also shown. Silverman et al. (2009) proposed an exosome-based pathway from the *Leishmania* parasite that is responsible for protein secretion and communication with mϕ. *L. donovani* exosomes are known to modulate human monocyte

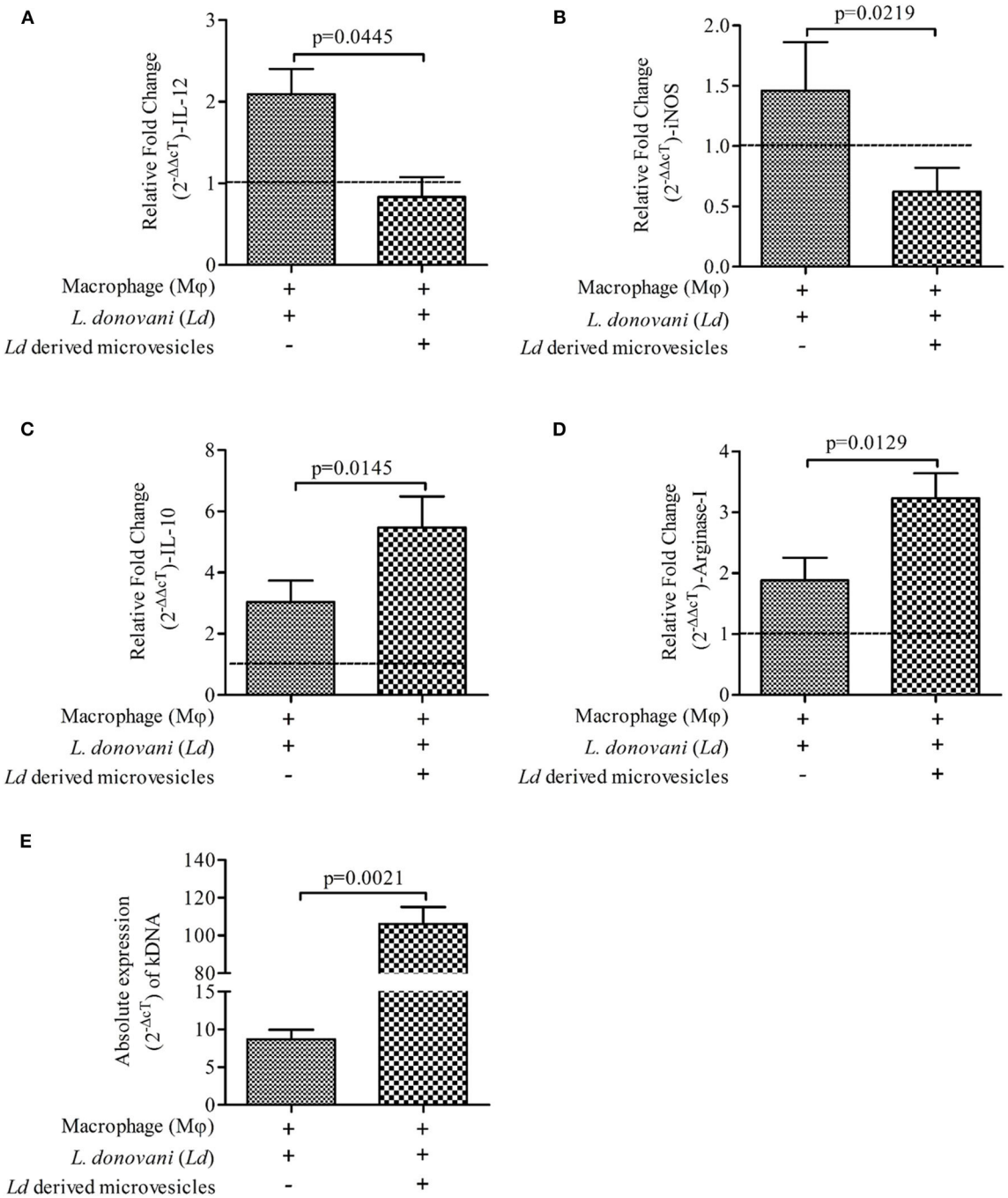

FIGURE 4 | Suppressive nature of *Leishmania donovani* (*Ld*) derived microvesicles (*Ld*Mv). Relative fold change ($2^{-\Delta\Delta cT}$) in the expressions of mRNA of **(A)** IL-12, **(B)** iNOS, **(C)** IL-10, and **(D)** Arginase-I genes are shown in three different conditions i.e., (i) mϕ (J774A.1) alone (uninfected control; horizontal line, fold change = 1), (ii) *Ld* infected mϕ, and (iii) *Ld* infected mϕ + *Ld*Mv. **(E)** Absolute levels ($2^{-\Delta cT}$) of kDNA are shown in two conditions i.e., (i) *Ld* infected mϕ (infected control) and (ii) *Ld* infected mϕ + *Ld*Mv. The results are representative of three independent experiments and each experiment was performed in triplicate. Data are expressed as Mean ± S. D. and significant differences are shown as the *p*-value on the graph.

cytokine responses by promoting Th-2 type immune response (IL-10) and inhibiting Th-1 type immune response (TNF-α, IL-12p70). Simultaneously, BALB/c mice exposed to *L. major* exosomes showed increased IL-4 production and decreased IFN-γ production at disease sites (spleen and draining lymph node) and exacerbation of *L. major* infection (Silverman et al., 2010).

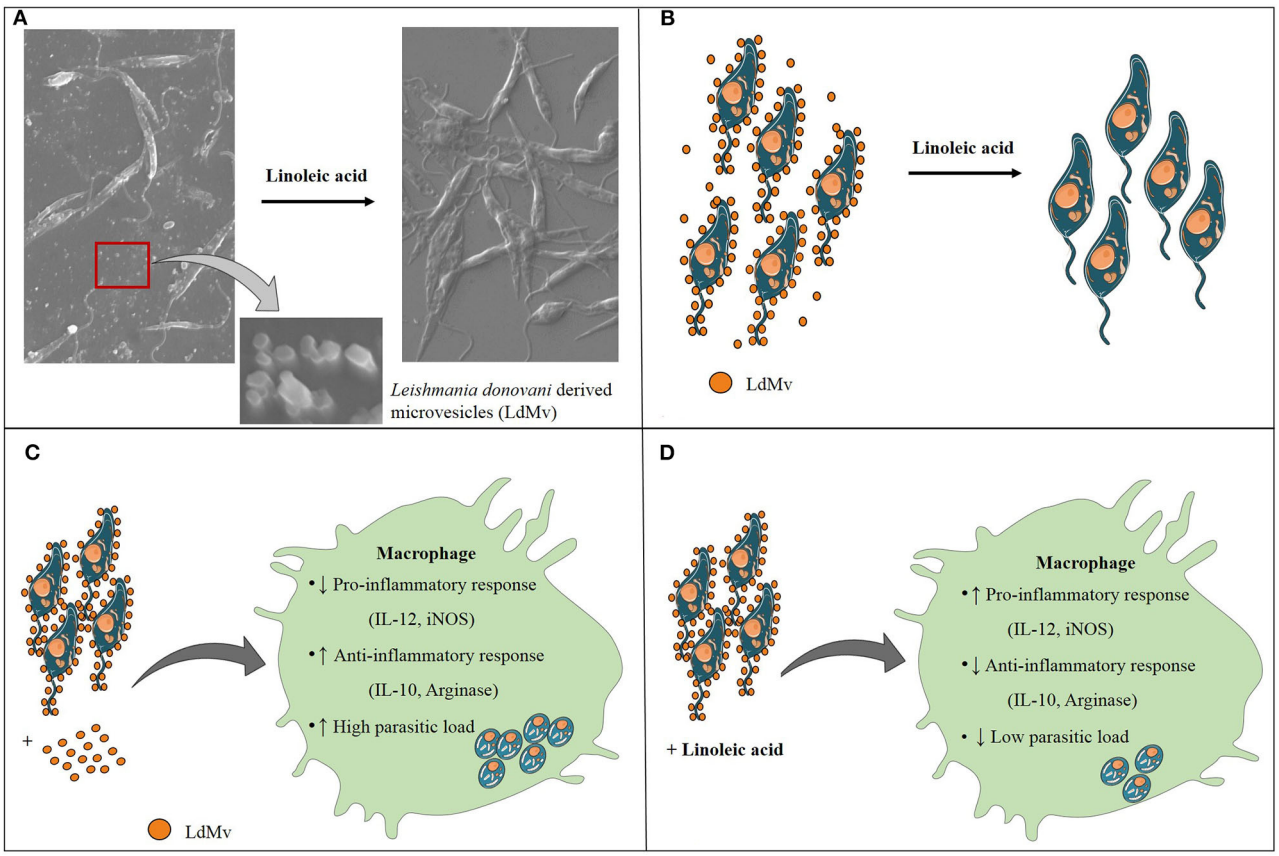

FIGURE 5 | Conclusion of the study. **(A)** Leishmania donovani (Ld) releases microvesicles (Mv), which are suppressed in the presence of linoleic acid. **(B)** Schematic diagram of LdMv inhibition by linoleic acid. **(C)** The infection of *Ld* in the presence of *Ld*Mv (exogenous) suppressed the protective response and up-regulated the inhibitory response, which helped in the dissemination of infection. **(D)** On the other hand, situation was reversed in the presence of linoleic acid which led to the restriction of *Ld* infection.

Hassani (2013) provide evidence about protein release from *L. mexicana* via exovesicles (40–100 nm, size range of exosome) during early moments of interaction with the mammalian host in order to that modulate signaling and functioning of the mϕ. It was further showed that GP63 bearing *L. major* exosomes have inflammatory properties and are capable of immune modulation at both signaling and gene expression levels in mϕ (Hassani et al., 2014). All these studies focused on exosomes, however other types of secreted extracellular vesicles like microvesicles (up to 1 μm) remain unexplored. Our study is the first of its kind which suggests the immunomodulatory activity of *Ld* secreted microvesicle (*Ld*Mv). Our findings indicated that the treatment with *Ld*Mv tilts the immune response toward M-1 type (IL-12 ↑ and iNOS ↑; IL-10 ↓ and Arginase-I ↓). Possibly, the release of *Ld*Mv tunes the mϕ and sets a conducive platform (Th-2 ↑) in the mϕ for the establishment of infection (**Figure 4**). This must also be noted that culture supernatant without *Ld*Mv had no effect on immune response against *Ld* infection (**Supplementary Figure 2**). Not only in the human host, but *Leishmania* parasite also secretes exosomes in the midgut of sand fly and are the part of sandfly's infective inoculum (Atayde et al., 2015). However, the release of Mv by the parasite and its role in the establishment of infection was not yet established. To the best of our knowledge, our study first time demonstrates the release and role of *Leishmania donovani* promastigote derived microvesicles in the establishment of infection. Moreover, its release is inhibited by the presence of LA i.e., a nourished condition, in the culture. We are suggesting that LA plays a dual role in the protective immune response against VL infection. It decreases the release of *Ld*Mv from the promastigote form of *Leishmania* parasite and promotes the pro-inflammatory response inside mϕ via the 5-lipoxygenase pathway to eliminate parasite present inside. This study highlights the immunomodulatory properties of *Ld*Mv and the possible role of nutrient i.e., LA in inhibiting their release which leads to the containment of *L. donovani* infection (**Figure 5**). Conclusively, it is believed that *Leishmania* secreted microvesicles exert modulation of immune responses.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SS performed all the experiments, analyzed as well as interpreted the data, and wrote the manuscript. AR conceptualized, designed the work, managed funding, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00406/full#supplementary-material

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Perils and Promises of Pathogenic Protozoan Extracellular Vesicles

Joshua Seun Olajide[1,2] *and Jianping Cai*[1]*

[1] State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, CAAS, Lanzhou, China, [2] Centre for Distance Learning, Obafemi Awolowo University, Ile-Ife, Nigeria

Extracellular vesicles (EVs) are membranous structures formed during biological processes in living organisms. For protozoan parasites, secretion of EVs can occur directly from the parasite organellar compartments and through parasite-infected or antigen-stimulated host cells in response to *in vitro* and *in vivo* physiological stressors. These secreted EVs characteristically reflect the biochemical features of their parasitic origin and activating stimuli. Here, we review the species-specific morphology and integrity of parasitic protozoan EVs in concurrence with the origin, functions, and internalization process by recipient cells. The activating stimuli for the secretion of EVs in pathogenic protozoa are discoursed alongside their biomolecules and specific immune cell responses to protozoan parasite-derived EVs. We also present some insights on the intricate functions of EVs in the context of protozoan parasitism.

Keywords: protozoa, extracellular vesicles, exosomes, effects, host cells, stressor

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***Correspondence:**
Jianping Cai
caijianping@caas.cn

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INTRODUCTION

Protozoa are single-celled eukaryotes with enormous structural complexity and diversity. The study of parasitic protozoa began in the 17th century (Cox, 2002) and, at the least, there are about 90 etiologic species of important human parasitic diseases (Coakley et al., 2015), while several other species affect economically important animals (Taylor, 2000). Pathogenic and amphizoic protozoa (Gonçalves and Ferreira, 2019) are dispersed within phyla amoeba, apicomplexa, metamonada, parabasalia and kinetoplastida (Szempruch et al., 2016a), which are known to cause a wide range of important diseases such as amoebiasis, malaria, babesiosis, toxoplamosis, leishmaniasis, trypanosomiasis, cryptosporidiosis, trichomoniasis, giardiasis, neosporosis, theileriosis, etc. Over the years, there have been continuous investigations on protozoan parasites' sub-cellular components, organellar structures, secretory/excretory molecules, and, recently, extracellular vesicles (Yanez-Mo' et al., 2015). As revealed from several studies, a substantial amount of parasitic molecules are carried by EVs secreted directly by parasites (Mantel and Marti, 2014), parasite-infected host cells (Atayde et al., 2015), and host cells stimulated by parasite antigens (Wu et al., 2019).

The trajectories of cellular and molecular involvement of pathogenic protozoan EVs during infection are being unfolded (Li et al., 2018a; Correa et al., 2019). Nevertheless, we have tasked ourselves on EVs that are directly secreted by pathogenic protozoa and those of protozoan parasite origin from parasitized host cells in the case of *Plasmodium* species. Briefly, we discuss the biogenesis of protozoan parasites EVs and the activating physico-chemical stressors that are involved in the formation and release of these vesicles. Intrinsic aspects of vesicular cargo content and functions are also discoursed with pathophysiological effects of pathogenic protozoan EVs (PPEVs) on the host cells and protozoan parasites after fusion and/or internalization.

PPEVs: FORMATION, CHARACTERIZATION, AND SUBCELLULAR ORIGIN

EVs are diverse, distinct membrane-bound structures that are formed and discharged as instruments of structural re-organization, stress response and survival among protozoa (Zhang et al., 2014). The release of EVs occurs either through direct budding from cell membranes (ectocytosis) or through the release of preformed vesicles from cellular compartments (exocytosis) (Sadallah et al., 2010). The systemic secretion of EVs is evolutionarily conserved among living organisms (Yanez-Mo' et al., 2015; Sampaio et al., 2017), and it is a constitutive cellular processes among protozoan parasites (Deolindo et al., 2013; Kehrer et al., 2016). Largely, the secretion of vesicles by parasitic protozoa maintains parasite-defensive mechanisms (Wowk et al., 2017), initiation of parasite infection and stronger interaction with host cells (Da Silveira et al., 1979; Ramirez et al., 2017; Moreira et al., 2019).

On the basis of biogenesis and size, and with respect to protozoan parasites, EVs are broadly classified into exosomes, ectosomes [microparticles or microvesicles (MVs)], and apoptotic bodies (Dong et al., 2019; Cronemberger-Andrade et al., 2020). Ectosomes are vesicles formed from protrusions on the plasma membrane (PM), while inward budding of endosomes forms microvesicle bodies (MVBs), which exocytically fuse with the plasma membrane to form exosomes (Lozano et al., 2017). Apoptotic bodies are formed through the condensation and segregation of the nucleus and the deterioration and blebbing of PM (Torró et al., 2018). Incidentally, simultaneous secretion of exosomes and plasma membrane blebs has been predicted among *Leishamania* spp (Montaner et al., 2014). The biogenesis and classification of exosomes and other EVs have been expertly reviewed in depth by Garcia-Silva et al. (2014), Colombo et al. (2014), and Gavinho et al. (2018). The internal volume of an exosome ranges between 20 and 90 nm^3 with a capacity to lodge an estimated 100 proteins and 10,000 nucleotides, values that should be higher in ectosomes and apoptotic bodies (Torró et al., 2018). EVs generally are between 20 nm and 1 μm (Mantel and Marti, 2014) but larger vesicles have been found among protozoan parasite species (Barbosa et al., 2018) (**Table 1**). Unlike the usual lipid bi-membranous layer of EVs, *Leishmania major* promastigote exosomes have their content protected by a phospholipid membrane (Leitherer et al., 2017), and vesicles of *Plasmodium falciparum*-infected red blood cells (*P. falciparum*-iRBCs) are mainly unilamellar (Sisquella et al., 2017).

Also, EVs are classified on the basis of biochemical properties (Kowal et al., 2016) and membrane surface proteins (Wu et al., 2019) which are often used as EV markers (Théry et al., 2018). All categories of EVs have tetraspanins (CD63, CD81, CD82), major histocompatibility (MHC) 1, integrins, endosomal sorting complex required for transport (ESCRT) I-III, ALIX proteins, heath shock protein (HSP) 70, cytoskeletal proteins, and GAPDH as surface markers (Yanez-Mo' et al., 2015; Théry et al., 2018). Importantly, in eukaryotes, CD63, CD9, HSP 70, TSG101, flotillin, and Rab5b are common markers for exosomes (Shao et al., 2018; Gill et al., 2019), whereas microvesicles can be identified by selectins, annexin V, flotillin-2, and CD40, and apoptotic bodies distinctively express annexin V, DNA histone, phosphatidylserine, and genomic DNA as specific markers (Couper et al., 2010; Shao et al., 2018; Wu et al., 2019). Specific transmembrane proteins (e.g., epidermal growth factor receptors) and adhesion proteins (e.g., epithelial cell adhesion molecules) are important pathophysiological EV biomarkers (Shao et al., 2018). Correspondingly, pathogenic protozoa such as *Leishamania* spp have expressed cytoskeletal protein (e.g., actin and tubulin), HSP70, HSP90, HSP83/90, and elongation factor-1 α (EF-1α) as EV markers (Silverman et al., 2010a; Castelli et al., 2019) and many soluble proteins that are contained in the vesicles (Ribeiro et al., 2018). However, a large proportion of the components within microparticles are yet undefined. Again, in some instances, EV markers may not be significantly expressed as observed with annexin V of *P. berghei* (Couper et al., 2010).

Common factors involved in EV secretion especially ESCRT have been shown in the secretion of *T. brucei* exosomes in which the suppression of Vps36, an ESCRT component, led to the compromise of *T. brucei* exosome secretion (Eliaz et al., 2017). Before this finding, it was reported among *Leishmania* spp that, vesicle secretion is rather homologous to the classical exosome secretion pathway found in higher eukaryotes (Atayde et al., 2015). Despite the absence of typical MVBs, *Giardia lamblia* trophozoites exosome-like vesicles were formed in the endosome/lysosome peripheral vacuoles with the involvement of ESCRT, Rab and ceramide (Moyano et al., 2019). Distinct functions of ESCRT in the formation of PPEVs may include mobilization, docking, and fusion (Reiner et al., 1996). The secretion of vesicles when MVBs fuse with lysosomes is also possible in parasitic protozoa (de Souza and Barrias, 2017). In spite of these varying reports, secretion of vesicles by *L. brucei* supposedly occurred by active exocytosis (Geiger et al., 2010), whereas findings on *Giardia intestinalis* microvesicle secretion supported the involvement of cholesterol (Evans-Osses et al., 2017), but it is not yet clear if this phenomenon occurs in all pathogenic protozoa.

In *Plasmodium*, some deviations in the formative process of EVs have been observed. Kehrer et al. (2016) reported that the changes in *P. berghei* exocytic inner membrane compartment led to the eventual fusion of the exocytic membrane and the parasite plasma membrane with subsequent formation of exosome-like structures. Another way of secretion of EVs in *P. berghei* is the reported selective clearance or degradation of some sporozoite organelles with temporal and spatial regulation of membrane components which are finally sorted and packed into vesicles (Jayabalasingham et al., 2010). Consequently, microvesicles from *P. falciparum*-infected red blood cells (RMVs) are distinct from post-rupture vesicles released before parasite egress from red blood cells (Mantel et al., 2013).

Vesicle formations by the budding process from the flagellar pocket are common with species of *Leishmania* and Trypanosomes. Additionally, all developmental stages of *Trypanosoma cruzi* and *T. brucei* perhaps have EVs bud off

TABLE 1 | Preparation and description of pathogenic protozoan-derived Evs.

Clade/species (strain)	Vesicle type	Activatory stimuli	Isolation methods	Sub-cellular origin	Size (mean or range)	Major vesicular content	References
Amoeba							
E. histolytica trophozoite (HM-1-IMSS)	Cytoplasmic vesicles EDG	Liver lesion/TYI-SS MD	?	Plasma membrane, cytoplasm cell periphery	0.1–1.0 μm 50–200 nm	Cationic and actin proteins	Chavez-Munguia et al., 2004
A. castellanii trophozoite (ATTC-30234)	EVs	Glucose MD	2, 5	?	31.9–467 nm 33.7–303.2nm	Serine protease, metalloproteinase, phospholipid, sterylesters, free fatty acids,	Gonçalves et al., 2018
A. castellanii	Exosome-like vesicles	PYG MD/page's Neff's saline	4,3,5	?	166.7 nm	IUNH, carboxylic ester hydrolase, peroxidase, aminopeptdase	Lin et al., 2019
Apicomplexa							
P. berghei (ANKA)	Microparticles	Parasitized RBCs	1,5	iRBCs	150–250 nm	?	Couper et al., 2010
P. berghei sporozoite and gametocyte	Secretory vesicles	Ookinete medium	1	Anterior end of parasite		Pantothenate transporter[1] osmiophilic bodies factor like G377, TRAP	Kehrer et al., 2016
P. falciparum (3D7)	EVs		4,2,5,7	infected RBCs	100–400 nm	Ago2, miRNA	Mantel et al., 2016
P. falciparum	Exosome-like vesicles	CM 2–4% haematocrit	1,6	Maurer's cleft/infected RBCs	∼70 nm	PfPTP2, DNA	Regev-Rudzki et al., 2013
P. falciparum (NF54)	EVs	iRBCS	4,2,7	iRBCs	50–350 nm	(ds) gDNA, tRNA, 5sRNA miRNA (hsa-miR-451a)$^{+}$,mRNA, DNA-binding protein H3, H4	Sisquella et al., 2017
P. falciparum (3D7 & CS2)	Microvesicles	iRBCs	4,2,7	iRBCs	100–250 nm	PVM, RESA, SBP1, Exp1, parasite invasion proteins	Mantel et al., 2013
P. yoelii(17X)	Exosomes	Mice-infection	4	iReticulocytes	∼56.8 nm	serine-repeat antigen, MZ surface protein 1&9, protease hsps, enzymes	Martin-Jaular et al., 2011
P. falciparum (3D7)	EVs	Modified RPMI	1,4,5	Infected cell	∼100	Glycophorine, CD63, PfMSP1, lactate dehydrogenase	Correa et al., 2019
N. caninum tachyzoite (Nc-1)	Vesicles	RPMI, 2% Exo-FBS	1,2,5	Parasite surface	50–150 nm	Functional proteins of ribos-omes, metabolism, RNA transport, hsp70&90, proteosome	Li et al., 2018c
T. gondii tachyzoite (ME49 & RH)	Exosomes	DMEM without serum	2,8	?	10–150 nm	hsp70,surface antigen 1 (SAG1)	Li et al., 2018a
T. gondii tachyzoite (RH)	EVs	RPMI without FBS	1,2,9	Membrane sur-face of parasite	138.2–171.9 nm	15–70 kDa protein spectrum	Silva et al., 2018
T. gondii tachyzoite (RH)	EVs	FBS free DMEM	1,2,8	?	130.8 ± 3.7 nm	Celullar, interaction, metabo-lic, regulation, response protei	Wowk et al., 2017
T. gondii tachyzoite (RH) highly virulent	Exosomes ectosomes	Sterile PBS at 37°C	4,5	Apical & posterrior end, PM	50–200 nm	MIC, ROP, GRA, phosphatase, metabolic proteins	Ramírez-Flores et al., 2019
Kinetoplastida							
T. cruzi epimastegote (Y)	Vesicles	Acetate, NaCl $CaCl_2$	4,5	FP, PM evagination	0.5 μm	Glycoproteins	Da Silveira et al., 1979
T. cruzi blood trypomastigote (Tcl)	EVs	FBS free RPMI	1,2,8		136.33 ± 86.3 nm	TcTASV-C secreted virulence factor	Caeiro et al., 2018
T. cruzi trypomasteg-(Tulahuen)	Exosomal vesicles, TESA EVs	FBS free EMEM	1,5,7	PM	60–100 nm	TESA, trans-sialidases, protease gp63, TolT, MASP, mucin-like protein TASV-C	Bautista-lópez et al., 2017
T. cruzi Epimastegote (Dm 28c clone)	Vesicles, reservosomes golgi-like vesic	Serum free/1% FBS in RPMI	4,5	Golgi complex, cytostome, FP	20–200 nm	TcPIWI-trypomastegote protein tsRNAs	Garcia-Silva et al., 2014
T. cruzi: E,P,A (PAN4 Tcl)	Vesicles	RPMI with 10% free-EV IFCS	5	Parasite surfa-ce, flagellum	50–100 nm	Mucin, MASP with signal peptide (SP)	Lozano et al., 2017

(Continued)

TABLE 1 | Continued

Clade/species (strain)	Vesicle type	Activatory stimuli	Isolation methods	Sub-cellular origin	Size (mean or range)	Major vesicular content	References
T. cruzi (Y, CL-14, YuYu)	Vesicles	RPMI with 5% glucose	1,2,9	membrane sur-face	≤200 nm	Proteins and terminal α-galact-osyl residues	Nogueira et al., 2015
T. cruzi: E, MT (Dm28c[27] clone)	Vesicles, MVs, LVs	DMEM without FBS	2,5	PM, FP	100–200 nm	Host-parasite interaction, signaling, transcription, hsps, chaperons, proteolytic proteins	Bayer-Santos et al., 2013
T. cruzi : T. (Y, CL-Brener)	Vesicles	HBSS	4,5	Cell body, FP	40–500 nm	Acid and alkaline phosphatases	Nievas et al., 2018
T. cruzi: T. (YuYu and Y)	EVs	DMEM with 2% glucose	1,2,10	?	2–3 μm	Transsialidases. MASPs, gp63 tubulin, hsp, mucins, proteases	Ribeiro et al., 2018
T. cruzi: E,T (clone Dm 28c)	EVs	FBS freeDMEM/TAU3AAG	2,5	?	?	rRNA, tRNA, CCD, snoRNA and snRNA	Bayer-Santos et al., 2014
T. brucei gambiense (Feo, Ok, and Biyamina)	Microvesicles	Secretion medium	1,2,5	PM, FP	50–100 nm	Degradation, nucleotide metabolism, folding protein	Geiger et al., 2010
T. b. gambienese(KETRI2482)	Nanotubules/EVs	RNAi-α-KDE1 complement active FBS, inaactvated serum	2,5	FP	70–165 nm	vSG, hsp70, glycerol kinase, matrix glycosomes, mitochondrial membrane protein	Szempruch et al., 2016b
T. brucei: procyclic	Exosomes	Trans-splicing inhibition (Vp36 silencing)	2,5,7	FP, membrane nanotubules	50–200 nm	SL RNA-associated proteins, p22, p27, and p58	Eliaz et al., 2017
L. infatum: P. (clone)	Vesicles	Miltefosine/apoptosis indicers, G418	5,3	?	30–100 nm	gp63, ribosomal protein, hsp70 elongation factor-1α, beta tubulin, β-fructofuranosidases[1]	Santarém et al., 2013
L. donovani, L. major, L. mexicana	Microparticles, (Exosomes, vesicles)	Neutral and acidic medium	4	PM, FP, phagol ysosome	30–70 nm	TESA, trans-sialidases, protease transport, metabolic protein	Silverman et al., 2010a
L. donovani HSP100−/- and wildtype)	Exosomes	RPMI with HEPES, MES	4,2,7	?	?	hsp100, 90, 70.4, gp63, histone, chaperonin proteins	Silverman et al., 2010b
L. major	Exosome-like	Insect	11	Membrane sur	50–120 nm	GP63, calpain-like cysteine peptida	Atayde et al., 2015
L. infantum P	Vesicles	Gut		Face,FP, MVB		se, HSP70, tryparedoxin peroxidase surface antigen protein	
L. infantum P,A	Exosome Vesicles	RPMI pepton Yeast	1,4	?	122 ± 56 nm 115 ± 65 nm	HSP70, HSP83/90, Acetylcholinesterase	Castelli et al., 2019
L. amazonensis P (-M2269)	Evs	RPMI/20% glucose	2,4	Whole body	180 nm	gp63, LPG	Barbosa et al., 2018
T. vaginalis (B7RC2&jtwild)	Microvesicle-like structure	Serum free TYM with $CaCl_2$	1,2,3	PM, Flagellum	100–1,000 nm (>1 μm)	Metabolic enzymes, ribosomal, cytoskeletal, endoplasmin Memebrane vacoule proteins	Nievas et al., 2018
Parabasalia							
T. vaginalis (B7RC2, G3, T1, RU38)	Exosome	TYM without serum	2,5,7	Large vesicular bodies	50–100 nm	Small RNAs, tetraspanins, Alix, Rabs, hsp70, signaling and metabolic proteins	Twu et al., 2013
Diplomonadida							
G. intestinalis	Microvesicles	Serum free YiS with $CaCl_2$	4,5	Trophozoite	201.4 nm	Nuclear, surface, cytoskeletal proteins, and chaperones	Evans-Osses et al., 2017

1. Centrifugation 2. Filtration, 3. Concentration by ultrafiltration/high molecular weight cut-off filter 4. Sequential/serial centrifugation 5. Ultracentrifugation, 6. Buoyant density on Optirep gradient fractionation 7. Buoyant density on sucrose gradient fractionation 8. precipitation by exo-prep kit 9. Gel exclusion chromatography, 10. Size exclusion chromatography 11. Dissection/Suspension in PBS FP, flagellar pocket, PM, plasma membrane T, trypomastegote, E, emastegote A, amastegote, [1]putative, CM, culture medium; MD, medium; EDG, electron dense granules; IUNH, inosine-uridine- preferring nucleoside hydrolase family protein; SAG, surface antigen protein; MIC, microneme proteins; RESA, trypomastigote excreted-secreted antigens; SBP1, skeleton binding protein 1; PVM, Parasitophorous vacuole membrane protein; GRA, dense granule antigens; ROP, Rhoptry protein; TcTASV-C, T. cruzi Trypomastigote Alanine, Valine and Serine rich proteins; PFMSP1, P. falciparum merozoite surface protein.

Summary: Combining filtration/concentration and ultracentrifugation through sucrose gradient cushion retain intact membrane vesicles. Commercial exosome purification kit which could precipitate a wider or more restricted range of vesicles has also been used for PPEVs isolation but its validation requires categorical proof. Populations of vesicles obtained by differential centrifugation and ultracentrifugation, most often provides a mixed population of EVs (Colombo et al., 2014) and soluble proteins that are usually not associated with vesicles (Bayer-Santos et al., 2013). Size exclusion chromatography has been advocated in situation of intended higher yield of PPEVs (Nievas et al., 2018). In the grossest sense, the method adopted to isolate EVs has considerable effects on its proteomic profile and thus compounds the difficulty to extrapolate findings between different proteomic studies of PPEVs. More so, recovered EVs after filtration of culture may not give complete representation of parasites extracellular products. Aside this, physicochemical stimuli also play important roles in the content and function of isolated EVs.

from the plasma membrane (Torrecilhas et al., 2012; Szempruch et al., 2016b). Incidentally, exosomes or microvesicle in *T. cruzi* conceivably have their origin from the tubular network of the endoplasmic reticulum and Golgi (Lozano et al., 2017). Also, there could be plasma membrane-derived vesicles and exosomes formed through fusion of MVBs with the flagellar pocket of epimastigote and metacyclic stages of *T. cruzi* (Bayer-Santos et al., 2013), whereas *Trichomonas vaginalis* microvesicles are derived from endocytic compartments or the plasma membrane (Rada et al., 2019).

Bizarre forms of *T. brucei* vesicles which are independent of ESCRT machinery and autophagy have been observed in addition to EV exocytosis from the flagellar pocket, parasite surface, and MVBs (Eliaz et al., 2017). *Leishmania* spp, on the contrary, use predominantly non-classical mechanisms to direct the release of microvesicles, exosome-like vesicles, apoptotic vesicles, and glycosomes (Silverman et al., 2008). *Giardia lamblia* bulbous excretory secretory vesicles (ESVs) were formed as clefts directly from the early dilation of rough endoplasmic reticulum cisternae (Lanfredi-Rangel et al., 2003). Apparently, the biogenesis and origination of PPEVs seem to be peculiar to parasitic protozoan species and en route differently from the parasite subcellular compartments (**Table 1**).

Pathogenic protozoa, at different developmental stages, can secrete mixed population of exosomes, microparticles (MPs), and apoptotic bodies (Garcia-Silva et al., 2014; Siedlar et al., 2017). Isolates of EVs from similar, but clinically divergent, species of *Leishamania* have displayed distinct profiles (Silverman et al., 2010a). Such distinct EV profiles depend on the life stage, strain, and population of *T. vaginalis* (Twu et al., 2013), *P. falciparum* (Regev-Rudzki et al., 2013), and *T. cruzi* (Moreira et al., 2019). Nevertheless, parasite-shed vesicles are an additional general mechanism that is central to parasite pathogenicity (Torrecilhas et al., 2009). Several specific terms for PPEVs are listed in **Table 1**.

STRESSORS FOR PPEV SECRETION

A large number of parasite niches in hosts and environmental factors are known to orchestrate the release of EVs (Torró et al., 2018). The complexities surrounding the secretion of PPEVs are due to diverse biochemical, physical, and mechanical stressors directed against the parasites *in vivo*. The secretion of PPEVs can be initiated by developmental changes in the parasite's life history as observed in *P. falciparum*, where developmental transition from the trophozoite, schizonts and the ring stages led to increasing MVs secretion (Barteneva et al., 2013). Human serum, at 10-fold bile concentration and pH 3 and 8, has been used to induce microvesicles (MVs) in *G. intestinalis* trophozoites (Deolindo et al., 2013). *Entamoeba histolytica* trophozoite EVs were secreted in liver lesion (Chavez-Munguia et al., 2004) feasibly after parasite exposure to varying physiological and physical conditions in the gut of mice. Physicochemical stressors in the vector mid-gut can also orchestrate the secretion of *T. cruzi*-derived EVs (Fernandez-Calero et al., 2015), and as reported by Gonçalves et al. (2018), *A. castellanii* EV secretion was triggered after re-cultivation in media without a protein source to mimic the physiological stress in the host's aqueous and vitreous humours.

Increasing parasite density could initiate the secretion of peculiar *P. falciparum* EVs *in vitro* (Correa et al., 2019). Congruently, nutrient-starved cultures are often being used to trigger EV secretion (**Table 1**) with claims that it mimics the hostile environment of the vector hind gut in the case of *T. cruzi* (Fernandez-Calero et al., 2015). On this premise, incubation of *T. cruzi* epimastigotes in culture media without fetal bovine serum (FBS) also triggered parasite transformation and disposal of some proteins via vesicle secretion (Bayer-Santos et al., 2013; de Souza and Barrias, 2017). However, chemical compositions of culture media will produce specific cargo that reflect the culture conditions and developmental stages of *T. cruzi* strains (Fernandez-Calero et al., 2015) just as comparable nutritional stress media triggered the release of different sizes of EVs among *T. cruzi* strains (Ribeiro et al., 2018). Apparently, serum starved media/chemically defined culture will trigger the formation of vesicles and induce apoptosis (Pope et al., 2013; Gonçalves et al., 2018) which may lead to myriad composition, size, and biosynthesis of PPEVs (**Table 1**).

Likewise, disintegration of *T. vaginalis* during *in vitro* cultivation produced vesicles which subsequently bind to the cell surface (Rada et al., 2019) and add to the existing subpopulations of EVs. The use of antibiotics as component of culture media may equally trigger typical secretion of EVs, although the implication of this was not clearly mentioned with the use of gentamicin as a component of culture media in the studies of *P. falciparum* EVs (Mantel et al., 2016; Sisquella et al., 2017; Castelli et al., 2019). Certainly, the biology of a specific parasite may play important roles in the preparation of culture media but it would be interesting to find out, if any, the functional difference (s) between PPEVs that are stimulated by drug and serum starvation and, also, the roles of drug-triggered EVs in parasite pathobiology in relation to drug selection and drug resistance because the involvement of HSPs in drug resistance among *Leishmania* strains has been speculated (Patino et al., 2019).

PPEV secretions have also been stimulated in culture media supplemented with calcium compounds (Cocucci et al., 2009). In fact, incubation of *T. vaginalis* with calcium chloride in regular media produced a 9-fold increase in parasite MVs (Nievas et al., 2018). The secretion of EVs by *G. lamblia* trophozoites has been recently shown to be stimulated by the addition of calcium into the culture medium (Moyano et al., 2019). Sodium compound at different concentrations and pH values have been shown to induce plasma membrane vesiculation in *T. cruzi* epimastigote (Da Silveira et al., 1979). Remarkably, purified tachyzoites of *Toxoplasma gondii* maintained in PBS at 37°C were able to secrete vesicles (Ramírez-Flores et al., 2019), but from the report of Barbosa et al. (2018), a temperature of 37°C impaired the secretion, biological effects, and cargo content of EVs released by *L. amazonensis* promastigote in culture.

Considerably high temperature at acidic pH induced the secretion of exosomes in *L. donovani/L. major* (Silverman et al., 2010b) and activation of *L. donovani* promastigote exosomes in parasitophorous vacuole (Deolindo et al., 2013), but only

pH was responsible for *in vitro* secretion of the *T. cruzi* vesicle (Da Silveira et al., 1979). Meanwhile, massive secretion of exosomes by *Trypanosoma brucei* subjected to heat shock has been established (Eliaz et al., 2017). Conversely, Deolindo et al. (2013) hypothesized that *Giardia intestinalis* trophozoites released MVs to resist pH change. There is also a report of exosome secretion by co-incubation of genetically modified and wild-type strains of *T. brucei*, but changes in environment factors prominently influenced the release of vesicle and cargo content (Torrecilhas et al., 2012). Likewise, the formation of vesicles by pathogenic protozoa can also occur in responses to host cell interaction (Nievas et al., 2018). The concern would be to find out if the degree of parasite virulence corresponds to vesicle secretion.

Relatively, centrifugation steps can disorganize tubules into vesicles similar to ectosomes and exosomes (Ramírez-Flores et al., 2019), indicating that laboratory treatment may influence extant populations of EVs, but whether tubule disorganization into the vesicle by gravitational shearing represents EV activation through cell–cell abrasions or cell–parasite contacts requires further clarification. Nonetheless, other stressors that can initiate the secretion of PPEVs include cell topography, apoptosis or autophagy (Yanez-Mo' et al., 2015), hypoxia, and irradiation (Torró et al., 2018). Substantial secretion of EVs by parasitic protozoa is due to nutritional stress, pathogenesis, anti-proteolysis, antigen presentation, and parasite growth. The consideration to find combinatory stressors that support the maximum secretion of EVs and the physiological/behavioral implications for a specific protozoan parasite will be good for the field. Nonetheless, *Acantamoeba castellanii* EVs could equally act as stressors and induce the secretion of EVs from the host cell membrane (Gonçalves et al., 2018).

FUNCTIONS OF PPEVs

The biological functions and cargo composition of PPEVs are dependent on the parasite from which they are secreted (Deolindo et al., 2013), and the amount of EVs secreted by *L. amazonensis* promatigote is dependent on the period of exposure to stressors (Barbosa et al., 2018).

Roles of PPEVs in the Parasite Community

The formation and release of vesicles enhance survival, transmission, and mitotic multiplication of pathogenic protozoa (Roditi, 2016). Also, vesicle-dependent and proportional 5-fold increase in absolute number of *T. cruzi* trypomastigote suggests that parasite-derived vesicles could initiate parasite replication (Garcia-Silva et al., 2014). Conversely, *P. falciparum*-infected cell-secreted EVs carried suicidal signals that could induce parasite death (Correa et al., 2019). Moreover, this raises the question: are PPEVs carrying death signals directed against other parasites in the population, or are they self-targeting?

PPEVs can actuate intra- and inter-specific quorum sensing. For instance, *T. vaginalis* vesicles interacted with other *T. vaginalis* strains and, in the process, enhanced cyto-adherence of the recipient strain (Nievas et al., 2018), which showcases EVs as mediators of intra-specific interactions (Twu et al., 2013). Likewise, tachyzoites of *T. gondii* invaded more cells when incubated with *T. cruzi* EVs. However, the incubation of *T. gondii* and EVs from *Crithidia mellificae* choanomastigotes did not orchestrate an increase in the number of infected cells (Moreira et al., 2019). Even high concentrations of MVs from *G. intestinalis* were unable to enhance the invasion of *T. cruzi* metacyclic trypomastigotes (Evans-Osses et al., 2017). Taken together, these evidences suggest that EVs can enhance parasite pathogenesis by exposing certain host cell surface ligands or molecules which the invading parasites can recognize, but how protozoan parasites distinguish EVs from unrelated species is yet to be determined.

EVs also play important roles as channels of intercellular communication within parasite population (Gavinho et al., 2018). Additionally, as suggested by Geiger et al. (2010), *T. brucei* may possibly use MVs to communicate between trypanosomes by exchanging non-protein cytosolic receptors or genomic information to harness survival strategies. Similarly, the vesicles of *T. vaginalis* might have functional roles in mediating communication between parasites during infection (Nievas et al., 2018). Intact exosomes can potentially regulate parasite migration by transmitting repulsive signals to facilitate communication and social migration of *T. brucei* (Eliaz et al., 2017). Secretory vesicles have likewise been shown to affect parasite motility and egress from infected cells. Specifically, certain G377-containing vesicles enhance parasite egress from parasitophorous vacuoles and RBC membranes. Inability of *P. berghei* to secrete such vesicles obliterated further transmission and motility (Kehrer et al., 2016). RMVs from *Plasmodium*-infected RBCs could as well stimulate and regulate gametocyte production (Mantel et al., 2013).

PPEVs in Host-Parasite Interactions

Szempruch et al. (2016a) suggested that communication between host cells and *T. brucei* occurred via assemblage of its fusogenic EVs which may serve as vehicles for parasite-host cell transfer of membrane proteins as *L. donovani* exosomes showed long-range communication with naive host cells (Silverman et al., 2010a). EVs derived from *P. falciparum*-iRBCs traverse the infected cells and are capable of promoting a parasite developmental switch (Mantel et al., 2016). Remarkably, exosome-like vesicles of *P. falciparum*-iRBCs transferred parasite DNA and acted as an emissary that induced sexual differentiation, parasite survival, and communication within the population of parasite-infected red blood cells (iRBCs) (Regev-Rudzki et al., 2013). *in vitro* incubation of EVs containing DNA with host cells revealed higher mRNA induction in host cells and is suggestive of the fact that PPEVs are carriers of signal molecules and could travel farther in cytosolic milieu (Sisquella et al., 2017) (**Figure 1**).

Shed vesicles represent an additional mechanism by which parasites present antigens to the host and play a pivotal role in acute parasitic disease. *T. cruzi* vesicles interact with target cells in ways that may be difficult for free molecules, and as such, exhibit the horizontal transfer of parasitic molecules and parasite extensions (Yanez-Mo' et al., 2015). Similarly, in the context of default survival plan by protozoan parasites, the release of EVs may be an efficient strategy employed by the parasite to

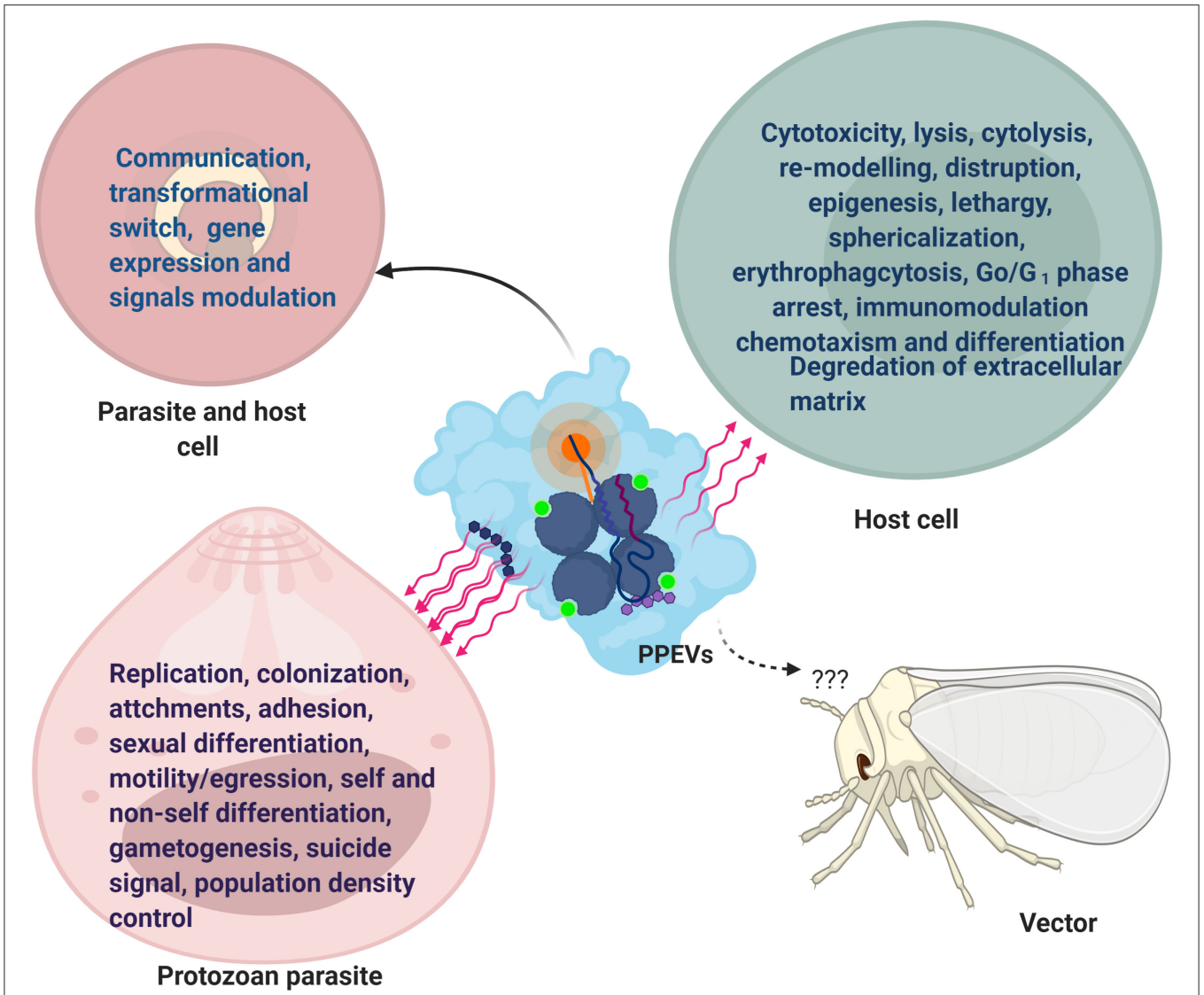

FIGURE 1 | Functional Effects of PPEVs. After-effects of PPEV interaction and internalization by recipient cells and protozoan parasites. The common effects in parasites and host cells are seen in communication, development, and gene expression.

protect parasitic biomolecules against extracellular degradation (Bayer-Santos et al., 2013). This is in accordance with the general function of MVBs to prevent cells from proteotoxicity through the formation and accumulation of intraluminal vesicles (ILVs) (Lozano et al., 2017). Also, co-egested *L. major* exosomes with *Leishmania* parasites during a blood meal by an infected sand fly possibly exert separate influence during transmission and early events of an infectious process in the host (Atayde et al., 2015), but the effects of parasite-derived EVs on vectors have not been reported (**Figure 1**).

EVs have the potential to increase parasitemia in host. For instance, prior inoculation of *T. cruzi*-derived EVs in mice showed over two times the number of parasites in blood and two times more amastigote nests in hearts (Lovo-Martins et al., 2018). The inoculation of *L. infantum* extracellular products potentiates dose-dependent infection *in vivo*, and EV populations significantly correlated with parasite numbers (Pérez-Cabezas et al., 2018). The role of EVs in host cell invasion is typified by the ability of *T. vaginalis* exosomes to prime the urogenital tract for the purpose of parasite colonization (Twu et al., 2013). Further still, the addition of *G. intestinalis* MVs to methyl-β-cyclodextrin-mitigated *G. intestinalis* trophozoites restored its attaching ability for subsequent invasion (Evans-Osses et al., 2017).

Moreover, the prior inoculation of *T. cruzi* vesicles in mice before parasite infection heightened pathogenicity to 100% mortality. These *T. cruzi*-shed membrane vesicles aggravated severe heart inflammation and increased the number of intracellular amastigote nests. It was shown further that *T. cruzi* vesicles could not directly induce significant pathology in mice, and injection of *T. cruzi* EVs into mice before trypomastigote

infection led to a transient but substantial increase in parasitemia (Torrecilhas et al., 2009). *L. major* exosomes possibly heightened the formation of a footpad lesion in mice as co-inoculation of *Leishmania* parasite and *Leishmania* exosomes exhibited a 3–4-fold increase in lesion volume than with parasites alone (Atayde et al., 2015). Barbosa et al. (2018) reported similar footpad lesion due to *L. amazonensis* EVs as well as increased parasitic load. Unassumingly, *T. cruzi*-derived vesicles may influence parasite proteolytic activity on the host tissue (Torrecilhas et al., 2009). However, the lytic effect of purified *T. cruzi* vesicles on host cells was transiently local to the site of inoculation in mice, suggesting that not all the RBCs in the circulation are equally vulnerable or probably lack certain corresponding molecular signatures (Roditi, 2016).

Perceptibly, initial host cell treatment with PPEVs would increase host cell parasitization. For instance, EVs of *T. cruzi* Y strain made the host cell more susceptible to parasite entry in the first moments of infection (Lovo-Martins et al., 2018). Dong et al. (2019) have lately reported that EVs have the capacity to favor infection and propagation of parasites in the hosts. Besides the fact that secreted vesicles have ability to fuse with susceptible host cells, parasite-derived MVs can fuse with host cell-derived MVs (Ramirez et al., 2017), and the amount of reacting EVs is proportional to the percentage of infected cells (Ribeiro et al., 2018). Such level of host-parasite interaction needs further elucidation. Furthermore, *Acanthoamoeba catellanii* EVs have shown cytotoxic effects and hampered mammalian epithelial cell viability by the action of its degrading enzymes. However, the extent of host cell damage was dependent on cell type and the dose of *A. castellani* EVs (Gonçalves et al., 2018) (**Figure 1**).

Similarly, there is report of cell sphericalization, disruption, reduction in adhesive ability, and cytolysis of rat glial cells following the exposure to *A. castellanii* EVs (Lin et al., 2019), just as *T. gondi* exosomes affected macrophage viability in dose-dependent mode (Li et al., 2018a) and EVs secreted by *T. cruzi* induced epigenetic changes in susceptible mammalian cells (Fernandez-Calero et al., 2015). When *T. brucei* EVs were fused with mammalian erythrocytes, erythrophagocytosis, the cause of anemia during acute trypanosomiasis, was reported (Szempruch et al., 2016a). In Chagas disease, *T. cruzi* EVs and the incorporated mucin and mucin-associated surface protein (MASP)-specific peptide inhibited host cell lysis facilitated by the human complement system (Lozano et al., 2017). Apart from reported pathological fall out, EVs from *P. falciparum*-iRBCs contributed to vascular dysfunction, endothelial activation/leakage, and parasite sequestration (Mantel et al., 2016) (**Figure 1**).

Experimental evidence suggests that shed vesicles of the *T. cruzi* sE48 strain significantly enhanced metacyclogenesis of the host cell (Garcia-Silva et al., 2014). Also, the incubation of EVs of the *T. cruzi* Pan4 strain with Vero cells induced intracellular mobilization of Ca^{2+}, causing the reversible disruption of the actin filaments and formation of filopodia, and finally halted cell cycle at G0/G1. Promastigote and amastigote exosomes of *L. infantum* caused host cell chemotaxism, and *L. infantum* amastigote exosomes specifically caused the differentiation of monocytes into macrophages (Castelli et al., 2019). Similarly, Moreira et al. (2019) demonstrated that *T. cruzi*-derived EVs could alter host cell architecture, membrane permeabilization, and exposure of epitopes to antibodies (**Figure 1**).

Nonetheless, the thermal treatment of *T. cruzi* EVs and enzymatic/chemical treatment with proteases and sodium periodate inhibited *in vitro* host cell parasitization by trypomastigotes of *T. cruzi* (Moreira et al., 2019). Moreover, disruption of the exosomal membrane and boiling abrogated the *L. major* exosome ability to enhance lesion size and decreased parasite load, suggesting the fact that intact exosome integrity plays important roles in diseases progression (Atayde et al., 2015). An example of a PPEV effect outside the host cell is the report of liberated electron dense granules proposed to be contained in vesicles of *E. histolytica* trophozoites, which could initiate the degradation of extracellular matrix through the action of its proteolytic enzymes (Chavez-Munguia et al., 2004) (**Figure 1**). It is not clear, however, whether there are degredative (lytic) and messenger PPEVs that are used to target host cells and protozoan parasites, respectively.

MECHANISMS OF PPEVs INTERNALIZATION

EVs have different half-life because they can quickly be taken up by target cells and thus exist only around the pathogen (Théry et al., 2009). Also, EV disappearance from circulation may be due to its uptake during interaction with the target cell and in the process becomes internalized (Mantel et al., 2016; Eichenberger et al., 2018). The disappearance of *Leishmania* exosomes is likely due to cellular uptake, membrane dissolution, or subsequent degradation after binding to naive cells (Silverman et al., 2010a). The approximate time for PPEV existence in circulation before cellular uptake is exemplified by *T. cruzi*-shed membranes that had a half-life of 3.5 h with respect to the half-life of Tc-85 protein released by the parasite (Torrecilhas et al., 2009). This is comparably consistent with other experimental evidence that posited 3 h as enough time for exosome uptake by the target cell (Cheng and Zeng, 2019). However, pathogenic protozoan exosomes have been hypothesized to have different kinetics in target cells (Silverman et al., 2010a).

PPEVs Internalization by Host Cells

There are indications that a large number of protozoan parasite exosomes can be internalized by host cells (Li et al., 2018a) with postulated mechanisms of receptor-mediated, fluid-phase endocytosis, or direct fusion with host cells (Szempruch et al., 2016b). *T. cruzi*-derived vesicles have been suggested to be endocytosed by host cells (Bayer-Santos et al., 2013). Hypothetically, vesicular content can be delivered to host cells through fusion of EVs with the host cell, endocytotic assimilation, or progressively by a control-delivery system (Roditi, 2016; Li et al., 2018b). The sequence of PPEV internalization might involve binding to host cells through receptor–ligand interactions, and in the process become putatively attached to the target-cell membranes before endocytosis (Valadi et al., 2007).

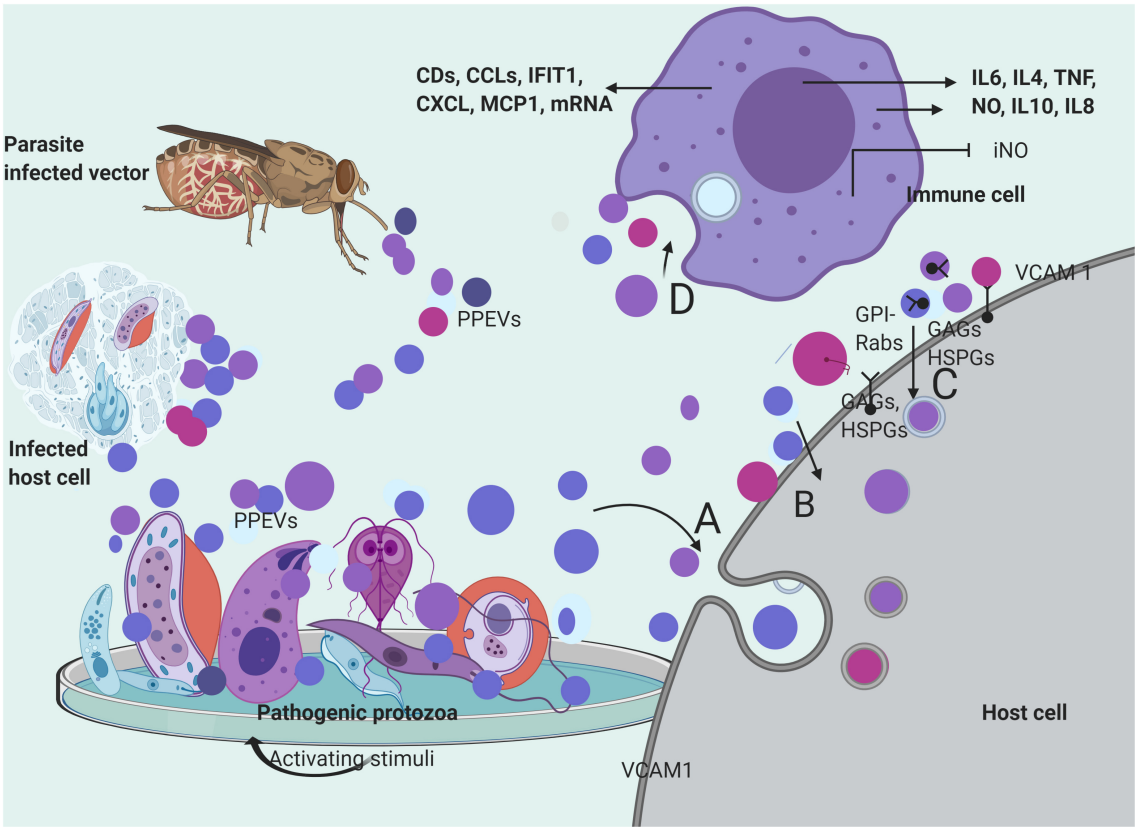

FIGURE 2 | Secretion and mechanisms of PPEVs internalization. PPEVs are formed after protozoan parasite exposure to various chemical and mechanical triggers in host cells, *in vitro*, and in the gut of vectors. After secretion, PPEVs are quickly taken up by susceptible cells, but various mechanisms have been proposed for internalization process. **(A)** bulk transport of PPEVs across the cell membrane via endocytic assimilation involving phagocytosis and pinocytosis. **(B)** PPEVs fuse with the host target cell before consequent internalization which may be mediated by Caveolin-dependent pathways. Lipid rafts, cholesterol, and lectin on PPEV membranes and host cells play significant roles in this respect. In the process of caveolin-dependent pathway, host cell caveolin-1 acts as regulator, and HSPGs acts as receptors for *T. vaginalis* EVs. Alternatively, using the mechanisms of membrane transporters, *L. donovani* exosomes could hijack the host retrograde trafficking pathway to directly dump exosomal cargo into the host cell (Garcia-Silva et al., 2014). **(C)** Possible uptake of PPEVs via receptor-ligand interactions involving receptors and proteins on host cells and membranes of PPEVs. Clathrin is a protein scaffolding found coating eukaryotic vesicles, which plays important role in receptor-mediated endocytosis of PPEVs through the plasma membrane associated with different adaptor proteins for clathrin-coated EVs. PPEV membrane protein can thus interact with receptors on the target cell and activate intracellular signaling. In this process, Rab5 protein mediates endocytosis and fusion of clathrin-coated vesicles. Also, GPI anchors on the vesicles may facilitate fusion to the host cell. Surface expression of vascular cell adhesion protein-1 (VCAM1) in endothelial cells has been demonstrated as a host cell response to iRBC EV uptake, which is significant in vascular dysfunction (Mantel et al., 2016). **(D)** Process by which immune cells interiorize PPEVs. A fundamental basis for PPEV internalization by immune cells has been linked to endocytosis and phagocytosis. There has been no empirical proof for ligand-receptor-mediated fusion and internalization of PPEVs by host immune cells. However, internalization of PPEVs by immune cells can redirect cytokine secretions and differential regulation of immune pathways.

Ramírez-Flores et al. (2019) reported that endosome-associated Rab proteins played some roles in the fusion of *L. infantum* vesicles to host cells and the formation of tubules even though electroporation of myelogenous leukemia cells with *T. cruzi* epimastigote EVs showed clear incorporation of labeled EVs following a series of endocytosis and exocytosis (Garcia-Silva et al., 2014) (**Figure 2**).

Glycosaminoglycans (GAGs), heparan sulfate proteoglycan (HSPGs) chains, and other unidentified host cell surface components mediated *T. vaginalis* EV uptake because the loss of host cell surface GAGs and HSPGs reportedly reduced, but did not completely block the uptake of *T. vaginalis* EVs (Rai and Johnson, 2019). Furthermore, the treatment of the host cells with a specific inhibitor of lipid raft-mediated endocytosis reduced *T. vaginalis* EV uptake to a considerable point. To this end, the cellular uptake of *T. vaginalis* EVs required cholesterol in addition to caveolin-dependent, lipid raft proteoglycan-mediated endocytosis (Rai and Johnson, 2019). Incidentally, Garcia-Silva et al. (2014) reported *T. cruzi* vesicles coated with clathrin from early endocytosis and the trans-Golgi membrane network. However, the inhibition of both Clathrin-mediated and caveolin-dependent endocytosis through Dynasore blocked EV uptake. This substantiates the endocytic internalization of *P. falciparum*-iRBC-derived EVs by endothelial cells (Mantel et al., 2016) and phagocytosis-like mechanisms through which *P. falciparum*-iRBC-derived microvesicles (RMVs) were enclosed in additional membranes after internalization (Mantel et al., 2013) (**Figure 2**).

Moreira et al. (2019) reported that *T. cruzi* EVs appear to adhere to the host cell through its surface lectin scaffolds, but the presumed enzymatic activities of glycosylated proteins of *T. cruzi* EVs during adhesion/internalization by the host cell require elucidation. Also, fusion with the host cell, before cargo delivery, has been reported in the case of *T. vaginalis* exosomes (Twu et al., 2013). *A. castellani* EVs were first found localized and accumulated within the phospholipid-rich membrane of epithelial cells before cytoplasmic phagocytosis. The elapsed accumulation of the amoebic EVs within the lipid epithelial membrane indicates the involvement of host cell lipid raft in the internalization process (Gonçalves et al., 2018) (**Figure 2**). However, the physiological mechanism that produced epithelial cell membrane associated EVs after *A. catellani* EV adhesion requires further elucidation. Similarly, Rat glial cells have internalized *A. castellanii* EVs but the mechanism involved remains unknown (Lin et al., 2019) (**Figure 2**).

In respect to PPEV internalization by immune cells, stained EVs of *Neospora caninum* have been found to randomly accumulate within the cytoplasm of macrophages (Li et al., 2018c), while fluorescent *P. falciparum*-iRBC-derived EVs were observed in the perinuclear region of the human bone marrow-derived endothelial cell (Mantel et al., 2016). Importantly, temperature could have facilitated this endocytotic internalization because *P. falciparum*-iRBC-derived EVs were significantly detected in monocytes at 37°C (Sisquella et al., 2017), as well as *G. intestinalis* MV internalization by immature dendritic cells (iDCs) which were later inhibited almost completely at 4°C and by the addition of cytochalasin D (Evans-Osses et al., 2017). In addition, iRBC-derived EV internalization by macrophages was sensitive to cytochalasin D (Mantel et al., 2016) (**Figure 2**). However, apart from the probable endocytotic process, it is yet to be determined if dyes confer additional properties on PPEVs to facilitate internalization by immune cells. In summary, the peculiarity of the host cell, plasma membrane architecture, PPEV lipid membrane, and cargo content plays significant roles during the internalization of protozoan parasite EVs (**Figure 2**).

PPEV Internalization by Protozoan Parasites

Studies have shown that protozoan parasites can as well-internalize EVs from related and unrelated species. *T. cruzi,* speculatively, makes use of a clathrin-mediated endocytosis machinery to internalize the tsRNA cargo of exogenous sources (Garcia-Silva et al., 2014), whereas, endocytic activity in *T. brucei* correlated with expression levels of the clathrin-independent pathway due to the enrichment of GPI-anchored proteins on the *T. bricei* plasma membrane (Allen et al., 2003) which is also found enriched in EVs (Raposo and Stoorvogel, 2013). It can be deduced, therefore, that the use of both autonomous clathrin-mediated endocytosis and clathrin-independent pathway (via GPI-anchors) may explain the short half-life and rapid disappearance of PPEVs. The incubation of exosomes from modified *T. brucei* with *T. brucei* wild-type revealed that the co-opted *T. brucei* exosomes were observed around or within the lysosomes of *T. brucei* wild-type (Eliaz et al., 2017).

Protozoan parasites may use contiguous receptor-independent endocytosis to internalize vesicles despite the array of protein anchors and membrane receptors on protozoan parasites. To substantiate this, Szempruch et al. (2016b) reported that the binding and uptake of *T. brucei* EVs by *T. b. brucei* was receptor-independent, which was first mediated by fusion with the mammalian erythrocyte membrane. Also, PKH67-labeled RMVs have been efficiently incorporated into *P. falciparum*-iRBCs with eventual accumulation in the parasite nuclear periphery, but only a subset of RMVs were internalized, signifying that not all *P. falciparum*-iRBCs were receptive to RMV uptake (Mantel et al., 2013). In congruence with EV fusion through lipid raft, EVs from *T. b. brucei*$^{SAR-Ty}$ fused with the membrane and flagellar pocket of adjacent *T. b. brucei*, which led to the internalization of the vesicles and associated protein in the endolysosome (Szempruch et al., 2016b). Furthermore, the internalization of EVs by parasitic protozoa can be aided by dissolution of parasite plasma membrane. For example, detergent treatment of *T. b. brucei* increased its membrane permeabilization and ensued the transfer of serum resistance-associated (SRA) proteins contained in *T. b. brucei* EVs (Szempruch et al., 2016b).

PPEVs BIOACTIVE MOLECULES: EXPORTS AND FUNCTIONS

Among parasitic protozoa, the composition of EVs includes proteins, carbohydrates, lipids, nucleic acids, virulence factors, resistant genes (Szempruch et al., 2016b; Sisquella et al., 2017), and unprocessed proteins (Lozano et al., 2017). Also, some PPEV-encapsulated biomolecules are classical EV markers (Mantel et al., 2013), immune modulators, mediators of intracellular signaling, host-parasite interactions, membrane fusion, transporters, and oxidation-reduction processes (Geiger et al., 2010; Moreira et al., 2019). However, the functions of PPEVs rely on preparation, time of reaction, temperature, pH and most importantly, species and strain of origin (Twu et al., 2013; Montaner et al., 2014; Nogueira et al., 2015; Silva et al., 2018; Moreira et al., 2019) (**Table 1**). PPEV bioactive molecules immensely contribute to parasite development, and it is likely that protozoan parasites secrete biochemically different EVs at every developmental stage so as to adapt to a changing environment as exemplified by *L. infatum* which has significant enrichment of ribosomal and RNA transport proteins during the parasite growth at the log phase but an abundance of cellular processes and oxidative phosphorylation proteins at the stationary phase (Santarém et al., 2013).

Nucleic Acids

The secreted nucleic acids in PPEVs vary with organisms, activating factors, and the subgroups of the EV population. Distinct types of small RNAs (sRNAs) in *A. castellanii* EVs were reported to be modulated by nutritional stress (Gonçalves et al., 2018). In addition, *T. cruzi* epimastigotes under nutritional

stress have a specific population of sRNA packaged into their vesicles for possible interactions with host cells (Fernandez-Calero et al., 2015). PPEVs are carriers of messenger RNAs (mRNAs), microRNAs (miRNAs), different types of mediators (de Souza and Barrias, 2017), RNA, and genomic DNA (gDNA) (Sisquella et al., 2017). Functionally, mRNAs in *T. gondii* EVs were recognized by the host immune system (Silva et al., 2018), and *T. cruzi*-secreted EVs contained sRNA, transfer RNA (tRNA), (small nucleolar) sno/(small nuclear) snRNAs, and specific Piwi proteins in complex association with ribonucleoprotein (Fernandez-Calero et al., 2015). The differential packing of sRNAs in PPEVs has revealed that the distinction between *Leishmania* epimastigote and metacyclic trypomastigote stages (Bayer-Santos et al., 2014). Garcia-Silva et al. (2014) demonstrated the release of nucleic acids from PPEVs by showing that tsRNAs in *T. cruzi* vesicles are delivered to adjacent *T. cruzi*. Mantel et al. (2016) reported that tsRNAs of *T. cruzi* epimastigote MVs have a longer nucleotide sequence than tsRNAs in the *T. cruzi* subcellular region. Conversely, secreted exosomes of *T. brucei* containing spliced leader RNA (SL RNA) affected the social motility of procyclic trypanosome with sheer dependence on perceptible genetic signal (Eliaz et al., 2017), and *P. falciparum*-derived vesicles altered cellular function via changes in EV-derived miR-451a-Argonaute2 complexes and target gene expressions (Mantel et al., 2016; Rivkin et al., 2017). Nevertheless, the potential functions of abundantly detected non-coding RNAs in the *P. falciparum*-EVs are yet to be clarified (Sisquella et al., 2017) as well as the extra nucleotide extension of tsRNAs of *T. cruzi* MVs.

Proteins and Virulence Factors

The secreted exosomes of *L. major* promastigotes and amastigotes function as the main protein secretory pathway (Leitherer et al., 2017), and the involvement of *T. vaginalis* vesicles in the export of adhesin proteins has been confirmed by immunofluorescence analyses (Rada et al., 2019). Of the total proteins released by *T. cruzi* EVs, prediction holds that about 57% were secreted through classical and non-classical pathways. This therefore lends credence to the evidence that the *T. cruzi* secretome is formed by proteins that are transported in EVs (Lozano et al., 2017). The proteomic analysis of *T. brucei*-derived EVs speculated that the flagellum might play a considerable role in the sorting and delivery of its biologically active molecules to neighboring cells (Szempruch et al., 2016b). *T. gondii* tachyzoite vesicles contained dense granular protein with an indication that dense granules and self-assembled vesicle-tubular structures are a potential source of proteins in the vesicle (Ramírez-Flores et al., 2019). Essentially, properties of *L. donovani* wild-type and mutant strain vesicles were influenced by the specificities of cargo packaging regulated by HSP100 (Silverman et al., 2010b). Additionally, the putative pantothenate protein (PAT) and HSP100 of *P. berghei* secretory vesicles were necessary for the expulsion of vesicular content into the parasitophorous vacuole (Kehrer et al., 2016), just as extracellular *T. gondii* tachyzoites constitutively secreted soluble components of the vesicles within the parasitophorous vacuole (Ramírez-Flores et al., 2019). Meanwhile, a subtle contrast has been found in *T. cruzi* where the predominant EV proteins were likely anchored on the parasite surface via GPI lipid or inserted into the EV membrane past a conserved C-terminal region (Bautista-lópez et al., 2017).

The functional array of molecules in pathogen-derived EVs has been concisely reviewed by Kuipers et al. (2018) (**Table 1**). Succinctly, EVs from *A. castellanii* are purportedly rich in aminopeptidase and proteases which contributed to the pathogenesis, host tissue damage, and cell death (Gonçalves et al., 2018; Lin et al., 2019). Enteric *Entamoeba histolytica* has also been reported to secrete vesicles containing actin and cationic proteins with proteolytic activities (Chavez-Munguia et al., 2004). As well, *T. vaginalis* exosomes contained surface proteins and proteases which enhanced parasite adherence (Twu et al., 2013), though the presence of proteases, kinases, and glycosidases in *A. castellani* EVs contributed to parasite establishment and the colonization of the host tissues (Gonçalves et al., 2018). *P. falciparum* lactate dehydrogenase with relative abundance in *P. falciparum*-iRBC-derived EVs had the capacity to communicate a suicidal signal (Correa et al., 2019).

Fundamentally, a large proportion of proteins and virulence factors are secreted in membrane-bound vesicles (Deolindo et al., 2013; Ribeiro et al., 2018), but at this point, it is needful to point out that the expression of virulence factors in PPEV cargoes may be connected with the relative abundance of certain biomolecules and other defining factors. Nogueira et al. (2015) reported that EVs from extremely virulent *T. cruzi* Colombiana expressed much less α-Gal epitopes than virulent strains, but it remains uncertain if EV-incorporated molecules correlates with virulence during host-parasite interaction given the condition of *in vitro* stimulation of EVs and host genetic factors, and the physiological condition within the vector. Identified virulence factors in *Leishamania* EVs include gp63, redox enzymes like tryparedoxin peroxidase, and HSPs (Montaner et al., 2014), whereas African trypanosome EVs contained and expressed serum resistance-associated (SRA) protein (Szempruch et al., 2016b). Virulence factors including the transsialidase family of glycoproteins, cruzipain, and MASPs have been found in *T. cruzi* EVs, which predicates *T. cruzi* pathogenesis (Lozano et al., 2017) and virulence (Ribeiro et al., 2018) (**Table 1**). Nevertheless, further clarifications on the roles of specific putative PPEV antigenic molecules and factors responsible for PPEV molecular sorting will be of tremendous addition to the study of parasitic protozoan EVs.

PPEVs could also contain specific antigenic proteins as observed in *T. gondii* exosomes which participate in parasite invasion and replication (Silva et al., 2018). Bautista-lópez et al. (2017) has also pointed out that phosphatases and membrane-bound proteins of *T. cruzi* EVs triggered Ca^{2+} signaling with lysosome mobilization and exocytosis that enhanced the formation of parasitophorous vacuoles and parasite invasion. *T. cruzi* membrane-shed vesicles contained trypomastigote surface glycoproteins (Torrecilhas et al., 2009) which may prime

toll-like receptors (TLRs) containing GPI-anchors on host cells for parasite invasion (Ribeiro et al., 2018).

PPEVs and Host Immune Responses

PPEVs can promote, re-direct, and suppress immune cell responses depending on the maturation of the immune cell, disease model, *T. brucei* EV concentration (Silverman et al., 2010b), amount of *T. cruzi* EVs (Cronemberger-Andrade et al., 2020), site of *T. cruzi* EV inoculation (Lovo-Martins et al., 2018), and time. PPEVs cannot cause infection *per se* but it can aid subsequent parasitization and diverse innate and chronic immune responses (de Souza and Barrias, 2017). *T. gondi*-derived EVs can elicit humoral and cellular immune responses separately or simultaneously in the host (Li et al., 2018b). Exacerbated immune response, in part, may occur when EVs are up-taken by immune cells and in the process elicits changes in the host cell transcriptomes leading to stronger immune cell recruitments than parasite-induced signals (Montaner et al., 2014). During infection, *T. gondi* EV-primed immune cells could acquire new membranous receptors, enzymes, and genetic material which might induce intracellular signaling (Li et al., 2018b). Thus, PPEVs are mediators of biological signals and immune responses (Fernandez-Becerra et al., 2014) (**Table 2**).

Components of protozoan parasite EVs that can affect innate immune response include agonists of pattern recognition receptors, mRNA, miRNAs, sRNAs, DNA, fibronectin, several pathogen-associated molecular patterns, and glycopeptidolipids (Yanez-Mo' et al., 2015; Castelli et al., 2019). The packaging of these molecules in PPEVs may prevent their recognition by the host immune system (Roditi, 2016), and the specific EV protein composition can considerably affect the phenotypic responses of cytokines (Silverman et al., 2010b). For instance, when mice were immunized with rex, a purified exosomal protein from *P. yoelii*, 83% of the mice survived the primary challenge and remained immunoprotected (Fernandez-Becerra et al., 2014). A similar down-regulation of immune cells with longer parasite survival time had been reported in mice immunized with non-lethal *P. yoelii* 17X-derived exosomes with a significant increase of reticulocytosis and changes in the parasite tropism (Martin-Jaular et al., 2011).

Twu et al. (2013) reported a potential critical role of dampening interleukin 8 (IL-8) response secreted by ectocervical cells after an exposure to *T. vaginalis* exosomes in order to establish successful chronic infections. In another instance, *T. vaginalis* exosome-like vesicles modified cytokine production in macrophages and ameliorated inflammatory process in mice model of trichomoniasis (Olmos-Ortiz et al., 2017). It has been reported also that *Leishmania* exosomes selectively induced IL-8 secretion to suppress host response (Silverman et al., 2010a). An early signal of lL-10, an anti-inflammatory cytokine, after the incubation of *T. gondi*-derived exosomes with macrophages shows that EVs promote parasite survival (Li et al., 2018a). *L. donovani* wild-type exosomes also promoted the secretion of IL-10 to create an infectious environment for parasite survival, but such property was not exhibited by mutant HSP100$^{-/-}$ *L. donovani* exosomes (Silverman et al., 2010b), and intracellular *T. cruzi* vesicles induced local reduction of inducible nitric oxide (iNO) activity which supported higher tissue parasitism (Torrecilhas et al., 2009) (**Table 2**). Thus, specific PPEVs can commonly impact the phenotypic responses of cytokines during protozoan parasite infection.

During malaria infection, parasite-derived MPs, or RMVs dominantly drive macrophage activation by either causing pathological inflammation or initiating anti-malaria immune responses which contributed to the local, systemic, and EV-dose-dependent production of pro-inflammatory cytokines and chemokines (Mantel et al., 2013, 2016; Kehrer et al., 2016). iRBC-derived vesicles induced pro-inflammatory cytokines such as IL-6 and IL-1 in human bone marrow-derived endothelial cells (Mantel et al., 2016). MPs have been shown to be responsible for macrophage activation when co-cultured with iRBC MPs with the significant up-regulation of cluster of differentiation CD 40 and production of tumor necrosis factor (TNF). In the process, the induced CD40 on antigen-presenting cells primed T cells for effector functions (Couper et al., 2010). Likewise, *L. infantum* EVs recruited more macrophages and dendritic cells than did other extracellular products or the parasite (Pérez-Cabezas et al., 2018). The derived mucin-like glycoproteins and glycoinositol phospholipids in *T. cruzi* trypomastigote EV were likely responsible for the induction of inflammatory responses in macrophages (Cronemberger-Andrade et al., 2020).

T. cruzi vesicle-derived cruzipain has been described to enhance the production of pro-inflammatory IL-4 and IL-5 cytokines (Torrecilhas et al., 2009). Equally, *A. castellanii* EVs triggered the transcription of pro-inflammatory cytokines in monocytes (Lin et al., 2019), but no specific antigenic product was identified. Parasite specific virulent molecules in *T. cruzi* EVs induced different levels of pro-inflammatory TNF-α, IL-6, and NO responses under the same treatment (Nogueira et al., 2015). Meanwhile, the quantification of cytokine secretion by ectocervical cells demonstrated that *T. vaginalis* exosomes induced IL-6 as *T. vaginalis* and promoted acute inflammation (Twu et al., 2013). *T. gondii* exosomes have been shown to affect the progress of intracellular infections with an onward regulation of inflammatory cytokines (IL-12, IFN-γ, and TNF-α in macrophages) and Th1 responses (Li et al., 2018a). There was also a significant increase in the production of IL-4 and TNF-α by *L. amazonensis* in the presence of *L. amazonensis* EVs (Barbosa et al., 2018) (**Table 2**). The enhanced inflammation observed in mice co-injected with *L. major* exosomes was attributed to the possible intermediation of Th17 cells in the lymph node (Atayde et al., 2015). Remarkably, EVs from *T. cruzi* Colombiana and Y strains induced a more pro-inflammatory reaction than those of YuYu and CL-14 strains (Nogueira et al., 2015).

Major humoral immune response elicited by PPEVs has been reported after mice immunization with *T. gondii* exosomes in which a high level of total IgG reminiscent of Th1 cells was detected (Li et al., 2018a). As well, exosomes obtained from *P. yoelii*-infected mouse reticulocytes elicited IgG2a and IgG2b isotype antibodies that recognized *Plasmodium*-infected RBCs (Martin-Jaular et al., 2011). MASPs in *T. cruzi* trypomastigote EVs triggered a rapid humoral IgM response but limited IgG class-switching during infection (Bautista-lópez et al., 2017). A

TABLE 2 | Immune cell functional responses after interaction with PPEVs.

Disease	Parasite EVs	Study type	Target cell	Functional response	References
keratitis	Ac EVs	*in vitro*	Human	Increased IL-6 and IL-12	Lin et al., 2019
Granulomatous amoebic			Monocytes		
Meningoencephalitis					
Malaria	Pf RMVs	*in vitro*	PBMCs macrophage neutrophils	Upregulation of CD40, CD54, and CD86; decreased IL-10 IL-10 and TNF-α	Mantel et al., 2013
	Pg MPs	*in vitro*	Macrophage	Increased CD40 and TNF expression	Couper et al., 2010
	pf RMVs	*in vitro*	Macrophage/PBMCs	Activation of IL-6, IL-12, IL-1β, and IL-10	Mantel et al., 2013
	Pf EVs	*in vitro*	Monocytes	mRNA induction of CCL5, of CCL5, CXCL10, IFNα, IFNB, IFIT1	
Neosporosis	Nc EVs	*in vitro*	Macrophage	IL-12p40, TNF-α, IL-1β, IL-6	Li et al., 2018c
			BMDMs	IFN-γ, and IL-10 increased	
Toxoplamosis	Tg exosome	*in vitro*	Macrophage	IL-12, TNF-α and IFN-γ signicantly increased	Li et al., 2018b
	Tg EVs	*in vitro*	Splenocytes	Significant high IFN-γ, IL-12; CD8+ subset of T cells	Li et al., 2018c
	Tg EVs	*in vitro*	Murine	mRNA expression of IL-10	Li et al., 2018c
			Macrophage	TNF-α, iNOS up-regulated	
Trypanosomiasis	Tc EVs	*in vivo*	BMDMs	Decreased TNF-α IL-6, NO,	Lovo-Martins et al., 2018
		in vitro	Spleen cells	TNF-α,IL-6,IL-12p70, IFN-γ, MCP-1, IL-10*	
			Murine	Induction of LB and PGE_2	
			Macrophage		
	Tc vesicles		Splenocytes, macrophages, B cell	higher TNF-α, IFN-γ, IL-6, IL-10, NO, IL-10 CD4+ and CD8+	Nogueira et al., 2015
			DC	higher T lymphocytes, TNF-α	
	Tc vesicles	*in vivo*	Splenocytes	Higher IL-10 levels, not IL-4 and NO	Torrecilhas et al., 2009
			Mice heart	induced IL-10 and IL-4 mRNA	
	Tc EVs	*in vitro*	CHO/CD14	lower IL-1β and higher IL-6 inductions, and TNF-α*	Cronemberger-Andrade et al., 2020
Leishamaniasis	Li exosome	*in vitro*	DC macrophage	MHC-II basal, decresed CD40 and CD 86	Pérez-Cabezas et al., 2018
	Ld exosomes	*in vitro*	MoDCs	increased TNF-α, IL-6, IL-8 reduced CD80, CD86, HLA-DR increased IFN-γ, IL-10, IL-17	Silverman et al., 2010b
		in vitro	Splenocytes	higher IFN-γ, IL-4(CD4)Tcells	
			Spleen nymph node	lower IFN-γ (CD4 T cells) and Foxp3	
	Li exosomes	*in vitro*	Human	Inducted IL-10	Castelli et al., 2019
			Monocytes	Reduced IL-18	
	Lm exosomes	*in vivo*	Lymph node	Inducted IL-17a, IL-4, IL-23, INF-y	Atayde et al., 2015
	La EVs	*in vitro*	Macrophage	Increased IL-6, IL-10	Barbosa et al., 2018
		in vitro	B-1 cell	Increased IL-6, decreased IL-10	
Trichomaniasis	Tv exosome	*in vitro*	Macrophage	increase NO, IL-6, IL-8 IL-10, IL-17,IL-22 and TNF-α expression	Olmos-Ortiz et al., 2017
Giardiasis	Gi microvesicles	*in vitro*	Dendritic cel	CD25, T cell alloproliferation	Evans-Osses et al., 2017

PBMCs, peripheral blood mononuclear cells; Pg, P. berghei; Ac, A. castenallii; N. caninum; Tg, T. gondii; Tc, T. cruzi; Li L, infatum; Lm, L. major; La, L. amazonensis; Ld, L. donovani; Tv, T,vaginalis; Gi, G. intestinalis; DC, dendritic cell; MoDCs, momocyte derived DC; LB, lipid body; and PGE_2, Prostaglandin E_2 CHO/CD14; TLR2, transfected macrophage cell line.
**No change in expression level.*

rather significant role of EVs in immune modulation was seen with *T. cruzi*-derived EVs, which induced lipid body formation and prostaglandin E_2 in murine macrophages (Lovo-Martins et al., 2018) (**Table 2**).

Interaction of PPEVs and protozoan parasites can heighten immune responses and pathogenesis. In respect to this, Lovo-Martins et al. (2018) had shown that the pre-inoculation of *T. cruzi* trypomastigote vesicles before parasite infection produced IL-4 which was dependent on parasite strain. Also, the inoculation of *T. cruzi*-derived EVs following *T. cruzi* infection resulted in the induction of high levels of TNF-α, interferon gamma (IFN-γ), monocyte chemoattractant protein-1 (MCP-1), and IL-6 cytokines (Lovo-Martins et al., 2018). *T. cruzi* trypomastigote-derived EVs elicited increase in TNF-α and IL-6 release in bone-marrow macrophage response (Choudhuri and Garg, 2020). This establishes the concept

that a protozoan parasite and its derived EVs may work in tandem to establish infection. Also, EV pro-parasitic actions are progressively being shown to be strain-specific. Ribeiro et al. (2018) reported that macrophages pre-treated with EVs from *T. cruzi* Y strain showed increased trypomastigote invasion, whereas pre-treatment with EVs from the *T. cruzi* YuYu strain displayed increased intracellular parasite proliferation. However, inoculation of mice with EVs of *T. cruzi* YuYu and CL-14 strains without subsequent infection did not stimulate inducible nitric oxide in the macrophage or spleenocytes, and EVs of the *T. cruzi* Y strain induced a local reduction of iNOs with subsequently higher tissue parasitism (Nogueira et al., 2015).

Secreted molecules in PPEVs may also be deployed by a parasite for immune evasion or to avoid extracellular degradation (Caeiro et al., 2018). On this basis, EVs from an intracellular and extracellular protozoan parasites promote growth and induce host immune evasion by manipulating the microenvironment for adaptive responses or inhibition of inflammation (Mantel and Marti, 2014). Kinases and glycosidases found in EVs of *A. castellanii* could act on extracellular matrix to favour the escape of *A. castellanii* from immune cells (Gonçalves et al., 2018). It has been speculated that *T. brucei* microvesicles may serve as antigenic epitopes deployed by the parasite to overwhelm the host immune system (Geiger et al., 2010), but more importantly, *L. infantum* promastigotes interacted with their extracellular products to initiate eventual immune evasion by modulating bone marrow-derived DC (BMDC) and impairing macrophage ability to eliminate *L. infantum* (Pérez-Cabezas et al., 2018). GPI-anchored tetraspanin proteins of EVs may also protect pathogenic protozoa from complement-mediated lysis as they support parasite evasion (Lozano et al., 2017) (**Table 2**).

The release of immune molecules during protozoan parasite infections has a correlation with different immune pathways. *T. cruzi* EVs stimulated the JAK/STAT signaling pathway through cytokine receptor-linkage wherein there were expressions of STAT1 and STAT3 mRNAs in macrophages (Cronemberger-Andrade et al., 2020). Available data suggest that *N. caninum* EV could activate the mitogen-activated protein kinases (MAPK) signaling pathway in bone marrow-derived macrophages (BMDMs) through a component of secretory proteins in its EVs by phosphorylation of mitogen-activated proteins (P38, ERK, and JNK) via TLR 2. Also, EVs of *T. cruzi* Y strain and Colombian strain activated MAPKs via TLR2 in peritoneal macrophages (Nogueira et al., 2015). Prior exposure of *T. cruzi* Y strain trypomastigote EVs to human macrophages transfected with TLR2 expressed CD25 and activated NF-κB via TLR2 (Cronemberger-Andrade et al., 2020). Meanwhile, TLR2 might be activated by *N. caninum* EVs in BMDMs because it contains some pathogen-associated molecular patterns (PAMPs) (Li et al., 2018c). A similar work showed that *T. gondii* exosomes induced elevated expression of JNK mRNA, activated the nuclear translocation of phosphorylated JKN-protein, and eventually activated the MAPK pathway (Li et al., 2018b). An entirely novel TLR-4/MyD88-mediated activation of macrophages by microparticles of *Plasmodium*-parasitized RBCs has been reported in malaria inflammatory responses (Couper et al., 2010). In particular, *T. cruzi*-derived EVs from different strains have been shown to activate ERK 1/2, JNK, and p38 via its protein and α-galactosyl contents (Nogueira et al., 2015). RNA and gDNA contained in *P. falciparum*-iRBCs EVs translocate into the monocytes to stimulate STING-TBK1 (protein kinase)-IRF3 (transcription factor 3)-dependent gene induction (Sisquella et al., 2017). The MAPK pathway is essential for the production of inflammatory cytokines in parasitic infections, but the translational roles of PPEVs in MAPK, STING, and TLR stimulations need to be further probed.

PERSPECTIVES AND CONCLUSION

Serum-starved culture has often been used to induce PPEVs, but the exact process of EV secretion in appropriate hosts might not have been comprehensively captured given anatomical, genetical, and physiological interplays in hosts and vectors. Can the inability of protozoan parasites to secrete EVs in certain hosts/vectors justify the existence of paratenic hosts or the mark of parasite dead end? PPEVs cause cellular distress and orchestrate multiple pathophysiological processes. Are there functional selective secretion mechanisms for PPEVs or causal mechanisms of genetic/epigenetic reprogramming by which PPEVs confer virulence on non-pathogenic species? Additionally, the biological process that grounds the signaling events of PPEVs in parasite-parasite interaction and epigenetic effects of EV expulsion on protozoan parasites needs to be investigated.

Wittingly, heterogeneous population of PPEVs requires functional and reproducible sorting into distinct sub-populations. Asymmetric flow field-flow fractionation has been used to separate distinct vesicles called exomere from EVs aggregates (Zhang et al., 2018) and it stands as a promising technology to separate PPEVs into distinct sub-types. In addition, lipids play important roles in EV biosynthesis, but studies aimed at elucidating PPEV lipidomics are underrepresented, and the specific roles of sugar molecules during internalization or adhesion of PPEVs need validation because sugar can specifically bind to lectin-like receptors on parasites. Also, EV-associated polysaccharides and lipid moiety are important therapeutic targets as they can induce protective and pro-inflammatory immune responses (Nogueira et al., 2015; Kuipers et al., 2018).

Inflammasomes are molecular structures of the innate immune system which induce inflammation in response to infectious microbes and molecules (Abal, 2017; Cypryk et al., 2018), but their roles have not been established in inflammatory responses to PPEVs. Studies on antigenic regions of PPEV proteins and specific immune response (Pablos et al., 2016) require further consideration especially by *in vivo* methods because parasite molecules are much more expressed in definitive hosts (Ramírez-Flores et al., 2019) (**Table 2**). Considering the physiological stress under which PPEVs and tsRNAs are formed and the biological functions of tsRNA in post-transcriptional regulations (Dou et al., 2019), the exact roles and vesicular

loading process of tsRNAs, non-coding RNAs, and DNA into PPEVs call for elucidation.

From this review, it is obvious that the composition and function of PPEVs are a reflection of the species of origin and the activating stimuli. PPEVs can be internalized by host cells and protozoan parasites using contiguous receptor-dependent and receptor-independent mechanisms to cause various cellular distresses and to provide genetical cues. PPEVs have been shown to induce differential cytokine expression depending on cell type, infection model, dose and origin of EVs. In extreme cases, PPEVs may present a similar effect as protozoan parasites or, at least, act in concert. The secretion of EVs by protozoan parasites comparatively represents parasite constitutive encryptions with which they harness developmental stimuli, nutritional materials, digestive enzymes, and control of maturation. We have only unveiled the phenomenal responses of pathogenic protozoa to stressors, secretion and internalization of EVs, and vesicular peculiarities with the hope that it would help to address fundamental questions on parasite biology.

AUTHOR CONTRIBUTIONS

JC proposed the contents and paper frame and provided critical feedback. JO drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Proteomic Analysis of Exosome-Like Vesicles Isolated From Saliva of the Tick *Haemaphysalis longicornis*

*Mohsin Nawaz, Muhammad Irfan Malik, Houshuang Zhang, Ibrahim A. Hassan, Jie Cao, Yongzhi Zhou, Mudassar Hameed, Zulfiqar Hussain Kuthu and Jinlin Zhou**

Key Laboratory of Animal Parasitology of Ministry of Agriculture, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China

Extracellular vesicles (EVs), are considered as vehicles of cellular communication. Parasites usually release EVs in their excretory-secretory products to modulate host environment. However, little is known about the secretion of EVs by ticks. In this study, we show for the first time that the tick *Haemaphysalis longicornis* secretes EVs in saliva that resembles exosomes. EVs were purified from pilocarpine induced saliva of partially engorged *H. longicornis* ticks. Electron microscopy analysis revealed the presence of exosome-like vesicles with a size of 100 nm. Proteomic analysis by LC-MS/MS identified a total of 356 proteins in tick-derived EVs. Proteome data of tick-derived EVs was validated by Western blot analysis. Immunodetection of Hsp70 and GAPDH proteins indicated that the proteomics data of tick-derived EVs were highly reliable. Bioinformatics analysis (Gene Ontology) indicated association of certain biological and molecular functions with proteins which may be helpful during tick development. Likewise, KEGG database revealed involvement of vesicular proteins in proton transport, detoxification, ECM-receptor interaction, ribosome, RNA transport, ABC transporters, and oxidative phosphorylation. The results of this study provide evidence that EVs are being secreted in tick saliva and suggest that tick saliva-derived EVs could play important roles in host-parasite relationships. Moreover, EVs could be a useful tool in development of vaccines or therapeutics against ticks.

Keywords: exosomes, extracellular vesicles, saliva, proteomics, *Haemaphysalis longicornis*

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****Correspondence:***
Jinlin Zhou
jinlinzhou@shvri.ac.cn

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INTRODUCTION

Haemaphysalis longicornis, also known as bush tick or Asian longhorned tick, belongs to the tick family Ixodidae. Although this tick is native to China, Russia, and Japan, it is now established in Pacific islands including New Zealand, Australia, and the eastern states of the USA (Heath, 2016; Berenbaum, 2018). *Haemaphysalis longicornis* is an important vector of human disease-causing agents such as thrombocytopenia syndrome virus, *Rickettsia japonica*, *Ehrlichia chaffeensis*, *Babesia microti*, and *Anaplasma bovis* (Mahara, 1997; Luo et al., 2015; Wu et al., 2017). Likewise, *H. longicornis* transmits theileriosis to cattle, thereby causing considerable blood loss and death of calves (Heath, 2016). Meanwhile, 25% reduction in dairy products has been observed in Australia and New Zealand. Parthenogenetic reproduction of *H. longicornis* allows a single female to generate progeny without mating, resulting in massive host infestations (Heath, 2016).

Extracellular vesicles (EVs) are small membrane vesicles derived from the endocytic compartment of cells. EVs have emerged as key players in intercellular communication and can be divided into exosomes, microvesicles, apoptotic bodies, and oncosomes (Devhare and Ray, 2018). Exosomes, formed by the fusion of multivesicular bodies (MVBs) were discovered in 1983 in reticulocytes (Harding et al., 2013). They were first considered as garbage bins being used by cells to discard their waste products (Johnstone, 1992). In some studies, these nano-sized vesicles were also considered as apoptotic blebs, cellular debris, or signs of cell death (Bobrie et al., 2011; Pant et al., 2012). However, the discovery of exosomes as carriers of genetic material (proteins, lipids and miRNAs) and their involvement in cellular communication has opened new horizons. Interaction of exosomes with target cells by binding with the receptors of other cells, fusion with their membranes and releasing their contents into the cytosol of target cells have made them powerful agents of cellular communication (Mathivanan et al., 2010). In addition, exosomes can even be used as vaccine candidates and biomarkers for the diagnosis and treatment of diseases (Zhang et al., 2018).

Parasites use excretory-secretory products to communicate with their host environment. Discovery of EVs within the excretory-secretory products of parasites such as *Fasciola hepatica*, *Echinostoma caproni*, *Heligmosomoides polygyrus*, *Dicrocoelium dendriticum*, *Schistosoma mansoni, Trichuris muris, Echinococcus granulosus, Leishmania amazonensis, Trichomonas vaginalis, Plasmodium vivax*, and *Trypnosoma cruzi* has gained considerable interest over the last few years (Marcilla et al., 2014; Coakley et al., 2015; Barbosa et al., 2018; Eichenberger et al., 2018; Gualdrón-López et al., 2018; Nicolao et al., 2019; RAI and Johnson, 2019). EVs can be purified form secretory products by series of steps by centrifugation, ultracentrifugation, precipitation kits, ExoChip, immunoprecipitation, acoustic nanofilter, size exclusion chromatography (SEC) column purification, and sucrose density gradient techniques (Raposo et al., 1996; Tauro et al., 2012; Kanwar et al., 2014; Lee et al., 2015; Zeringer et al., 2015; Wu et al., 2019). EVs can play a decisive role in parasite-host interactions by transferring their inner contents (virulence factors and effector molecules) from parasites to hosts (Wu et al., 2019). The content of these vesicles consists of a variety of proteins, miRNAs and lipids. Among miRNAs, high abundance of miR-71 and miR-72 has been observed in parasites suggesting their involvement in embryo development, growth, and metabolism of parasite (Chen et al., 2011; Cai et al., 2013). Furthermore, single-stranded DNA, mitochondrial DNA, double-stranded DNA and oncogene amplifications have been identified in microvesicles (Balaj et al., 2011; Thakur et al., 2014). Similarly, proteins like GAPDH, enolase, and Hsp70, usually involved in parasite survival, reproduction, and growth, have been associated within the parasite-derived EVs (Sotillo et al., 2008, 2010). In addition to miRNAs and proteins, lipids are critical components of EVs. Exosomes-like vesicles are highly enriched in an array of lipid species, including sphingomyelin, glycosphingolipids, cholesterol, and ceramide (Skotland et al., 2017; Brzozowski et al., 2018; Chen et al., 2019; Sun et al., 2019). Uptake of lipid contents by the parasites helps them to develop protective mechanisms against host immunity, support parasitic survival, and promote growth (Yesuf and Kenubih, 2019). Therefore, identification of lipid enriched EVs led us to speculate that parasite-derived EVs could provide a mechanism to modulate hosts immune responses.

EVs have been isolated from excretory-secretory products of some parasites but, to the best of our knowledge, ticks have not been studied. Here, we report for the first time that the saliva of the tick *H. longicornis* secreted exosome-like vesicles. Proteomics analysis of tick-derived EVs revealed the presence of some significant proteins such as GAPDH, heat shock proteins, thioredoxin peroxidase and proteases, which may be used by ticks in modulating host-parasite interactions.

METHODS

Ethics Approval and Consent to Participate

All experiments carried out during the study were approved by the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute (IACUC No: SHVRI-SOP-1104-003). Rabbits were maintained at the animal house (SHVRI) under normal conditions of regulated temperature (22°C) and light with free access to feed and water. Rabbits were kept in cages in compliance with the guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China).

Ticks

A colony of *H. longicornis* ticks (parthenogenetic strain) was collected from Shanghai Wildlife Park, China. The tick colony was established after maintenance of three generations in the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China. After feeding the ticks on New Zealand white rabbits, rearing was done in the laboratory at 25°C (92% humidity) in a dark incubator (Mulenga et al., 1999; Zhou et al., 2006). Finally, the parthenogenetic ticks were used for the collection of saliva.

Collection of Saliva

For infestation, 40 adult ticks were attached per rabbit ear and maintained with the help of ear bags made of cotton cloth. The ear bags were held onto the rabbit ears with the help of surgical stitches and adhesive tape. After attachment, rabbits were placed in cages made of steel. A total of 55 female rabbits (4 months old) were used in this study. After feeding for 4 days, ticks were removed and saliva was collected as previously described (Patton et al., 2012). After washing with sterile distilled water, ticks were attached to glass slides with adhesive tape. Pilocarpine was injected (0.5–1 μl) posterior to fourth coxae in the region of epidermal and anal plates of the tick. Ticks were placed at 37°C in 85% humidity chamber. Saliva was collected with pipette tip after an interval of 20 min. Saliva collected from partially fed ticks was mixed with equal quantity of PBS and stored at −80°C.

Isolation of EVS

For isolation of EVs from saliva of *H. longicornis*, a protocol described by Abdi et al. (2017) was followed with slight modifications (**Supplementary Figure 1**). Briefly, saliva was

mixed with an equal amount of PBS and centrifuged at 2,600 g for 30 min at 4°C to remove cellular debris. Cell free medium (supernatant) was filtered through 0.22 μm filter (Merck Millipore) to remove contaminating apoptotic bodies and cell debris. Supernatant was centrifuged at 140,000 g for 3 h at 4°C in OptimaTM L-100 XP ultracentrifuge (Beckman Coulter) using an SW 60 (44.5) rotor. Supernatant was removed carefully and pellet was collected. Pellet was washed twice by re-suspending in cold PBS and centrifuging at 150,000 g for 2 h after each wash. A discontinuous gradient was prepared by diluting a stock solution of OptiPrep™ (60% w/v) with 0.25 M sucrose/6 mM EDTA, 60 mM Tris (pH 7.4). The gradient was formed by layering 40, 20, 10, and 5% gradient solutions on top of each other into 4 ml open top thin wall polyallomer (Beckman Coulter). Pellet was loaded on top of gradient and centrifuged at 250,000 g for 18 h. One milliliter fractions were collected from top of the gradient and transferred to 1.5 ml Eppendorf tubes. Weights of the tubes were measured to estimate the density of purified vesicles. A total of 5 fractions were collected, each fraction was diluted in PBS to 4 ml and centrifuged at 150,000 g for 2 h. Resulting pellet was collected and stored at −80°C. Confirmation of EVs within the saliva samples was accessed by electron microscopy.

Electron Microscopy

The pellet was analyzed by electron microscopy at Shanghai Veterinary Research Institute, China. EV sample was fixed 1:1 with 2% glutaraldehyde. A 200 mesh copper grid with carbon-coated formvar film (Agar Scientific, Essex, UK) was incubated onto 5 μL of fixed sample for 30 min. Excess liquid was removed by blotting and grids were allowed to dry at room temperature. Grids were washed with water and stained with phosphotungstic acid for 1 min. After staining, grids were washed with ethanol (70%) followed by four washes with molecular grade water. Finally, the grids were loaded onto a sample holder of a transmission electron microscope (FEI T12 equipped with AMT XR51 CCD camera system) and exposed to 80 kV electron beam for image capture.

SDS-PAGE Analysis and Western Blot

Purified EVs were homogenized with lysis buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl pH 8.0, protease inhibitor). After 3 min incubation in boiling water, the homogenate was sonicated on ice. The crude extract was then incubated in boiling water again and clarified by centrifugation at 16,000 × g at 25°C for 10 min. The concentration of proteins was determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer's specifications and using BSA (Thermo Fisher Scientific) as a standard. Five micrograms of protein per lane were processed by SDS-PAGE (12% polyacrylamide linear gradient gels; Bio-Rad Laboratories, Hercules, CA, USA) and stained with Coomassie Brilliant blue R-250. Gels were scanned using a Bio-Rad Molecular Imager FX system (Bio-Rad Laboratories).

For western blot analysis, 7 μg of proteins were subjected to 12% SDS-PAGE. It was followed by the transfer of proteins to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk diluted in PBS/0.05% Tween (PBST) for 2 h at 37°C. Membranes were washed three times (5 min/washing) with PBST. Blots were incubated overnight at 4°C in rabbit anti-Hsp70 antibody diluted to 1:1,000 in PBST (cat. no. ab79852; Abcam) and rabbit anti-GAPDH antibody diluted to 1:1,000 in PBST (cat. no. ab37168; Abcam). Blotted membranes were washed three times with PBST (10 min/washing) and incubated in the presence of goat anti-rabbit IgG antibody (horseradish peroxidase-conjugated; dilution, 1:2,000; Bethyl Laboratories, Inc., USA) for 1 h at 37°C. Washing with PBST (10 min/washing) was done before visualization of bands. Protein signals were detected with an Enhanced Chemiluminescent

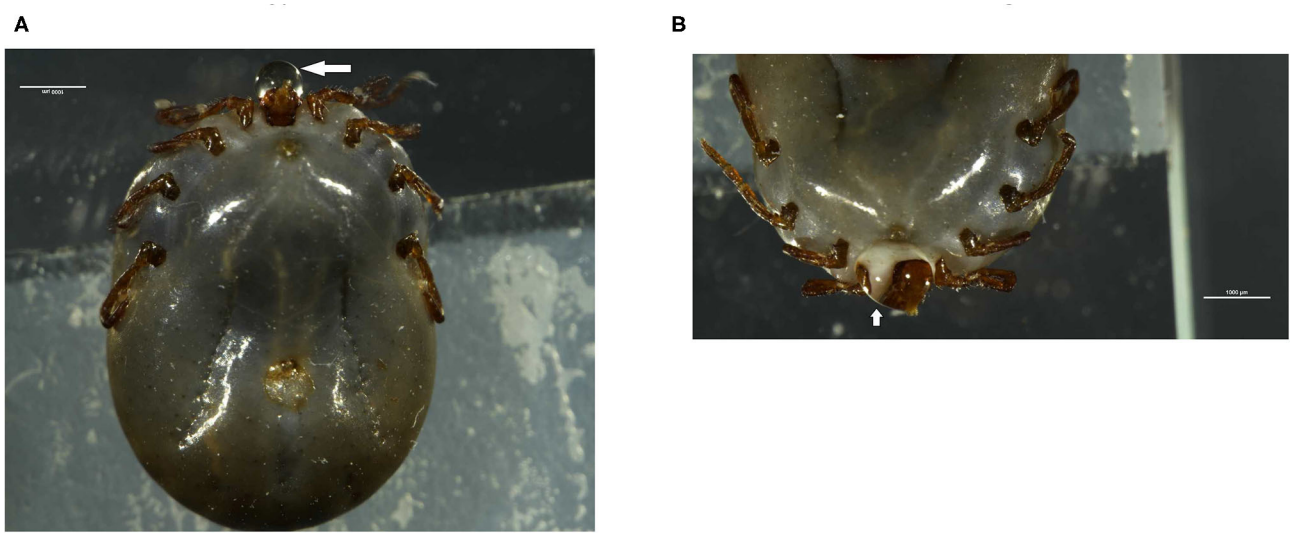

FIGURE 1 | Collection of saliva from partially engorged *H. longicornis* ticks. **(A,B)** Ticks were injected with pilocarpine and 0.5–1 μl of saliva was collected. Arrows indicate the saliva secreted by ticks.

Substrate Reagent Kit (NCM Biotech, Sunzhou, China) and were visualized under a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology, Shanghai, China).

Liquid Chromatography Mass Spectrometry (LC-MS/MS)

Purified samples (EVs) dissolved in PBS were diluted in 30 μl SDT buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) and boiled for 5 min. The detergents (DTT and other low-molecular-weight components) were removed using 200 μl UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) by repeated centrifugation (14,000 × g for 15 min). After centrifugation, the concentrates were mixed with iodoacetamide (IAA, 50 mM IAA in UA) and incubated in darkness for 30 min at room temperature. After 15 min centrifugation, filters were washed three times with 100 μL UA buffer and then 100 μL of dissolution buffer (50 mM triethylammonium bicarbonate, pH 8.5) twice. The tryptic peptides resulting from the digestion were extracted with 0.1% formic acid in 60% acetonitrile. Protein suspension was digested with 2 μg trypsin (Promega, Madison, USA) in 40 μL 25 mM NH4HCO3 overnight at 37°C. The extracts were pooled and completely dried using a vacuum centrifuge.

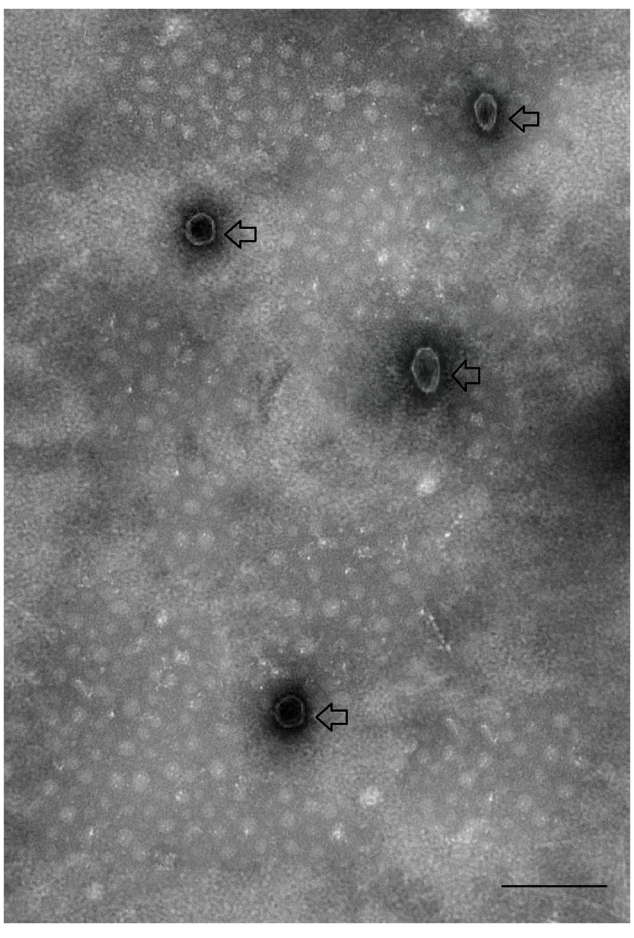

FIGURE 2 | Transmission electron micrograph of exosomes-like vesicles derived from tick saliva. Arrows indicate tick-derived EVs. Scale-bar: 100 nm.

For protein identification, liquid chromatography-mass spectroscopy assay (LC-MS/MS, Thermo Fisher Scientific) was performed using Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer coupled on Easy NLC system 1000 (Thermo Fisher Scientific). Five micrograms of proteins were used by LC-MS/MS. Trypsin digested peptides were desalted on Zorbax 300SB-C18 peptide traps (Agilent Technologies, Wilmington, DE, USA) and separated on a C18-reversed phase column (0.15 × 150 mm, Column Technology Inc., Fremont, CA, USA). Mobile phases A (0.1% formic acid in HPLC-grade water) and B (0.1% formic acid in 84% acetonitrile) were delivered using an Easy nLC system (Thermo Fisher Scientific) with a linear gradient of 4–50% B (50 min), 50–100% B (4 min), and 100% B (6 min) at a flow rate of 250 nl/min. A data-dependent method, based on 10 most abundant precursor ions for HCD fragmentation was used to acquire mass spectrometry data. For survey scans (m/z 300–1,800), the target value was determined based on predictive Automatic Gain Control at a resolution of 70,000 at m/z 200 and dynamic exclusion duration of 25 s. The resolution set for the HCD spectra was 17,500 at m/z 200. Normalized collision energy was 27 eV and the under-fill ratio was set as 0.1%.

For in gel protein identification, Ettan™ MDLC controlled by UNICORN™ software (GE Healthcare), was used for desalting and separation of peptides. Peptide mixtures were desalted on RP trap columns and then separated on a C18-reversed phase column. Mobile phase A (0.1% formic acid in HPLC-grade water) and mobile phase B (0.1% formic acid in 84% acetonitrile) were selected. Tryptic peptide mixtures were loaded onto the columns, and separation was done at a flow rate of 2 μL/min by using the linear gradient buffer B described above. LTQ Velos (Thermo Scientific) equipped with a micro-spray interface was connected to the LC setup for eluted peptides detection. Data-dependent MS/MS spectra were obtained simultaneously. Each scan cycle consisted of one full scan mass spectrum (m/z 300–1,800) followed by 20 MS/MS events of the most intense ions with the following dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 90 s.

Database Searching and Protein Identification

MaxQuant software (version 1.3.0.5.) suite was used to process Raw MS files. Peak list files were analyzed using Mascot search algorithm (Matrix Science Matrix Science, London, UK; version 2.2) against the UniProtKB Ixodoidea database (downloaded at July 06, 2018, with 190,922 entries) containing both forward and reverse protein sequences. The search parameters were: trypsin enzyme; two missed cleavages; a fragment ion mass tolerance of 0.10 Da; mascot score >20 and peptide tolerance of 20 ppm. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification whereas oxidation of methionine was specified as variable modification. Proteins with at least two peptide (false discovery rate< 0.01) uniquely assigned to the respective sequence were considered.

Gene ontology (GO) is a universally acknowledged functional enrichment database and is generally used to search for enriched GO terms. Differently expressed proteins were classified into GO annotations according to molecular function, biological process, and cellular component. Additionally, KEGG database was mapped to analyse the pathways of the proteins involved.

Statistical Analysis

Right-tailed Fisher's exact test was used to access Gene enrichment of three ontologies (biological processes, cell components, and molecular functions) and KEGG pathway enrichment analysis. GO analyses were performed using DAVID (v6.8; https://david.ncifcrf.gov/) and Cytoscape online software (https://cytoscape.org/) (Shannon et al., 2003; Sherman and Lempicki, 2009; Xing et al., 2016). Unpaired *t*-tests were used to perform all statistical analyses. $P < 0.05$ was considered to indicate significant differences. All assays were performed in three replicates.

RESULTS

Haemaphysalis longicornis Saliva Produce Exosome-Like Vesicles

Saliva was collected from partially fed (4 days post-feeding) *H. longicornis* after injecting with pilocarpine as shown in **Figures 1A,B**. A total of 2,500 μl of saliva was collected from ∼4,000 ticks. EVs, isolated from saliva were observed by electron microscopy. Vesicles appeared within saliva were having a size of 100 nm, which is usually the range of exosomes (**Figure 2**). Exosomes appeared as typical spherical structures released into the saliva, as previously observed in the case of other parasites (Samoil et al., 2018). To ascertain whether these vesicles were also present in supernatants after ultracentrifugation, we next carried out purification of these structures from supernatants using TEM to visualize them. However, structures resembling EVs were not found in supernatant.

Tick EVs Contain Proteins

Proteomics (LC-MS/MS) was carried out to identify proteins present within purified EVs. SDS-PAGE followed by Coomassie blue staining indicated an enrichment of specific proteins (**Figure 3A**). Protein identification using MASCOT confirmed the presence of 356 proteins (**Supplementary Table 1**). Most of the proteins like nuclear proteins (elongation factors and histones), cytoskeletal proteins (actin, tubulin), and stress-related proteins (HSPs) have been identified in previous studies. Proteins have been classified in groups based on function and/or protein families (**Table 1**). In addition, EVs isolated from tick saliva were found to be enriched with host proteins. MASCOT searches identified the presence of 225 host proteins mainly corresponding to immunoglobulins, histones as well as metabolic proteins (**Supplementary Table 2**). However, deep analysis revealed slight differences (peptide counts and cover percent) between host proteins and vesicular proteins, e.g., peptide counts for GAPDH from host proteins were 3 while only 2 counts were observed for EVs-derived GAPDH. Similar differences were also observed in other proteins like histones.

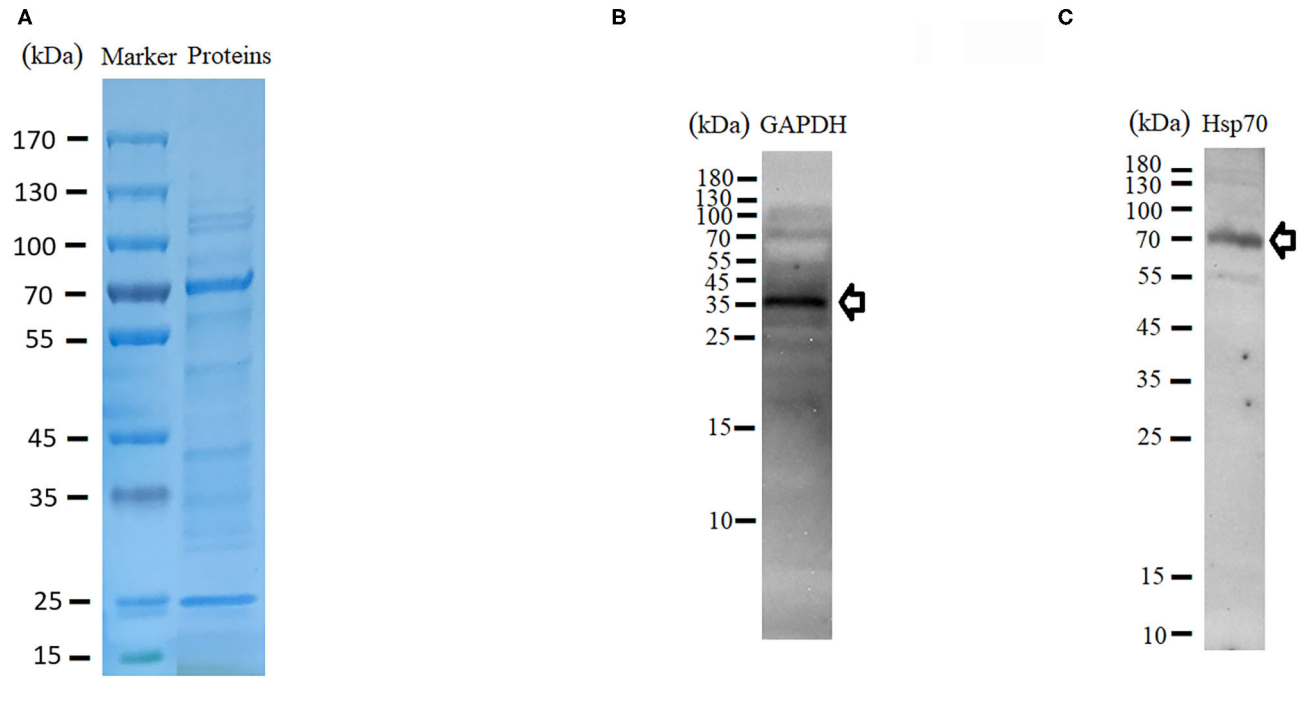

FIGURE 3 | Characterization of protein contents of tick-derived EVs. **(A)** SDS-PAGE and Coomassie blue stained. **(B,C)** Validation of GAPDH and Hsp70 through Western blot.

TABLE 1 | Proteins identified in tick-derived EVs by LC–MS/MS.

Uniprot ID	Description	Uniprot ID	Description
Protein synthesis machinery			
A0A2R5LAZ1	Putative 40s ribosomal protein s3	A0A2R5LH55	Ribosomal protein s18
A0A131Z199	E3 ubiquitin-protein ligase TRIP12	A0A131ZAN1	Small subunit ribosomal protein S27Ae
A0A1E1XMP5	Putative e3 ubiquitin-protein ligase herc2	A0A023FFH9	Mitochondrial ribosomal protein mrp-s35
A0A1E1XUQ4	RBR-type E3 ubiquitin transferase	A0A023FHE7	Ribosomal protein l34
B7PFT8	U4/U6 small nuclear ribonucleoprotein Prp4	A0A131YU15	Small subunit ribosomal protein S7
A0A2R5LAX2	60s ribosomal protein l17	A0A1E1XHY1	Ribosomal protein l3
A0A2R5LH55	Ribosomal protein s18	A0A1Z5LGI7	60s ribosomal protein L10
Heat shock proteins			
A0A023FJK7	Putative heat shock protein	B7Q057	HEAT repeat-containing protein
A0A023GP15	Putative heat shock protein	M9WB33	Heat shock protein 90
A0A131YY80	Heat shock 70 kDa protein		
Translation regulation			
A0A147BCU5	Translation initiation factor 5b eif-5b	A0A293N8J1	Elongation factor 1-alpha
A0A1E1XCT0_	Translation initiation factor eif-2b subunit delta-like isoform 1	A0A147BP71	Elongation of very long chain fatty acids protein
V5I557	Negative regulation of translation involved in protein silencing by mirna		
Nuclear regulation			
A0A023GER7	Histone H2A	A0A2R5L410	Endothelial zinc finger protein induced by tumor necrosis factor alpha
A0A131XUE0	Component of histone deacetyl	V5IF43	Lamin
A0A240EVT3	Histone H3	A0A2R5LHU4	Transcription factor a mitochondrial
A0A0C9RRX6	Histone H2B	A0A131YIB9	Transcriptional regulator ATRX
A0A023FTG4	Histone H4	A0A293LF12	Zinc finger protein
Signal transduction			
A0A293MX67	Ras-related protein	V5HIK9	Ras-related protein rab-11a
Glycine rich			
A0A023FQ63	Glycine-rich cell wall structural protein 1.8		
Lipocalins (4)			
A0A023GD09	Lipocalin-2 1	A0A131Z256	Lipocalin
A0A131YQ62	Lipocalin	A0A147BW77	salivary lipocalin
Cement (2)			
A0JC33	Cement-like antigen	A0JC35	Cement-like antigen
Cytoskeletal (11)			
L7LY53	Myosin light chain binding protein	A0A1E1XF25	Microtubule-actin cross-linking factor 1-like protein
A0A147BK70	Myosin-16	A0A109QJ05	Tropomyosin
A0A1E1X2T4	Myosin-2 heavy chain	A0A147BWI1	Tropomyosin-2 isoform 4
L7LY53	Myosin light chain binding protein	A0A2R5L9T9	rhoa gtpase effector dia/diaphanous
A0A1E1X349	Actin related protein 1	A0A293M7V9	Tubulin alpha chain
A0A023FMG5	Actin-binding cytoskeleton protein filamin		
Serpins (3)			
A0A090X8Z2	Serine protease inhibitor	B7Q8M7	Serpin-2 precursor
A0A131YIT6	Pancreatic trypsin inhibitor		
Enzymes (68)			
A0A1Z5L0Z6	*Enoyl-CoA hydratase*	A0A023GFD5	Carboxypeptidase
B5M758	3-hydroxyacyl-coa dehyrogenase	A0A023GK80	cd73 ecto-5′-nucleotidase
A0A023G073	2-oxoglutarate dehydrogenase e1 subunit	A0A0C9S1T0	gdp-l-fucose synthetase
A0A0K8RQB8	Dihydrolipoyl dehydrogenase	A0A0K8R7B1	Glutathione s-transferase 1
A0A131XU72	Hydroxysteroid dehydrogenase-like protein 2	A0A1E1XJE7	Sumo1/sentrin specific peptidase 6a

(Continued)

TABLE 1 | Continued

Uniprot ID	Description	Uniprot ID	Description
A0A131YNN3	15-hydroxyprostaglandin dehydrogenase (NAD)	A0A293LZ27	GDP-fucose protein O-fucosyltransferase 1
A0A1Z5KY25	Malate dehydrogenase	A0A2R5LGC2	Glyoxalase
A0A293MCU1	Glutaryl-CoA dehydrogenase	A0A2R5LGN0	Dual specificity phosphatase
A0A0K8RBY4	Peptidyl-prolyl cis-trans isomerase	A0A2R5LJB8	rna polymerase-associated protein ctr9
A0A131YPA8	Peptidylprolyl isomerase	A0A2R5LK45	Argininosuccinate synthase
A0A147BS85	Putative prolyl 4-hydroxylase alpha subunit	A0A2R5LN87	ran gtpase-activating protein
A0A2R5LKC5	Putative cytosol aminopeptidase	B7P6A7	ATP synthase subunit alpha
A0A023FM97	Aminopeptidase	B7PB22	Calcium-dependent cysteine protease
A0A131XGJ9	Putative ftsj-like rna methyltransferase	B7Q0Z1	GTPase
A0A131XZ89	Alpha-mannosidase	B7Q2U6	Nicotinamide N-methyltransferase
A0A131YR95	Amidase	A0A131ZD23	Reprolysin
A0A147B8Y3	Thioredoxin peroxidase	A0A224YV60	O-phosphoseryl-tRNA(Sec) kinase
A0A147BSL3	Putative thymidylate synthase	B7PXE6	Phosphatidylinositol 4 kinase
A0A1E1X8M7	ATP synthase subunit beta	B7Q6K8	Phosphatidylinositol 3-kinase catalytic subunit
A0A293N4N6	Catalase	A0A1E1XUQ4	RBR-type E3 ubiquitin transferase
A0A2R5L8K4	Putative abc transporter atp-binding protein/permease	Q86GZ5	Midgut cysteine proteinase 2
A0A2R5LGC2	Putative glyoxalase	A0A1Z5L983	Sulfotransferase
A0A2R5LGN0	Putative dual specificity phosphatase	A0A224YIB1	ATP-dependent RNA helicase DHX33
A0A2R5LK45	Putative argininosuccinate synthase	B7Q3E5	Citrate lyase beta chain
A0A2R5LKC5	Putative cytosol aminopeptidase	A0A224Z2B8	Malonyl coaacp transacylase
B7PB22	Calcium-dependent cysteine protease	A0A293MCU1	Glutaryl-CoA dehydrogenase
V5H108	DNA-directed RNA polymerase III subunit	A0A293MQS4	NADPH-dependent diflavin oxidoreductase 1
B7Q0Z1	GTPase, putative	L7MCS1	Putative mrna splicing factor atp-dependent rna helicase
B7Q2U6	Nicotinamide N-methyltransferase, putative	Q4R1A6	Metalloprotease
B7QCU5	Sulfotransferase, putative	*Q86GZ5*	*Midgut cysteine proteinase 2*
A0A131YGI5	RHIAP Angiotensin-converting enzyme	V5HY02	Putative tick metalloprotease
L7S6B3	Glutathione peroxidase	V5HZQ9	Putative endoribonuclease dcr-1
A0A023FMK0	Aspartate aminotransferase	A0A023FVG3	Angiotensin-converting enzyme
G8C7A0	Lysosomal acid phosphatase	A0A293LC34	Farnesyltransferase alpha subunit
A0A2P1DPZ4	Glyceraldehyde-3-phosphate dehydrogenase		
Secreted proteins (24)			
A0A023G1E9	Putative secreted protein	A0A023FEZ7	Putative secreted mucin
A0A023G2A9	Putative secreted protein	A0A1E1XQT0	5′-nucleotidase
A0A023GE86	Putative secreted protein	A0A1E1X7A9	cd73 ecto-5′-nucleotidase
A0A090X9H5	Putative secreted protein	A0A023GD61	glycosyl hydrolase family 38
A0A090XCY0	Putative secreted protein	A0A0K8RNX7	m13 family peptidase
A0A0K8R869	Putative secreted protein	A0A1Z5LIJ2	Thioredoxin-dependent peroxide reductase mitochondrial
A0A131Y3N9	Putative secreted protein	A0A2R5LK45	Argininosuccinate synthase
A0A1E1WY51	Putative conserved secreted protein	A0A147BG74	Acyltransferase required for palmitoylation of hedgehog hh family of secreted signaling
A0A1E1X1V4	Putative secreted protein	A0A023FL62	f0f1-type atp synthase alpha subunit
F0J8F4	Hypothetical secreted protein 1752	V5ICE2	Putative secreted protein
V5HCM2	Putative secreted protein	A0A131XF49	Putative secreted salivary gland peptide
V5I529	Putative secreted protein	A0A131XLI6	Putative secreted metalloprotease
Transporters (25)			
M5AYG7	Ferritin	A0A131YI50	Vitellogenin-3
Q6WNX5	Ferritin	A0A023FUV2	Vitellogenin-2
E1CAX9	Vitellogenin-1	A0A023FYX2	Vitellogenin-2

(Continued)

TABLE 1 | Continued

Uniprot ID	Description	Uniprot ID	Description
B1B544	Vitellogenin-2	A0A023GCA7	Vitellogenin-2
E1CAY0	Vitellogenin-3	A0A2R5L9R5	Vitellogenin-1
G9M4L6	Vitellogenin-B	A0A1E1X1H2	Vitellogenin-c
A0A023GME3	Vitellogenin-1	Q19V51	Hemelipoglycoprotein
A0A023GMC7	Vitellogenin-1	B5ABL8	Hemelipoglycoprotein 2
V5H7G7	Vitellogenin-2	A0A023FKG8	Lipid exporter abca1
A0A023GNW9	Vitellogenin-2	A0A023FWZ5	Nuclear pore complex protein nup85
A0A034WXH7	Vitellogenin 4	A0A1Z5L0J3	Aquaporin
A0A023GPB4	Vitellogenin-2	A0A224Z507	Nuclear pore complex protein Nup188
A0A2R5L4N6	Spatacsin		
Immunity related (5)			
A0A1E1XEL3	Alpha-macroglobulin	G3BJU6	Immunogenic protein
A0A023FNM2	Alpha-2-macroglobulin-like protein	A0A0K8R7R0	Ixodes 26 kDa salivary protein
A0A224YHA0	Alpha-2-macroglobulin splice variant 1		

Proteins were classified in groups based on function and/or protein families.

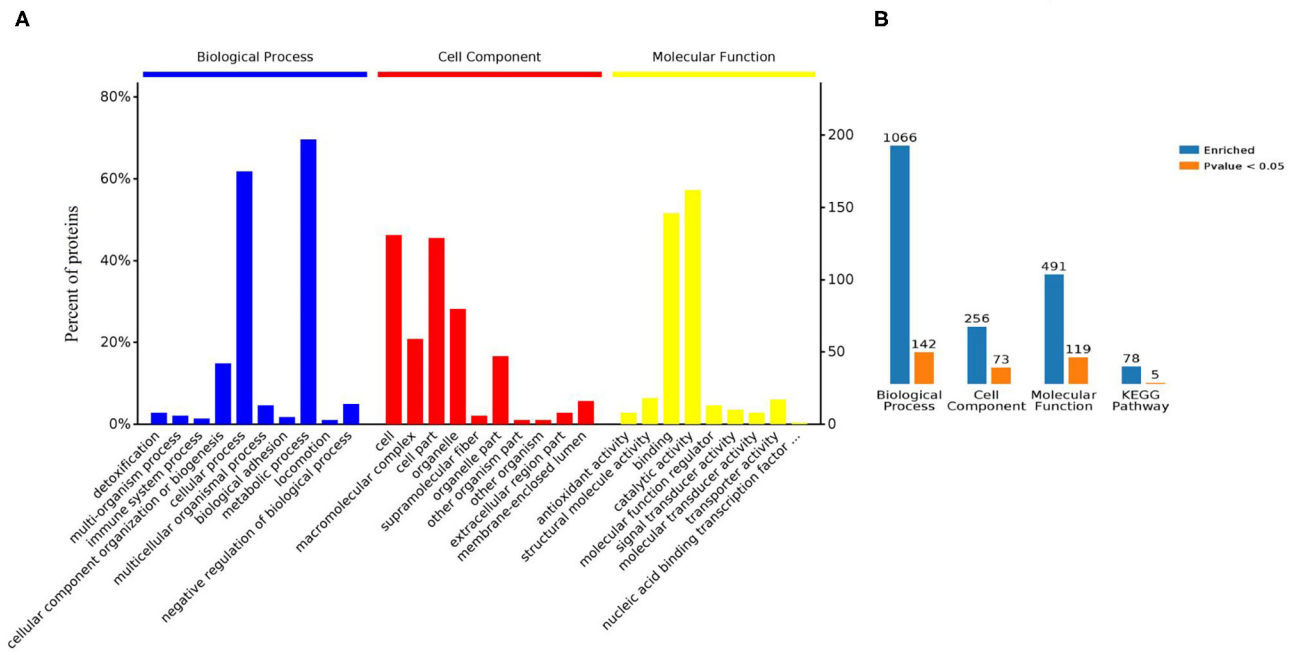

FIGURE 4 | Bioinformatic analyses of the proteins derived from tick EVs. **(A)** Enrichment analysis by biological process, cell component, and molecular function. **(B)** Enriched GO terms and KEGG pathways analysis of differently expressed proteins: blue: enriched; orange: significantly enriched.

The presence of host-derived proteins suggests that tick EVs are involved in host-parasite relationships.

In order to validate proteome data of tick-derived EVs, the supernatant collected during centrifugation was also examined for the presence of proteins within it. LC-MS/MS identified 490 proteins within supernatant, which may be considered as tick saliva proteins (**Supplementary Table 3**). Presence of these proteins within supernatant suggests that the proteins within pellet are specific to tick EVs. Likewise, western blot analysis was carried out to get further confirmation. For this purpose, HSP70 and GAPDH proteins were selected. Immunodetection of proteins resulted in thick bands of 36 and 70 kDa. The predicted size of GAPDH is 35.6–36 kDa, which matches the observed band (**Figure 3B**). Similarly, the band observed at 70 kDa matches to Hsp70 (**Figure 3C**). Validation of the proteins by Western blot indicated that the proteomics data of tick-derived EVs were highly reliable.

Gene ontology (GO) analysis of differentially expressed proteins showed significant enrichment for 142 proteins associated with "Biological process," 73 associated with

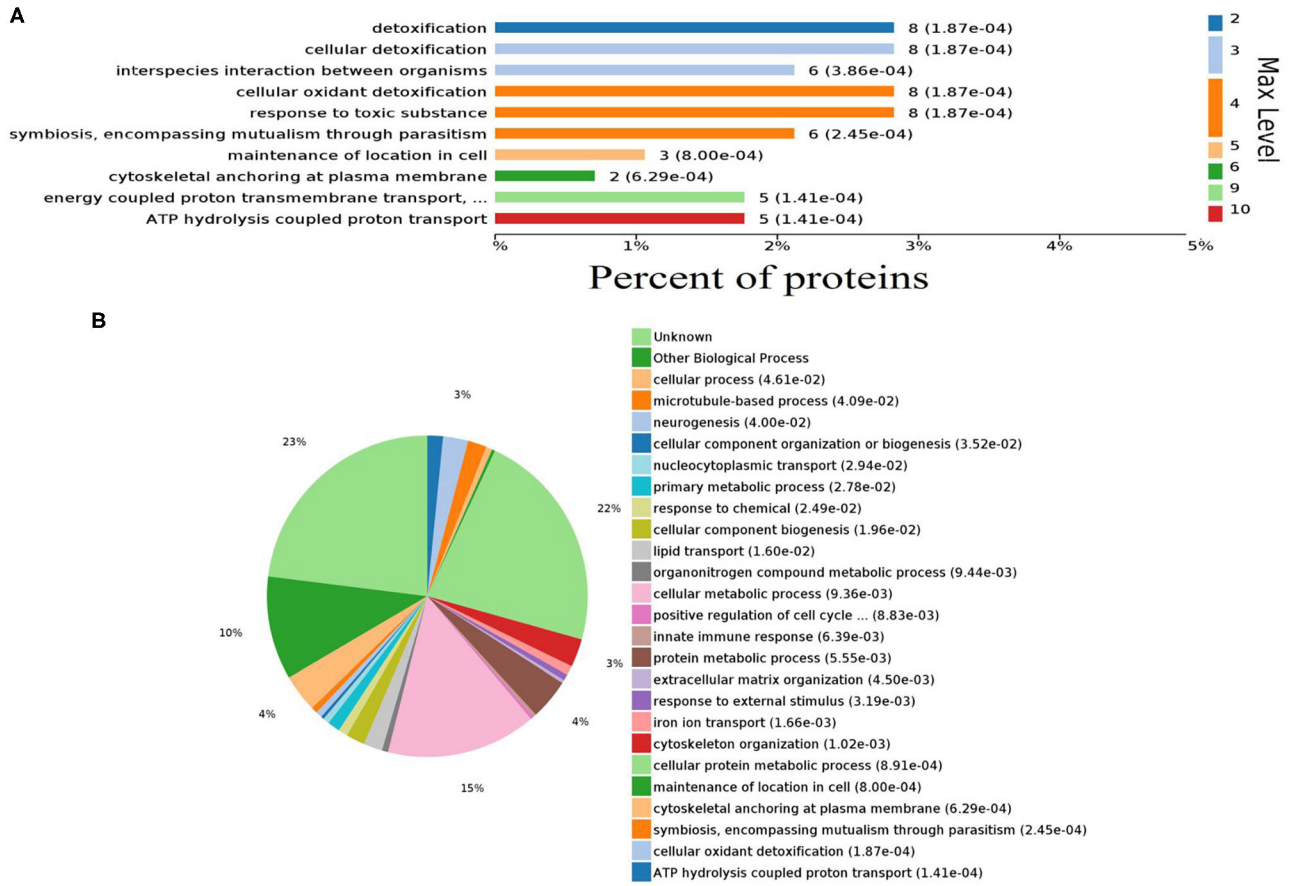

FIGURE 5 | Abundantly represented gene ontology biological process terms. **(A)** Bar chart showing the percentage of proteins involved in biological process. **(B)** Pie chart distribution of enriched biological processes.

"Cellular components," and 143 associated with "Molecular function" (**Figures 4A,B**, **Supplementary Table 4**). In terms of biological process, the *P*-value ($P < 0.05$) indicated that ATP hydrolysis coupled proton transport, energy coupled proton transmembrane transport, cellular oxidant detoxification, detoxification, and cellular detoxification were more significant (**Figures 5A,B**, **Supplementary Figure 2A**). Analysis of "Cellular components" indicated that most of the proteins were associated with the "cell" category. In addition, further subdivisions revealed that differentially expressed proteins were involved in intracellular parts, cytoplasm, proton-transporting two-sector ATPase complex, and cytoskeleton (**Figures 6A,B**, **Supplementary Figure 2B**). Significant "Molecular functions" associated with vesicular proteins were antioxidant activity, peroxidase activity, hydrolase activity, pyrophosphatase activity, and ATPase activity (**Figures 7A,B**, **Supplementary Figure 2C**).

The KEGG database is a collection of various pathways, representing the molecular interactions and reaction networks. To identify the pathways involved, we mapped the KEGG database and found that identified proteins were enriched in 78 pathways (**Figure 8A**, **Supplementary Table 5**). Further analysis of the *P*-values ($P < 0.05$: **Figures 8B,C**) revealed that differentially expressed proteins were mainly involved in ECM-receptor interaction, ribosome, RNA transport, ABC transporters, and oxidative phosphorylation.

DISCUSSION

EVs are known to transfer intracellular information from one cell or tissue to other (Nawaz et al., 2019; Wu et al., 2019). The information (proteins, miRNAs) transferred by EVs are thought to mediate cellular activity and pathways in recipient cells (Zhu et al., 2016). Recent studies revealed that parasites such as *Schistosoma japonicum*, *Leshmania infantum*, *Toxoplasma gondii*, and *Fasciola hepatica* release EVs in their excretory-secretory products (Cwiklinski et al., 2015; Zhu et al., 2016; Li et al., 2018; Marshall et al., 2018). In the context of ticks, Zhou et al. (2018) demonstrated that tick embryonic cell line *Ixodes scapularis* ISE6 is also capable of secreting extracellular vesicles including exosomes. However, it remained unknown whether ticks such as *H. longicornis*, a major agent causing severe pathology of theileriosis, could secrete vesicles in saliva as well. Here, we isolated vesicles from saliva of *H. longicornis*. Transmission electron microscopy was carried out to examine

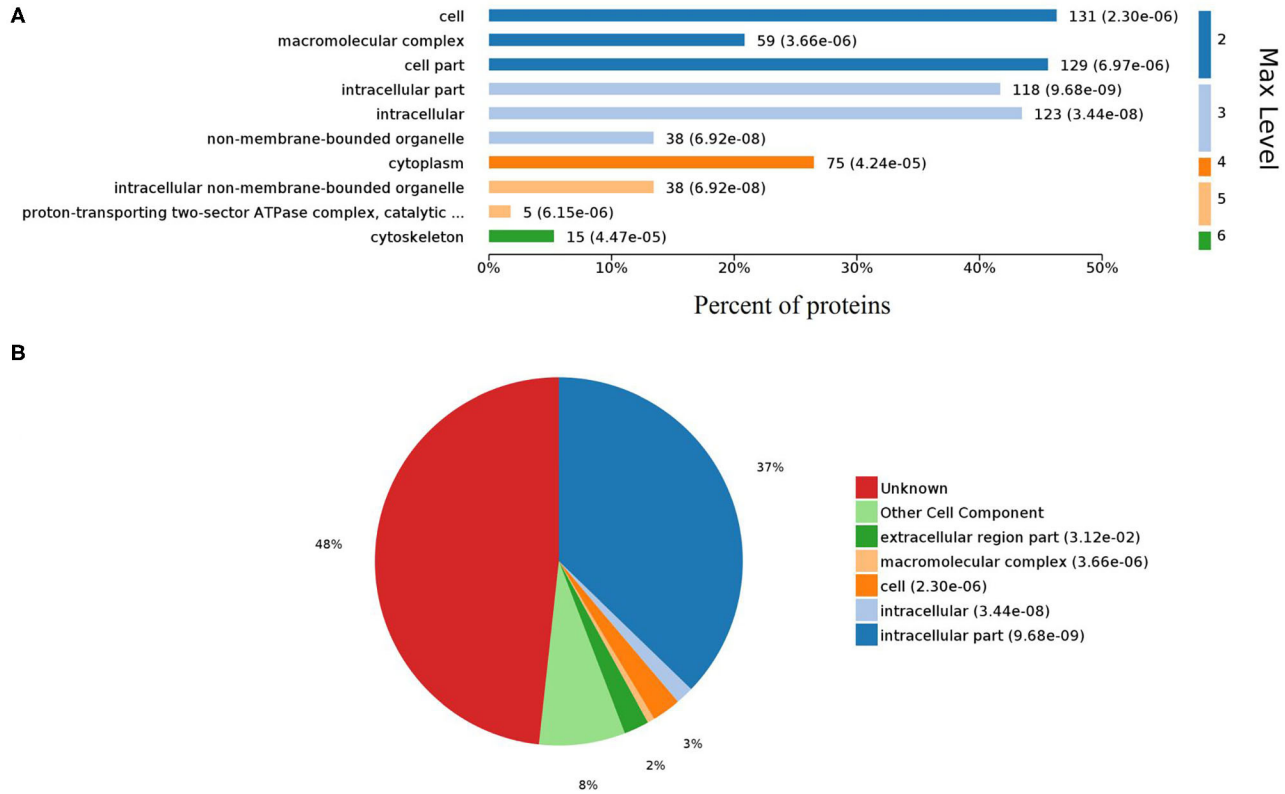

FIGURE 6 | Gene Ontology analysis of cellular components. **(A)** Bar chart representing the levels of cell components. **(B)** Pie chart showing the classification of cell components.

EVs. Consistent with previous reports, we demonstrated that *H. longicornis*-derived EVs are similar in size (100 nm) and shape to other parasite-derived exosomes.

LC-MS/MS showed a wide variety of proteins within tick-derived EVs. The major protein groups included structural proteins, metabolic proteins, nuclear proteins, transporters, enzymes, and some proteins for which no homologs (**Table 1**). Structural/cytoskeletal proteins such as myosin, tropomyosin, actin, and tubulin alpha chain found in our study have been recorded with high scores previously in parasite-derived EVs such as *B. malayi*, *S. japonicum* and *L. infantum* (Atayde et al., 2015; Zamanian et al., 2015; Zhu et al., 2016). Actin, representing 1.1% of proteins identified, has been known to play immunogenic role in *Echinostoma caproni* (Sotillo et al., 2010). Likewise, myosin proteins (1.7%) localized beneath the plasma membrane exhibit biophysical properties required to generate fast movements in parasites such as *T. gondii* and other apicomplexans (Sibley et al., 1998; Herm-Götz et al., 2002). The presence of structural proteins suggests that they may be associated with the production of vesicles (Wubbolts et al., 2003).

The vitellogenin group of proteins, representing 4.5% of proteins identified in our study, have not been previously identified in EVs of other parasites. Vitellogenin (Vg), also considered as female-specific protein, is synthesized as a high molecular-mass precursor in ovaries, gut, and fat body of ixodid ticks (Rosell and Coons, 1992; Thompson et al., 2007; Boldbaatar et al., 2010). After its release into the haemolymph, Vg is taken up by oocytes through receptor mediated endocytosis, and is then accumulated in yolk granules.Vg is considered as a source for embryo development and egg formation during tick reproduction (Xavier et al., 2018). Antioxidant property of Vg, diminishing heme-induced lipid peroxidation has been reported. Importantly, silencing of Vg in ticks feeding on sheep resulted in reduced engorgement and oviposition rates (Esteves et al., 2017). Likewise, the protein ferritin identified within the tick EVs, has been known to play crucial role in blood-feeding and reproduction in *H. longicornis* and *Ixodes ricinus* (Hajdusek et al., 2009; Galay et al., 2013). Moreover, insect ferritin was also implicated in iron transport, immune response and oxidative stress (Orino et al., 2001; Ong et al., 2006). As these proteins have been identified in saliva of ticks, therefore, it has been believed that tick saliva is beneficial for reproduction and blood-feeding of ticks. However, the association of these particular proteins with tick EVs still requires further confirmation.

Some other proteins like heat-shock proteins (HSPs), thioredoxin peroxidase, metalloprotease, glyceraldehyde-3-phosphate dehydrogenase, and glutathione S-transferase have also been identified in the context of parasite-derived EVs. HSPs play key roles in differentiation, adaptation, and protection of parasites from killing mechanisms of hosts such as low pH

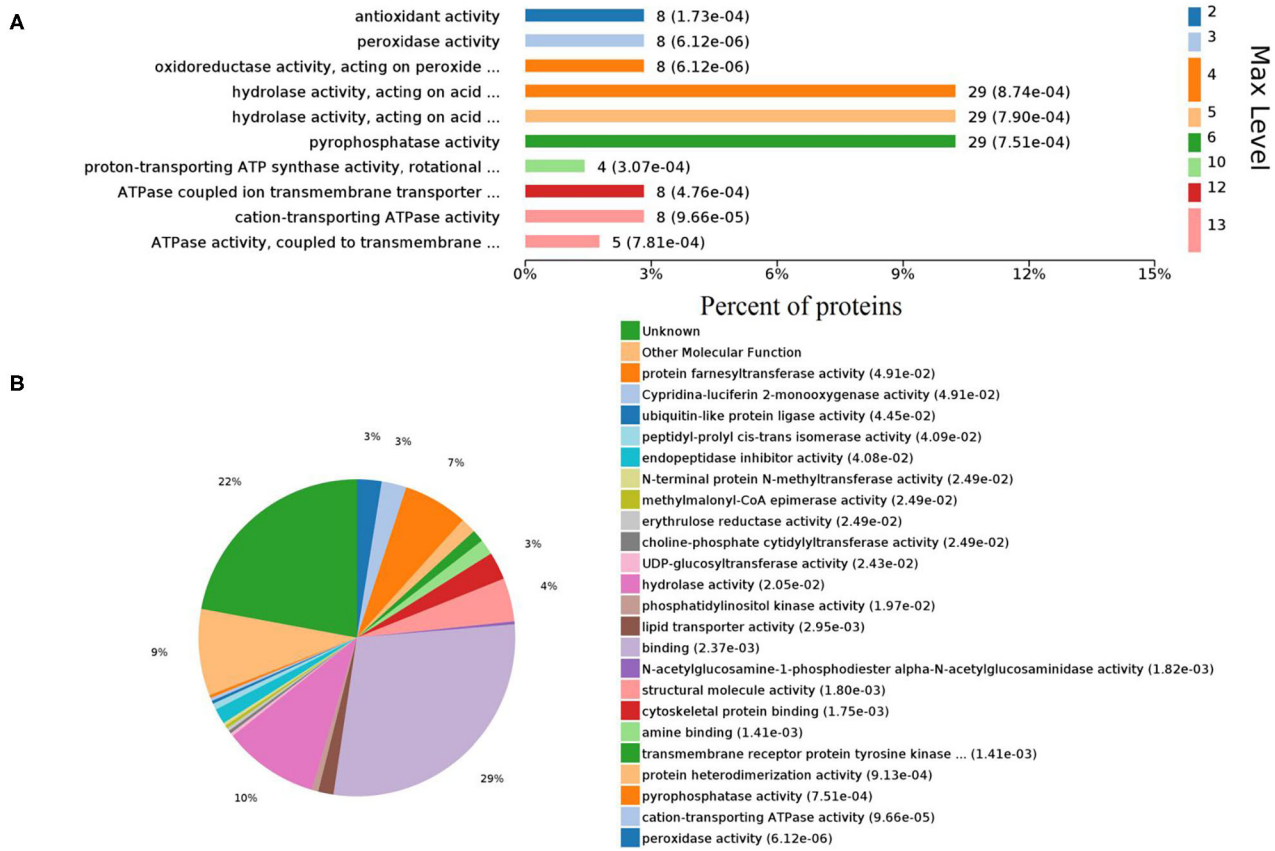

FIGURE 7 | Enriched GO terms of molecular functions. **(A)** Different levels of molecular functions. **(B)** Pie chart denoting the distribution summary of molecular functions.

and reactive oxygen metabolites (Johnson and Brown, 2009). Moreover, heat-shock proteins (HSP70) are thought to induce transformation of promastigote stage of *Leishmania donovani* to its amastigote stage (Wiesgigl and Clos, 2001). Thioredoxin peroxidase, an antioxidant enzyme, is also identified in parasite-derived exosomes (Tzelos et al., 2016). It has been proposed that these vesicle-derived enzymes direct the immune system of host toward Th2 immune response, which is thought to be favorable for parasite development within the host (Robinson et al., 2010; Dalton et al., 2013). In addition to the proteins described above, MS spectra of tick-derived EVs were also analyzed for the presence of host proteins within these EVs. These host proteins have been known to be present in saliva of tick species and parasite-derived EVs (Buck et al., 2014; Kim et al., 2016; Samoil et al., 2018). In recent studies, it has been proposed that the host proteins like fibrinogen, serum albumin, and serotransferrin are likely associated with the events toward tick feeding (Kim et al., 2016). Therefore, presence of such proteins within tick-derived EVs clearly implicates these structures in host-parasite communication processes. However, their role in tick EVs requires further investigation.

Moreover, the proteome of *H. longicornis* tick saliva-derived EVs showed similarity with proteins identified from tick saliva (**Supplementary Table 6**). Proteins such as enolase, histones, heat shock proteins, lipocalin, thioredoxin, and vitellogenin have been identified within saliva of *Ornithodoros moubata, Ixodes scapularis, Rhipicephalus sanguineus*, *Amblyomma americanum, Dermacentor andersoni*, and *Haemaphysalis longicornis*. (Díaz-MartÍN et al., 2013; Oliveira et al., 2013; Mudenda et al., 2014; Tirloni et al., 2015; Ren et al., 2019). Due to the similarty in proteomes, it has been proposed that ticks could use these nano-sized vesicles to produce saliva (Díaz-MartÍN et al., 2013). Therefore, the findings of current study gave further confirmaton to assumptions derived in previous studies.

Gene Ontology (GO) database determines the functional annotation of gene products with a vocabulary of ontological terms describing their biological processes, molecular functions, and cellular components of the cell (Ashburner et al., 2000; Consortium, 2004; Thomas, 2017). GO data revealed that the possible outcomes of vesicular proteins were hydrolysis coupled proton transport, energy coupled proton transmembrane transport, and detoxification. "Biological process" indicated role of proteins in proton transport as well as in removal of harmful toxins accumulated within the body. In "cell component" category, highly enriched category was found

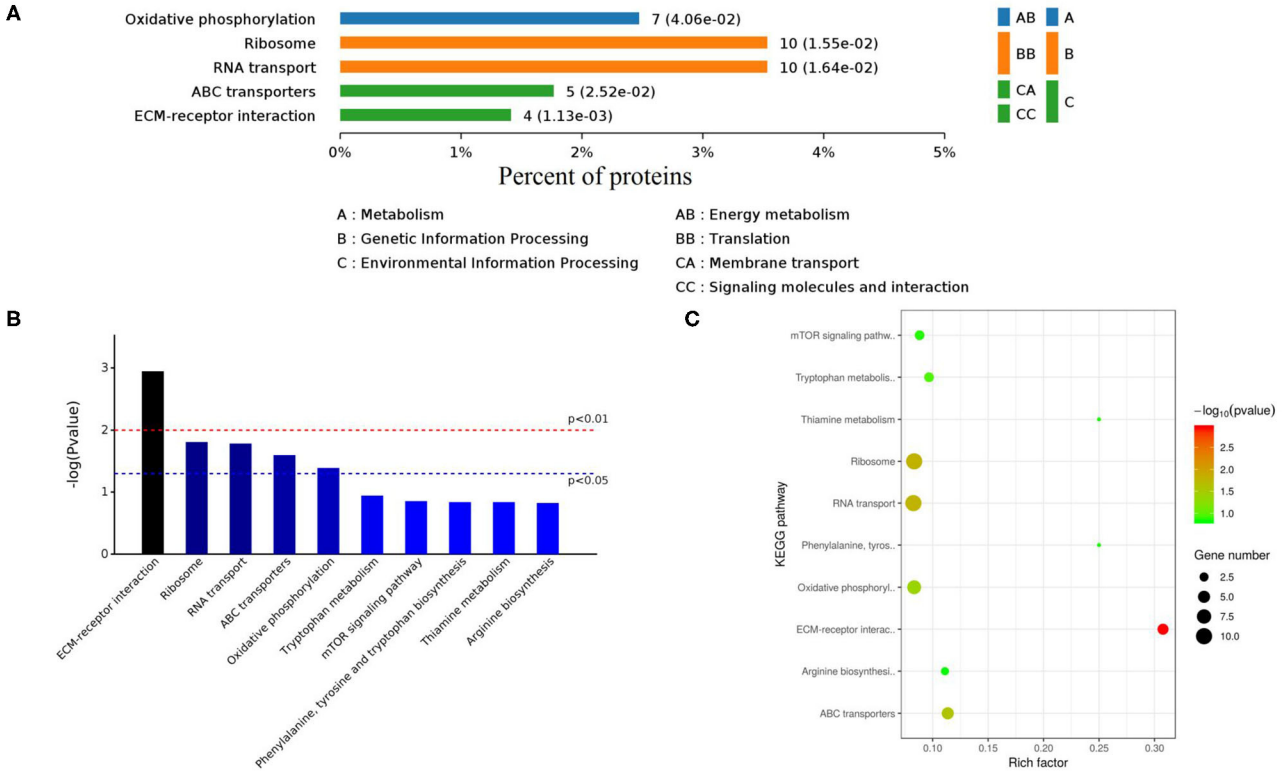

FIGURE 8 | KEGG enrichment pathways of differently expressed proteins. **(A)** Classes of enriched KEGG pathways. **(B)** Distribution of KEGG pathways (Top 10). **(C)** Scattered plot of differently expressed proteins. X-axis indicates the rich factor; Y-axis indicates the name of KEGG pathway. The dot size means the protein number and dot color indicates the *q*-value.

to be "cell." This analysis revealed that major biological as well as metabolic functions occur within cell. "Molecular function" analysis indicated that proteins were mainly involved in binding and catalytic activity. However, other significant functions associated with proteins were the transfer of ions across the membranes as well as inhibition of oxidation. KEGG pathways analysis showed that proteins regulated several pathways associated with ribosome biosynthesis (ribosome), transport of RNA species (RNA transport pathway), and regulation of oxidative pathway as well as generation of ATPs (oxidative phosphorylation). Bioinformatics analysis of vesicular proteins revealed that proteins are associated with certain biological as well as molecular functions which may be beneficial for reproduction and survival of ticks.

To the best of our knowledge, we report for the first time that the tick *H. longicornis* is also capable of secreting exosome-like vesicles. However, some limitations have been associated with the present study. This study deals with ticks, and saliva is the main excretory source of ticks, however, it is quite difficult to collect enough amount of saliva to be used separately for all the standard techniques. In addition, EVs isolated from saliva were just enough to be used for SDS-PAGE, electron microscopy, BCA, Western blot, and LC-MS/MS. Therefore, characterization of exosomes by nano-particle tracking analysis is not provided. At this stage, we would like to present the interesting results at an early stage to other researchers. Further categorization of tick-derived EVs is required to improve knowledge about the proteins within EVs and their associated functions.

CONCLUSIONS

In summary, the present study constitutes the first analysis of secretion of EVs from ticks. The present study indicates that EVs may be a useful pathway for ticks for the transfer of genetic materials to host cells, thereby helping ticks in modulating host immune responses. However, better understanding of how EVs increase tick attachment to host skin as well as modulation of host immune responses will be helpful in pathogenesis and development of therapeutics and vaccine against ticks.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ProteomeXchange (accession number PXD020300).

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute.

AUTHOR CONTRIBUTIONS

JZ conceived and designed the experiments. MN performed the experiments. HZ, JC, and YZ completed the data analysis. MM, IH, MH, and ZH contributed reagents, materials, analysis, and tools. JZ and MN wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.542319/full#supplementary-material

Supplementary Figure 1 | Schematic representation of steps followed to purify tick EVs.

Supplementary Figure 2 | Histograms representing significantly enriched ($P \leq 0.05$) biological processes **(A)**, cellular components **(B)** and molecular functions **(C)**.

Supplementary Table 1 | Protein content of tick-derived EVs identified using MASCOT search engine.

Supplementary Table 2 | Host proteins identified in purified EVs.

Supplementary Table 3 | Proteins present in supernatant of tick-derived EVs.

Supplementary Table 4 | Details of Biological processes, cellular components and molecular functions.

Supplementary Table 5 | KEGG Pathway analysis of proteins identified from tick-saliva derived EVs.

Supplementary Table 6 | Common proteins between tick saliva and tick-derived EVs.

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Leishmania amazonensis Promastigotes or Extracellular Vesicles Modulate B-1 Cell Activation and Differentiation

Natasha Ferraz de Campos Reis[1], *Talita Vieira Dupin*[1], *Carolina Rizzaro Costa*[1], *Maytê dos Santos Toledo*[1], *Vivian Cristina de Oliveira*[2], *Ana Flavia Popi*[2], *Ana Claudia Torrecilhas*[1] *and Patricia Xander*[1]*

[1] *Laboratory of Cellular Immunology and Biochemistry of Fungi and Protozoa, Department of Pharmaceutical Sciences, Federal University of São Paulo, São Paulo, Brazil,* [2] *Department of Microbiology, Immunology and Parasitology, Paulista School of Medicine, Federal University of São Paulo, São Paulo, Brazil*

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***Correspondence:**
Patricia Xander
patricia.xander@unifesp.br

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B-1 cells are considered an innate-like B cell population that participates in effective innate and adaptive responses to pathogens. B-1 cells produce immunoglobulins, cytokines, chemokines, migrate to inflammatory sites, and differentiate into mononuclear phagocyte-like cells. Murine B-1 cells phagocytosed *Leishmania in vitro* and *in vivo* and participate in immunity against *Leishmania*. Our group showed that B-1 cells or their extracellular vesicles (EVs) led to a resistance to experimental infection by *L. amazonensis.* However, the B-1 cells' responses to *Leishmania* or EVs isolated from parasites are still poorly characterized. Studying the activation and differentiation of B-1 cells *in vivo* can contribute to a better understanding of how these cells participate in immunity to *L. amazonensis.* Thus, we evaluated the expression of myeloid (M-csfr, G-csfr, Spi-1) and lymphoid (EBF, E2A, IL-7R) lineage commitment factors, Toll-like receptors (TLRs), activation cell surface markers, nitric oxide (NO) and reactive oxygen species (ROS) production in murine peritoneal B-1 cells collected after 24 or 48 h post-infection with *Leishmania (Leishmania) amazonensis* promastigotes or EVs released by the parasites. Our results demonstrated that *L. amazonensis* infection did not stimulate the expression of CD40, CD80, CD86, F4/80, and MHC II in B-1 cells, but a significant decrease in the production of NO and ROS was observed. The infection induced a significantly higher arginase expression in B-1 cells, but the stimulation with EVs led to a decrease in this gene expression. TLR-2 and TLR-6 had significantly higher expression in B-1 cells from mice intraperitoneally stimulated with the parasite. The TLR-9 expression was higher in animals infected or stimulated for 48 h with EVs. Interestingly, in B-1 cells the stimulus with *L. amazonensis* led to a substantial increase in the expression of myeloid restricted transcription factors. Thus, our study suggests that the parasites or EVs differently modulated the activation and differentiation of B-1 cells.

Keywords: *Leishmania*, extracellular vesicles, B-1 cells, innate immune response, activation

INTRODUCTION

Leishmania release virulence factors in extracellular vesicles (EVs) that can interact with host cells, modulate host immune systems, contributing to the *Leishmania* infection (Silverman and Reiner, 2011; Atayde et al., 2016). Many groups have been studying the role of EVs in *Leishmania* infection, focusing on parasite-host cell interaction, and innate immune response (Santarém et al., 2013; Atayde et al., 2015; Atayde et al., 2019; Dong et al., 2019).

EVs are components secreted by mammalian cells (Théry et al., 2018), bacteria (Lee et al., 2016), fungi (Vallejo et al., 2012a; Vallejo et al., 2012b; Ikeda et al., 2018), and parasites (Torrecilhas et al., 2012; Marcilla et al., 2014; Campos et al., 2015; Evans-Osses et al., 2017; Ribeiro et al., 2018). They represent a new type of intercellular communication since they are composed of DNA, RNA, proteins, lipids, and cellular metabolites (Kalra et al., 2016; Théry et al., 2018). *In vitro*, EVs released by *Leishmania* modulated cytokine production, cell surface marker expression, and microbicidal molecule production by human and murine phagocytic cells (Silverman et al., 2010a; Silverman et al., 2010b). *In vivo*, experimental models of leishmaniasis have shown that the inoculation of the parasites in the presence of the EVs led to an increase in the lesion size and inflammation, supporting the hypothesis that EVs released by the *Leishmania* have a role in parasite infection (Atayde et al., 2015; Barbosa et al., 2018).

In tropical regions are an estimated 1.3 million new cases of leishmaniasis diagnosed annually with 20,000 to 30,000 deaths (Alvar et al., 2012; Burza et al., 2018). *Leishmania* spp. are protozoan parasites, etiologic agents of leishmaniasis, a debilitating, and often disabling disease (Burza et al., 2018). The clinical forms of leishmaniasis depend on the *Leishmania* species (Subramanian and Sarkar, 2018), vector characteristics (Rogers, 2012), and the host's immune response (Kaye and Scott, 2011; Scott and Novais, 2016). The macrophages are the central cells in immunity against *Leishmania* infection (Tomiotto-Pellissier et al., 2018). The production of microbicide molecules, such as NO and ROS, inflammatory cytokines, and the upregulation of TLRs have been related to the leishmanicidal activity of human and murine macrophages (Faria et al., 2012). However, *Leishmania* has developed several strategies to evade the immune response in the vertebrate host (Atayde et al., 2016; Scott and Novais, 2016). Lipophosphoglycan (LPG) and the glycoprotein metalloprotease gp63 (gp63) are important virulence factors produced by the parasites that act in different mechanisms of host immune response providing an environment permissive for the establishment of *Leishmania* infection (Olivier et al., 2012; Forestier et al., 2014; Barbosa et al., 2018).

Besides macrophages, other cell types can be infected by *Leishmania* and influence the immune response against the parasite (Hurrell et al., 2017; Martínez-López et al., 2018). Our group and others have demonstrated that B-1 cells, a subtype of B lymphocytes, participate in the immune response against *Leishmania* (Arcanjo et al., 2015; Gonzaga et al., 2015; Geraldo et al., 2016). Murine B-1 cells express unusual cell surface markers ($CD19^{+}CD23^{lo}IgM^{hi}IgD^{lo}CD45^{lo}CD11b^{+/-}CD43^{+/-}$ (Baumgarth, 2011; Baumgarth, 2017) and human B-1 cells express $CD20^{+}CD27^{+}CD43^{+}$ (Griffin and Rothstein, 2012; Quách et al., 2016); they participate in homeostasis and immune response by producing cytokines (mainly IL-10) (O'Garra et al., 1992), and host humoral response (natural and inducible antibodies) (Baumgarth, 2017; Savage et al., 2017). Intriguingly, B-1 lymphocytes express both myeloid and lymphoid commitment transcription factors, which allows them to differentiate into cells with phenotypic, physiological and molecular characteristics of mononuclear phagocytes (Almeida et al., 2001; Popi et al., 2009).

In leishmaniasis, whether B-1 cells participate in resistance or susceptibility depends upon the *Leishmania* species and strain (Gonzaga et al., 2015; Gonzaga et al., 2017). Animals with severe X-linked immunodeficiency (XID) (due to mutation in Bruton's tyrosine kinase -Btk) had a significant reduction in peritoneal B-1 cells (Hayakawa et al., 1983; Khan et al., 1995). These animals showed more resistance to chronic infection with *Leishmania infantum* as compared to control (Gonzaga et al., 2015). In contrast, BALB/XID mice were more susceptible to infection by *L. amazonensis* (MHOM/BR/1973/M2269 strain) (Gonzaga et al., 2017). Also, EVs released by B-1 cells led to a reduction in the parasite load in mice experimentally infected by the parasite (Toledo et al., 2020). *In vitro*, stimulation with *L. amazonensis* promastigotes or EVs released by the parasite modulated the cytokines production by B-1 cells (Geraldo et al., 2016; Barbosa et al., 2018). Thus, although the mechanisms are not yet fully understood, it is possible to consider that B-1 cells participate in immunity to *L. amazonensis*. However, the ability of the parasite or their EVs to induce B-1 cell activation and/or differentiation into phagocytes *in vivo* have not yet been evaluated.

Thus, this work investigated the differentiation and activation of B-1 cells after stimulation with *L. amazonensis* or EVs released by the parasites. We evaluated the production of microbicidal molecules, the expression of TLRs, cytokines, and lymphoid and myeloid commitment factors in peritoneal B-1 cells after intraperitoneal stimulation with *L. amazonensis* promastigotes or EVs released by the parasites. Understanding how B-1 cells are activated after stimulation by the parasite and its components can help better to understand the role of B-1 cells in leishmaniasis. This study can unveil the relationship of B-1 cells and *Leishmania* and how they can be related to cutaneous leishmaniasis progression and protozoa parasite-host B-1 communication and activation.

MATERIAL AND METHODS

Animals

Six- or 8-week-old female BALB/c mice were purchased from Center for the Development of Experimental Models for Medicine and Biology (CEDEME, Universidade Federal de São Paulo - UNIFESP, São Paulo, SP, Brazil). The mice were housed under pathogen-free conditions, as recommended by the National Council for Control Animal Experimentation

(CONCEA) of Brazil. All procedures were approved by the Committee on Ethics of Animal Experiments (CEUA) of UNIFESP, under protocol number 8762030718.

Parasites

L. amazonensis (MHOM/BR/1973/M2269 strain) parasites were kindly provided by Dr. Clara Lúcia Barbieri of UNIFESP, São Paulo, Brazil. The parasites were aseptically cultured as promastigotes at 26°C in 199 medium (Gibco, Life Technologies Brand, Grand Island, NY, United States) supplemented with 4.2 mM sodium bicarbonate, 4.2 mM HEPES, 1 mM adenine (Sigma, St. Louis, MO, United States), 5 μg/ml hemin (bovine type I) (Sigma) plus 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, United States). Promastigotes in the stationary growth phase were recovered by centrifugation and used for infection or obtaining EVs.

Obtaining and Isolating of EVs Released by *L. amazonensis*

L. amazonensis promastigotes were cultured *in vitro* until the stationary phase. Parasites were recovered, washed 5 times in phosphate-buffered saline (PBS), and 10^8 parasites were distributed in each microtube containing 1 ml of medium 199 added with 2% D-dextrose. The parasites were incubated at 26°C for 4 h for EVs releasing (Barbosa et al., 2018). Then, the cultures were centrifuged to remove parasites and EVs were recovered from the supernatants. Afterward, the supernatants were recovered and filtered through 0.45-μm sterile cartridges and submitted to sequential centrifugation at 4°C: 500 g for 10 min, 1,500 g for 10 min, 10,000 g for 10 min, and twice at 100,000 × g for 1 h to pellet parasite EVs. Sorvall WX Ultra Thermo Scientific (rotor T890) was used in the ultracentrifugation steps (Barbosa et al., 2018). The pellets were washed once and then diluted in sterile filtered PBS.

The protein concentrations of isolated EVs were evaluated by using the Micro BCA protein assay kit (Thermo Scientific, Waltham, MA, United States). The concentration of particle number/ml and size distribution of EVs were determined using Nanoparticle Tracking Analysis (NTA) in a Nanosight NS300 instrument (Malvern Instruments Ltd., Malvern, United Kingdom). Samples were diluted 10- to 100-fold in sterile PBS and captured in triplicate for 30 s with the camera level set to 14 using the same threshold along all analyses. The data acquired were examined using the NTA software (version 2.3 build 0017) as previous described (Barbosa et al., 2018).

Intraperitoneal Inoculation of BALB/c Mice With Parasites or EVs

BALB/c mice were intraperitoneally inoculated with 10^8 parasites or with 4 μg of EVs (corresponding to 2.39×10^6 particles ± 1.25×10^6). Parasites were cultured as promastigotes until stationary phase. After washing with sterile PBS, parasites were resuspended at a concentration of 10^8 parasites/500 μl and then intraperitoneally inoculated in mice (adapted from Geraldo et al., 2016). Sterile EVs resuspended in PBS were intraperitoneally inoculated at a concentration of 4 μg/mouse (Barbosa et al., 2018). Animals inoculated with sterile PBS were used as a negative control. 24 or 48 h after inoculation, the animals were euthanized, and peritoneal cavities were washed with ice-cold sterile PBS to collect total peritoneal cells.

Flow Cytometry

Peritoneal cells were washed with cooled sterile PBS and counted in the Neubauer chamber. The cell suspensions were distributed in 1.5 ml microtubes and diluted up to 100 μl PBS (1×10^6 cell/microtube). The purified rat anti-mouse CD16/CD32 (2.4G2 clone, BD Fc Block™, BD Bioscience, San Jose, CA, USA) was used to block non-antigen-specific binding of immunoglobulins to the Fc receptors. Cell suspensions were incubated with Fc block for 60 min at 4°C. Samples were then incubated with anti-CD19 coupled with allophycocyanin (APC) (clone 1D3, BD) and anti-CD23 coupled with phycoerythrin (PE) (clone B3B4, BD). B-1 cell population was identified as $CD23^-CD19^+$. The following monoclonal antibodies were used to evaluate other cell surface markers: fluorescein-isothiocyanate (FITC)-conjugated anti-mouse CD80 (anti-CD80 FITC, clone 16-10A1, BD), anti-CD86 FITC (clone GL1, BD), anti-CD40 FITC (clone 3/23, BD), anti-F4/80 coupled with peridinin-chlorophyll-protein - PerCP (clone BM8) (BioLegend, San Diego, CA, USA). To evaluate the expression of MHC II, we used anti-MHC II, and B-1 cells were identified with anti-CD19 APC (BD), anti-CD23 FITC (BD). All antibodies were used at 1:100 dilutions. The samples were incubated with fluorochrome-conjugated antibodies for 30 min at 4°C, washed with PBS, resuspended in 500 μl of PBS, and analyzed with BD FACSCalibur flow cytometer (BD Bioscience).

Intracellular NO production was evaluated using the fluorescent reagent 4-amino-5-methylamino-2,7-difluorescein diacetate (DAF2-DA, Sigma) and the production of intracellular ROS was evaluated using the fluorescent reagent H_2DCFHDA (Sigma). Total peritoneal cells were labeled with anti-CD19 APC and anti-CD23 PE. Then, cells were washed with PBS and followed incubated with 5 μM of DAF2-DA or H_2DCFHDA for 30 min at 37°C in the dark. Cells were washed with PBS and resuspended with 500 μl of PBS. The data acquisition was performed with the FACSCalibur cytometer (BD), and the results were analyzed with FlowJo software version 10.6.2 (FlowJo, LLC, FlowJo™ Software for Mac, Ashland, OR, USA). The gating strategy is shown in **Supplementary Figure 1**.

Enrichment of Peritoneal B-1 Cells

Total peritoneal cells from mice stimulated or not with *L. amazonensis* promastigotes or EVs released by the parasite were subjected to centrifugation at 161 × g for 5 min. The pellets containing the cells were resuspended in PBS pH 7.2 supplemented with 0.5% FBS, and 2 mM ethylenediaminetetraacetic acid—EDTA (MACS buffer) and the cells were counted in a Neubauer chamber. For enrichment 90 μl of MACS were added to every 10^7 total cells. Cell suspensions were incubated with 2 μl of Fc block per 6×10^6 total cells for 60 min at 4°C. Then, cells were washed with sterile PBS, resuspended in MACS, and subjected to negative selection with anti-CD23 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by positive selection with anti-CD19

microbeads (Miltenyi Biotec) ($CD23^-$ $CD19^+$). All procedures were performed according to the manufacturer's instructions. The purity of the cell suspension was evaluated by flow cytometry using anti-IgM APC (II/41 clone, BD) and anti-CD11b PE (M1/70 clone, BD) cell surface markers. The data were acquired in the FACSCalibur cytometer (BD Bioscience) and analyzed with FlowJo software version 10.6.2 (FlowJo, LLC).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

qRT-PCR was employed to analyze the expression of arginase enzyme, Toll-like receptors (TLRs), cytokines, and myeloid and lymphoid commitment transcription factors in B-1 cells from mice intraperitoneally infected with parasites or inoculated with EVs from parasite. All qRT-PCR followed the recommendations of the Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). First, peritoneal B-1 cells were enriched, and then total RNA was extracted using PureLinkRNA Mini Kit (Ambion; Thermo Fisher Scientific Brand, Grand Island, NY, United States™) following the manufacturer's recommendations. The quantification of total RNA was performed by evaluating their UV absorption in a spectrophotometer (Nanodrop 2000c, Thermo Fisher). Samples with reading ranges of 1.8–2.0 at 260/280 nm and 260/230 nm were analyzed for their integrity by electrophoresis in 1.5% agarose gels. Then, identical amounts (in μg) of the total RNA samples with high quality and integrity were treated with DNAse (RQ1 RNase-free DNase; Promega, Madison, WI, United States) to remove possible contamination with genomic DNA. The cDNA synthesis was performed with the Proto Script First Strand cDNA Synthesis Kit (New England Biolabs Inc., MA, United States). Treatment with DNAse and cDNA preparation was developed by following the instructions of the manufacturer. All the qRT-PCR were performed with equal amounts of each cDNA using SYBR Green Real-Time PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the apparatus StepOnePlus (Applied Biosystems). The following conditions were used in all qRT-PCR reactions: 1 μl of cDNA, 5.0 μl of SYBR Green Master Mix (Thermo Fisher), and 2.0 μl of each oligonucleotide (1.0 μM); the cycle parameters 10 min at 50°C for enzyme activation, denaturation for 5 min at 95°C, and 40 cycles of 95°C for 30 s and 60°C for 1 min. Internal negative controls were performed by using two approaches: I) reactions were performed with samples without addition of the transcriptase reverse for cDNA preparations; and II) not adding template for qPCR reaction (NTC- no template control). In both cases, no amplifications were detected.

To analyze, first, we checked the quality of the reaction based on the dissociation curves (melting curves), and we always detected the presence of only one peak in each reaction. The baseline was adjusted to two or three cycles before the detection of the fluorescent signal. The cycle threshold (Ct) was defined as follows: I) above the background fluorescence baseline, to avoid the amplification plot crossing the threshold prematurely due to background fluorescence; II) in the log phase of the amplification plot, avoiding the plateau phase; and III) at a position where the log phases of all amplification plots are located in a parallel line.

Before performing the gene expression assays, the efficiencies of all primers used throughout this study were analyzed. The efficiency of primers was evaluated by constructing standard curves with the dilution of the samples. The standard curve always contained four or five different points. The Ct values of each dilution point were determined and used to make the standard curve and to calculate the efficiency of primers using the equation E = 10(-1/slope)-1 (E corresponds to the efficiency, and slope is the slope of the standard curve). Then, we compared the efficiency of primers of target genes and normalizers to validate the best normalizers. The sequences of the primers used for each target gene are shown in the **Supplementary Table 1**. The primers sequences were obtained according PrimerBank (Spandidos et al., 2010).

The relative quantification of genes analyzed was calculated according to the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). All experiments were performed in triplicates with at least three biological samples. Differences in the relative expression levels of target genes (fold change) were determined by comparing B-1 cells from mice injected with PBS as reference samples (control) with B-1 cells from mice stimulated with parasites or EVs. The gene expression for the reference sample was always adjusted to 1.

Statistical Analysis

Experiments were carried out with at least three biological replicates. Results from representative experiments are shown. The data are shown as the mean ± standard deviation (SD). Paired one-tailed Student's t-tests were used to perform statistical analysis. P-values < 0.05 were considered significant. All statistical tests were performed using Graph Pad Prism version 7 for Mac (GraphPad Software, La Jolla, CA, United States).

RESULTS

Intraperitoneal Stimulation With *L. amazonensis* Promastigotes or EVs Modulated the Expression of Arginase Enzyme and the Production of NO and ROS in Murine B-1 Cells

In this work, we evaluated the modulation of peritoneal B-1 cells after intraperitoneal stimulation with *L. amazonensis* promastigotes or EVs released by the parasite. All experiments were performed with *L. amazonensis* stationary promastigotes. The EVs from parasites were obtained as described in the *Material and Methods* section. Isolated EVs exhibited mean diameter sizes of 180.0 ± 12.4 nm (data not shown). No differences in mean size were observed in all EVs purification experiments. In this work, we used the total shed of parasite as previously described (Barbosa et al., 2018). The size of the EVs was larger, 180 nm, than the EVs sizes reported in previous studies (Silverman et al., 2010a; Silverman et al., 2010b;

Hassani and Olivier, 2013). However, EVs released from *L. amazonensis* with larger sizes have already been reported (Barbosa et al., 2018; Sauter et al., 2019). As expected, no particles were found in the medium alone (negative control, data not shown). All experiments described in this session are summarized in the workflow in **Figure 1**.

BALB/c mice were inoculated with parasites (1×10^8/mouse) or EVs (4 μg/mouse, corresponding to 2.39×10^6 particles ± 1.25×10^6) for 24 or 48 h (**Figure 1**). Then, the total peritoneal cells were collected, and B-1 cells were enriched using magnetic selection. The expression of the arginase was evaluated in the enriched B-1 cell population, and a significant increase was observed in B-1 cells of infected animals, compared to cells obtained from uninfected mice at both time points (**Figures 2A, B**). However, the intraperitoneal stimulation with EVs induced a significant decrease in the expression of this enzyme after 24 or 48 h of inoculation (**Figures 2C, D**, respectively).

Arginase catalyzes the hydrolysis of L-arginine to L-ornithine and urea. L-arginine is a substrate for inducible nitric oxide synthase (iNOS) to produce NO. Total peritoneal cells were

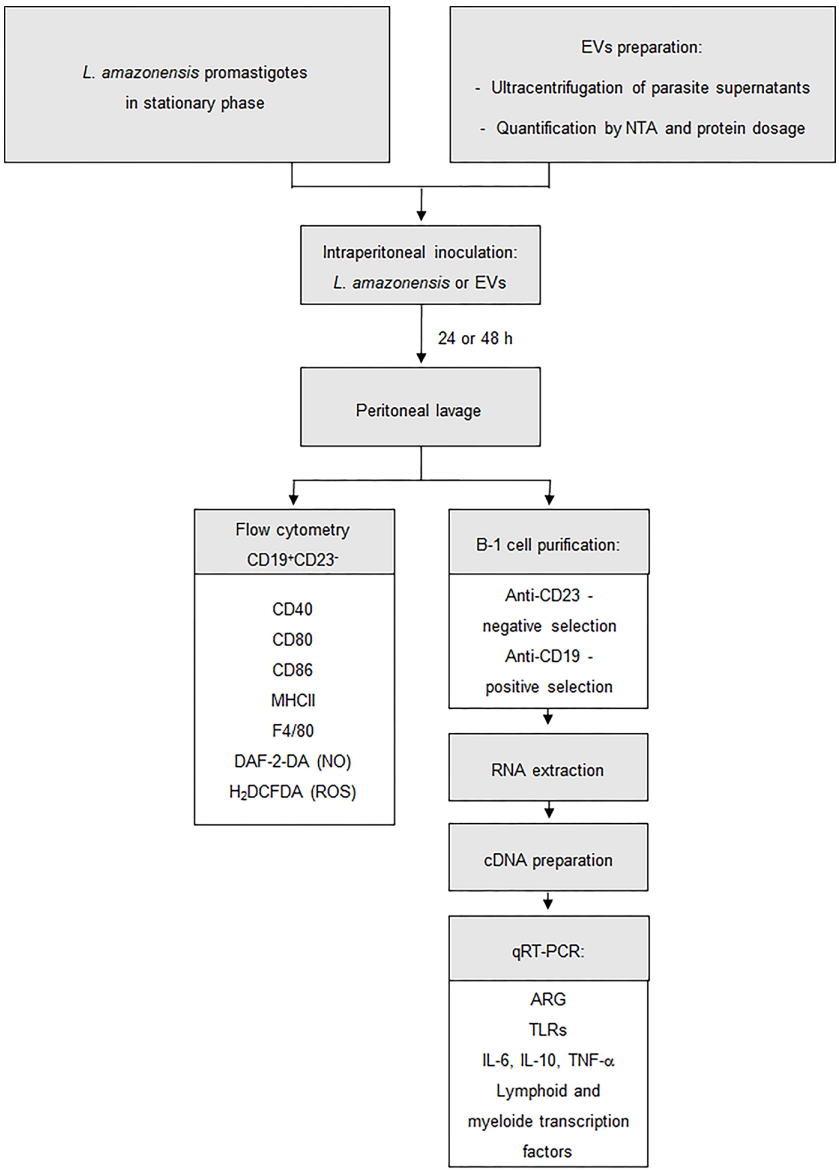

FIGURE 1 | Procedures employed for the enrichment and stimulation assays. Workflow used to develop this study. BALB/c mice were injected intraperitoneally with 1×10^8 *L. amazonensis* promastigotes or 4 ug of EVs (corresponding to 2.39×10^6 particles ± 1.25×10^6). After 24 or 48 h, the total peritoneal cells were collected from mice and then analyzed by flow cytometry or were used to enrich B-1 cells. In flow cytometry, B-1 cells were evaluated for the presence of CD80, CD86, CD40, MHC II, F4/80, NO, and ROS. Enriched B-1 cells were used to analyze the gene expression of arginase, TLRs, cytokines and myeloid and lymphoid transcription factors. *Material and Methods* section explains the details of the methodology employed.

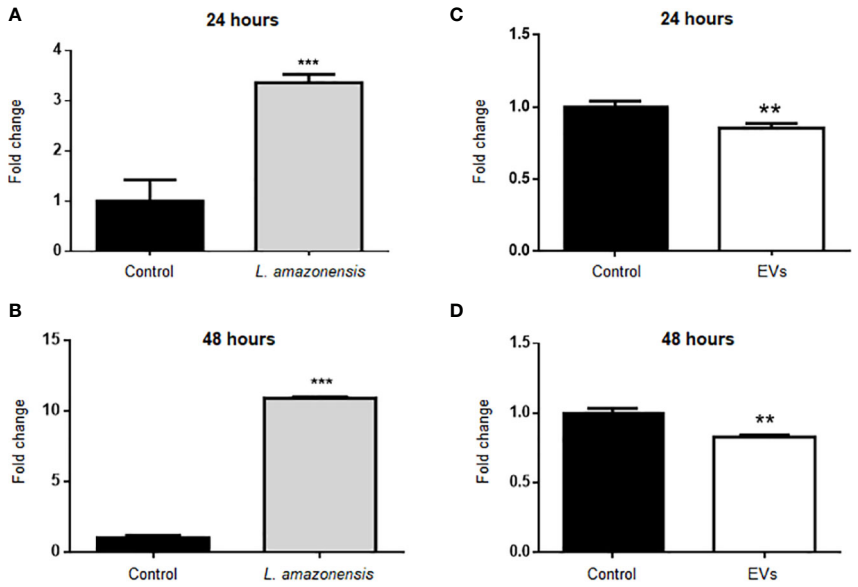

FIGURE 2 | Relative expression of arginase in B-1 cells after intraperitoneal stimulation for 24 or 48 h with *L. amazonensis* promastigotes (left panel) or EVs (right panel). B-1 cells were negatively selected with anti-CD23 microbeads and positively with anti-CD19 microbeads. Animals inoculated with PBS were used as control. The RNA was extracted, the cDNA obtained, and qPCR analyses were performed to verify the arginase enzyme's gene expression. Infection for **(A)** 24 h and **(B)** 48 h with the parasite, **(C, D)** stimulation for 24 or 48 h with the EVs, respectively. The bars indicate the average of triplicates, and the error bars the standard deviation. The graph is representative of three independent experiments. Test t-student, $^{**}P < 0.01$; $^{***}P < 0.001$.

collected and labeled with antibodies anti-CD19 APC and anti-CD23 PE to identify B-1 cells ($CD23^{-}CD19^{+}$) and with a fluorescent probe to evaluate NO. BALB/c mice inoculated with PBS were used as the control group. **Figure 3** shows the graphs with the mean values of mean fluorescent intensity (MFI) for intracellular NO in the population of B-1 cells. The results showed a significant decrease in MFI in B-1 cells of animals infected for 24 or 48 h of infection with *L. amazonensis* promastigotes, compared to the uninfected group (**Figures 3A, B**, respectively). B-1 cells from animals stimulated with EVs had an increase in the production of NO after 24 h (**Figure 3C**). However, after 48 h of stimulation (**Figure 3D**) no differences in NO production were observed.

We also analyzed the production of ROS in B-1 cells stimulated with parasites and EVs. **Figure 4** shows the graphs with the MFI values of ROS in B-1 cells after 24 or 48 h of the stimulus with the parasite (**Figures 4A, B**, respectively). The results show a significant decrease in MFI in B-1 cells of animals infected for 48 h with *L. amazonensis* promastigotes, compared to the uninfected group (**Figure 4B**). No differences were detected in ROS production in B-1 stimulated for 24 or 48 h with EVs (**Figures 4C, D**). We also evaluated the expression of CD80, CD86, CD40, MHC II, F4/80 in B-1 cells from mice, but no differences were detected in B-1 cells from infected or non-infected animals (data not shown).

Taken together, these results suggest that intraperitoneal stimulation with parasites induced a decrease in the production of microbicidal molecules (NO and ROS) by B-1 cells. On the other hand, intraperitoneal inoculation with EVs stimulated NO production only 24 h after stimulation.

Expression of TLRs and Cytokines in B-1 Cells of Mice Intraperitoneally Stimulated With *L. amazonensis* Promastigotes or EVs

TLRs can interact with *Leishmania* and participate in innate immunity to a pathogen, such as *Leishmania* spp. (Tuon et al., 2010). In addition, *Leishmania* induces upregulation of TLRs in human and murine macrophages (Faria et al., 2012; Chauhan et al., 2017). Herein, TLRs expression was evaluated in B-1 cells of mice intraperitoneally stimulated with parasites or EVs. Enriched B-1 cells obtained 24 or 48 h after i.p. stimulation were used to analyze TLR-2, TLR-6, and TLR-9 expression by qRT-PCR. TLRs showed significantly higher expression levels in B-1 cells of animals stimulated with the parasite for 24 or 48 h, compared to cells from control (**Figures 5A, B**). No changes were detected in TLR-2, TLR-6, and TLR-9 in B-1 cells from animals stimulated for 24 h with parasite EVs (**Figure 5C**). However, after 48 h, a significant increase in the expression of TLR-9 was detected, as compared with control mice (**Figure 5D**). Altogether, our data revealed the differential expression of TLR2, 6, and 9 in B-1 cells recovered from BALB/c mice stimulated intraperitoneally with *L. amazonensis* or their EVs.

The expression of IL-6, IL-10, and TNF-α was evaluated in B-1 cells of BALB/c mice inoculated with *L. amazonensis* promastigotes. B-1 cells showed a significant increase in the expression of these cytokines after the stimulus for 24 and 48 h (**Figures 6A, B**, respectively). It is noteworthy the pronounced increase in IL-6 after 48 h, while the expression of IL-10 and TNF-α had a decrease after 48 h, as compared with 24 h. The peritoneal stimulation with

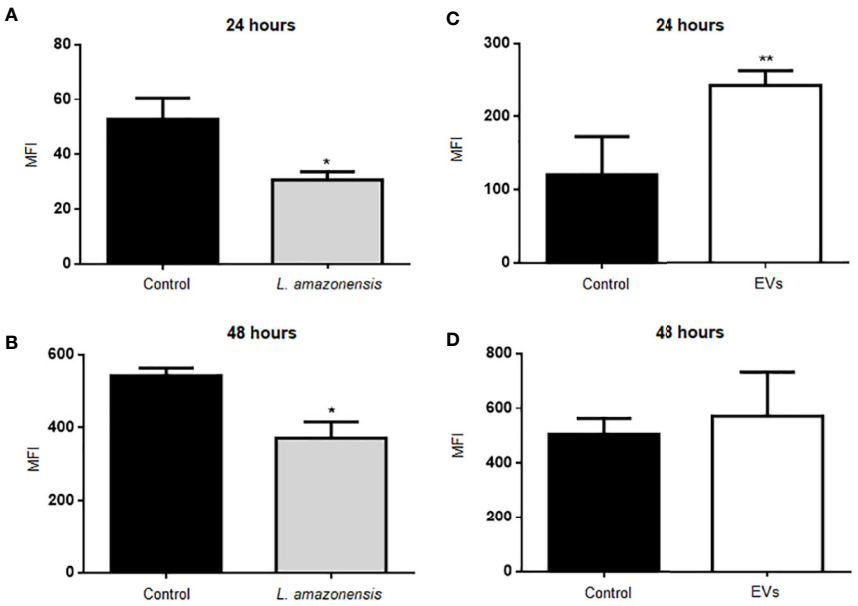

FIGURE 3 | Intracellular NO production in B-1 cells after 24 or 48 h of intraperitoneal inoculation with *L. amazonensis* promastigotes (left panel) or EVs (right panel). **(A)** 24 h of infection with the parasite, **(B)** 48 h of stimulation with the parasite, **(C)** stimulation for 24 h with EVs, and **(D)** 48 h of stimulation with EVs. Total peritoneal cells were collected and labeled with anti-CD19 coupled with APC, anti-CD23 coupled with PE and DAF-2DA. Data acquisition was performed using a FACSCalibur cytometer. Subsequent analyzes were performed using the FlowJo software. The graphs represent the median fluorescence intensity (MFI) for the DAF probe in $CD19^{+}CD23^{-}$ cells. The bars represent the mean of the duplicates, and the error bars the standard deviation. The graph is representative of two independent experiments. Test t-student, *P < 0.05; **P < 0.01, comparing stimulated (with EVs or parasites) and unstimulated B-1 cells.

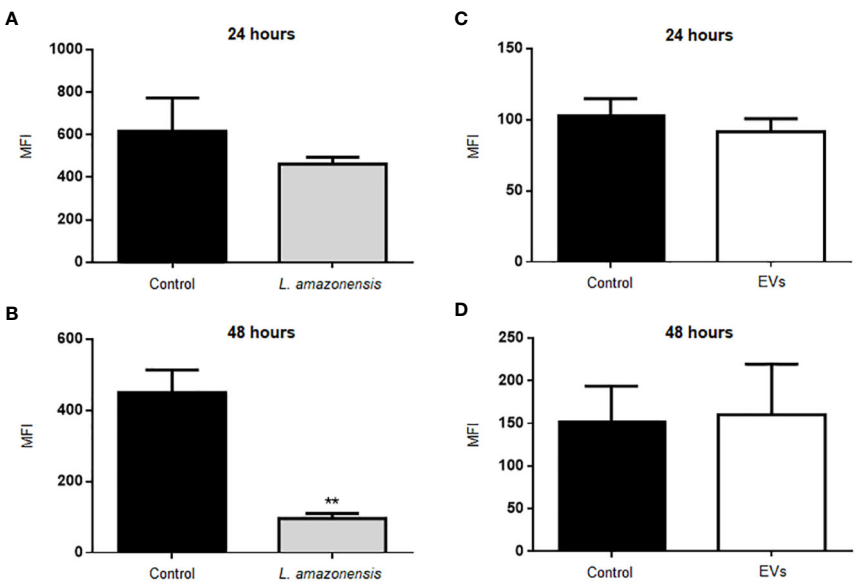

FIGURE 4 | Production of ROS in B-1 cells after 24 or 48 h of stimulation with *L. amazonensis* promastigotes (left panel) or the parasites EVs (right panel). **(A)** infection for 24 h with *L. amazonensis* promastigotes, **(B)** 48 h of stimulation with the parasite, **(C)** 24 h of stimulation with EVs, and **(D)** 48 h of stimulation with EVs. Total peritoneal cells were collected and labeled with anti-CD19 coupled with APC, anti-CD23 coupled with PE, and H_2DCFHDA. Data acquisition analyzes were performed using a FACSCalibur cytometer, followed by analyzes in FlowJo software. The graphs represent the median fluorescence intensity (MFI) for the H_2DCFHDA probe in $CD19^{+}CD23^{-}$ gating cells. The bars represent the mean of the duplicates, and the error bars the standard deviation. The graph is representative of two independent experiments. Test t-student, **P < 0.01.

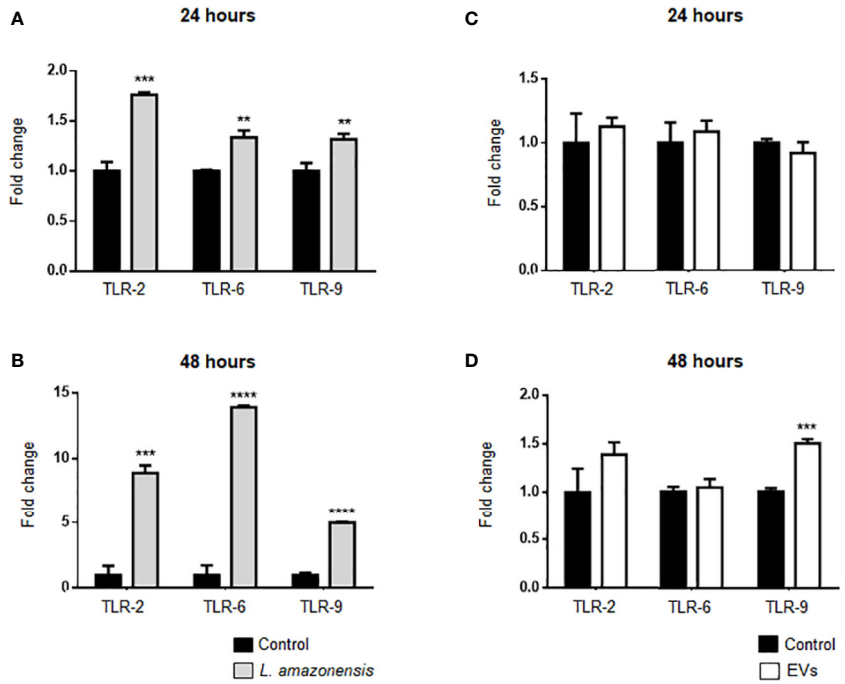

FIGURE 5 | Expression of TLRs in B-1 cells after 24 or 48 h of intraperitoneal stimulation with *L. amazonensis* promastigotes (left panel) or parasite EVs (right panel). **(A, B)** stimulation for 24 and 48 h with parasites, respectively; **(C)** 24 and **(D)** 48 h of stimulation with EVs. B-1 cells were negatively selected with anti-CD23 microbeads and positively selected with anti-CD19 microbeads. Mice intraperitoneally inoculated with PBS were used as control. The RNA was extracted, the cDNA obtained, and qPCR analyses were performed to verify the gene expression of TLR-2, TLR-6, and TLR-9. The bars indicate the average of triplicates, and the error bars the standard deviation. The graph is representative of three independent experiments. Test t-student, $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$, comparing stimulated (with EVs or parasites) and unstimulated B-1 cells.

EVs released by the parasites led to a significant decrease in IL-6, IL-10, and TNF-α expression in enriched B-1 cells after 24 h of stimulation (**Figure 6C**). However, after 48 h of EVs intraperitoneal inoculation, we detected in B-1 cells a decrease in the IL-6 expression, no changes in the IL-10 mRNA levels, and an increase in the TNF-α expression (**Figure 6D**). Based on these data, IL-6, IL-10, and TNF-α were differentially expressed in B-1 cells after intraperitoneal stimulation with *L. amazonensis* or EVs. Interestingly, the increase in cytokine expression was observed in the group with the highest expression of TLRs.

Intraperitoneal Stimulation With *L. amazonensis* Promastigotes or EVs Alters the Expression of Myeloid and Lymphoid Impairment Transcription Factors in Mouse B-1 Cells

B-1 cells express both lymphoid and myeloid commitment transcription factors (Popi et al., 2009) and differentiate into phagocytes *in vitro* and *in vivo* (Almeida et al., 2001; Popi et al., 2012). Phagocytes derived from B-1 cells phagocyted *Leishmania in vitro* and released cytokines and EVs, which influence the activation of other cells such as macrophages (Arcanjo et al., 2015; Geraldo et al., 2016; Toledo et al., 2020). However, it has not yet been demonstrated whether this differentiation occurs *in vivo* after stimulation with the parasite or their EVs. Thus, we evaluated the effects of intraperitoneal stimulus with *L. amazonensis* or EVs on the differential expression of myeloid or lymphoid commitment transcription factors in the B-1 cells. The expression of myeloid impairment genes (transcription factor PU.1—Spi1, macrophage colony-stimulating factor 1 receptor—M-csfr, and granulocyte colony-stimulating factor 3 receptor—G-csfr) and lymphoid impairment genes (early B cell Factor - EBF, transcription factor 3—E2A, and Interleukin-7 receptor—IL-7r) were analyzed by qRT-PCR. **Figure 7A** shows a significant increase in the expression of all target genes analyzed after 24 h of intraperitoneal stimulation with the parasites, except for the E2A gene that the increase in expression was not significant. However, we observed a more pronounced increase in the expression of myeloid-impaired genes (Spi1, M-csfr, and G-csfr). After 48 h, a decrease in the lymphoid impairment genes (EBF and E2A) was observed, but the myeloid genes remained increased (**Figure 7B**). EVs led to an increase in the expression of M-csfr in B-1 cells after 24 h of stimulation (**Figure 7C**). After 48 h, the EBF levels significantly decreased while the expression of IL -7r and G-csfr showed a significant increase (**Figure 7D**), compared with the control group. Taken together, our results showed that intraperitoneal stimulation with the parasite led to an increase in the myeloid commitment factor, suggesting that B-1 cells differentiated in a myeloid profile. On the other hand, EVs

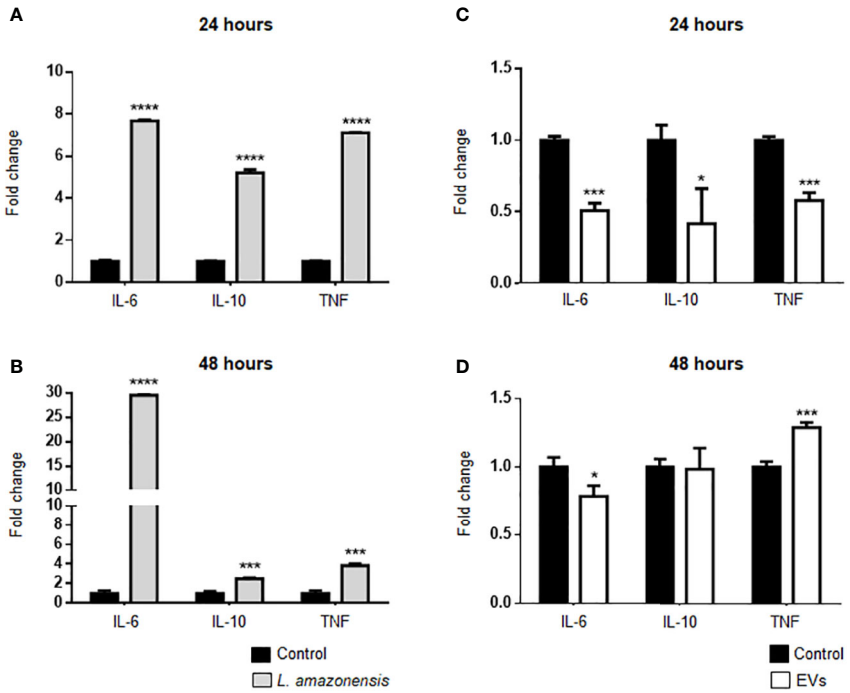

FIGURE 6 | Expression of IL-6, IL-10, and TNF-α in B-1 cells after stimulation for 24 or 48 h with *L. amazonensis* promastigotes (left panel) or EVs (right panel). **(A)** and **(B)** stimulation with the parasites for 24 or 48 h, respectively; **(C)** 24 and **(D)** 48 h of stimulation with EVs. B-1 cells were enriched using negative selection with anti-CD23 microbeads and positive selection with anti-CD19 microbeads. Animals inoculated with PBS were used as control. The RNA was extracted, the cDNA obtained, and qPCR analyses were performed to verify the gene expression of IL-6, IL-10, and TNF-α cytokines. The bars indicate the average of triplicates, and the error bars the standard deviation. The graph is representative of three independent experiments. Test t-student, *P < 0.05; ***P < 0.001; ****P < 0.0001, comparing stimulated (with EVs or parasites) and unstimulated B-1 cells.

were not efficient in stimulating the differentiation of B-1 cells into phagocytes.

DISCUSSION

B-1 cells are a group of B cells that can modulate and participate in innate and adaptive host immune responses (Baumgarth, 2011; Novaes e Brito et al., 2019). However, the response of these cells to parasites or EVs released by the parasite are poorly understood. Our group showed that B-1 cells participate in immunity against *L. amazonensis* (Gonzaga et al., 2017). Mice deficient in B-1 cells (XID mice) were more susceptible to chronic infection with *L. amazonensis* as compared to the background BALB/c (Gonzaga et al., 2017). The previous treatment of mouse footpad with EVs released by B-1 cells led to a decrease in the parasite load and the inflammatory marks in mice challenged with *L. amazonensis* promastigotes (Toledo et al., 2020). Herein we analyzed the changes in peritoneal B-1 cells from mice intraperitoneally stimulated with *L. amazonensis* or parasites EVs. A similar model was used to evaluate the population and activation of B-1 cells after intraperitoneal stimulation with LPS, *Francisella tularensis*, heat-killed *Propionibacterium acnes* as well as their components (Cole et al., 2009; Mussalem et al., 2012; Popi et al., 2012; Gambero et al., 2016). This model shows the changes in B-1 cells *in vivo* and minimizes the artifacts that can be induced by *in vitro* stimulation.

Our results showed a decrease in the NO production by B-1 cells from mice inoculated with *L. amazonensis* after 24 or 48 h of protozoa infection. Also, we detected an increase in the expression of arginase in B-1 cells from mice intraperitoneally infected. Arginase is an enzyme that inhibits the production of NO and is involved in the production of ornithine, a precursor to hydroxyproline and polyamines. Classically activated macrophages (M1 macrophages) do not induce arginase activity and, therefore, convert arginine to NO, one of the components used to eliminate intracellular pathogens (Fleming and Mosser, 2011). *Leishmania* parasites can regulate the increase in arginase activity in the host cells (Wilkins-Rodríguez et al., 2020) and the decrease of NO production, leading to the persistence of the pathogen (Gregory and Olivier, 2005; Nandan and Reiner, 2005). Thus, our data demonstrate intraperitoneal *Leishmania* infection induced a reduction in NO production in B-1 cells, which is similar to what has been previously shown for infection macrophages.

On the other hand, the intraperitoneal stimulation with EVs released by *L. amazonensis* induced a different behavior in B-1

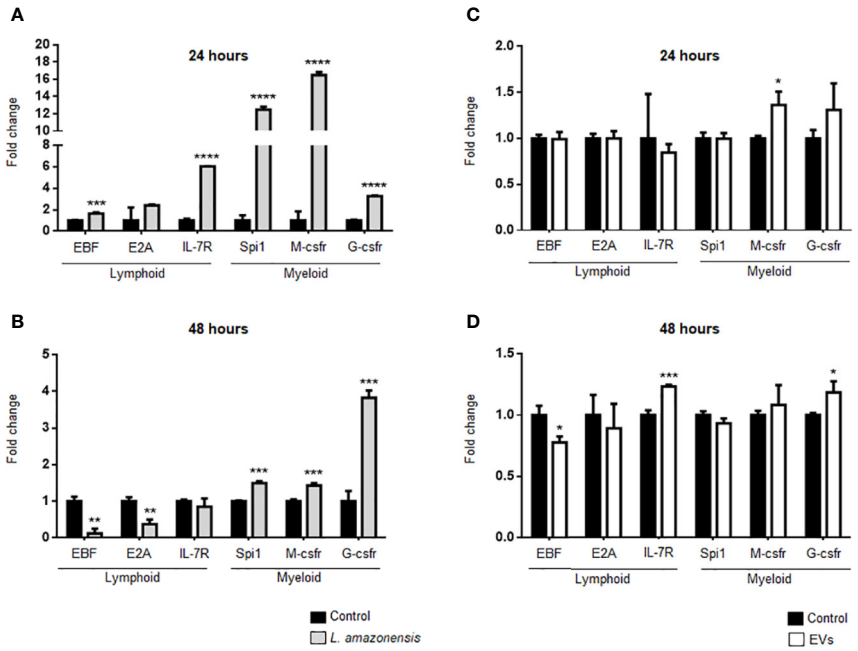

FIGURE 7 | Expression of lymphoid and myeloid restricted transcription factor in B-1 cells after stimulation for 24 or 48 h of intraperitoneal stimulation with *L. amazonensis* promastigotes (left panel) or parasite EVs (right panel). **(A)** infection for 24 h with the parasite, **(B)** stimulation for 48 h with parasite, **(C)** 24 h, and **(D)** 48 h of stimulation with EVs, respectively. B-1 cells were enriched using negative selection with anti-CD23 microbeads and positive selection with anti-CD19 microbeads. Animals inoculated with PBS were used as control. The RNA was extracted, the cDNA obtained, and qPCR analyses were performed to verify the gene expression of EBF, E2A, IL7-r, Spi1, M-csfr, and G-csfr. The bars indicate the average of triplicates and the error bars the standard deviation. The graph is representative of three independent experiments. Test t-student, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, comparing stimulated (with EVs or parasites) and unstimulated B-1 cells.

cells. After stimulation with EVs, B-1 cells reduced the expression of arginase after 24 and 48 h of stimulation and showed an increase in the NO production 24 h of intraperitoneal stimulation. EVs released by *Leishmania* may contain different parasite molecules, such as LPG and nucleic acids (Srivastava et al., 2013), that can interact with different cell surface receptors such as TLRs (Faria et al., 2012; Chauhan et al., 2017). B-1 cells express TLRs, and the *in vitro* stimulation with several TLRs agonists increased the NO production (Tumurkhuu et al., 2010). Although the *in vitro* stimulation of macrophages with exoproteome or exosomes of *Leishmania* led to an inhibition of NO production (Hassani et al., 2011; Hassani and Olivier, 2013), our results showed that B-1 cells respond differently after *in vivo* stimulation with EVs. In our model, B-1 cells were stimulated with EVs *in vivo*, and their interaction with other cells and cytokines most probably occurred. A better understanding of the activation mechanisms of B-1 cells and their interaction with parasites, their components, and the immune system can lead to uncover their role in leishmaniasis.

ROS can also participate in the elimination of intracellular pathogens (Nüsse, 2011). In mitochondria, ROS may be produced by NADPH oxidases that reduce oxygen using NADPH as the electron donor to produce superoxide anion (Babior, 1999). Superoxide radicals can be converted to hydrogen peroxide (H_2O_2), which is toxic to many pathogens (Ganguli et al., 2019). Non-activated macrophages infected *in vitro* with *L. amazonensis* showed low or insufficient ROS production to eliminate *L. amazonensis* (Scott and Novais, 2016). Our results showed no differences in the ROS production by B-1 cells after 24 h of the intraperitoneal infection with *L. amazonensis*. However, after 48 h of intraperitoneal stimulation with the parasite, a significant reduction in the ROS production was detected in B-1 cells. The stimulation with EVs had no changes in the detection of ROS by B-1 cells. Thus, the intraperitoneal stimulation with the parasite induced a decrease in the production of two important microbicidal molecules by B-1 cells but the stimulation with EVs increased the NO production. B-1 cells participate in immunity against *L. amazonensis* since the presence of these cells or their EVs induce resistance to infection (Gonzaga et al., 2017; Toledo et al., 2020). How these cells act in response to parasites or their components is essential to understand the mechanisms involved in this resistance. Thus, probably B-1 cells participate in immunity to *L. amazonensis* by different mechanism.

Regarding the expression of cell surface receptors, the change in the expression of TLRs by B-1 cells from intraperitoneally infected animals was notable. Toll-type receptors (TLRs) are transmembrane glycoproteins with an important role in the innate and adaptive immune response (Tuon et al., 2010; Gurung and Kanneganti, 2015; Fitzgerald and Kagan, 2020).

They are found in macrophages, dendritic cells, NK cells, T and B lymphocytes, including B-1 cells (Arancibia et al., 2007; Popi et al., 2016). TLR-2 and TLR-4 have been considered important in the development of the inflammatory response and pathology in several infectious diseases, such as tuberculosis, malaria, and toxoplasmosis (Mukherjee et al., 2016). Several studies have shown the recognition of *Leishmania* spp. by different TLRs (Becker et al., 2003; de Veer et al., 2003) and an important correlation in the protective responses against *Leishmania* parasites with high expression of TLR-2, -4, and -9 in parasite infections were identified (Martínez-Salazar et al., 2008; Faria et al., 2012). A higher expression of TLR-2 was identified in monocytes from patients with cutaneous leishmaniasis (CL) (Carneiro et al., 2016; Polari et al., 2019). The frequency of monocytes expressing TLR-9 was related to lesion size (Vieira et al., 2013) and granuloma formation (Tuon et al., 2010) in patients with CL. Besides parasites, recently, an interaction between TLR9 and EVs released by *L. amazonensis* amastigotes had been reported in macrophages stimulated *in vitro* (Sauter et al., 2019). Our study demonstrated an increase in the expression of TLR-2, -6, and -9 in B-1 cells after intraperitoneal stimulation for 24 and 48 h with *L. amazonensis* promastigotes. However, intraperitoneal stimulation with EVs induced an increase in the expression of TLR-9 in B-1 cells after 48 h. The presence of nucleic acids in parasite EVs can explain the increase in TLR-9 expression in B-1 cells. Analysis of the content present in *L. amazonensis* EVs are ongoing in our laboratory.

IL-10, IL-6, and TNF-α play an important role in *Leishmania* infection. IL-10 is produced by many cell types and plays a regulatory role in the immune response, by inhibiting the production of inflammatory mediators, and the activation of monocytes and macrophages (Sabat, 2010). *L. amazonensis* stimulates the IL-10 expression making a permissive environment that favors the intracellular parasite survival and growth (Castellano et al., 2015). IL-6 is a pleiotropic cytokine produced by several cell types. Their biological effects include differentiation of macrophages, participation in Th2 polarization, and inhibition of Th1 differentiation leading to a non-protective immune response against *Leishmania* infections (Dienz and Rincon, 2009; Velazquez-Salinas et al., 2019). On the other hand, TNF-α is a pro-inflammatory cytokine that is related to active disease (Nateghi et al., 2016). An increase in cytokine expression has also been identified in B-1 cells from intraperitoneally infected animals. The mRNA levels of IL-6, IL-10, and TNF-α had increased in B-1 cells from animals stimulated with the parasite for 24 and 48 h, compared to cells from control animals (inoculated with PBS). It is notable that after 48 h there was a very pronounced increase in IL-6 expression. Our group demonstrated that the interaction between *Leishmania* and B-1 cells *in vitro* and *in vivo* induced a significant increase in cytokine expression (Geraldo et al., 2016; Barbosa et al., 2018). The activation of TLRs can activate different cell signaling pathways leading to an increase in cytokine production (Fitzgerald and Kagan, 2020). In our model, we observed an increase in the expression of TLR-2, -6, and -9 in B-1 cells from mice intraperitoneally infected, which can be related with the increase in the expression of cytokines in these cells.

On the other hand, after 24 h of stimulation with EVs released by *L. amazonensis* promastigotes, a significant decrease in the cytokine expression were detected in B-1 cells. After 48 h, there was a significant decrease in IL-6 expression, no changes in IL-10 mRNA levels, and a significant increase in the TNF-α expression. No differences in the TLRs were detected in B-1 cells stimulated with EVs except for TLR-9 after 48 h of stimulation. TLR-9 is located intracellularly and activated by DNA sequences. Some experimental models have related the activation of this receptor to the production of TNF-α (Lim et al., 2006; Ivory et al., 2008; Grassin-Delyle et al., 2020). Our results showed an increase in the expression of TLR-9 and TNF-α in B-1 cells from animals intraperitoneally inoculated with EVs. Although additional studies need to be carried out, the correlation in the expression of these two genes cannot be ruled out.

Molecular studies have shown that B-1 cells express mRNA of genes for the lymphoid and myeloid compromising profile (Popi et al., 2009). In our experimental model, the intraperitoneal stimulation with *L. amazonensis* promastigotes for 24 h led to a significant increase in the expression of myeloid and lymphoid-impaired genes in B-1 cells. However, the increase in the expression of myeloid genes was significantly higher compared to the lymphoid genes in these cells. After 48 h of infection, we detected a decrease in the expression of lymphoid genes, while myeloid maintained their higher expression. A similar mRNA expression profile was observed in B-1 cells from mice intraperitoneally stimulated with the *P. acnes* and the soluble polysaccharide fraction of the bacterium (Mussalem et al., 2012) and was related with the early induction of B-1 cells into phagocyte-like cells. The intraperitoneal treatment with LPS also induced differentiation of B-1 cells into phagocytes (Popi et al., 2012). Our group and others have shown that phagocytes derived from B-1 cells can phagocytize *Leishmania* promastigotes more efficiently than macrophages (Arcanjo et al., 2015; Geraldo et al., 2016). In addition, a recent work showed that B-1 cells stimulated *in vitro* with the *Leishmania* parasites secreted more EVs and displayed a morphological change with macrophage characteristics (Toledo et al., 2020). Herein we showed that the intraperitoneal stimulation with *L. amazonensis* can induce the *in vivo* differentiation of B-1 cells into phagocyte-like cells by increasing the expression of myeloid compromising factors.

The intraperitoneal stimulation with EVs induced minimal changes in the expression of lymphoid and myeloid transcription factors. After 24 h, there was a significant increase in the M-csfr gene (myeloid). However, after 48 h, the expression of EBF decreased and IL-7r increase (both lymphoid genes). The myeloid gene G-csfr showed an increase in expression after 48 h of intraperitoneal stimulation with *Leishmania* derived EVs. Thus, although intraperitoneal stimulation with parasites induced a change in gene expression for a myeloid profile in B-1 cells, the inoculation of EVs was not as efficient in inducing a pronounced increase in the expression of myeloid compromising genes. Additional studies to better assess the involvement of B-1 cells for the lymphoid/myeloid profile are underway by our group.

Several studies have shown the role of B-1 lymphocytes in the immune response to different pathogens (Baumgarth, 2011; Baumgarth, 2017; Novaes e Brito et al., 2019). Collectively our results showed that the intraperitoneal inoculation of *L. amazonensis* promastigotes or EVs released by the parasites differentially modulated B-1 cells, altering the production of microbicidal molecules, expression of TLRs, cytokines, or lymphoid/myeloid compromising factors. When combined with other studies, our findings offer a better understanding of the B-1 cells after intraperitoneal stimulation. B-1 cells can produce cytokines, differentiate into phagocytes and modulate innate and adaptative immune response. Since B-1 cells are able to differentiate into phagocytes, part of the phagocyte population presents during *Leishmania* infection can be derived from B-1 cells and can act differently than macrophages to promote immune response and to eliminate the parasite. This work showed by molecular evidences that this differentiation can occurs *in vivo*. Besides parasites, our study with EVs and their role in the immunobiology of infectious diseases has brought relevant and important information for the knowledge on the pathogenesis of diseases, as well as the pathogen-host relationship.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Ethics of Animal Experiments (CEUA) UNIFESP, protocol number 8762030718.

AUTHOR CONTRIBUTIONS

NR, TD, and CC performed the experiments. MT and VO assisted with real-time PCR experiments. AP, AT, and PX helped with data analysis and discussion of results. NR and PX wrote the manuscript. PX conceived and designed the study, and sought funds for this project. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.573813/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | (S1). Gating strategy for flow cytometry. The lymphocyte gate was analyzed for their expression of CD19, and CD23 cell surface markers. $CD19^{+}CD23^{-}$ cells were gated and the expression of CD80, CD86, CD40, F4/80, MHC II, NO (labeled with DAF-2DA) and ROS (labeled with H_2DCFDA) analyzed. This figure is representative of labeling with DAF-2DA.

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The Role of Small Extracellular Vesicles in Viral-Protozoan Symbiosis: Lessons From *Trichomonasvirus* in an Isogenic Host Parasite Model

Yashini Govender[1], *Tiffany Chan*[1], *Hidemi S. Yamamoto*[1], *Bogdan Budnik*[2] *and Raina N. Fichorova*[1]*

[1] Laboratory of Genital Tract Biology, Department of Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School, Brigham & Women's Hospital, Boston, MA, United States, [2] Mass Spectrometry and Proteomics Resource Laboratory, FAS Division of Science, Harvard University, Cambridge, MA, United States

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***Correspondence:**
Raina N. Fichorova
RFICHOROVA@BWH.HARVARD.EDU

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The protozoan parasite *Trichomonas vaginalis* (TV), exclusively adapted to the human genital tract, is one of the most common sexually transmitted pathogens. Adding to the complexity of the host-pathogen interactions, the parasite harbors TV-specific endosymbiont viruses (*Trichomonasvirus*, TVV). It was reported that small extracellular vesicles (sEVs) released by TV play a role in host immunity; however, the role of the viral endosymbiosis in this process remained unknown. We hypothesized that the virus may offer evolutionary benefit to its protozoan host at least in part by altering the immunomodulatory properties of sEVs spreading from the site of infection to non-infected immune effector cells. We infected human vaginal epithelial cells, the natural host of the parasite, with TV natively harboring TVV and an isogenic derivative of the parasite cured from the viral infection. sEVs were isolated from vaginal cell culture 24 h post TV infection and from medium where the isogenic TV strains were cultured in the absence of the human host. sEVs from TVV-negative but not TVV-positive parasites cultured alone caused NF-κB activation and increase of IL-8 and RANTES expression by uterine endocervical cells, which provide innate immune defense at the gate to the upper reproductive tract. Similarly, mononuclear leukocytes increased their IL-8, IL-6 and TNF-α output in response to sEVs from virus-negative, but not isogenic virus-positive parasites, the latter exosomes being immunosuppressive in comparison to TV medium control. The same phenomenon of suppressed immunity induced by the TVV-positive compared to TVV-negative phenotype was seen when stimulating the leukocytes with sEVs originating from infected vaginal cultures. In addition, the sEVs from the TVV-positive infection phenotype suppressed immune signaling of a toll-like receptor ligand derived from mycoplasma, another frequent TV symbiont. Quantitative comparative proteome analysis of the secreted sEVs from virus-positive versus virus-negative TV revealed differential expression of two functionally uncharacterized proteins and five proteins involved in Zn binding, protein binding, electron transfer, transferase and catalytic

activities. These data support the concept that symbiosis with viruses may provide benefit to the protozoan parasite by exploiting sEVs as a vehicle for inter-cellular communications and modifying their protein cargo to suppress host immune activation.

Keywords: *Trichomonasvirus*, *T. vaginalis*, exosomes, extracellular vesicles, immune modulation, cytokines, proteomics

INTRODUCTION

The extracellular protozoan parasite *Trichomonas vaginalis* (TV) causes trichomoniasis, one of the most common sexually transmitted infections affecting over 200 million men and women each year (Patel et al., 2018). The infection is recurrent, asymptomatic in more than half of those diagnosed and often lasting over a long period of time in the urogenital tract while causing a myriad of complications including infertility, preterm birth, low birth weight, bacterial vaginosis (BV), and increased risk of cancer, human papillomavirus (HPV) persistence, and HIV infection (Fichorova, 2009; Kissinger, 2015; Meites et al., 2015). The poor understanding of the molecular mechanisms used by this parasite to avoid clearance by a robust inflammatory immune response is a critical barrier to prevention of TV-associated health risks and conditions. Adding to the immunological complexity, the majority of the TV clinical isolates stably harbor endosymbiont double-stranded (ds) RNA viruses from the genus *Trichomonasvirus* (TVV) (Goodman et al., 2011a; Goodman et al., 2011b; Fichorova et al., 2017). TVV shed by the parasite is sensed by the toll-like receptor (TLR)-3 in female genital tract epithelial cells where it can induce selective virus-stress response (Fichorova et al., 2012), which however appears insufficient to cause clinically effective inflammatory response to clear the parasite (Graves et al., 2019). Consequently, other molecular mechanisms may be built into the virus-protozoan symbiotic relationship to counteract immune activation and to provide evolutionary advantage to the parasites harboring the virus. We hypothesized that the virus may offer an evolutionary benefit to its protozoan host by altering the immunomodulatory properties of small extracellular vesicles (sEVs) spreading from the site of infection. sEVs are nano-sized membrane-bound particles (<150 nm) which contain a mix of exosomal and non-exosomal vesicles (Mastoridis et al., 2018). sEVs are universally released into mucosal secretions and the blood circulation by both host cells and eukaryotic parasites and mediate cell-cell communications through the sEV molecular cargo and signal transduction (Coakley et al., 2015). However, the role of sEVs in subversion of host immunity by viral-protozoan symbiosis in trichomoniasis has not been elucidated to date.

METHODS

T. vaginalis Culture

The TVV positive TV clinical isolate 347V+ and its TVV-cured derivative 347V- were provided by John F. Alderete (University of Texas Health Science Center, San Antonio, TX) (Wang et al., 1987). The progeny strain 347V- was rendered virus-negative after continuous passage (Benchimol et al., 2002). For some experiments, we also used the TVV negative laboratory strain B7RC2 (ATCC® 50167™) (Fichorova et al., 2012), and the vaginal isolates were previously characterized for their naturally occurring TVV status including UR1 (TVV positive), isolated from the University of Rochester STI Clinic (Goodman et al., 2011a), OC7 (TVV negative) and OC8 (TVV positive), isolated at the Onondaga County Health Department STI Clinic (Syracuse, NY) (Fichorova et al., 2012). Parasites were cultured in modified Diamond's trypticase-yeast extract-maltose medium supplemented with 10% horse serum (TV medium) at a concentration of 4×10^5 TV/ml at 35°C under anaerobic conditions as described (Fichorova et al., 2012). We did not deplete sEVs from the TV medium prior to culture because we observed suboptimal growth of the parasites in sEV-depleted medium (**Supplementary Figure S1**). To account for sEVs contributed by the horse serum and other medium supplements, we isolated and tested in each experiment sEVs from the complete culture medium alone, which served as a control to culture supernatants after allowing TV to grow for 24 h.

Vaginal Epithelial Cell Culture and *T. vaginalis* Infection

Human vaginal epithelial cells (Vk2/E6E7) were cultured in modified antibiotic-free, keratinocyte serum-free medium (KSFM) as described (Fichorova et al., 1997). For infection experiments, TV was harvested in late log phase (24 h) by centrifugation (1,000 × g for 5 min) and the TV pellet was re-suspended in KSFM at 4×10^5 TV/ml. Vk cells grown to 80–90% confluency were infected and incubated under anaerobic conditions on a shaker at 50 rpm and 35°C for 24 h, as described (Fichorova et al., 2012).

Isolation of Small Extracellular Vesicles

Supernatants from 24 h (i) 10×10^6 TV monoculture (ii) Vk + 10×10^6 TV co-culture and (iii) medium alone (TV medium or KSFM) were centrifuged at 2,000 × g for 30 min and passed through a 0.22 μm filter. sEVs were isolated using the Total Exosome Isolation Reagent for cell culture medium (Invitrogen, Carlsbad, CA) per manufacturer's protocol. The sEV pellets were re-suspended in either PBS or KSFM medium. Exosome Spin Columns (Invitrogen, Carlsbad, CA) were used to remove low molecular weight contaminants (MW<3000) from sEV samples per manufacturer's protocol. Once purified, samples were sterilized through 0.22 μm filters and frozen at -20°C until use. The sEV size was confirmed by nanotracking. Briefly, sEV

preparations were diluted 1000× and 50,000× in PBS and quantified using the ZetaView® (Particle Metrix, Meerbusch, Germany) by translational diffusion size distribution. Nanotracking analysis confirmed presence of sEVs from TV cultures in the peak size range of 76.6–106.9 nm similar to those previously reported for *T. vaginalis* (Twu et al., 2013; Olmos-Ortiz et al., 2017; Nievas et al., 2018) and at peak concentrations ranging from 1.1E9–1.65E11 particles/ml (**Supplementary Figure S2A**). Transmission electron microscopy with immunogold labelling of CD63 confirmed the presence of exosomes in the sEV samples (**Supplementary Figure S2B**).

sEV Treatment of Non-Infected Bystander Cells

Human endocervical epithelial cell (End1/NFκB) were cultured in KSFM. Cells were seeded into 96-well flat-bottom plates at a density of 5×10^5 cells/ml. sEV treatment dose was calculated based on the sEV equivalent of the parasite load applied to the vaginal infection model. Blood was obtained from five healthy anonymous donors, after written informed consent, at Research Blood Components LLC (Brighton, MA). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll®-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient separation as per manufacturer's protocol. PBMCs were cultured in RPMI-1640 medium with L-glutamine (Corning™, Manassas, VA) and 10% heat inactivated newborn calf serum (Gibco™, Life Technologies, Grand Island, NY) at a density of 2×10^6 cells/ml in round-bottom 96-well plates. PBMCs were treated with sEVs for 24 h in the presence or absence of 25 nM TLR2/TLR6 ligand mycoplasma-derived macrophage-activating lipopeptide-2 (MALP-2) (Enzo Life Sciences, NY).

NF-κB Activity

A stably transfected endocervical epithelial cell line, which expresses NF-κB firefly luciferase reporter (End1/NF-κB) previously generated in our laboratory (Singh et al., 2009) was used for measurement of NF-κB activation as described (Fichorova et al., 2012). After 24 h of exposure to sEVs, cells were lysed with GloLysis buffer (Promega, Madison, WI) and luciferase activity was determined using the Bright-Glo Luciferase Assay System (Promega, Madison, WI) as per manufacturer's protocol.

Biomarkers of Immune Modulation

Levels of soluble immune mediators were measured in cell supernatants using electrochemiluminescence multiplex assays on a Sector Imager S600 (Meso Scale Discovery (MSD), Gaithersburg, MD). IL-8 and RANTES were measured simultaneously in epithelial culture supernatants by an MSD 2-plex. IL-8, IL-6, IL-10, and TNF-α were measured simultaneously in PBMC supernatants using an MSD multiplex.

Cell Viability

Epithelial and PBMC cell viability were assessed by the non-radioactive CellTiter96 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, Madison, WI) and TV viable counts were obtained microscopically with the trypan blue exclusion test (Fichorova et al., 2012).

sEV Proteomic Analysis

Secreted sEVs were isolated from TV medium, three strains of virus negative TV (347V-, B7RC2 and OC7) and three strains of virus positive TV (347V+, UR1, and OC8). sEV pellets were transferred into Covaris® microTUBE- 15 (Woburn, MA) microtubes with Covaris® TPP buffer. Samples were lysed in Covaris S220 Focused-ultrasonicator instrument with 125W power over 180s with 10% max peak power. Lysed samples were then chloroform/MeOH precipitated and weighed then digested *via* filter aided sample preparation (FASP) digest. Promega® Sequencing Grade Trypsin was used for an overnight digestion at 38 °C. Tandem-Mass-Tag (TMT) peptide labeling was performed using Thermo Scientific TMT Reagents (Thermo Fisher Scientific, San Jose, CA). The pooled sample was fractionated into 10 fractions and submitted for LC-MS/MS experiment that was performed on a Orbitrap Lumos (Thermo Fisher Scientific, San Jose, CA) equipped with Ultimate 3000 (Thermo Fisher Scientific, San Jose, CA). Peptides were trapped onto a 150 μm inner diameter microcapillary trapping column packed first with approximately 3 cm of C18 Reprosil resin (5 μm, 100 Å, Dr. Maisch GmbH, Germany) followed by 50 cm micro Pillar Array Columns (μPAC™) analytical column (PharmaFluidics, Belgium). Separation was achieved through applying a gradient from 5–27% ACN in 0.1% formic acid over 90 min at 200 nl min^{-1}. Electrospray ionization was enabled through applying a voltage of 1.8 kV using a home-made electrode junction at the end of the microcapillary column and sprayed from stainless still needle (PepSep, Denmark). The LTQ Orbitrap Lumos was operated in data-dependent mode for the mass spectrometry methods. The mass spectrometry survey scan was performed in the Orbitrap in the range of 395–1,800 m/z at a resolution of 6×10^4, followed by the selection of the twenty most intense ions (TOP20) for CID-MS2 fragmentation in the Ion trap using a precursor isolation width window 2 Da for CID scans, AGC setting of 10,000, and a maximum ion accumulation of 200 ms. Singly charged ion species were not subjected to fragmentation. Normalized collision energy was set to 35 V and an activation time of 10 ms. Ions in a 10 ppm m/z window around ions selected for MS2 were excluded from further selection for fragmentation for 60 s. The same TOP20 ions were subjected to HCD MS2 event in Orbitrap part of the instrument. The fragment ion isolation width was set to 0.7 m/z, AGC was set to 50,000, the maximum ion time was 200 ms, normalized collision energy was set to 27V and an activation time of 1 ms for each HCD MS2 scan. Raw data were submitted for analysis in Proteome Discoverer 2.4 (Thermo Scientific, CA) software. Assignment of MS/MS spectra was performed using the Sequest HT algorithm by searching the data against a protein sequence database including all entries from Uniprot_Human2018_SPonly, Uniprot_Trichomonas_vaginalis, Uniprot_ Saccharomyces_cerevisiae databases, and other known contaminants such as human keratins and common lab contaminants. For the peptide screening, we included the yeast database because TV medium contains yeast

extracts. Sequest HT searches were performed using a 20 ppm precursor ion tolerance and requiring each peptides N-/C termini to adhere with Trypsin protease specificity, while allowing up to two missed cleavages. 11-plex TMT tags on peptide N termini and lysine residues (+229.162932 Da) was set as static modifications, while methionine oxidation (+15.99492 Da) was set as variable modification. A MS2 spectra assignment false discovery rate (FDR) of 1% on both protein and peptide level was achieved by applying the target-decoy database search. Filtering was performed using a Percolator (64bit version, reference 6). For quantification, a 0.02 m/z window centered on the theoretical m/z value of each of the six reporter ions and the intensity of the signal closest to the theoretical m/z value were recorded. Reporter ion intensities were exported in result file of Proteome Discoverer 2.2 search engine as an excel table.

The raw data are available at https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=d972dafadff24186af5c36bd6b171f11

Bioinformatics and Statistical Analyses

NFκB activation, MTT and cytokine data were analyzed by analysis of variance (ANOVA) with Tukey's multiple comparisons test (GraphPad Prism, v.7) and p values <0.05 were considered significant. For quantitative proteomics, principal component analysis and differential expression analysis was performed using homemade R package. Data sets underwent two-way ANOVA analysis and proteins with p values <0.05 were considered significant for down or upregulated hits. **Supplementary Table S1** contains the protein list of TV phenotype 1 (virus-negative) vs. phenotype 2 (virus-positive) with statistics and the gene ontology (GO) analysis.

RESULTS

sEVs Derived From TVV-Negative but Not TVV-Positive Parasites Activated NF-KB and Cytokine Production by Uninfected Bystander Epithelial Cells and Effector Immune Cells

Previous evidence showed that sEVs from TV laboratory strains modulate innate immunity in human cervical cells (Twu et al., 2013). We showed using multiple clinical TV isolates that TVV induced NF-kB activation and cytokine upregulation in endocervical, ectocervical and vaginal human epithelial cells (Fichorova et al., 2012). Therefore, to decipher the effect of TVV on TV sEV-mediated immunity, we first treated the same endocervical cells tested previously with sEVs derived from a native TVV positive TV strain (347V+) and its TVV negative isogenic derivative (347V-), and measured NF-κB activation and levels of IL-8 and RANTES. IL-8 was chosen because it is the most abundantly expressed epithelial chemokine for neutrophils involved in clearing protozoan infection (Mercer et al., 2018). RANTES was chosen because its levels are low at baseline in the epithelial cells but are significantly upregulated in response to dsRNA, including TVV genomic dsRNA upon exposure to both purified RNA and whole intact virions (Fichorova et al., 2012). Thus, the RANTES response served as a control for viral contamination of the sEV preparations from the TVV+ strain.

The endocervical cells responded to the sEVs from 347V- but not 347V+ with significant NF-κB activation (>2-fold increase over baseline, $p < 0.0001$) and increased levels of both IL-8 (>20-fold, $p < 0.0001$) and RANTES (~2-fold, $p < 0.0001$) (**Figure 1**). The lack of RANTES response to the 347V+ sEVs confirmed lack of dsRNA contamination of the sEV preparation.

We next treated PBMCs with 347V+ and 347V- sEVs and measured levels of IL-8, IL-6 and TNFα as markers of leukocyte activation. None of the sEV preparations reduced leukocyte viability below 100% of the medium control (data not shown). 347V- sEVs significantly increased levels of all three cytokines compared to both baseline and 347V+ sEVs, while 347V+ sEVs had no significant effect (**Figure 2** and **Supplementary Figure S3**).

sEVs Derived From TV Infected Vaginal Cells Activated Leukocytes in a Manner Dependent on the TVV Status of the Parasite

In the natural course of infection, both the human host cells and the extracellular parasite adherent to them can contribute to the sEV pool released by the infected vaginal epithelium. To determine whether the TVV endosymbiont will continue to exert its immunomodulatory effect in the context of the host-parasite sEV pool, we isolated sEVs from the vaginal

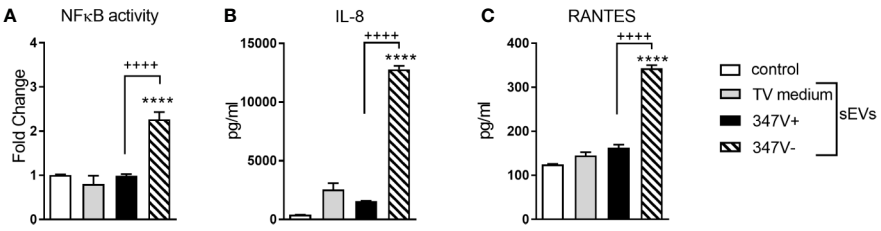

FIGURE 1 | TVV infection status modifies the immunostimulatory properties of TV sEVs in bystander cervical cells. Endocervical cells were treated with control no sEV, sEVs derived from TV medium alone, 347V+, or 347V- for 24 h. **(A)** NFkB activity was measured by a luciferase reporter assay and shown as fold change from control. **(B)** IL-8 and **(C)** RANTES protein levels by MSD assays. Data represent two independent experiments performed in triplicate and plotted as mean ± standard deviation. ****$p < 0.0001$ represent sEVs different from control. $^{++++}p < 0.0001$ represent 347V- different from 347V+.

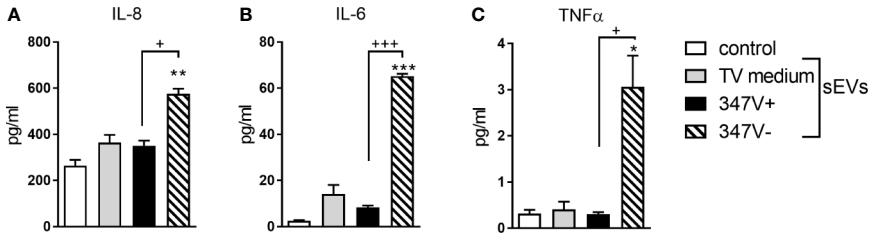

FIGURE 2 | sEVs from virus-negative TV but not virus-positive parasites induce multi-cytokine response in immune cells. PBMCs were treated with control no sEV, sEVs derived from TV medium, 347V+, and 347V- TV strains for 24 h. **(A)** IL-8, **(B)** IL-6, and **(C)** TNF-α protein levels were measured by MSD assays. Graphs represent data from PBMC donor X in duplicate and plotted as mean ± standard error of mean. Results are shown as protein levels normalized to cell viability. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ represent sEVs different from control. $^{+}p < 0.05$ and $^{+++}p < 0.001$ represent 347V- different from 347V+.

supernatants in our vaginal TV infection model. In this model in addition to the 347V+ and 347V- isogenic pair, we used a second previously well-characterized TVV+ strain UR1 (Goodman et al., 2011a; Goodman et al., 2011b; Fichorova et al., 2012) and also stimulated the vaginal epithelium with synthetic dsRNA poly(I:C) to mimic the TVV dsRNA effects in the absence of TV.

As shown in **Figure 3A**, sEVs from the vaginal infection with both TVV+ strains suppressed IL-8 production by the PBMCs in comparison to sEVs derived from uninfected vaginal cells ($p = 0.0296$ and $p = 0.0015$ for 347V+ and UR1 infection, respectively). This immunosuppressive effect was reversed when the 347V+ strain was cured from the virus. The sEVs from the 347V- infection phenotype induced similar activation as the sEVs from uninfected vaginal cells ($p > 0.99$). sEVs derived from vaginal cells exposed to poly(I:C) had a similar immunosuppressive effect ($p < 0.0016$) as the sEVs from the TVV-positive infection phenotype suggesting that the vaginal sEV manipulation may be at least in part attributable to direct effect of TVV dsRNA. IL-10 followed the same pattern of decreased expression in MALP-2-treated PBMCs as IL-8 (**Figure 3B**). The decrease in cytokine output occurred in the absence of cell toxicity (**Figure 3C**).

We then tested the effect of the vaginal infection sEVs on PBMCs in the presence of MALP-2, a TLR2/6 ligand derived from mycoplasma, another frequent TV endosymbiont (Fichorova et al., 2017). MTT data confirmed lack of toxicity under all experimental conditions (**Figure 3C**). As expected, and shown in **Figure 3A**, MALP-2 induced a 3-fold increase of IL-8 production by the PBMCs in the absence of exposure to sEVs from the vaginal infected cells. Exposure of the PBMCs to vaginal sEVs from the TVV+ infection phenotype significantly suppressed the IL-8 response to MALP-2 (MALP-2 alone versus combined with UR1+ and 347V+, $p < 0.0164$ and $p < 0.0254$, respectively). sEVs from vaginal cells activated with poly (I:C) showed similar immunosuppressive effect ($p < 0.0022$). Similarly to the effects of sEVs in the absence of MALP-2, the removal of the virus from the 347V+ strain reversed the immunosuppressive effect (347V- > 347V+, $p < 0.0001$) and 347V- led to a higher activation status compared to MALP-2 alone ($p = 0.0014$), similar to that induced by sEVs from uninfected vaginal cells.

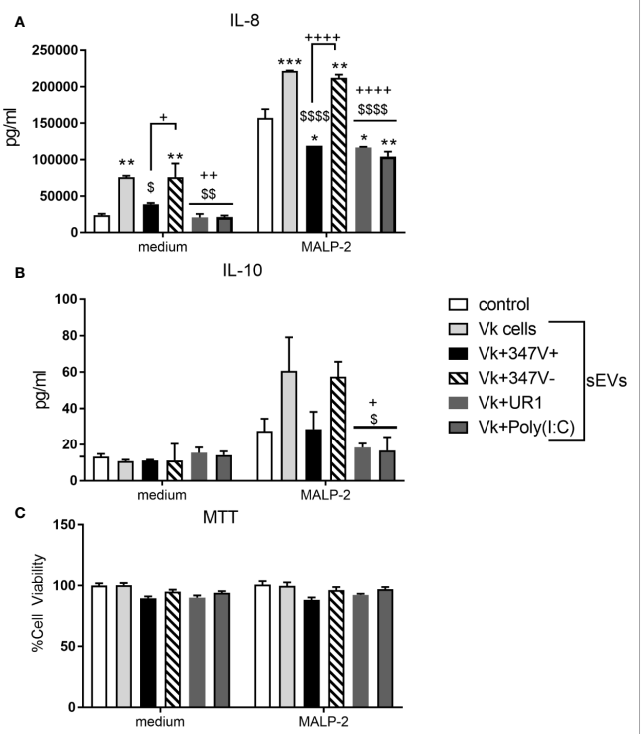

FIGURE 3 | sEVs from TV-infected vaginal cells regulate cellular immunity in a manner dependent on the viral status of the parasite. PBMCs were treated with control no sEV, sEVs derived from vaginal cells (Vk cells), Vk+347V+, Vk+347V-, Vk+UR1, and Vk+Poly(I:C) with or without MALP-2 for 24 h. **(A)** IL-8 and **(B)** IL-10 protein levels were measured by MSD assay. **(C)** MTT assays were performed to confirm cell viability after 24 h treatment relative to control. Graphs represent data from PBMC donor A in duplicate and plotted as mean ± standard error of mean. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$ represent sEVs different from control. $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; and $^{\$\$\$\$}p < 0.0001$ represent comparisons different to Vk cells sEVs. $+p < 0.05$; $++p < 0.01$; and $^{++++}p < 0.0001$ represent comparisons different to Vk+347V- sEVs.

The immune-suppressive effects of the sEVs of the TVV+ infection phenotype in both absence or presence of MALP-2 were reproduced with PBMCs from multiple donors (**Supplementary Figure S4**).

Quantitative Proteomic Analysis of sEVs Derived From *T. vaginalis*

To identify proteins that may be involved in this differential immune response, we examined the expression of proteins packaged into sEVs released by virus-positive and virus-negative TV. Quantitative proteome analysis using TMT-labeling compared sEVs derived from three virus-positive TV strains (347V+, UR1 clone, and OC8) and three virus-negative TV strains (347V-, OC7, and B7RC2). The sEVs from the 6 TV strains clustered separately from the sEVs isolated from culture medium as a background control (**Figure 4**). A total of 241 proteins were identified with 192 consistently present in the sEV preparations from all 6 TV strains. Of those 11% (21 proteins) were identified as common contaminants. Of the remaining 171 proteins detected in the TV sEVs, 67% (114 proteins) were matched to the UniProt human database suggesting high homology with human proteins, 6% (11 proteins) were matched to the yeast database, and 27% (46 proteins) were identified as unique TV proteins with no homology to human or yeast. The 171 TV sEV proteins were characterized by molecular function and protein class according to the PANTHER classification system, which uses Gene Ontology (GO) (Mi et al., 2019). According to the predicted function assigned by GO; 23.95% are proteins involved in catalytic activity and 21.06% are involved in binding (**Figure 5**). In smaller proportion, assigned protein functions included molecular function regulators, transporters, structural molecules, transcription and translation regulators and molecular transducers. TV proteins categorized by protein class were assigned into 17 classes, with the larger proportions belonging to protein binding and modifying, metabolite enzyme, extracellular matrix, and defense/immunity. Majority of proteins identified were not assigned to a functional group and this could partly be due to a large number of TV proteins being uncharacterized. Differential expression analysis showed seven proteins were significantly differentially expressed (DE) ($p < 0.05$) between virus-negative vs. virus-positive TV (**Figure 6**). The relative abundance values of the DE proteins show that the protein expression data from the TV strains tightly clustered by TVV phenotype (**Figure 7**). According to UniProt database (Uniprot Consortium, 2018), the three proteins (TVAG_071310, TVAG_483690, and KTR1) that were more abundant in the virus-negative phenotype are involved in zinc ion binding (GO:0008270), electron transfer activity (GO:0009055), and transferase activity (GO:0016740), while the human protein MGAM that was less abundant in that phenotype is involved in catalytic activity (GO:0003824), metabolic process (GO:0008152), and neutrophil degranulation (GO:0043312). The other three proteins (TVAG_201690, TVAG_249950, and TVAG_079150) that were less abundant in the virus-negative sEV phenotype are considered uncharacterized hypothetical proteins. According to TrichDB genome database (Aurrecoechea et al., 2009), TVAG_249950 hypothetical protein has a predicted function of protein binding but no functional information is available for TVAG_079150 or TVAG_201690. Protein BLAST analysis (Uniprot Consortium, 2018) revealed TVAG_201690 shares sequence similarity to five different integrase core domain proteins from *Tritrichomonas foetus* and therefore may be involved in DNA integration, while TVAG_079150 shares sequence similarity to multiple uncharacterized proteins.

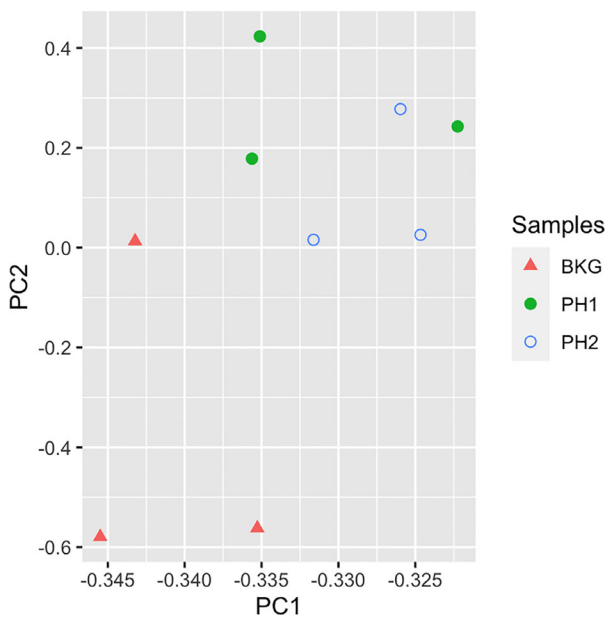

FIGURE 4 | TV sEVs cluster separately from background medium control. Principal component analysis was used to compare sEVs on the log2-transformed abundance values of all proteins identified. Triangles represent background medium (BKG), filled circles represent virus-negative TV phenotype (PH1), unfilled circles represent virus-positive TV phenotype (PH2).

DISCUSSION

Our study is the first to show in human vaginal epithelial cells, the primary *T. vaginalis* host can release immunosuppressive sEVs in response to infection with TVV-positive TV and that these immunosuppressive effects are eliminated and replaced by immune stimulation upon cure of the parasite from the symbiont virus. It is also first to compare the sEV effects and their protein cargo in virus-positive versus virus-negative phenotypes derived from isogenic parasites.

Extracellular vesicles (EV) secreted by protozoan parasites have been shown to facilitate parasite-host as well as parasite-parasite communications and functions including parasite adherence, tissue tropism, drug resistance, and differentiation (Marti and Johnson, 2016; Wu et al., 2018). EVs originating from *Leishmania* spp., *Trypanosoma cruzi*, and erythrocytes infected with *Plasmodium* spp. have been implicated in immunomodulation, most of them showing upregulation of cytokines, while some, e.g., EV from *Leishmania* spp., causing downregulation of IL-8 and TNF-α in

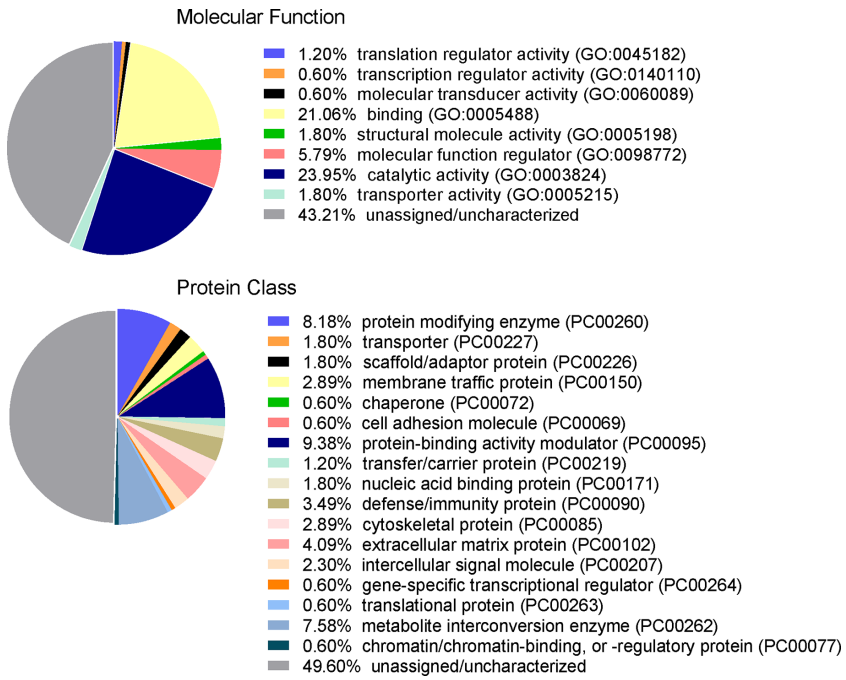

FIGURE 5 | Functional categorization of TV sEV proteome. The identified TV sEV proteins were sorted into functional groups for molecular function with gene ontology (GO) terms and for protein class (PC) using PANTHER classification system (pantherdb.org).

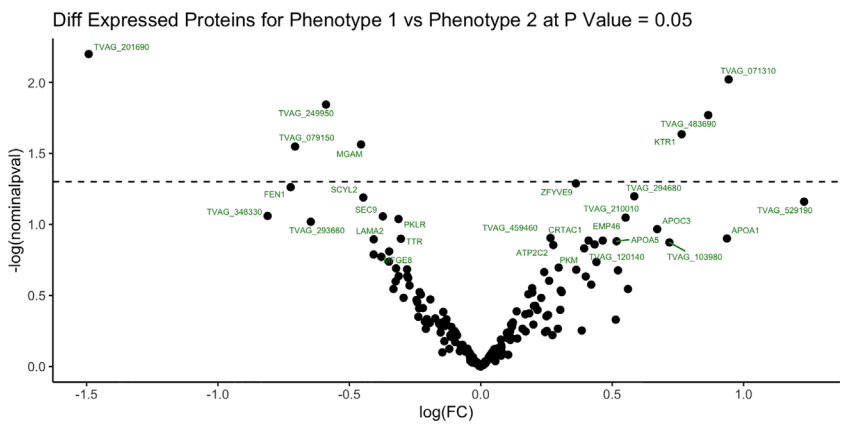

FIGURE 6 | Differentially expressed sEV proteins between virus-negative TV vs. virus-positive TV. Differential expression analysis compared log2-transformed abundance values of all sEV proteins identified between virus-negative TV vs. virus-positive TV. Dashed line represents p value = 0.05. X-axis represents log2-transformed fold change (FC). Y-axis represents -log2 transformed P value.

monocytes, but these phenomena were not investigated in the context of symbiont viruses (Szempruch et al., 2016). Our finding of suppressed IL-8 by sEVs from the TVV+TV infection may have clinical significance for the extracellular parasite adaptation since IL-8 is involved in the recruitment of neutrophils (Shaio et al., 1995)—the immune cells primarily responsible for clearance of TV parasites (Mercer et al., 2018; Bhakta et al., 2020).

Only a few studies to date have described sEVs released by TV, limited to parasites with either unknown or negative TVV status. A study by Twu et al. showed that sEVs released from TV upregulated IL-6 and IL-8 protein levels in human ectocervical epithelial cells in the absence of parasite, but IL-8 was lower when the cells were infected after sEV pre-incubation (Twu et al., 2013). Twu et al. listed several TV strains in the method section

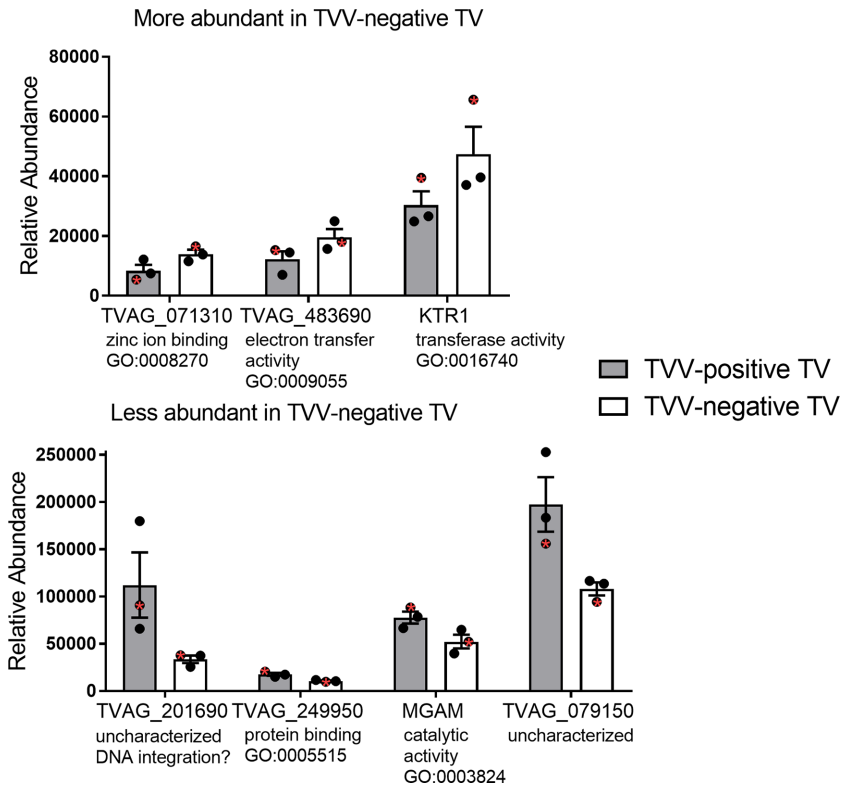

FIGURE 7 | Relative abundance of the differentially expressed sEV proteins. Differential expression analysis compared log2-transformed abundance values of all sEV proteins identified between virus-negative TV vs. virus-positive TV. Graphs represent relative abundance values of the 7 significantly differentially expressed ($p < 0.05$) proteins between three virus-negative TV strains vs. three virus-positive TV strains and plotted as mean ± standard error of mean. The isogenic strains 347V+ and 347V- are noted with a red asterisk within each phenotype. For each protein, gene ontology (GO) terms for molecular function are annotated.

but did not specify, which one was used in each experiment and did not provide TVV status of the strains. Of note, the human ectocervical epithelial cell line used by Twu et al. is isogenic to the endocervical epithelial cell line in this study, both generated from the same female donor (Fichorova et al., 1997). A mouse infection model where sEVs from a TV strain GT-21 of unknown TVV status upregulated mRNA levels of IL-10, and slightly IL-6 and TNF-α in macrophages but sEV pre-treatment prior to TV infection showed mixed effects, e.g., increased IL-10, inhibited IL-13 and IL-17, and delayed inhibition of IL-6 (Olmos-Ortiz et al., 2017). Our study provided evidence that the symbiont virus is needed by the parasite to generate an immunosuppressive sEV phenotype. IL-10 is known for its immunosuppressive effects in other protozoan parasitic infections e.g. *Leishmania* (Kane and Mosser, 2001). However, our data suggest that the immunosuppressive effect of exosomes secreted by vaginal cells in response to TVV-positive TV and in response to the viral dsRNA mimic (poly-IC) occurs in the absence of IL-10 upregulation and, moreover, blunts IL-10 expression by PBMCs. In our study, the human PBMC's responded to TLR2/6 stimulation by MALP-2 with IL-10 upregulation as expected. However, this IL-10 response was selectively suppressed upon exposure to sEVs from poly(I:C) (dsRNA) treated vaginal cells as well as vaginal sEVs from the TVV+TV but not the TVV-TV infection. Thus, mechanisms other than IL-10 are responsible for the immunosuppressive effects of circulating exosomes driven by viral dsRNA and virus positive TV phenotype. PBMCs isolated by the gradient method applied in our study typically contain 70–90% lymphocytes (T-cells, B-cells, and NK cells), 10–20% monocytes and 1–2% dendritic cells (Kleiveland, 2015). Thus, our findings primarily apply to the lymphocyte pool. Various cell types in the tissue context may respond differently as it is known that the IL-10 signaling depends on the cellular source (Iyer and Cheng, 2012). Since, we confirmed the immunosuppressive effect of the TVV-positive sEVs phenotype using multiple donors, it is unlikely that it is necessarily driven by T memory cells to TV proteins. The role of immune memory and cell types remains to be elucidated by future studies.

Various components of the sEV molecular cargo may mediate the observed immunomodulatory effects of the TV sEVs. Proteins, RNAs and episomal DNA have been found to mediate host-parasite interactions in protozoan infection models (Szempruch et al., 2016). One recent study showed that

in a mouse infection model, *Leishmania* spp. can package their symbiont virus (LRV1) into EVs to facilitate viral transmission between the parasites which exacerbated the mucocutaneous lesions typical for the clinical presentation of leishmaniasis (Atayde et al., 2019). This study, however, did not measure cytokines and did not characterize other immunologic aspects of hijacking the *Leishmania* exosomal pathway by the virus. It is theoretically possible that whole TVV virions or genomic dsRNA may be carried by the TV sEVs. The TVV virions are at most 45 nm in diameter (Parent et al., 2013) and thus may fit into sEVs of ~100 nm size used in this study. We have, however, not observed them within our sEV preparations by electron microscopy (data not shown) suggesting that sEV-mediated transmission may be rare event in TV making it unlikely that the immunologic effects we observed are mediated by the uptake of virions wrapped in TV sEV membranes. The different ways protozoan-viral symbiosis exploits the sEV pathways in leishmaniasis and trichomoniasis may be explained by adaptation to different parasitic lifecycles. In contrast to TV, the *Leishmania* spp. are intracellular parasites that infect phagocytes relying on both myeloid and T cell responses for clearance of infection (Tripathi et al., 2007; Liu and Uzonna, 2012). In contrast to the role of virus in leishmaniasis, in trichomoniasis the viral symbiont does not appear to show a more severe symptomatology (Graves et al., 2019), which supports our hypothesis and findings that in TV the virus makes the host more permissive by blocking leukocyte responses that can clear the extracellular parasite.

It is also unlikely that the effects specific for the TVV-positive sEV phenotype were mediated by direct signaling of genomic viral dsRNA. The presence of such dsRNA in the sEVs if taken up by the endocervical epithelial cells by either membrane fusion or endocytosis would have caused a significant RANTES upregulation through the TLR3 signaling pathway as we have previously shown (Fichorova et al., 2012). However, this was not observed in our experiments when the cells were exposed to sEVs of the TVV-positive phenotype. In fact, the sEVs from TVV-negative strains induced RANTES upregulation while the sEVs from the TVV-positive phenotype were immunosuppressive. However, other pathways of dsRNA processing and effects may be involved since we were able to reproduce the immunosuppressive phenotype of the vaginal sEVs by stimulating with poly(I:C), a synthetic dsRNA mimicking genomic TVV in the absence of the parasite and the parasite-derived sEVs. Studies are under way to further investigate the role of RNAs including micro-RNAs in regulating the effects of sEVs generated in TV infection.

Prior research has presented evidence that TV sEVs contain proteins of significance for TV pathogenesis (Twu et al., 2013). Here we show for the first time that TV proteins packaged in the protozoan sEVs may depend on the endosymbiont status. Also, for the first time we report a quantitative comparative proteome analysis of sEVs originating from different TV strains. Our approach of using labeled proteomic analysis allowed us to quantitatively compare TV sEV proteomes in a mix of multiple virus-negative and virus-positive TV strains.

Our proteome analysis identified a total of 241 proteins and after comparison to the human and yeast databases and removal of common contaminants, 171 proteins were identified as consistently expressed by TV, with 114 shared with the human database and 46 uniquely matched to the TV database. Twu et al. identified a total of 215 proteins, with only 2 proteins that overlapped with those reported in this study. Multiple factors may account for these differences. The proteome analysis performed by Twu et al. was initial gel separation followed by multidimensional protein identification technology (MudPit), followed by a label-free quantitation of the proteome of a single strain. In contrast, we used a quantitative TMT labeled approach which allowed us to analyze a mix of three strains per phenotype (6 replicates). Firstly, differences in sample processing between these two methodologies may account for the differences in the number of proteins identified. The gel separation followed by MudPit mass spectrometry might lead to higher resolution and depth/coverage due to more fractionation and/or higher single sample input. In our study, we did not perform deep fractionation to achieve depth of the coverage but rather our goal was to show differences in sEV proteomes using labeling and triplicates in each group to have the statistical power to show proteins that differentiate between virus-negative and virus-positive TV phenotypes. Secondly, protein identification based on peptide sequences might have identified different proteins due to different databases screened. Both, our study and Twu et al. screened the same TV proteome database. However, our study also simultaneously screened the human and yeast databases, while Twu et al. did not report screening against those databases. Third, differences in TV growth conditions such as TV density, culture medium composition, and incubation time may account for differences in TV protein expression. In the study reported here, TV strains were grown in non-EV depleted Diamond's medium to allow optimal growth conditions over a period of 24 h. In contrast, Twu et al. exposed TV strain to an EV-depleted TYM medium and harvested the sEV for protein analysis in only 4 h, allowing limited protozoan replication. Furthermore, in the present study, TV cultures were started at a 2.5-fold lower density to reduce stress derived from dying protozoa (4×10^5 parasites/ml vs. 1×10^6 parasites/ml by Twu et al), which could have also affected the sEV protein cargo.

Since protein identification in this study was done at the peptide-level, which may be indistinguishable by species, the 114 TV-expressed proteins identified as "human" are most likely TV proteins highly homologous to human and thus matched with higher confidence to the human proteome database which is much larger than the TV proteome database. The 46 proteins identified as TV were not homologous to human proteins and thus have unique peptides that belong to TV proteins only. Our findings suggest that 67% (114/171) of the sEV proteins expressed by TV are homologous to human proteins that are not common contaminants. These findings are in agreement with previous findings that TV exosome proteins contained 73% mammalian orthologs when compared to the ExoCarta database (Twu et al., 2013). Our analysis revealed that 54% (92/171) of the

proteins identified in the TV sEVs were matched to proteins previously found in human exosomes described in the ExoCarta database (Keerthikumar et al., 2016). Similarly, another report showed a 56% proteome overlap between TV microvesicles and human EVs when compared to EVpedia (Nievas et al., 2018).

Our study showed enrichment for functions including catalytic, transcription, translation and transducer activities and protein classes including immune/defense proteins. Of the proteins identified as TV in our study, 30% (13/46 proteins) were previously identified in the TV secretome (Stafkova et al., 2018) and their functional relevance to host immunity is still to be elucidated. The single human protein (MGAM) more abundant in the virus-positive TV phenotype is involved in neutrophil degranulation. Neutrophils are important in resolving protozoan parasitic diseases (Mercer et al., 2018; Diaz-Godinez and Carrero, 2019) using killing mechanisms including degranulation whereby they release inflammatory mediators, proteases, and cytolytic factors (Lacy, 2006). Whether MGAM facilitates virus-positive TV sEVs in dampening the immune response should be investigated since it may have broader implications for neutrophil regulation. Less is known about the three TV proteins (TVAG_201690, TVAG_249950 and TVAG_079150) more abundant in the virus-positive phenotype and whether they may be involved in regulation of immune responses, therefore further investigation into the functions of these proteins is needed.

Another finding with important potential clinical implications is that sEVs derived from the vaginal infection with TVV-positive TV also suppressed signaling by a major mycoplasma protein (MALP-2). This phenomenon suggests that the parasite-viral symbiosis may dampen the immune surveillance against mycoplasma—another frequent symbiont of the TV parasite, which provides metabolic benefit to the parasite and facilitates the dysbiotic vaginal condition characteristic for trichomoniasis (Fichorova et al., 2017).

Collectively, our results suggest that endosymbiont virus-positive parasites may use the sEV pathways to evade host immunity. Future studies should continue dissecting the sEV cargo in the context of TV infection in symbiosis with *Trichomonasvirus* and mycoplasma to identify the molecular pathways of immune modulation and further implications for the human host.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the MassIVE Repository (a member of the ProteomeXchange consortium), ID PXD021696.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Partners Human Research Affairs-Partners Human Research Committee, Brigham and Women's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RF conceived the study. Data were generated by TC, YG, HY, and BB. Data analysis was performed by RF, YG, BB, and TC. The manuscript was drafted by YG and RNF. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.591172/full#supplementary-material

SUPPLEMENTARY TABLE 1 | Proteomics data analysis. Data analysis sheets contain (i) the protein list of TV phenotype 1 (virus-negative) vs. phenotype 2 (virus-positive) with statistical analyses and includes UniProt accession number, gene symbol, log2 fold change (FC), FC, p-values and identification of common contaminants and (ii) the gene ontology (GO) analysis for protein class and molecular function using PANTHER classification system (pantherdb.org).

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Conflict of Interest: BB is a scientific adviser for Merck.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Pathogenesis of Viral Hepatitis-Induced Chronic Liver Disease: Role of Extracellular Vesicles

Hong Kiat Lim [1], Gary P. Jeffrey [2,3], Grant A. Ramm [1,4] and Carolina Soekmadji [1,4]**

[1] Hepatic Fibrosis Group, Department of Cellular and Molecular Biology, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia, [2] Faculty of Health and Medical Sciences, University of Western Australia, Perth, WA, Australia, [3] Sir Charles Gairdner Hospital, Nedlands, Hepatology Department and Liver Transplant Service, Perth, WA, Australia, [4] Faculty of Medicine, The University of Queensland, Brisbane, QLD, Australia

Extracellular vesicles are encapsulated lipid nanoparticles secreted by a variety of cell types in living organisms. They are known to carry proteins, metabolites, nucleic acids, and lipids as their cargoes and are important mediators of intercellular communication. The role of extracellular vesicles in chronic liver disease has been reported. Chronic liver disease such as viral hepatitis accounts for a significant mortality and morbidity burden worldwide. Hepatic fibrosis has been commonly associated with the chronic form of viral hepatitis, which results in end-stage liver disease, including cirrhosis, liver failure, and carcinoma in some patients. In this review, we discuss the potential role of extracellular vesicles in mediating communication between infectious agents (hepatitis B and C viruses) and host cells, and how these complex cell-cell interactions may facilitate the development of chronic liver disease. We will further discuss how understanding their biological mechanism of action might be beneficial for developing therapeutic strategies to treat chronic liver disease.

Keywords: extracellular vesicles, chronic liver disease, hepatic fibrosis, therapy, biomarker

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****Correspondence:***
Carolina Soekmadji
Carolina.Soekmadji@qimrberghofer.edu.au
Grant A. Ramm
Grant.Ramm@qimrberghofer.edu.au

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INTRODUCTION

Extracellular vesicles (EV), first described over four decades ago (Chargaff and West, 1946; Wolf, 1967), have now gained recognition as an important mediator of intercellular communication in chronic liver disease (CLD) (Ramakrishnaiah et al., 2013; Hirsova et al., 2016; Devhare et al., 2017; Banales et al., 2019). These membrane-bound nanoparticles are secreted by a variety of cell types in a living organism and are known to carry cargoes such as proteins, metabolites, nucleic acids, and lipids, which mediate complex cell-cell communications. The specific nature of EV-derived cargo has led to an immense interest in using EV as a tool for disease diagnosis and as a target for therapeutic intervention (Banales et al., 2019; Soekmadji et al., 2020).

The CLD is an umbrella term used for reference to any pathological condition where progressive destruction of liver tissue occurs over 6 months or more. CLD is a significant health concern worldwide; chronic hepatitis C, chronic hepatitis B, alcoholic or non-alcoholic fatty liver disease, and autoimmune hepatitis account for a significant global mortality and morbidity burden. It is estimated that about 844 million people are living with CLD (2017), and at least 2 million deaths per year are associated with CLD globally (Byass, 2014). Cell-cell interactions amongst cell types such as

liver progenitor cells (LPC), hepatic stellate cells (HSC), and Kupffer cells have been implicated in the development of liver fibrosis and CLD, which eventually progress into an end-stage liver disease including cirrhosis, liver failure and carcinoma (Dwyer et al., 2014; Pozniak et al., 2017). Recently, EVs have been shown to mediate these complex cell interactions among these cell types in the liver, particularly in the context of CLD, which shed light on their potential roles in the development of this disease (Deng et al., 2017).

This review discusses the potential role of EVs in mediating communication between infectious agents and host cells, and how EV-mediated cell-cell interactions facilitate the development of liver disease. We will further discuss how understanding their biological mechanism of action might be beneficial for developing therapeutic strategies to treat chronic liver disease.

LIVER STRUCTURE AND FUNCTION

The liver is the largest visceral organ in the body, making up an estimated 2–5% of the adult body weight with roughly 10% of the body's blood flowing through at any one time (Vekemans and Braet, 2005). The liver performs a myriad of homeostatic roles associated with metabolism, digestion, immunity, and the endocrine system. Microscopically, the liver is composed of two main cell types, parenchymal and non-parenchymal cells (Trefts et al., 2017). Parenchymal cells, including hepatocytes and cholangiocytes, form the majority of cell types in the liver. Hepatocytes, along with the liver sinusoidal endothelial cells (LSEC), line the sinusoids and are the primary epithelial cells of the liver (Trefts et al., 2017). Hepatocytes and LSECs are separated by the perisinusoidal space, also known as space of Dissé. Cholangiocytes line the bile ducts and are involved in the production and modification of bile composition (**Figure 1**).

In the injured liver, a unique subset of stem-like cells are induced termed hepatic or liver progenitor cells (LPC), also described as oval cells in rodents, which have the potential to reconstitute liver mass by differentiation into hepatocytes or cholangiocytes (Tirnitz-Parker et al., 2010; Köhn-Gaone et al., 2016). LPCs are proposed to originate in the canal of Hering in relatively small numbers at steady state but quickly expand through rapid proliferation following chronic hepatic injury (Dwyer et al., 2014). While their origin remains controversial, recent lineage tracing studies have reported that activated Sox9^{+}-ductal cells proliferate and differentiate into liver progenitor cells (LPCs) after sustaining chronic hepatic insults (Furuyama et al., 2011).

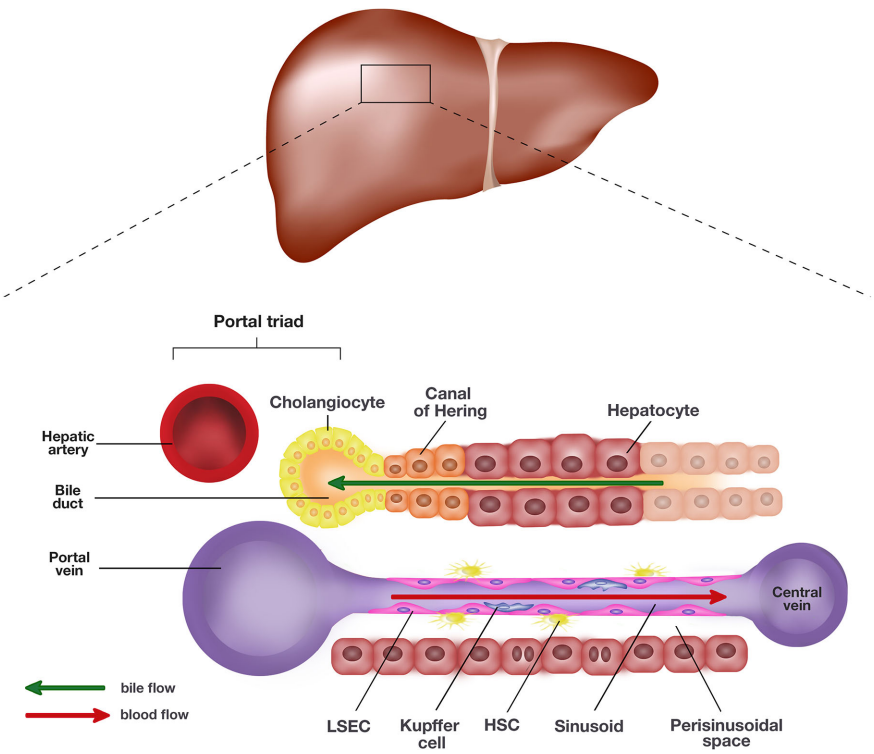

FIGURE 1 | Schematic representation of the liver and the single end of its lobule. The portal triad comprising the hepatic artery, bile duct, and portal vein sits at each end of the hepatic lobule. Blood collected from the portal vein and hepatic artery flows toward the central vein through the hepatic sinusoids lined by liver sinusoidal endothelial cells (LSEC) and hepatocytes. The hepatocytes and LSECs are separated by the perisinusoidal space, also known as space of Dissé where hepatic stellate cells (HSC) are located. The hepatocytes produce bile which empties into the bile duct lined by cholangiocytes. The canal of Hering is positioned in the junctional region between cholangiocytes and hepatocytes, where liver progenitor cells (LPC) are proposed to originate.

Nonparenchymal cells of the liver comprise liver myofibroblast precursors called hepatic stellate cells (HSC), resident liver macrophages or Kupffer cells and LSECs. HSCs which usually reside in the perisinusoidal space are liver-specific mesenchymal cells rich in vitamin A (Higashi et al., 2017). They exist at the ratio of 3.6 to 6 cells per 100 hepatocytes in human liver, and their primary function in the normal liver appears to involve vitamin A storage (Moreira, 2007). Studies have also reported their regulatory role in regulating hepatic blood flow and portal venous pressure at steady state (Geerts, 2001). LSECs are specialized endothelial cells, which form the highly fenestrated sinusoidal endothelium needed for the exchange of fluid, nutrients, and solutes between the sinusoidal blood and hepatocytes (Ni et al., 2017). They are highly endocytic and have a well-developed clathrin-mediated endocytosis system (Simon-Santamaria et al., 2010). Finally, Kupffer cells are specialized macrophages in the liver. They form part of the reticuloendothelial system and are involved in clearing senescent cells and pathogens such as bacteria and viruses. They are one of the key players in hepatic immunity.

PATHOPHYSIOLOGY OF CHRONIC LIVER DISEASE

While the liver represents an organ with enormous regenerative potential, chronic hepatic insults from pathogens, metabolic insults, and other toxic agents can lead to the development of CLD where the ability of the liver to heal and regenerate diminishes as a consequence of hepatic scarring (fibrosis) and eventually results in deterioration of liver function. The development of CLD is a complex multifactorial process involving many different cell types. Following a hepatic insult, the liver attempts to repair the injured tissue through the normal wound healing process. Paracrine stimulatory signals, including inflammatory mediators, from other cell types such as LPCs, LSECs, Kupffer cells, and hepatocytes within the liver microenvironment activate quiescent dormant HSCs to proliferate and migrate into the primary site of insults. These activated α-smooth muscle actin- (SMA) and collagen type I-expressing HSCs transdifferentiate into myofibroblasts, which produce collagen and extracellular matrix needed for the wound healing process (Higashi et al., 2017). They rapidly lose their ability to store Vitamin A, causing the amount of Vitamin A within the cell to decrease as it divides and distributes Vitamin A-lipid droplets into two daughter cells (Higashi et al., 2005). In CLD, these HSC-derived myofibroblasts contribute to excessive deposition of collagen and extracellular matrix in response to chronic liver injury; thus they are responsible for hepatic fibrosis in a variety of different CLDs in adults including chronic hepatitis C virus (HCV), alcoholic liver disease, non-alcoholic fatty liver disease (Friedman, 2008), liver cancer (Bridle et al., 2001) haemochromatosis (Ramm et al., 1997), and in pediatric liver disease such as biliary atresia (Ramm et al., 1998) and cystic fibrosis-associated liver disease (Lewindon et al., 2002).

LPCs can rapidly proliferate and differentiate in response to liver injury in a process called the ductular reaction. However, the role of LPCs in liver regeneration and repair seems to be restricted to chronic injury where the replication of mature hepatocytes has been impaired, or the hepatic microenvironment has been substantially changed (Dollé et al., 2010; Best et al., 2013). Clouston *et al.* showed that inflammatory cytokines such as interferon (IFN)-γ inhibited proliferation of hepatocytes, resulting in an expansion of LPCs (Clouston et al., 2005). One possible mechanism for LPC expansion involves tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor-inducible 14 (Fn14)-signaling (TWEAK/Fn14 signaling) (Dwyer et al., 2014). Binding of TWEAK secreted by macrophages or natural killer (NK) cells to Fn14 expressed on the surface of LPCs activates the downstream NFκB signaling pathway, which switches the genes involved in proliferation on, leading to the expansion of LPCs (Tirnitz-Parker et al., 2010; Viebahn et al., 2010; Bird et al., 2013).

Interactions between HSCs and LPCs have been shown to drive hepatic fibrogenesis in CLD. Notch signaling has previously been implicated in the biliary specification of Notch1/Notch2$^+$ LPCs through interactions with Jagged1$^+$ myofibroblasts (Boulter et al., 2012). The study demonstrated a decrease in expression of biliary genes in LPCs when Notch inhibitor was used in co-cultures of LPCs and HSCs. Interestingly, another study demonstrated impairment in the differentiation of LPCs into cholangiocytes when Notch2 liver-specific knockout mice were treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Fiorotto et al., 2013). Previous studies in this field identified a novel regulatory mechanism of HSC/LPC crosstalk involving lymphotoxin beta (LTβ) and its receptor, LTβR (Ruddell et al., 2009; Tirnitz-Parker et al., 2014). LTβ expression has been shown to increase in several animal models of chronic liver injury (Lowes et al., 2003; Akhurst et al., 2005; Knight et al., 2005; Lee et al., 2005; Dwyer et al., 2014). One study found that LTβR$^{-/-}$ animals fed with choline-deficient, ethionine-supplemented (CDE) diet to induce biliary fibrosis had decreased levels of inflammatory cytokines, which paralleled the reduction in fibrosis and activated HSC numbers, compared to the wild-type animals (Ruddell et al., 2009). Moreover, LPCs were shown to express LTβ, while LTβR expression was detected on quiescent/activated HSCs in that study, further suggesting the possible role of LTβ/LTβR signaling in HSC/LPC crosstalk (Ruddell et al., 2009).

In CLD, interactions between HSC and LSEC have also been previously reported (Horn et al., 1987; DeLeve et al., 2008b). LSECs undergo phenotypic changes in a process called capillarization prior to HSC activation and fibrosis (Horn et al., 1987; DeLeve et al., 2008b). Activated LSECs defenestrate and form a continuous basement membrane liken to the phenotype of capillaries in order to restrict the movement of toxic molecules which would otherwise be harmful to the liver (Deleve et al., 2008a; Ni et al., 2017). Unfortunately, this safety mechanism also prevents an adequate exchange of solute or fluid between sinusoids and hepatocytes, which further exacerbates the fibrotic process in the liver (Bartneck et al., 2014). While

healthy LSECs prevent activation of HSC and initiate changes of HSC from an activated to a quiescence state, capillarized LSECs promote HSC activation (Deleve et al., 2008a).

VIRAL HEPATITIS AS A CAUSE OF CHRONIC LIVER DISEASE

Viral hepatitis is one of the most common causes of CLD affecting close to 397 million individuals worldwide with both hepatitis B virus (HBV) and HCV infection cases combined (Trepo et al., 2014; Westbrook and Dusheiko, 2014; Ferri et al., 2016). There are five main types of hepatitis viruses, including types A, B, C, D, and E, of which, types B and C remain prevalent globally, albeit differing in the geographical distribution of disease prevalence. Infections associated with hepatitis types B, C, D, and E viruses cause CLD at varying rates with type E being the rarest, although co-infection of type B is still required for type D to become chronic. While hepatitis viruses primarily infect hepatocytes, some studies have reported binding of hepatitis types C viruses to other cell types such as peripheral blood mononuclear cells (PBMC) (El-Awady et al., 2005; Yamada et al., 2005), which raises the possibility that other cell types could play a more significant role in the pathogenesis of viral hepatitis. Most of the pathophysiology of viral-induced CLD is attributed to the exacerbated host immune response to the virus rather than the viral replication in the cells, although more work has to be done to clarify the mechanisms involved (Nakamoto et al., 1998; Buchmann et al., 2013; Ringelhan et al., 2017). This review will focus on HBV and HCV, given their high degree of clinical relevance in CLD and disease prevalence.

HBV, an enveloped partially double-stranded DNA virus, belongs to the Hepadnaviridae family, genus Orthohepadnavirus (Sekiba et al., 2018). The infectious viral particles are double-shelled and spherical. They consist of an outer lipid envelope embedded with hepatitis B surface antigens (HBsAg), and a nucleocapsid which comprises hepatitis B core antigens (HBcAg), viral polymerase, and DNA genome (Liang, 2009). The HBV genome is a 3.5kb long relaxed circular DNA (rcDNA), consisting of four overlapping reading frames, which encodes envelope proteins, structural core, viral polymerase/reverse transcriptase, and regulatory x protein (HBx) (Liang, 2009). HBV first enters the cells using the sodium taurocholate co-transporting polypeptide receptor (Yan et al., 2012; Ni et al., 2014; Tong and Li, 2014). After entry, the virion un-coats in the cytoplasm, allowing rcDNA to be transported into the host nucleus where rcDNA is converted into covalently closed circular DNA (cccDNA) (Grimm et al., 2011). The host RNA polymerase II transcribes cccDNA into viral messenger RNA (mRNA) transcripts containing viral pregenomic RNA (pgRNA), which are then re-exported back into the cytoplasm of the host cell for translation of viral proteins (Rajbhandari and Chung, 2016). Assembly of nucleocapsids occurs in the cytoplasm where pgRNA is co-packaged with viral polymerase/reverse transcriptase for reverse transcription of pgRNA into rcDNA. Mature nucleocapsids are enveloped through the endoplasmic reticulum (ER) and Golgi apparatus before secretion from the host cell *via* exocytosis (Rajbhandari and Chung, 2016). While the majority of pgRNA is reverse transcribed into rcDNA, a small proportion (10%) gets synthesized into double-stranded linear DNA (dslDNA) which can integrate into the host genome. However, unlike retroviruses, integration of HBV DNA is not essential for viral replication (Sekiba et al., 2018).

HBV is known to cause both acute and chronic forms of hepatitis. However, it is estimated that 10% of acute infection progresses to chronic disease (McKeating et al., 2018). The clinical manifestations of acute hepatitis include flu-like symptoms, dark urine, and jaundice, although some cases may appear asymptomatic. While the majority of HBV-infected people recover from the infection completely, some people remain chronically infected with HBV. In chronic hepatitis, a persistent unproductive immune response to HBV is responsible for substantial necroinflammation of the liver. It has been shown that T helper (Th) 2 type cytokines such as IL-4 and IL-10 are associated with persistent HBV infections resulting in more severe liver damage (Lee et al., 1999). Indeed, the importance of a protective T helper (Th) 1 rather than a tolerant Th2 immune response in clearing HBV has been demonstrated by several studies (Marinos et al., 1995; Maini et al., 2000). Akbar et al. demonstrated the importance of IFN-γ in controlling HBV infections by showing a reduction in HBV DNA in the liver and sera after dendritic cells were treated with Th1 type cytokine; IFN-γ (Akbar et al., 1996). In addition to host immune response, studies have reported the involvement of viral component HBx in the progression of chronic HBV. It was first shown by Lee et al. that HBx antigen was able to inhibit $CD8^+$ T cell response by reducing the production of IFN-γ and inducing apoptotic program in $CD8^+$ T cells (Lee et al., 2010). Other findings have also shown that HBx induces innate pro-inflammatory IL-6, IL-8, and TNF-α but often not at the level sufficient for viral clearance in chronic HBV (Mahé et al., 1991; Lara-Pezzi et al., 1998; Lee et al., 1998). In particular, it has been shown that IL-6 initiates a switch from acute to chronic inflammation by recruiting monocytes to the inflammation sites (Gabay, 2006). Furthermore, HBx was found to activate and promote the proliferation of HSCs (Martín-Vílchez et al., 2008; Bai et al., 2012).

While it is widely accepted that a substantial number of chronic HBV-associated liver cirrhosis cases develop hepatocellular carcinoma (HCC) as an end-stage complication of the infection (El-Serag and Mason, 1999; Block et al., 2003; El-Serag and Rudolph, 2007), little is known about the process in which the malignant transformation occurs. Persistent hepatic necroinflammation as a result of viral-host immune interaction is, however, recognized as the primary driver of HCC development (Ringelhan et al., 2017; Chen and Tian, 2019). Interestingly, as opposed to HCV-driven HCC, which develops mainly in the presence of cirrhosis, only about 20% of HCC driven by chronic HBV infection occur with cirrhosis (Chayanupatkul et al., 2017). Indeed, the development of HCC in HBV-infected individuals in the absence of inflammation shed light on alternative mechanisms for tumorigenesis.

Epidemiology data revealed that 85–90% of HBV-associated HCCs contained HBV DNA integrated into the host genome (Minami et al., 2005), raising the possibility that the integrated viral DNA might be involved in HCC development. Integration of HBV viral DNA was previously shown to activate expression of oncogenes such as cyclin E1 (CCNE1), telomerase reverse transcriptase (TERT), and mixed-lineage leukemia 4 (MLL4) (Sung et al., 2012). Moreover, genetic instability leading to chromosomal translocation and accumulation of genetic mutations after integration of viral HBx into the host genome has been reported (Lee and Rho, 2000; Bonilla Guerrero and Roberts, 2005; Feitelson and Lee, 2007).

HCV is an enveloped positive-sense single-stranded RNA virus belonging to the Flaviviridae family, genus hepacivirus. The HCV virions contain E1 and E2 glycoproteins within the viral envelope that surrounded the core protein and nucleocapsid. The HCV genome is 9.6kb long, consisting of two untranslated regions (UTR) 5'-UTR and 3'-UTR and an open reading frame (ORF). The ORF encodes a polyprotein, which is further processed into various viral proteins including core protein, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The life cycle of HCV, while only partially elucidated, starts with the attachment of HCV to a host receptor. Several host receptors, including lipoprotein (LDL) receptor, scavenger receptor B1 (SR-B1), and CD81 tetraspanin (which is also an EV marker) (Théry et al., 2018) have been proposed to be involved. Once bound to its receptor, HCV-receptor complex is internalized through clathrin-mediated endocytosis, releasing the nucleocapsid into the cytoplasm. Viral genomic RNA is freed from the nucleocapsid after uncoating, which then undergoes genomic replication *via* a negative-sense RNA intermediate and translation of polyproteins at the ER for further post-translational processing. Assembly and maturation of virion take place in the ER and Golgi apparatus before releasing from the host cell by exocytosis.

About 80% of acute HCV cases develop into chronic HCV infection due to the inability of the host immune system in clearing the virus (McKeating et al., 2018). Several mechanisms responsible for the persistence of HCV in the host have been reported. First, the error-prone and high replicating characteristic of HCV RNA polymerase results in a high acquisition of mutations in the viral genome, leading to the generation of many HCV quasispecies with mutated epitopes, which cannot be recognized by cytotoxic $CD8^+$ T cells (Irshad et al., 2013). Consequently, the cell-mediated cytotoxic killing of infected host cells is prevented, allowing the viruses to persist (Irshad et al., 2013). Moreover, persistent antigenic stimulation in chronic infection results in T cell exhaustion, which involves a loss of virus-specific effector T cells and increased expression of inhibitory molecules such as PD-1 (Penna et al., 2007; Radziewicz et al., 2007). For example, Wedemeyer *et al.* reported defective IFN-γ production and proliferative capacity with HCV-$tetramer^+$ T cells in patients with chronic HCV infection (Wedemeyer et al., 2002). Second, there is evidence that HCV proteins drive Fas-mediated apoptosis of virus-specific immune cells while promoting infiltration of peripheral T cells into the liver (Soguero et al., 2002). It is thought that the apoptosis of T cells in the liver might be partially responsible for liver injury mediated by inflammation (Soguero et al., 2002). Third, it has been reported that HCV E2 glycoprotein suppresses the non-specific cytotoxicity function in NK cells, allowing HCV to persist in affected patients (Crotta et al., 2002). As with HBV infections, persistent hepatic necroinflammation in chronic HCV infections creates a microenvironment which favors the development of cirrhosis and malignancies. It has been reported that $CD8^+$ T cells and NK cells are directly involved in the pathogenesis of HCV related liver cirrhosis and HCC (Cooper et al., 1999; Karidis et al., 2015; Khatun and Ray, 2019).

THE ROLE OF EXTRACELLULAR VESICLE (S) IN THE PATHOGENESIS OF VIRAL DISEASE

The extracellular vesicle (EV) is a collective term for "particles released from cells that are delimited by a lipid bilayer and cannot replicate" (Théry et al., 2018). While EVs have been previously categorized into exosomes, microparticles, and apoptotic bodies, what constitutes each subtype has been ambiguous. Since no consensus has been reached on their specific markers, this review will refer to EV subtypes based on the recommendations from the International Society for Extracellular Vesicles (ISEV) (Théry et al., 2018). In terms of size, EVs can be defined as small (<100 or 200nm) and medium/large (>200nm) EVs (Théry et al., 2018). Small EVs are highly enriched in a class of membrane proteins called tetraspanins such as CD9, CD63, CD81, as well as cytosolic proteins TSG101 and Alix. These proteins also play diverse roles in cell biology and physiology (Andreu and Yáñez-Mó, 2014; Lozano-Andrés et al., 2019). In addition, EVs can be categorized based on their biochemical composition and conditions or cell where they originate (Théry et al., 2018).

Biogenesis of EVs differs among exosomes, microparticles and apoptotic bodies and has been extensively reviewed elsewhere (Akers et al., 2013; Schorey et al., 2015; Abels and Breakefield, 2016; Hirsova et al., 2016). In general, exosomes are described as vesicles that are formed by inward budding at the multivesicular endosomes. Multivesicular endosomes destined for degradation or exocytosis will either fuse with lysosomes for degradation of their contents, or with the plasma membrane for exocytosis (Abels and Breakefield, 2016). Exosome biogenesis involves either endosomal-sorting complex required for transport (ESCRT)-dependent or independent mechanisms. ESCRT is a type of multi-subunit molecular machinery and comprises five complexes (ESCRT-0, I, II, III and VPS4) with specific roles assigned to each ESCRT complex (Hirsova et al., 2016). These complexes are involved in cargo recognition, recruitment, vesicle maturation, and secretion (Hirsova et al., 2016). ESCRT independent mechanism, on the contrary, is largely lipid raft and ceramide-based and was first described in oligodendroglial cells (Trajkovic et al., 2008; Colombo et al., 2014).

The Potential Role of Extracellular Vesicles in Viral Infectious Disease

The association between viral particles and EVs is indicated by the presence of viral elements in EVs isolated from infected cells (**Table 1**). Early studies in human immunodeficiency virus (HIV) have demonstrated the presence of viral transactivating response (TAR) element RNA in EVs isolated by gel filtration Sephadex G-10 spin column and Nanotrap particle A for $CD63^+$ vesicles from sera of HIV-1 infected patients and supernatants of cultured infected J1.1 cells respectively (**Table 1**) (Narayanan et al., 2013; Sampey et al., 2016). It was shown that these TAR RNA-containing EVs prevented apoptosis and enhanced viral replication in recipient cells, causing these cells to be more susceptible to HIV-1 infection (Narayanan et al., 2013). Their role was further confirmed by a study, which demonstrated decreased susceptibility of recipient cells to HIV-1 infection after the release of those EVs was inhibited (Sampey et al., 2016). Furthermore, HIV virulence factor, Nef detected in plasma EVs of HIV-infected patients using sucrose density gradient ultracentrifugation was shown to correlate with the low T cell counts in patients (**Table 1**) (Lee et al., 2016). Indeed, another study reported the induction of $CD4^+$ T cell apoptosis *in vitro* by EV-associated HIV Nef (**Table 1**) (Lenassi et al., 2010). Like the EVs from HIV-infected cells, the EVs isolated from Epstein-Barr virus (EBV)-positive A-type lymphoblastoid cells contain biomolecules such as latent membrane protein 1 (LMP1) which was found to inhibit NK and T cell functions (**Table 1**) (Dukers et al., 2000; Flanagan et al., 2003). In the context of human papillomavirus (HPV), it was found that the amount of EVs secreted by HeLa cells increases when E6/E7 oncogene expression is inhibited (Honegger et al., 2013). Moreover, the inhibition of E6/E7 oncogene expression leads to a reduction in the levels of EV cargo survivin, a negative regulator of apoptosis, although the E6/E7 oncogene protein itself has not been detected within the EVs (Honegger et al., 2013). Further studies are needed in part due to the possibility of co-isolation of virus during EV preparation as shown by the presence of retrovirus and xenotropic murine leukemia virus-related virus in isolated EVs using ultracentrifugation (Knouf et al., 2009; Soekmadji et al., 2017). It should be noted that there is an overlap in the mechanism by which viruses and EVs may share in biogenesis, entry, and secretion mechanisms, as shown by enveloped viruses that co-utilize the host's cellular machinery during infection (Nolte-'t Hoen et al., 2016). One cellular mechanism being investigated is the ESCRT which, as mentioned above, forms an integral part of the cellular endosomal system for EV biogenesis (Abels and Breakefield, 2016). Strickland *et al.* demonstrated inhibition of HIV-1 replication and budding through depletion of an ESCRT-I protein called TSG101 in the human embryonic kidney, HEK 293 cells (Strickland et al., 2017). TSG101 is also widely utilized as an EV marker (Soekmadji et al., 2013). Interestingly, knocking down TSG101 was shown to increase HBV production in human hepatoma, Huh-7 cells (Stieler and Prange, 2014). They demonstrated the importance of ESCRT-II subunits EAP30, EAP45, and EAP20 for HBV replication and showed that extracellular HBV was substantially reduced after knockdown of these ESCRT-II components (Stieler and Prange, 2014). Moreover, several studies have reported a role of tetraspanin EV markers in the life cycle of several viruses such as HIV-1 (Pelchen-Matthews et al., 2003; Fu et al., 2015), HCV (Bartosch et al., 2003; Fénéant et al., 2014), human papillomavirus (HPV) (Spoden et al., 2008; Scheffer et al., 2013), and influenza A virus (IAV) (Earnest et al., 2015; Earnest et al., 2017), which further suggests the potential convergence of EV and virus biogenesis pathways.

The Role of Extracellular Vesicles in Virus-Associated Chronic Liver Disease

While it remains unclear to what extent the similarities between viruses and EV biogenesis contributes to the disease progression of viral infections, it is possible that viruses may hijack the mechanism of EV biogenesis to increase infectivity and

TABLE 1 | Types of viral elements present in extracellular vesicles (EVs) released from cells infected by HIV or Epstein-Barr virus (EBV).

Causative agent	Cargo	Isolation process	EV characterization	Source	References
HIV*	TAR element RNA	Filtration and ultracentrifugation ExoQuick™ reagent and gel filtration Sephadex G-10 spin column	Western blot (CD63, CD45, Hsp70#, and Alix) Transmission electron microscopy	Culture supernatant of Jurkat^ and J1.1^ cells Patient sera	(Narayanan et al., 2013)
		Size-exclusion chromatography and Nanotrap particle A for $CD63^+$ vesicles	Western blot (CD63 and Hsp70#)	Culture supernatant of Jurkat^ and J1.1^ cells	(Sampey et al., 2016)
	Nef protein	Ultracentrifugation	Western blot (CD9, CD63, and CD81)	Culture supernatant of plasmid Nef-transfected HeLa.CIITA^, Jurkat^ and SupT1^ cells	(Lenassi et al., 2010)
		Differential centrifugation and column-based bead isolation	Western blot (CD63 and CD81)	Culture supernatant of monocytes	(Lee et al., 2016)
		Sucrose density gradient ultracentrifugation		Patient sera	
EBV*	LMP1	Sequential centrifugation	Immunoelectron microscopy	Culture supernatant of DG-75^ and QIMR NB-B95-8^	(Flanagan et al., 2003)

HIV, human immunodeficiency virus; EBV, Epstein-Barr virus.

^Jurkat; immortalized human T lymphocyte cell line, J1.1; HIV-1 lymphadenopathy associated virus (LAV) infected Jurkat E6 cell line, HeLaCIITA; immortalized human cervical cell line transfected with class II transactivator, SupT1; human T cell lymphoblastic lymphoma cell line, DG-75; Burkitt's lymphoma cell line, QIMR NB-B95-8; EBV-positive A-type lymphoblastoid cell line.

#Hsp70 is not commonly used as EV markers despite being found in EVs (Théry et al., 2018).

TABLE 2 | Involvement of extracellular vesicles (EVs) in chronic liver disease.

Causative agent or disease state	Origin of cargo	Cargo	Isolation process	EV characterization	Source	References
HBV*	Viral	HBV RNA	Total and CD81$^+$ exosome isolation kits (Thermo Fisher Scientific)	Western blot (CD9, CD63, and CD81)	Culture supernatant of pHBV-transfected HepG2^ and Huh-7^ cells	(Kouwaki et al., 2016)
		HBsAg, HBeAg, and HBV DNA	Differential centrifugation	Flow cytometry (different size latex beads)	Patient platelet-free plasma	(Sukriti et al., 2019)
		HBV rcDNA and HBV RNA (HBx and HBs/p)	Ultracentrifugation and CD63-labeled Dynabeads® positive selection (Life Technologies)	Flow cytometry (CD81) Electron microscopy	Patient sera	(Yang et al., 2017)
		HBV DNA	Sequential centrifugation and ultracentrifugation	Immunoprecipitation (CD9, CD63, and CD81) Stimulated emission depletion microscopy (CD81)	Culture supernatant of HBV-infected PXB^-cells	(Sanada et al., 2016)
		HBsAg, HBcAg, and HBV DNA	Ultracentrifugation and density gradient separation	Western blot (CD9 and CD63)	Culture supernatant of HepAD38^ cells	(Kakizaki et al., 2018)
	Host	miR-21 and miR-29a	Total and CD81$^+$ exosome isolation kits	Western blot (CD63)	Culture supernatant of pHBV-transfected HepG2^ cells	(Kouwaki et al., 2016)
HCV*	Viral	HCV RNA	Sequential centrifugation	Western blot (CD63 and CD81)	Culture supernatant of HCV-infected Huh-7.5.1c2^ hepatocytes	(Dreux et al., 2012)
	Host	Galectin-9	ExoQuick™ method (System Biosciences)	–	Culture supernatant of HCV-infected Huh-7.5.l^ hepatocytes	(Harwood et al., 2016)
		miR-19a	Sequential centrifugation and ExoQuick™ method (System Biosciences)	–	Culture supernatant of HCV-infected IHH^ cells	(Devhare et al., 2017)
Hepatocellular Carcinoma	Host	miR-221 and miR-222	ExoQuick™ Exosome Precipitation Solution (System Biosciences)	Western blot (CD63, CD9, and calnexin#)	Patient sera	(Sohn et al., 2015)
		miR-21	Sequential centrifugation and ultracentrifugation	Western blot (CD63, CD81, and CD9) Transmission electron microscopy	Conditioned medium of HepG2^, Hep3B^, SNU-449^ and Huh-7^ cells	(Cao et al., 2019)
			Filtration and ExoQuick™ Exosome Precipitation Solution (System Biosciences)	Western blot (CD63 and Tsg101) Transmission electron microscopy	Patient sera	(Tanaka et al., 2013)
			Total Exosome Isolation Reagent (Invitrogen)	Western blot (CD63) Transmission electron microscopy	Patient sera	(Wang et al., 2014)
Hepatic Fibrosis	Host	miR-214	Sequential centrifugation	Transmission electron microscopy Zeta potential analysis and dynamic light scattering Western blot (CD9)	Conditioned medium of activated passage 6 mouse pHSC^	(Chen et al., 2014)
		miR-199a-5p	Sequential centrifugation	NanoSight nanoparticle tracking analysis Western blot (CD81)	Conditioned medium of activated passage 6 mouse pHSC^	(Chen et al., 2016)
		miR-122, miR-192, and miR-200b	Total Exosome Isolation Reagent (Thermo Fisher Scientific)	Western blot (CD63 and Tsg101)	Patient plasma	(Lambrecht et al., 2017)

**HBV, hepatitis B virus; HCV, hepatitis C virus.*

^HepG2; human hepatocellular carcinoma cell line, Huh-7; human hepatocellular carcinoma cell line, Huh-7.5.1 or Huh-7.5.1c2; subclone of Huh-7 cell line with HCV strain JFH-1 subgenomic replicon, PXB; human primary hepatocytes from liver of humanized mice, HepAD38; HepG2 cell line with a stable integration of an HBV genome, IHH; immortalized human hepatocytes, Hep3B; human hepatocellular carcinoma cell line which contains an integrated HBV genome, SNU-449; human hepatocellular carcinoma cell line which contains an integrated HBV genome, pHSC; primary hepatic stellate cells.

#Calnexin is not commonly used as EV markers despite being found in EVs (Théry et al., 2018).

transmissibility due to their similarities with EVs. In addition, EVs could be utilized as a modulator of the host immune response against viruses. There is evidence to suggest a potential role of EVs in CLD associated with HBV and HCV infections (**Table 2**; **Figure 2**). The earliest strong evidence of EV involvement in viral-induced liver disease comes from studies that reported the presence of viral genetic materials co-isolated with EVs from culture supernatant using magnetic beads coated with antibodies specific for an EV marker CD81. Kouwaki *et al.* found that $CD81^+$ EVs containing HBV-RNA isolated from plasmid pHBV-transfected HepG2 were able to induce expression of NKG2D ligands on macrophages, which has been known to simulate IFN-γ from NK cell (**Table 2**; **Figure 2**) (Kouwaki et al., 2016). Interestingly, a similar observation was made with HCV-RNA packaged EVs isolated from the culture supernatant of HCV-infected Huh-7.5.1c2 hepatocytes using sequential centrifugation, where these EVs were shown to activate plasmacytoid dendritic cells which produce type I IFN (**Table 2**; **Figure 2**) (Dreux et al., 2012). These observations suggest that EVs may be involved in the innate immune response against the virus. Further, while the contamination of EV preparation from pHBV-transfected HepG2 with free-floating viral genetic materials is possible, the contaminating viral genetic materials are unlikely to have affected the expression of NKG2D ligands on macrophages (**Table 2**; **Figure 2**). Although discrimination of virions and free-floating viral genetic materials from EVs has long been a challenging process, purer EV preparations could be obtained by immunoprecipitation using antibodies against EV specific markers (Zhang et al., 2019; Shahjin et al., 2020). As HBV DNA and proteins were found in EVs isolated using both differential centrifugation (Sukriti et al., 2019) and magnetic beads specific for CD63 (Yang et al., 2017) from the plasma/serum of patients with chronic HBV infection (**Table 2**), this strongly suggests that viral cargo could be present in EVs. These EVs containing HBV DNA were taken up by uninfected hepatocytes HepG2, leading to detectable HBV DNA levels (Sukriti et al., 2019) and expression of HBsAg and HBcAg in hepatocytes (Yang et al., 2017), which suggest a potential mechanism for viral transmission (**Table 2**; **Figure 2**). Indeed, findings by Sanada *et al.* demonstrating that HBV DNA-containing EVs isolated from HBV-infected primary hepatocytes of humanized mice were able to transmit to naïve hepatocytes, paralleled results reported in earlier studies (**Table 2**; **Figure 2**) (Sanada et al., 2016). This study also demonstrated the resistance of EVs to antibody neutralization, suggesting the possibility for EVs to act as a physical barrier between the infectious viral particles and the immune system, allowing viruses to establish persistence in chronic patients (Sanada et al., 2016).

Immune modulation in the form of evasion and suppression represent a fundamental process whereby viruses establish persistent infection (**Figure 2**). Indeed, viruses have been able to co-evolve with their hosts successfully for centuries as obligate intracellular parasites using various immune evasion and suppression strategies. However, utilization of EVs as a mechanism for immune modulation by viruses is a relatively new concept put forward by various groups. It has been shown that EVs isolated using immunomagnetic separation from patients with chronic HBV infection were able to inhibit NK

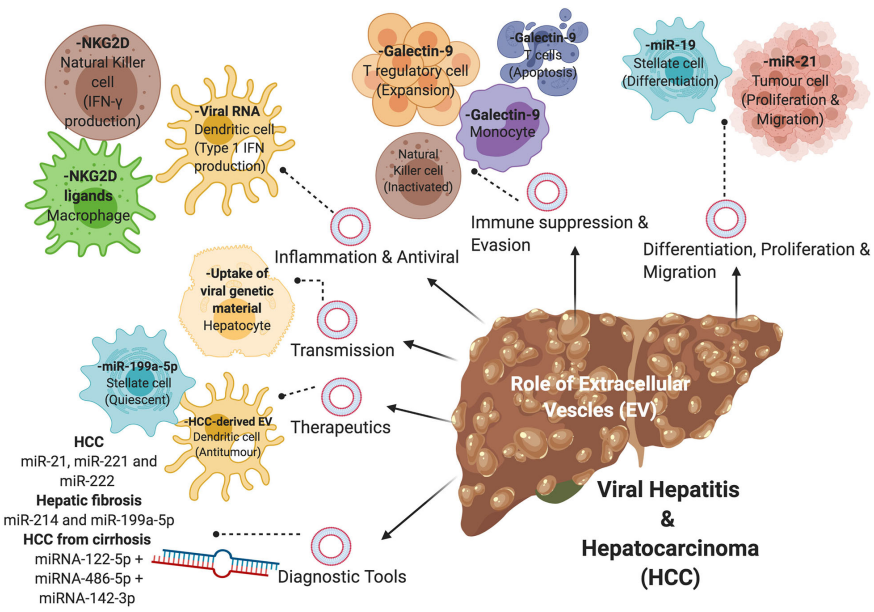

FIGURE 2 | The role of extracellular vesicles (EVs) in viral-associated liver disease. EVs containing nucleic acids, proteins, or metabolites are involved in the disease progression of viral hepatitis and HCC *via* various mechanisms, including differentiation of hepatic stellate cells, proliferation and migration of tumor cells, immune suppression and evasion, inflammation and antiviral properties, and transmission of virions. EVs can also be used in the diagnosis and treatment of viral-associated liver disease.

cell functions (**Table 2**) (Yang et al., 2017). Interestingly, these EVs were also able to transfer HBV rcDNA and HBV RNA (HBx and HBs/p) into NK cells from healthy donors when co-cultured (Yang et al., 2017). A similar finding was reported with HCV, where HCV-containing EVs from Huh-7.5.1 hepatocytes infected with HCV were able to induce expression of galectin-9 on monocytes (**Table 2**; **Figure 2**) (Harwood et al., 2016). Galectin-9 was previously found to be upregulated on Kupffer cells in livers of HCV-infected patients, which coincides with an elevated level of galectin-9 in chronic HCV patients. It was reported that galectin-9 promotes the expansion of $Foxp3^+$ regulatory T cells (Tregs) and apoptosis of HCV-specific T cells (Mengshol et al., 2010), resulting in immunosuppression.

EVs harvested from HepAD38 expressing HBV pgRNA (using density gradient centrifugation) were able to immunosuppress monocytes by upregulating expression of programmed death-ligand-1 (PD-L1) (Kakizaki et al., 2018). PD-L1 is known to bind to PD-1 expressed on T cells and inhibits T cell activation and proliferation, which was evidenced by a decrease in expression of an early marker of lymphocyte activation, CD69 in that study (Kakizaki et al., 2018). Furthermore, microRNAs (miRNAs) associated with EVs have been implicated in the modulation of the host immune response by HBV. In particular, EV-associated miR-21 and miR-29a, which were isolated from pHBV-transfected HepG2 cells, downregulate IL-12 production and depress the immune response to HBV (**Table 2**) (Kouwaki et al., 2016). IL-12 is a known key regulator for Th1 differentiation and NK cell activation (Scharton-Kersten et al., 1995). Importantly, IL-12 also stimulates IFN-γ secretion from activated T and NK cells (Gately et al., 1998). The expression of miR-21 in the context of HCC development has also been shown to positively correlate with HCC progression (**Table 2**). Cao et al. found that EV-associated miR-21 positively regulates proliferation and migration of HCC cells, resulting in tumor growth and metastasis (Cao et al., 2019). In another study, Liu et al. has shown that EV-associated miR-92a-2-5p isolated from THP-1 macrophages were able to increase liver cancer cell invasion by decreasing the expression of androgen receptors on cancer cells (Liu et al., 2020). It is noteworthy that the role of EV-associated miRNA in disease progression is, however, not restricted to immune cells in CLD. EVs derived from HCV-infected hepatocytes were reported to contain miR-19a that activated HSCs for differentiation into myofibroblasts (Devhare et al., 2017). It was found that miR-19a was highly upregulated in the sera of chronic HCV patients (Devhare et al., 2017).

Taken together, we propose that viruses move from the acute phase of infection to the chronic phase of infection by virtue of their biology, immune evasion mechanisms, and modulation of the host immune response. While the initial innate immune response provides the first line of defense against invading viruses such as HBV and HCV, its ability for viral clearance is limited (Szabo and Dolganiuc, 2008; Tang et al., 2018). As such, it is possible that EVs act as a sanctuary for viral components of HBV and HCV during the acute phase of infection where it would be difficult for virus-specific antibodies or immune cells to have access to them (Sanada et al., 2016). This provides the viruses with an excellent mode of transmission to uninfected cells, allowing viral persistence to be established (Yang et al., 2017), while the immune system continuously attempts to eliminate the viruses without any success. Persistent unproductive immune response results in an inflammatory microenvironment within the liver that is favorable for the development of liver fibrosis, cirrhosis, and HCC transformation (Ringelhan et al., 2017; Chen and Tian, 2019). Indeed, it would be interesting to test whether blocking the EV biogenesis pathway would decrease the risk for the development of liver cirrhosis and HCC since studies in cell culture systems have already shown that HCV titer is lowered when the release of EVs is blocked (Ramakrishnaiah et al., 2013; Shrivastava et al., 2013). To this end, it is important to emphasize that the current understanding of the role of EVs in viral-associated liver disease is incomplete and more research is required to bridge knowledge gaps.

The Role of Extracellular Vesicles as a Biomarker for Virus-Associated Chronic Liver Disease

EVs from blood and urine may be used as a potential non-invasive diagnostic tool for early detection of viral-associated CLD and HCC, which remain challenging to diagnose in part because most patients remain asymptomatic in early stages of disease pathogenesis. Of note, a large proportion (55-85%) of HCV patients have been reported to develop chronic hepatitis from the initial acute phase of the infection (Ringelhan et al., 2017). It is estimated that 20–30% of chronic HCV patients develop liver cirrhosis 15–25 years after infection (Lingala and Ghany, 2015) and 67–91% of these patients die from liver-associated causes such as HCC and liver failure if they do not receive timely antiviral therapy (Fattovich et al., 2002; Kobayashi et al., 2006; Toshikuni et al., 2009). Furthermore, poor prognosis of HCC is often associated with a late diagnosis of the disease. The 5-year survival rate for HCC is 12%, with a median survival of an estimated 6 to 20 months following diagnosis (McGlynn and London, 2011; McGlynn et al., 2015; Golabi et al., 2017). The 5-year survival rate increases to > 70% for patients if the diagnosis is made at an early stage (Tsuchiya et al., 2015).

HBV infection is currently detected *via* a serological assay for viral antigen, followed by a confirmatory real-time PCR test for viral DNA (Krajden et al., 2005; WHO, 2017). HCV is detected *via* a serological assay for viral antibody followed by confirmatory real-time PCR for RNA (Krajden et al., 2005; WHO, 2017). Subsequent staging of liver disease upon confirmation of viral infection involves assessment for clinical features of advanced liver disease/cirrhosis and non-invasive tests such as aspartate aminotransferase (AST)-to-platelet ratio index (APRI) and transient elastography (FibroScan) (WHO, 2017). However, clinical assessment, APRI and transient elastography have inherent limitations and failure rates; for instance, APRI is not liver-specific and does not discriminate intermediate fibrosis stages well, while transient elastography may have high failure rates and false positive results due to obesity, non-fasting state, acute hepatitis and inflammation, and inexperienced operators (Patel and Sebastiani, 2020).

In terms of diagnosis of HCC, it is unique cancer in that the diagnosis can be made solely on the basis of specific imaging characteristics of liver lesions using computer tomography imaging or magnetic resonance imaging without the need for liver biopsy (Cartier and Aubé, 2014). However, a definitive diagnosis of HCC in a non-cirrhosis disease background may still require an invasive liver biopsy. While liver biopsy remains the reference standard for the diagnosis of HCC, it is a costly procedure that only allows a small part of the liver to be examined and interpreted (Sumida et al., 2014; Sung et al., 2018). This increases the chance of sampling errors in heterogeneous solid liver tumor (Sung et al., 2018). Moreover, this procedure is associated with known morbidities, such as pain and complications. Therefore, it has been reserved for more complicated cases of HCC which cannot be definitively diagnosed using non-invasive imaging methods (Cartier and Aubé, 2014).

To circumvent the limitations associated with the current diagnostic regime for early detection of viral-associated CLD and HCC, various different types of "liquid biopsy" have been proposed as a potential diagnostic tool by several groups (Weis et al., 2019; Jeffrey et al., 2020). Liquid biopsies may contain various different combinations of analytes, circulating tumor cells, cell-free tumor DNA, mRNA, miRNAs, proteins, or metabolites (Mattox et al., 2019), as demonstrated for the diagnosis of HBV-derived HCC by Qu et al. (2019; Jeffrey et al., 2020). Weis and colleagues recently proposed in a pilot study the use of a serum miRNA panel comprising miR-122-5p, miR-486-5p, and miR-142-3p to discriminate HCV-derived HCC from mild disease and cirrhosis (sensitivity of 80%, a specificity of 95%, the negative predictive value of 82%, the positive predictive value of 74%, and overall accuracy of 78%) (Weis et al., 2019), although these miRNAs were not identified as EV-associated. More recently, there has been a growing interest in EVs as biomarkers for viral-associated CLD and HCC. An EV-based liquid biopsy is useful because EVs from diseased individuals often carry specific proteins, nucleic acids, and metabolites that can reveal the status of disease (Whiteside, 2017; Hoshino et al., 2020). Unlike standard liver biopsy, liquid biopsy is non-invasive and less costly to perform. Furthermore, as EVs contain molecules that are specific to a particular organ or disease, EV-based liquid biopsy can be optimized to be highly disease-specific. Such non-invasive tests also allow for a complementary or standalone test, providing rapid diagnosis or prognosis of diseases, such as the FDA approved EV-based biomarker for prostate cancer ExoDX (Tutrone et al., 2020).

Several EV-associated miRNAs such as miR-21, miR-221, and miR-222 have been linked to HCC (**Table 2**; **Figure 2**) (Meng et al., 2007; Varnholt et al., 2008; Yang et al., 2014; Sohn et al., 2015). Of interest, Tanaka et al. reported a higher level of EV-associated miR-21 in the sera of cancer patients compared with healthy individuals (Tanaka et al., 2013). Furthermore, the expression of miR-21 was found to be significantly higher in the EVs than that in the EV-depleted sera of patients with HCC, which further suggests that the highly enriched EV-associated miR-21 could be a more reliable diagnostic parameter than free circulating miR-21 in these patients (**Table 2**) (Wang et al., 2014).

Given that viral-induced liver fibrosis in HCV-infected patients is the primary cause of viral-associated HCC, early detection of liver fibrosis could prove beneficial for the long-term surveillance of HCC. Specifically, EV-associated miR-214 and miR-199a-5p have been reported as potential fibrosis-related EVs in the liver (**Table 2**; **Figure 2**). In one study, downregulation of miR-214 was shown to increase connective tissue growth factor (CCN2), which drives fibrogenesis in activated primary murine HSCs and human HSCs, LX-2 (Chen et al., 2014). Similarly, it was found that miR-199a-5p also works in a similar fashion as miR-214, resulting in reduced levels of EV-associated miR-199a-5p in fibrotic mouse livers and activated primary murine HSCs (Chen et al., 2016). Furthermore, Lambrecht et al. investigated the use of EV-associated miR-122, miR-192, and miR-200b for the early staging of hepatic fibrosis in chronic HBV and HCV patients and reported increased levels of all three miRNAs in total plasma of early-stage chronic patients (Lambrecht et al., 2017).

Extracellular Vesicles as Therapeutics for Chronic Liver Disease

In recent years, EVs have attracted enormous attention as a delivery system for therapeutic molecules. Unlike liposomes and nanoparticles, the current preferred carrier choices for drug delivery, EVs are naturally occurring nano-sized biological carriers in a eukaryotic system (Akuma et al., 2019). Due to their cellular origin and small size, EVs are biocompatible, non-toxic, and less immunogenic than liposome-based drug delivery, which make them better candidates than the artificially made liposomes and nanoparticles as drug delivery agents (Wu et al., 2019). Furthermore, they are able to carry a wide range of biological molecules across biological barriers such as the blood-brain barrier, increasing the bioavailability of the molecules in the biological system and reducing the dosage required for therapeutic benefit (Akuma et al., 2019). The structural composition of EVs also provides a protected enclosed space, making it an ideal delivery carrier for gene therapy, anti-fibrotic and cancer treatments (Wang et al., 2019). However, it is worth mentioning that the use of EVs as a drug delivery system is not without limitations. The feasibility to scale-up primary cell culture to obtain sufficient EVs for clinical use is an important consideration and also a significant challenge (Raimondo et al., 2019). While tumorigenic or immortalized cell lines offer an easier alternative to primary cells for large scale production of EVs, EV-derived from cancer cell lines may inherently pose a risk of undesirable horizontal gene transfer which may have dire consequences in the event that oncogenes are transferred into EVs (Raimondo et al., 2019). Isolating and purifying EVs can also be extremely costly, labor-intensive and time-consuming, although the technologies have been continuously improved (Soekmadji et al., 2020).

At present, one of the challenges with the treatment of HCC is the lack of effective therapeutic agents which can successfully improve the overall survival of HCC patients. There are currently six approved drugs available for treatment of HCC with only moderate success (Jindal et al., 2019). Due to the heterogeneity of

the patient population and increasing number of advanced HCC patients showing drug resistance (Niu et al., 2017; Namee and O'Driscoll, 2018; Wang et al., 2019), there is an urgent need to develop innovative therapy for HCC (Jindal et al., 2019). Takahashi *et al.* found a role of EV-associated long non-coding RNAs, linc-ROR, in HCC cells which mediate chemoresistance through attenuation of drug-induced apoptosis and inhibition of p53 expression (Takahashi et al., 2014). In light of the need for new and effective cancer therapy, EVs have shown great promise in delivering a common chemotherapeutic agent, paclitaxel to treat multiple drug resistance cells (Kim et al., 2016). Kim et al. demonstrated that there was at least a 50-fold increase in cytotoxicity in drug-resistant cells using the EV-based treatment (Kim et al., 2016). Several studies have also utilized EVs to deliver anti-tumor therapeutics such as methotrexate and doxorubicin to destroy HCC cells (Tang et al., 2012; Tian et al., 2014). Immunotherapy involving EVs as carriers is a promising alternative treatment for HCC. Rao et al. demonstrated a significant reduction in tumor growth in HCC mice treated with HCC-derived EVs which were able to elicit a strong dendritic cell-mediated anti-tumor immune response (**Figure 2**) (Rao et al., 2016). In addition, the tumor microenvironment was also found to have substantially higher levels of infiltrating $CD8^+$ T cells and inflammatory cytokines (Rao et al., 2016).

Viral-induced liver fibrosis and cirrhosis is one of the major risk factors for the development of HCC. Therefore, slowing or halting the progression of hepatic fibrosis will likely be beneficial in reducing the risks for malignancy transformation. Chen *et al.* found that EV-associated miR-199a-5p derived from quiescent HSCs was able to reduce the expression of fibrogenic genes and proteins in activated HSCs (**Figure 2**) (Chen et al., 2016). Furthermore, EVs released from adipose-derived mesenchymal stem cells (MSC) transfected with miR-122 were able to inhibit activation and proliferation of HSCs. Interestingly, these MSC-derived EVs were also able to inhibit fibrosis in the livers of mice exposed to carbon tetrachloride (Lou et al., 2017). Thus, EVs may have the potential to be used as therapeutics to treat CLD, with further research in this area clearly warranted.

CONCLUSIONS AND FUTURE DIRECTIONS

EVs represent a novel class of nanoparticles, which could be involved in the pathogenesis and progression of CLD. Indeed, evidence gathered from recent studies has shed light on the role of EVs in viral-associated CLD and HCC. EVs have emerged as an important, yet poorly understood mechanism utilized by viruses and the host immune system for disease pathogenesis of viral-associated CLD and HCC. While there is evidence that EVs have the potential to play a role as non-invasive diagnostic tools for early detection of disease and as carriers for therapeutics, a more thorough understanding of EV biogenesis and disease pathogenesis and better, internationally standardized technologies for the isolation and enrichment of EVs are warranted before they can be widely used in the clinic for the treatment of CLD.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Cross-Kingdom Extracellular Vesicles EV-RNA Communication as a Mechanism for Host–Pathogen Interaction

Isadora Filipaki Munhoz da Rocha[1], *Rafaela Ferreira Amatuzzi*[1], *Aline Castro Rodrigues Lucena*[2], *Helisson Faoro*[2] *and Lysangela Ronalte Alves*[1]*

[1] Gene Expression Regulation Laboratory, Carlos Chagas Institute, Oswaldo Cruz Foundation (FIOCRUZ), Curitiba, Brazil, [2] Laboratory for Applied Science and Technology in Health, Carlos Chagas Institute, Oswaldo Cruz Foundation (FIOCRUZ), Curitiba, Brazil

The extracellular vesicle (EVs) traffic has been highlighted as a very important pathway of cellular communication. EVs are produced by prokaryotes and eukaryotes organisms and can carry molecules to help maintain homeostasis, responding to general disbalance, infections, and allowing rapid modulation of the immune system. In the context of infection, EVs from both the host and the pathogen have been identified as playing roles in the recruitment of immunological molecules that can lead to the resolution of the infection or the host's defeat. Bacterial vesicles RNA cargo play roles in the host cell by regulating gene expression and modulating immune response. In fungi the RNA molecules present in EVs are diverse and participate in communication between the host and pathogenic fungi. Little is known about how cross-kingdom sRNA trafficking occurs, although in recent years, there has been an increase in studies that relate EV participation in sRNA delivery. This review aims to elucidate and update the reader concerning the role of extracellular vesicles, with emphasis in the RNA content. We describe the EVs during infection from the host point-of-view, as well as the bacteria and fungi pathogens producing EVs that help the establishment of the disease.

Keywords: extracellular vesicles, host–pathogen interaction, bacteria, fungi, infection

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****Correspondence:***
Lysangela Ronalte Alves
lysangela.alves@fiocruz.br;
lys.alves@gmail.com

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INTRODUCTION

Cell communication is crucial for organisms to maintain homeostasis and respond to adverse conditions, as during an infection. Distinct pathways are involved in cell communication, such as direct cell contact and molecular secretion and the transfer of extracellular vesicles (Raposo and Stoorvogel, 2013). Extracellular vesicles (EVs) are defined as cell-derived particles delimited by a lipid bilayer that cannot replicate and can carry different cargos across the organism; EVs include entities such as apoptotic bodies, exosomes, and microvesicles (Koliha et al., 2016; Théry et al., 2018).

EVs are produced by several organisms, ranging from prokaryotes to mammals and plants (Yáñez-Mó et al., 2015), and every known cell is capable of producing them (Mardahl et al., 2019). The secretion

of extracellular vesicles is regulated by various mechanisms that culminate with shedding at the plasma membrane, which can occur spontaneously or in response to certain conditions (Théry et al., 2009). The EV content is closely related to cell-type specificity and is influenced by the physiological or pathological state of the cell (Kalluri and LeBleu, 2020)

Regarding EV classification, there is still no consensus. The difficulty in classifying EVs is attributed to their overlapping sizes, the similar composition of EV subclasses and the lack of knowledge about their biogenesis as well as isolation technique limitations. Currently, EVs are classified into three different subgroups: apoptotic bodies, microvesicles, and exosomes (Théry et al., 2018). Microvesicles and apoptotic bodies are released by plasma membrane budding and their size varies from approximately 50 nm to 1 μm in diameter (Kalluri and LeBleu, 2020). Exosomes have endosomal origin; therefore, they are involved in specific intracellular regulatory processes that determine the exosome content. The size ranges from approximately 40 to 200 nm in diameter (Kalluri and LeBleu, 2020). The biogenesis of exosomes depends on several cell stages (Yáñez-Mó et al., 2015). First, the plasma membrane undergoes invagination associated with soluble molecules from the extracellular milieu, and then, the early-sorting endosome forms. Next, early endosomes mature into late-sorting endosomes, which form multivesicular bodies with intraluminal vesicles in the lumen of the organelle (Yáñez-Mó et al., 2015). When multivesicular bodies fuse with the plasma membrane, they release the exosomes into the extracellular milieu (Hessvik and Llorente, 2018). ESCRT (endosomal sorting complexes required for transport) machinery and tetraspanins are important for exosome biogenesis (Colombo et al., 2013). Their depletion is related to exosome-secretion reduction (Hurwitz et al., 2016; Hurwitz et al., 2017). Other molecules, such as ceramides and sphingomyelinases, are also essential in vesicular transport and are involved in exosome biogenesis processes, such as membrane deformation, fission and fusion (McMahon and Boucrot, 2015). The origin of an extracellular vesicle may be traced by the surface proteins that resemble the cell. Among them is the tetraspanin family, whose proteins are commonly used as EV markers, for example, CD63, CD81, CD9 (Raposo and Stoorvogel, 2013), and the more recently discovered HSP70 (Van Niel et al., 2018). Importantly, so far, there is not a single marker—or a set of markers—that can identify all types of vesicles, and the distribution of tetraspanins among these subtypes is variable (Koliha et al., 2016). The characteristics and composition of EVs differ even depending on the cellular and environmental state (Yáñez-Mó et al., 2015).

The main functions of EVs related to cell communication include molecular transfer, changes in gene expression and cell surface rearrangement (Raeven et al., 2018). In humans, EVs are involved in several physiological processes, such as tissue regeneration (Teng et al., 2015), reproductive biology (Simon et al., 2018) and blood coagulation (Heijnen et al., 2015). They can also act in presenting antigens, stimulating immune responses and tolerogenic effects, immunosuppression, angiogenesis, tumor progression, and the transmission of virulence factors (Raposo and Stoorvogel, 2013). Moreover, they have already been linked with the aging process, cancer (Joncas et al., 2019) and stem cells differentiation (Tatischeff, 2019). Animal and plant pathogen/parasite EV secretion is used to aid survival, cell communication and pathogenesis (Yáñez-Mó et al., 2015). Inside the host, EVs derived from the parasite are related to host modulation, allowing the recruitment of specific immune cells and contributing to the parasite's life cycle and reproduction (Yáñez-Mó et al., 2015; Dong et al., 2019).

EVs are also studied for drug delivery purposes, due to their biocompatible composition and availability in the organism, as it occurs with encapsulated compounds. The variety of compounds carried inside EVs also makes them attractive in biomarker research, especially in liquid biopsies, which are less invasive and methodologically demanding to execute than other diagnostic procedures (Kalluri and LeBleu, 2020).

Many different biomolecules are carried by EVs, including proteins, lipids and nucleic acids (Yáñez-Mó et al., 2015). In mammalian cells, RNA molecules present in the EVs have been highlighted for their capacity to be internalized by the recipient cell and regulate gene expression (O'Brien et al., 2020). The RNAs identified in EVs include mRNA (messenger RNA), microRNA, rRNA (ribosomal RNA), tRNA (transfer RNA), sRNA (small RNA), and lncRNA (long noncoding RNA) (Im et al., 2018; Turchinovich et al., 2019). Differences in the RNA composition of EVs are based on the cellular state and the producing cell, such as cancer and infection, and can, therefore, be used as biomarkers of certain cellular conditions (Mittelbrunn et al., 2011; Baglio et al., 2015; Shah et al., 2018; Turchinovich et al., 2019). The mechanism that directs the RNA species into the EVs is not understood. The RNA could be passively incorporated due to an RNA abundance in the cytosol; by RNA-binding proteins (RBPs) through motif recognition or RNA secondary structure recognition, and by specific modifications in RNA or RBPs, such as uridylation, ubiquitylation, sumoylation, and phosphorylation (Gibbings et al., 2009; McKenzie et al., 2016; Mateescu et al., 2017; Ragusa et al., 2017).

In this review, we provide an overview of RNAs enclosed in extracellular vesicles, with an emphasis on both the host and the bacteria and fungi pathogens, that are common agents of infection worldwide.

EVs AND INFECTION: THE HOST RESPONSE

In an infectious environment, host-originated EVs can promote both immunostimulant and immunosuppressive events; they can carry antigenic molecules as well as Major Histocompatibility Complex (MHC) molecules (Mardahl et al., 2019). Antigen presenting cells (APCs) are at the center of studies involving host-originated extracellular vesicles. They are believed to mediate interactions with T and B cells to: a) activate naïve T cells through MHC molecules, b) serve as exogenous antigens for APCs, c) present antigens directly to CD4+ T cells, and d) present antigens to dendritic cells (DCs), which will be loaded into their own MHCs. EVs originating from host cells have also been reported to inhibit IL-8 and TNF, reducing inflammation. Additionally, the immune response can be

promoted by EV content through antigen-independent mechanisms (Mardahl et al., 2019; Raposo and Stahl, 2019). On the other hand, EVs can mediate protective messages between cells during stress situations (Tatischeff, 2019). In a model of *Conidium* infection, the authors identified different EV cargo compositions derived from PMN cells enriched with antimicrobial molecules to respond to the infection (Shopova et al., 2020). It has been shown that the microRNA signature of EVs is different from the in-microRNA signature of the cell (Mittelbrunn et al., 2011; Roderburg et al., 2013). In the context of the immune system, some micro-RNAs, like miR-760, miR-632, miR-654-5p, and miR-671-5p, were loaded into activated T cell EVs and they were more abundant in the EVs compared to the parental cell during immune synapsis (Mittelbrunn et al., 2011). Additionally, vesicles from different species can lead to distinct protection against diseases. For example, EVs from *Nippostrongylus brasiliensis*, a hookworm that causes chronic gastrointestinal infection in humans, were isolated and injected in mice. After the EV injection, colitis was induced and the EVs protected the animals against the inflammation. However, EVs from the whipworm *Trichuris muris* did not present the same protective pattern. From RNA-Seq data of *N. brasiliensis*, 52 differentially expressed microRNAs were identified, and it was shown that they play an important role in inflammatory cytokine suppression and secretion of anti-inflammatory IL-10 in the host cell (Eichenberger et al., 2018).

Within the gut microbiome, host vesicles have been reported to enter microorganisms and regulate their gene expression and growth (Lee, 2019). The gut microbiota is composed of 100-200 distinct bacterial species. Depending on genetics, diet and disease state this composition may vary, and a series of correlations have been described regarding the microRNA-mediated control of the gut microbiota. Liu et al. (2016) identified the most abundant miRNAs present in the feces of human and mice and compared to those observed inside the Evs. The most abundant miRNAs observed in the EVs were miR-1224, miR-2146, miR-2134, miR-483, miR-710, miR-2141, miR-720, miR-155, and miR-34c. The authors showed that some miRNAs were able to enter the bacterial cells and regulate gene expression by RNA alignment. The host miRNA targeted rRNA and ribozyme (RNaseP), but the expression could be induced or repressed, depending on the species analyzed. In addition, using mice defective in the miRNA pathway (Dicer knockouts), it was shown that the KO mice were more susceptible to induced colitis than the Wild type and that fecal miRNA transplantation could help to restore the gut microbiota (Liu et al., 2016). The EV traffic also helps communication between components of the microbiota through biofilm formation or aggregation-derived quorum sensing, promoting the homeostasis of the host and even prevent and fight infections (Morales and Hogan, 2010). The quorum sensing process uses extracellular signals to communicate and coordinate social activities. In *Pseudomonas aeruginosa* the molecule PQS (Pseudomonas quinolone signal) is an important quorum sensing molecule that is transported into vesicles. When this molecule was removed, it led to problems with cell-cell communication (Mashburn and Whiteley, 2005).

EVs are recognized as important players in the pathological process of sepsis, influencing aspects such as coagulation and hyperinflammation disturbances. A study showed that several microRNAs were dysregulated in septic shock, highlighting exosomal miR-125b-5p that was validated as a survival predictor and miR-26b-5p and miR-199b-5p, which were able to differentiate healthy individuals from septic patients (Reithmair et al., 2017). Bacterial vesicles were introduced intraperitonially in mice that afterwards presented symptoms of sepsis-like inflammation and eventually died, highlighting that the EVs were sufficient to trigger the host inflammatory response (Park et al., 2018). Additionally, it has been described that the pathogen vesicle formation can be affected by the antibiotic choice in the treatment of sepsis—a great contributor to treatment success—making it even more important to understand the phenomena surrounding the EV-cargo-microbe-host system (Dagnelie et al., 2019).

In addition to the host cell response to infections, one must consider the role of the EVs being shed by the pathogens; the next topics are focused on the EVs produced by common pathogens identified in worldwide infections—bacteria and fungi.

MICROORGANISMS—EV SHEDDING AND THEIR ROLES IN CELL COMMUNICATION AND DISEASE

Bacterial Membrane Vesicles

The bacterial membrane vesicles (MVs) are composed of a lipid bilayer and have a size ranging from 20 to 400 nm. As described for eukaryotes, there are different MV categories, varying according to their structure, composition and origin (Toyofuku et al., 2019). The outer membrane vesicles (OMVs) are a class of vesicles produced by gram-negative bacteria, derived from the outer membrane, and due to its origin, they are covered by lipopolysaccharides (LPS) (Schwechheimer and Kuehn, 2015). The OMVs biogenesis is still unclear, however some models are proposed to explain the process. One of these models is based on the dissociation of the covalent linkage between the outer membrane and the peptidoglycan layer, being the absence of these bonds associated with the growth of the outer membrane leading to the formation of OMVs (Kulp and Kuehn, 2010; Schwechheimer and Kuehn, 2015). Another model proposes that OMVs are formed by protuberances that appear on the outer membrane due to an increased pressure in the periplasmic space caused by the accumulation of misfolded proteins and fragments of peptidoglycans (Kulp and Kuehn, 2010; Schwechheimer and Kuehn, 2015). A third model is related to an enrichment of curvature-inducing molecules, such as quinolone PQS of *P. aeruginosa* (Mashburn-Warren et al., 2008). Roier and coworkers proposed a novel method based on phospholipid accumulation as a result of deletion or downregulation of *vacJ* and/or *ybr* genes (Roier et al., 2016). More recently a group of genes involved in OMVs biogenesis in *Salmonella enterica* Serovar Typhi were identified (Nevermann et al., 2019). This group contains some genes related to envelope stability, LPS synthesis, peptidoglycan synthesis and remodeling, stress sensor and transcription regulator. Gram-negative bacteria also produce outer-inner membrane vesicles (OIMVs), that are originated from the inner membrane and were firstly observed in *Shewanella vesiculosa* M7T and other

pathogenic bacteria (Pérez-Cruz et al., 2013; Pérez-Cruz et al., 2015). These OIMVs contain both the outer membrane and the inner membrane, as well as cytoplasmic components. Gram-positive bacteria also produce MVs; however, the mechanisms of generation and release through the cell wall are not well known (Brown et al., 2015; Liu et al., 2018). Although the internal content is similar, the MVs of gram-positive and gram-negative bacteria have different glycoconjugates (Gill et al., 2019).

Several functions are attributed to bacterial MVs, including communication with other bacteria. The distribution of antimicrobial resistance genes is an example of this interaction, being considered a type of horizontal gene transfer (Chatterjee et al., 2017; Domingues and Nielsen, 2017). The presence of DNA in MVs has been reported in several cases, and even the acquisition of resistance in sensitive bacteria has been observed after exposure to OMVs from resistant bacteria in *E. coli* (Kim et al., 2018). MVs can also mediate the host cell invasion process and act in competition with other pathogens, due to the presence of several virulence factors and toxins. In *P. aeruginosa* OMVs, multiple virulence factors that are involved with the host colonization process were identified, such as β-lactamase associated with host peptide degradation, alkaline phosphatase involved in biofilm formation, hemolytic phospholipase C, and Cif related to *P. aeruginosa* virulence (Bomberger et al., 2009). Toxins involved in cell death induction were found in OMVs from enterohemorrhagic *Escherichia coli* (EHEC) O157, such as cytolethal distending toxin V and EHEC-hemolysin (Bielaszewska et al., 2017). In addition, MVs can induce the host immune response through interaction with pathogen-associated molecular pattern (PAMP) receptors. Molecules such as LPS, peptidoglycan, lipoprotein, DNA, and RNA are recognized by host cell receptors, such as Toll-like receptors (TLRs), resulting in the induction of signaling cascades and the production of proinflammatory molecules (Pathirana and Kaparakis-Liaskos, 2016). OMVs from *P. aeruginosa* stimulate the production of IL-8 in A549 human lung epithelial carcinoma cells (Bauman and Kuehn, 2012).

As already mentioned, interaction between the host vesicles and the microbiome in the intestine have been described. It was observed that the MVs contribute with gut homeostasis by enhancing innate immunity, since OMVs of the microbiota are involved in the activation of NOD1 signaling pathways in intestinal epithelial cells (Cañas et al., 2018). In fact, it was demonstrated that OMVs from *B. fragilis* induce immunomodulatory effects and prevent colitis. Inside OMVs, capsular polysaccharide A was detected, which interacts with dendritic cells *via* TLR2, enhancing regulatory T cells and the production of cytokine IL-10 (Shen et al., 2012). However, it is also suggested that vesicles play a role in nutrient acquisition for the entire microbiota community, since they are enriched with hydrolytic enzymes, such as glycosidases and proteases (Elhenawy et al., 2014). The mechanism of vesicle content packaging has not yet been elucidated; however, it seems to be a regulated process. When the RNA profile of *Salmonella* OMVs was analyzed under different environmental conditions, it was observed that some mRNAs were enriched in OMVs when compared to intracellular fractions, reinforcing the concept that MVs packaging is not a passive process, but tightly regulated (Malabirade et al., 2018).

Bacterial MVs can transport different types of RNA molecules, including mRNA, tRNA, rRNA and ncRNA (Dauros-Singorenko et al., 2018) (**Figure 1** and **Table 1**). Studies characterizing the MV and extracellular RNA-content of *Escherichia coli* K-12 substrain MG1655 (OMVs and OMVs-free) identified mainly tRNA and rRNA fragments (3S rRNA, 16S rRNA, and 5S rRNA), as well ncRNAs (Ghosal et al., 2015). In OMVs from *Vibrio cholerae* strain A1552, ncRNA was the most abundant class of RNA identified (Sjöström et al., 2015). In *V. cholerae* O395, the enrichment of rRNA and sRNA, including CsrB1, CrsB2 and CrsB3 was also observed (Langlete et al., 2019).

Blenkiron and coworkers demonstrated that bacterial MVs RNA content is transferred to the recipient cell and directed to the cytoplasm of epithelial cells and could be also found in the nucleus, reinforcing the hypothesis of inter-kingdom communication (Blenkiron et al., 2016). In fact, MVs also show regulatory activity in the host through MV-RNA molecules that are functional in the host's cell. RNA sequencing analysis identified "microRNA-like" in *E.* coli and *Streptococcus mutans,* speculating that these small RNA molecules may play a role in bacteria similar to miRNAs in eukaryotes (Lee and Hong, 2012; Kang et al., 2013). As mentioned, MVs can trigger immune response in the host, however, in *P. aeruginosa* it was identified an interaction between an sRNA molecule present in the OMV and the host cell that led to a reduction in IL-8 secretion (Koeppen et al., 2016). Using RNA-Seq, Koeppen and coworkers identified the sRNA5320 present in OMVs from *P. aeruginosa*. Different assays were performed *in vitro* and *in vivo*, showing that this sRNA is transferred to cells *via* OMVs and plays a role in modulating the immune response by decreasing IL-8 secretion. Similarly, in *Helicobacter pylori*, sncRNAs sR-2509025, and sR-989262 can reduce the LPS-mediated induction of IL-8 protein secretion in human gastric adenocarcinoma cells (Zhang et al., 2020).

In addition, distinct sRNAs have been identified in the vesicles of three different bacteria that cause periodontitis. The transfection of synthetic copies of these sRNAs into Jurkat T cells resulted in decreased expression levels of IL-5, IL-13, and IL-15 (Choi et al., 2017). Afterwards, for *Aggregatibacter actinomycetemcomitans*, which is one of the causes of periodontitis, it was observed that the sRNA of OMVs can be incorporated by the host's RISC system, contributing to the alteration in gene expression of the host (Han et al., 2019). The potential of sRNAs to act in the pathogen-host interaction has been proposed as a target to be explored for the discovery of new biomarkers for bacterial diseases (Wang and Fu, 2019).

Fungi EVs and the RNA Content

EVs have been described in several fungal species, both in yeast and in filamentous fungi such *Saccharomyces cerevisiae* (Zhao et al., 2019), *Candida albicans* (Vargas et al., 2015), *Histoplasma capsulatum* (Albuquerque et al., 2008), *Paracoccidioides brasiliensis* (Ganiko et al., 2011), *Cryptococcus neoformans* (Rodrigues et al., 2007), *Aspergillus fumigatus* (Souza et al., 2019), *Sporothrix brasiliensis* (Ikeda et al., 2018), *Malassezia sympodialis* (Rayner et al., 2017) and *Alternaria infectoria* (Silva et al., 2014).

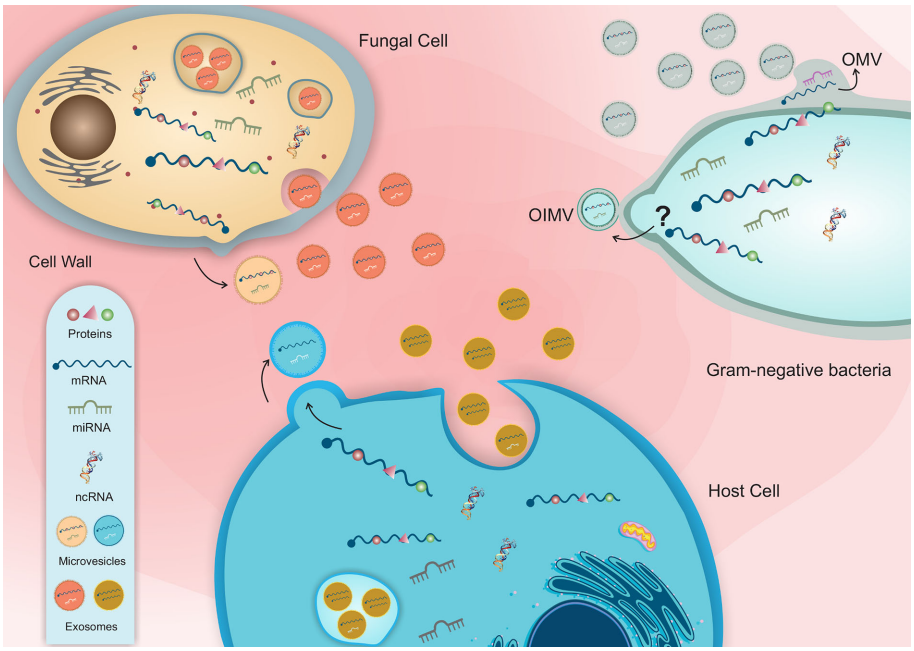

FIGURE 1 | Schematic representation of host-pathogen EVs interaction and the distinct classes of RNA molecules in bacterial and fungal vesicles.

TABLE 1 | Summary of the role of RNA molecules present in extracellular vesicles on pathogen and host communication.

Origin	RNA molecule	Function	Reference
Host to pathogen	miR-199b-5p, miR-125b-5p	Those miRNAs were able to differentiate healthy individuals from septic and predict their prognosis	Reithmair et al., 2017
	miR-1224, miR-2146, miR-2134, miR-483, miR-710, miR-2141, miR-720, miR-155 and miR-34c	Human and mice miRNAs were able to enter bacterial cells and regulate gene expression	Liu et al., 2016
	sRNAs	*Arabidopsis* secretes EVs to deliver sRNAs into fungal cells to silence virulence-related genes	Cai et al., 2018
Pathogen	ncRNAs, tRNAs and rRNAs (3S rRNA, 16S rRNA, and 5S rRNA)	*Escherichia coli* K-12 OMVs can carry distinct types of RNA molecules	Ghosal et al., 2015
	ncRNAs, rRNAs and sRNAs (CsrB1, CrsB2 and CrsB3)	*Vibrio colerae* strain A1552 carries a great variety of ncRNA molecules	Sjöström et al., 2015; Langlete et al., 2019
	mRNAs	*Salmonella enterica* Serovar Typhimurium mRNAs were diferrentially enriched in OMVs	Malabirade et al., 2018
	microRNA-like molecules	Those molecules were identifeid in OMV from *E. coli* and *Streptococcus*	Lee and Hong, 2012; Kang et al., 2013
	microRNA-like, snoRNA snRNAs and mitochondrial tRNAs	Those RNA molecules were identified in EVs from *P. brasiliensis, C. neoformans, C. albicans* and *S. cerevisiae*	Peres da Silva et al., 2015
	mRNAs	mRNAs habe been found in fungal EVs which were involved in essential processes for survival and pathogenesis	Peres da Silva et al., 2015
	anti-sense ncRNAs and tRNAs	In *Histoplasma capsulatum*, anti-sense ncRNAs were found inside EVs	Alves et al., 2019
Pathogen to host	rRNAs, sRNAs	The RNA content of Escherichia coli strain 536 OMVs was found inside the recipient cell, reinforcing the hypothesis of inter-kingdom communication	Blenkiron et al., 2016
	sRNA (sRNA52320)	This sRNA led to a reduction in IL-8 secretion in the host cell	Koeppen et al., 2016
	sncRNAs sR-2509025 and sR-989262	In *Helicobacter pylori*, these ncRNAs reduced the LPS-mediated induction of IL-8 protein secretion in the host cell.	Zhang et al., 2020
	sRNAs	In periodontitis-causing bacteria, sRNAs in OMVs led to decreased levels of IL-5, IL-13 and IL-15	Choi et al., 2017
	sRNAs and allergens	*Malassezia sympodialis*' EVs carry allergen molecules and sRNAs to host cells	Rayner et al., 2017
	RNA content	EVs from a more virulent strain of C. gatti increased the proliferation of a less virulent strain inside the macrophages	Bielska et al., 2018
	mRNAs	*Paracoccidioides* mRNAs present in the EVs could be actively translated	Peres da Silva et al., 2019

As described for mammalian cells, fungal EVs are also classified according to their biogenesis, being able to release both exosomes and microvesicles (Kwon et al., 2019). The EVs, biogenesis process in fungi and how the sorting of their cargo occurs remains unknown. However, there are preliminary studies that report the relevance of EVs regulation pathways in molecular traffic across the cell wall (Bielska et al., 2018). In *C. albicans* EVs are key players to biofilm matrix production. ESCRT (endosomal sorting complexes required for transport) defective mutations caused reduced EV production and consequently biofilm thickness reduction and increased sensitivity to the antifungal drug (Mitchell et al., 2018). Mutations in genes that encode components of ESCRT machinery in *S. cerevisiae* also led to the reduction in EVs population and changes in the EVs proteomic profile (Zhao et al., 2019). The deletion of Sec6, a component of the exocyst complex involved with vesicles fusion with the plasma membrane, prevents EVs production and laccase secretion to extracellular milieu, and decreased virulence in mice (Panepinto et al., 2009). In addition, mutations in protein of the ESCRT complex, Vps27, led to MVB accumulation and decreased laccase transport to the cell wall (Park et al., 2020).

Fungal EVs have been related to several functions, such as biofilm matrix production (Mitchell et al., 2018), the delivery of virulence factors (Bielska et al., 2018; Ikeda et al., 2018; Konečná et al., 2019), cell wall remodeling (Zhao et al., 2019), host response and host–pathogen interaction (Rayner et al., 2017; Bitencourt et al., 2018; Johansson et al., 2018) (**Figure 1** and **Table 1**). The EVs are composed of polysaccharides (Rodrigues et al., 2007), lipids (Vallejo et al., 2012), allergens (Johansson et al., 2018), pigments (Frases et al., 2009), cytosolic and membrane proteins (Gil-Bona et al., 2015), and nucleic acids such RNA molecules (Peres da Silva et al., 2015; Rayner et al., 2017; Alves et al., 2019; Peres da Silva et al., 2019).

Several studies have identified different RNA species loaded in fungal EVs that could perform different functions in recipient cells. The commensal yeast *Malassezia sympodialis*, which colonizes human skin and is associated with common skin disorders, can secrete vesicles ranging from 50 to 600 nm, which carry allergens related to inflammatory responses and small RNAs ranging from 16 to 22 nucleotides in length (Rayner et al., 2017). For the human pathogenic fungi *P. brasiliensis*, *C. neoformans*, *C. albicans*, and *S. cerevisiae* EVs, several miRNA-like sequences, as well as small nucleolar RNAs (snoRNAs) and nuclear RNA and mitochondrial tRNAs, were identified in high abundance (Peres da Silva et al., 2015). Messenger RNAs have also been found in fungal EVs; they are involved in essential processes, such as vesicle-mediated transport, metabolic pathways, cellular responses to stress, transcriptional regulation and cell cycle control (Peres da Silva et al., 2015). The pathogenic fungi *H. capsulatum* also produces EVs enriched with different ncRNA populations; the most abundant were tRNA fragments. It was also identified anti-sense ncRNAs, with 25 nt in length, that aligned with specific regions of the transcripts and could act in gene expression regulation as a silencing mechanism (Alves et al., 2019). In fungi, ncRNAs are important players in gene expression regulation in the fungal cells. For example, during the transition phase in dimorphic fungi, a process already recognized as important in the context of infection and virulence. Antisense transcription of ncRNAs have been linked to hyphae and spore formation in *Ustilago maydis*, and the loss of such RNAs resulted in virulence attenuation (Morrison et al., 2012).

In an elegant work performed by Bielska and coworkers, it was shown that EVs from a more virulent strain of *C. gatti* could increase the proliferation of less virulent fungal cells inside the macrophages, thus promoting pathogen survival instead of clearance by the host cell. The protein and RNA fractions were required for this transference process (Bielska et al., 2018).

Although it is not clear whether EV mRNAs are translated into functional peptides, the presence of functional mRNAs in fungal EVs was confirmed from two species of *Paracoccidioides* by *in vitro* translation (Peres da Silva et al., 2019). The presence of these transcripts suggests that they can be internalized by fungal or host cells and alter gene expression regulation and play a role in the host-fungal interaction. Most of the mRNAs present in the EVs are associated with virulence, like heat shock proteins Hsp 70 and Hsp 90-like, that have a role during infection of dimorphic fungi (Peres da Silva et al., 2019). It is possible to speculate that EV mRNA can be translated into the host cell, inducing gene expression alterations that could aid pathogen infection and survival.

Small RNAs can induce gene silencing by binding to argonaute proteins, directing the RNA-induced silencing complex (RISC) to target mRNAs for their repression. In host and pathogenic fungi interactions, sRNA molecules can participate in cross-kingdom communication (Weiberg et al., 2013; Chen et al., 2014; Weiberg et al., 2015; Cai et al., 2018). In plants, the EVs can act as effectors that suppress fungal pathogens (Cai et al., 2018). Phytophogenic (Weiberg et al., 2013; Chen et al., 2014; Wang et al., 2016) and entomopathogenic fungi (Cui et al., 2019) interfere in plant and insect immunity, respectively. They play a role in silencing host immunity genes by hijacking the host's RNAi machinery to facilitate infection (Weiberg et al., 2013; Wang et al., 2016). The entomopathogenic fungus *Beauveria bassiana* exports miRNA-like molecules loaded in vesicles to the host's mosquito *Anopheles stephensi*, attenuating host immunity, and facilitating infection (Cui et al., 2019).

Conversely, the host can also suppress fungal pathogenesis. The extracellular vesicles of plants play an essential role in sRNA trafficking between *Arabidopsis* and the pathogen *Botrytis cinerea*. *Arabidopsis* secretes EVs to deliver sRNAs into fungal cells to silence virulence-related genes (Cai et al., 2018).

CONCLUDING REMARKS

In summary, this unconventional pathway of communication is gaining more attention because it is involved in all aspects of

cell life regarding its important, if not essential, role in organism homeostasis. Regarding infection, it was shown that EVs of both hosts and pathogens play a role in either promoting or fighting it where the EVs influence antigen presentation, immunological stimulation and suppression as well as transmission of virulence factors. Additionally, one of the most widely studied aspects of EVs is their RNA cargos, mainly because of their capacity to regulate gene expression in the recipient cell, the microRNA pathway, leading to gene silencing that can favor the pathogen, and also the mRNA in the EVs that can be translated and influence the cell metabolism. In addition, some physiological aspects regarding pathogenesis and host response were studied and are helping to understand how the EVs can be used for diagnostic and therapeutical purposes, the RNA present in EVs derived from bacteria and fungi and their role in the host are recent and still need to be further addressed for most of the work has been descriptive. The EV biogenesis and how specific molecules are sorted and directed to them are questions to be answered. Nevertheless, the promising results obtained so far are paving a new path for the study of RNA present in the vesicles and their important role in cell communication and gene expression regulation highlighting the potential of EVs and their role in the host during infection.

AUTHOR CONTRIBUTIONS

IM, RA, AL, HF, and LA discussed, wrote, and approved the manuscript in its current form. All authors contributed to the article and approved the submitted version.

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Extracellular Vesicles in Viral Infections: Two Sides of the Same Coin?

Sharon de Toledo Martins[1,2]* *and Lysangela Ronalte Alves*[1]*

[1] Gene Expression Regulation Laboratory, Carlos Chagas Institute, ICC-Fiocruz, Curitiba, Brazil, [2] Biological Sciences Sector, Federal University of Paraná (UFPR), Curitiba, Brazil

Extracellular vesicles are small membrane structures containing proteins and nucleic acids that are gaining a lot of attention lately. They are produced by most cells and can be detected in several body fluids, having a huge potential in therapeutic and diagnostic approaches. EVs produced by infected cells usually have a molecular signature that is very distinct from healthy cells. For intracellular pathogens like viruses, EVs can have an even more complex function, since the viral biogenesis pathway can overlap with EV pathways in several ways, generating a continuum of particles, like naked virions, EVs containing infective viral genomes and quasi-enveloped viruses, besides the classical complete viral particles that are secreted to the extracellular space. Those particles can act in recipient cells in different ways. Besides being directly infective, they also can prime neighbor cells rendering them more susceptible to infection, block antiviral responses and deliver isolated viral molecules. On the other hand, they can trigger antiviral responses and cytokine secretion even in uninfected cells near the infection site, helping to fight the infection and protect other cells from the virus. This protective response can also backfire, when a massive inflammation facilitated by those EVs can be responsible for bad clinical outcomes. EVs can help or harm the antiviral response, and sometimes both mechanisms are observed in infections by the same virus. Since those pathways are intrinsically interlinked, understand the role of EVs during viral infections is crucial to comprehend viral mechanisms and respond better to emerging viral diseases.

Keywords: extracellular vesicles, virus infection, host response, viral particles, immune response

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Armando de Menezes Neto, Oswaldo Cruz Foundation (Fiocruz), Brazil

***Correspondence:**
Lysangela Ronalte Alves
lysangela.alves@fiocruz.br
Sharon de Toledo Martins
sdt.martins@gmail.com

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EXTRACELLULAR VESICLES (EVs) IN VIRAL DISEASES

Extracellular vesicles (EVs) are membrane vesicles that have recently received considerable attention. EVs carry several RNA subtypes, proteins and DNA that can be functional in recipient cells after transfer. The smallest EV type, initially called an exosome (Johnstone et al., 1987), originates from multivesicular bodies, a part of the endocytic pathway. They were initially observed with a cup-shaped morphology through conventional transmission electron microscopy techniques, although nowadays it is known that this shape is a preparation artifact, and a round morphology is observed with more advanced cryo-EM techniques (Chuo et al., 2018). They are also enriched with

molecules such as CD63, CD9 and CD81, Alix and TSG101; however, the distribution of these surface molecules varies greatly depending on the cell type, and no canonical markers applicable to all types of EVs have been described to date (Théry et al., 2018). Several other types of EVs have been identified, such as apoptotic bodies, microvesicles and vesicles specific to certain cell types, such as oncosomes secreted by tumor cells (Raposo and Stoorvogel, 2013). The exact biogenesis pathway of these different subtypes is not totally understood. Although EV pathways are active in homeostasis, they are very important under disease conditions. It was previously shown that the EV composition changes drastically during infection, especially infections by intracellular pathogens such as viruses, and host RNAs contained in EVs can affect viral recognition by the immune system to induce or restrict viral propagation in recipient cells (Yoshikawa et al., 2019).

EVs can affect recipient cells through different mechanisms. Cargo delivery by membrane fusion can transport functional molecules such as RNAs into recipient cells (Montecalvo et al., 2012). The endocytic uptake mechanisms can involve clathrin-dependent or clathrin-independent pathways, and heterogeneous populations of EVs are probably internalized by multiple mechanisms (Mulcahy et al., 2014). The clathrin-independent mechanisms can be mediated by caveolin or lipid rafts, and EVs can also be internalized by phagocytosis and micropinocytosis. Proteins and glycoproteins present on the surface of EVs and in recipient cells can also influence these mechanisms (Mulcahy et al., 2014). In addition to cargo delivery by direct fusion or internalization, EVs can also influence target cells through interaction with different receptors, such as lectins (Barrès et al., 2010), heparan sulfate proteoglycans (Christianson et al., 2013), conexins and integrins (Shimaoka et al., 2019). Extracellular vesicles can bind to the cell surface and remain attached to proteins like integrins or trigger intracellular signaling. They also can be internalized and directed to the endosomal pathway until they reach multivesicular endosomes (MVEs), where they can fuse with lysosomes directing their content to degradation and recycling (Tian et al., 2010). Vesicles near the MVE membrane can release their contents on the cytoplasm by back fusion escaping degradation (Bissig and Gruenberg, 2014), and this can also happen to vesicles attached directly to the plasma membrane. This process is important to deliver nucleic acids present on the EVs to the recipient cells, although is still not well understood (Van Niel et al., 2018). There is also evidence that some vesicles can be re-secreted by fusion of MVEs with the plasma membrane or through the early endocytic recycling pathway (Heusermann et al., 2016).

EVs are secreted by most cells, travel long distances within the body, and can be found in several bodily fluids, having great potential as diagnostic tools and in therapeutic and preventive interventions such as vaccine production (Dogrammatzis et al., 2020), as already shown for the influenza virus (Keshavarz et al., 2019), porcine respiratory reproductive syndrome virus (Montaner-Tarbes et al., 2019), and SARS coronavirus, in which they were able to induce the production of a high level of neutralizing antibodies (Kuate et al., 2007). For example, therapeutic EVs derived from mesenchymal stem cells have the ability to induce the differentiation of anti-inflammatory macrophages, inactivate T cells and induce regulatory immune cells such as T and B lymphocytes and dendritic cells. These vesicles can be used to treat acute inflammatory conditions such as severe cases of COVID-19 (Tsuchiya et al., 2020).

CHALLENGES TO THE ISOLATION AND DETECTION OF EXTRACELLULAR VESICLES AND VIRAL PARTICLES

The methodologies used to isolate and characterize extracellular vesicles are very diverse, and each experimental model and scientific question poses its own challenges. Fortunately, efforts of researchers and scientific societies in the EV field are helping to identify better methods and standards to study EVs (Théry et al., 2018). Several established methods used for viral isolation, such as ultracentrifugation, precipitation with crowding reagents, cross-flow filtration, column chromatography and affinity purification, can also be used to isolate extracellular vesicles (McNamara and Dittmer, 2019). Although the ability to use these methods in both viral and EV fields is interesting, it poses difficulties for separating replicative viral particles from extracellular vesicles, especially because these vesicle/virus populations appear to exist on a continuum. Phenotypic characterization of those populations with antibodies, affinity purification after isolation and the use of strategies such as those involving viral replicons that do not secrete viral particles are potential research strategies. In addition, new techniques such as nanofacs and flow virometry seem promising in the search for better separation of these subpopulations (McNamara and Dittmer, 2019).

ROUTES OF EXTRACELLULAR VESICLE BIOGENESIS—EXOSOMES AND MICROVESICLES

Exosome biogenesis is very complex, can vary depending on the cargo, cell type and other stimuli received by the cell, with several mechanisms acting at the same time or sequentially (Edgar et al., 2014), generating an heterogeneous population of vesicles (Van Niel et al., 2018). Different sorting machineries can act on the same endosomal compartment (van Niel et al., 2011), or different machineries can target the same cargo, as observed for MHC class II (Buschow et al., 2009). For this reason, different subpopulations of EVs can coexist (Colombo et al., 2014). Viral infection can interfere with all cellular processes and the intervention with cellular metabolism and reorganization of internal membranes can end up crossing the pathways of EV biogenesis and viral budding, affecting early endosomal sorting machineries (Van Niel et al., 2018).

Exosomes are generated as intraluminal vesicles (ILVs) in the lumen of endosomes during their maturation to multivesicular endosomes (MVEs), involving several sorting mechanisms. They segregate content in membrane microdomains in the MVE membrane and generate smaller membrane vesicles by inward budding and fission (Van Niel et al., 2018). The ESCRT machinery was one of the first proteins to be discovered in this process (Hurley, 2008), acting in several steps, in which ESCRT-0 (also known as HRS) and ESCRT-1 gather ubiquitylated transmembrane cargos in microdomains, and ESCRT-II recruits ESCRT-III, responsible for fission and budding. Inactivation of the members of ESCRT family can affect the composition and release of vesicles (Colombo et al., 2013) and HRS seems to be required for exosome formation and secretion by dendritic cells (Tamai et al., 2010). Molecules like syntenin, ALIX and VPS32 are also important in this process (Baietti et al., 2012).

Exosomes can also be formed in an ESCRT independent manner. When the four ESCRT proteins are depleted, ILVs loaded with CD63 are still able to be formed (Stuffers et al., 2009). The first ESCRT independent pathway of exosome formation is mediated by neutral type II sphingomyelinase, that transforms sphingomyelin in ceramide (Trajkovic et al., 2008), allowing the formation of membrane subdomains (Goñi and Alonso, 2009) that create negative membrane curvatures. Ceramide can also be transformed in sphingosine-1-phosphate and activate a receptor that is crucial for cargo sorting (Kajimoto et al., 2013). Tetraspanins like CD81, CD83, CD9 and CD63 can also regulate biogenesis in an ESCRT-independent way, since they can form clusters and induce budding in membrane microdomains with tetraspanins and other transmembane and cytosolic proteins (Charrin et al., 2014). CD63 was also shown to be involved in endosomal sorting (van Niel et al., 2011; van Niel et al., 2015), cargo targeting and biogenesis of exosomes. CD81 presents a cone-like structure that can accommodate cholesterol inside it, and their clustering can induce inward budding. Tetraspanins can also regulate the intracellular route of cargo like integrins (Odintsova et al., 2013). The type of cargo can also affect the sorting on exosomes. Transmembrane cargos are heavily depending on endosomal machineries, and the affinity of molecules like GPI anchored proteins to lipid rafts could affect membrane properties and be involved in budding (De Gassart et al., 2003). Soluble proteins can be sequestered inside ILVs by co-sorting with chaperones (HSP70, HSC70) found in exosomes of different origins (Géminard et al., 2004). Also, proteins with certain modifications like ubiquitination or farnesylation are enriched in ILVs, but the mechanisms are still unknown. The sorting of nucleic acids is differential, since some types of miRNA motifs are preferentially sorted inside ILVs (Villarroya-Beltri et al., 2013), but passive loading can also occur. Machineries involved in nucleic acid sorting to EVs include the ESCR-II subcomplex that can have RNA-binding properties (Irion and St Johnston, 2007), sequestration of RBPs in membrane domains (Perez-Hernandez et al., 2013) or the presence of RNA silencing complexes like miRNA induced silencing complex (miRISC), argonaute 2 (AGO2), KRAS-MEK signaling, major vault protein and Y box binding protein (YBX1) (Gibbings et al., 2009; McKenzie et al., 2016; Shurtleff et al., 2016; Teng et al., 2017).

Although apoptotic bodies are known for a long time, the mechanisms of microvesicle release from the membrane of healthy cells are starting to be uncovered only recently. Rearrangements in the plasma membrane (lipid components, proteins, and CA+2 levels) are important in this process (Johnstone et al., 1987). Aminophospholipid translocases (flippases and floppases), scramblases and calpain are dependent on Ca2+ and rearrange membrane phospholipids, bending the membrane and restructuring the actin cytoskeleton, favoring membrane budding and microvesicle formation (Piccin et al., 2007). Defects in the scramblase can impair the exposure of phosphatidylserine and the production of platelet-derived procoagulant microvesicles (Piccin et al., 2007). Other lipids like cholesterol can also contribute to microvesicle biogenesis, since their depletion impair the formation of microvesicles in neutrophils (Del Conde et al., 2005). Cytoskeleton regulators that alter actin dynamics, like RHO GTPases and RHO-associated protein kinase (ROCK), can induce microvesicle biogenesis (Li et al., 2012). Metabolic changes can also affect their release, as seen for the Warburg effect, when the inhibition of glutaminase activity dependent of RHO GTPases can block microvesicle biogenesis (Wilson et al., 2013). For the cargo selection, lipids and other cargos with membrane affinity can localize to lipid raft membrane domains, as happen to oligomeric cytoplasmic proteins that are anchored in plasma membrane (Yang and Gould, 2013), and cytosolic components need to bind to the inner leaflet of the plasma membrane. This mechanism is very similar to the budding of HIV and retroviruses (Van Niel et al., 2018). The mechanisms of nucleic acid targeting to the cell membranes is still unknown, but is still unclear how nucleic acids, which are generally found in microvesicles, are targeted to the cell surface. The presence of zip code RNA sequence motifs in the 3′-UTR regions of mRNA can be one of the possible targeting mechanisms to microvesicles (Bolukbasi et al., 2012).

VIRUSES USE INTRACELLULAR MEMBRANES TO EVADE THE IMMUNE RESPONSE AND COMPLETE THEIR CYCLE

Viruses can exploit intracellular membranes to complete their cycles and propagate, creating structures called replicative organelles (Wolff et al., 2020) and using cellular secretion mechanisms to facilitate particle formation and budding. Positive sense RNA viruses, such as nidoviruses (Angelini et al., 2014), arteriviruses (Knoops et al., 2012), flaviviruses (Gosert et al., 2002), coronaviruses (Snijder et al., 2006; Ulasli et al., 2010), have an interesting mechanism of replication involving internal membrane rearrangements in host cells, generating double-membrane structures known as replicative organelles (den Boon and Ahlquist, 2010). These structures contribute to immune evasion by hiding viral components from the immune system and working as scaffolds that anchor viral replication and transcription complexes (V'kovski et al., 2015). This membrane

reorganization can be induced by viral proteins, as shown for SARS-CoV, that can induce membrane disorder and proliferation (through nsp3, in both full length and truncated forms, and nsp6), membrane pairing (with the synergic action of nsp3 and nsp4) and induction of perinuclear vesicles around the microtubule organizing center (through nsp6) (Angelini et al., 2013). The result of these rearrangements is demonstrated by the formation of double-membrane vesicles and convoluted membranes connected to the rough endoplasmic reticulum (Ulasli et al., 2010). Components of ER-Golgi cellular trafficking were also shown to be involved in the formation of these structures (Reggiori et al., 2010), and they are also involved in EV formation, being a possible point of overlap to allow the presence of viral components inside EVs. The degree of induction of intracellular membrane structures can vary between coronavirus strains, although it is not necessarily correlated with pathogenicity (Maier et al., 2016). This mechanism also indicates that infection changes cellular lipid metabolism and that some enzymes involved in lipid processing are crucial for the formation of these membrane structures. The inhibition of cytosolic phospholipase A2a significantly reduces the formation of coronavirus particles *in vitro*, suggesting that the formation of these internal membrane structures is essential for completion of the viral replication cycle (Müller et al., 2017).

VESICLES OR VIRAL PARTICLES? OVERLAP BETWEEN VIRAL BUDDING AND EV BIOGENESIS

In addition to secreting replicative viral particles, infected cells can also secrete other structures containing viral proteins and nucleic acids that can activate the immune system or impact recipient cells, favoring viral propagation (van der Grein et al., 2018). There is ongoing discussion about the classification of these particles, since they can be either host EVs containing viral molecules or defective viral particles. It is difficult to isolate pure populations of these different types of vesicles since they are of similar size, density and composition, and most isolation methods cannot be used to separate them (van der Grein et al., 2018). The replicative viral structures found inside host EVs can be complete viral particles or "quasi-enveloped" viruses (viruses that are classically nonenveloped but can be found "cloaked" inside host EVs) (Feng et al., 2014).

The virology field classifies some viruses as enveloped when their capsids are surrounded by host membrane; these viruses usually bud directly from the plasma membrane or through an exocytic pathway without necessarily promoting cell death. Examples of enveloped viruses are HIV, influenza, dengue and SARS-Cov2. Nonenveloped viruses, such as hepatitis A virus (HAV), coxsackievirus, norovirus, poliovirus and rhinovirus, typically promote cell lysis, which is required for their release, and are not surrounded by host membrane (Lindenbach, 2013; Altan-Bonnet, 2016). Nevertheless, in 2013, the distinction between enveloped and nonenveloped viruses became less clear when both types of particles were found *in vivo* and in the extracellular medium of liver cells infected in HAV (Feng et al., 2013). Cellular analysis was used to track them inside multivesicular bodies (MVBs) to their cells of origin, and the depletion of ESCRT proteins blocked their release, suggesting an overlap of viral particle release and the exosome biogenesis pathway (Altan-Bonnet, 2016).

Viral particle formation pathways sometimes include the endosomal machinery that produces EVs. Usually, viruses enter cells through endocytosis (although some enveloped viruses can fuse directly to cell membranes) and release their nucleic acids, which are undergoing replication/transcription, and new virions can bud through the cell membrane or are released after cell lysis (Urbanelli et al., 2019). In 2003, the "Trojan Horse" hypothesis was formulated by Gould and colleagues who showed that EVs secreted by dendritic cells infected with HIV were able to infect T CD4+ lymphocytes (Gould et al., 2003). Gag, an HIV structural protein, is known to directly recruit Alix and ESCRT-1 proteins (Votteler and Sundquist, 2013), important components of exosome formation machinery. The expression of coronavirus E and M proteins is sufficient to generate virus-like particles even in the absence of the other viral components (Maeda et al., 1999). Additionally, some herpesviruses can interact with the ESCRT machinery during the formation of the viral envelope in endosomal compartments and the trans Golgi network (TGN) (Sadeghipour and Mathias, 2017). For this reason, some nonenveloped viruses can be found in "quasi-enveloped" states inside exosomes when they are released, cloaked in host membranes and lack viral surface proteins, as observed for the hepatitis A virus (HAV) and hepatitis E (Nagashima et al., 2014). Recent research has demonstrated that collective viral spread involving viral aggregates can favor viruses and promote the evolution of defective interfering particles, and extracellular vesicles may also have a role in this process (Andreu-Moreno and Sanjuán, 2020), since aggregation can change the internalization route of EVs, favoring phagocytosis and micropinocytosis (Feng et al., 2010).

Even when envelopes are acquired in canonical pathways, the exosomal pathway can be exploited by viruses to facilitate its own transmission, as seen for porcine reproductive and respiratory syndrome virus (PRRSV) (Wang et al., 2018), herpes simplex virus HSV-1 (Bello-Morales and López-Guerrero, 2020), enterovirus 71 (Gu et al., 2020), and RVFV, which pack viral RNA and proteins inside vesicles (Ahsan et al., 2016); HIV, which facilitates macrophage infection through EVs (Kadiu et al., 2012); HBV, which can directly induce replication through EVs of infected cells (Li et al., 2019); and HTLV-1, which exports functional viral proteins inside EVs to uninfected cells (Jaworski et al., 2014). Cells infected with rhinovirus secrete EVs that induce the upregulation of viral receptors in monocytes, which allows the virus to infect alternative cell types (Miura, 2019). Mosquito cells infected with DENV secrete larger EVs than uninfected cells, and these structures contain virus-like particles that are able to infect other cells (Reyes-Ruiz et al., 2019). Quiescent CD4+ T lymphocytes are usually refractory to

HIV1 infection; however, EVs from infected cells can make them permissive to viral replication through the action of ADAM17 and Nef (Arenaccio et al., 2014). Additionally, Nef-containing EVs can modulate lipid rafts in recipient cells, facilitating the fusion of new viral particles with these cells and increasing infection (Dubrovsky et al., 2020). For HCV, it was shown that replication-competent sub genomic RNAs can be transferred through EVs and establish infection in recipient cells, even when complete viral particles are absent (Longatti et al., 2015). Thus, HCV infectivity was independent of classical HCV receptors or viral envelope proteins, rendering them partially resistant to antibody neutralization, which may be an immune evasion strategy used by several other viruses (Ramakrishnaiah et al., 2013). Vesicles containing genomic RNAs found in HCV patients also carried Ago2, HSP90 and miR-122, which facilitate viral stability and replication (Bukong et al., 2014; Altan-Bonnet, 2016). It was also shown that ZIKV is able to induce the amplification of EV production through increased expression and activity of SMPD3 and that EVs containing viral RNAs and proteins promote viral transmission (Zhou W. et al., 2019). FMDV (foot and mouth disease virus) can also be transmitted through host EVs that carry genomic RNAs and some viral proteins, and FMDV replication is not fully blocked by neutralizing antibodies, suggesting an immune evasion mechanism (Zhang et al., 2019). For SFTS (a tick-borne bunyavirus associated with hemorrhagic fever), exosomes from infected cells contained viable virions that were able to infect cells by an alternative route independent of classical receptors (Silvas et al., 2016). They enter cells by endocytosis, similar to naked virions, but they are uncoated, and their genome is released by different pathways. Viruses that usually have lytic cycles can also be released from cells in a nonlytic way (Rivera-Serrano et al., 2019). A scheme depicting an interplay between extracellular vesicles, viruses and subpopulations of viral particles can be observed in **Figure 1**.

SECRETORY AUTOPHAGY AND VIRAL BUDDING

Some nonenveloped viruses can exit the cell in vesicles that originate from autophagosomes instead of MVBs, as shown for rhinovirus, poliovirus and coxsackievirus (Bird et al., 2014; Robinson et al., 2014). Autophagosomes usually have a double membrane and are formed from the endoplasmic reticulum, mitochondria or plasma membrane (Cuervo, 2010), and through their natural degradation mechanism, they process large quantities of cytoplasm to provide nutrients during stress (Feng et al., 2015). However, instead of fusing with lysosomes, they can fuse with the plasma membrane to release vesicles containing viral particles. This "secretory autophagy" is observed in uninfected cells (Ponpuak et al., 2015) from which useful molecules and organelles are released into the extracellular space (Altan-Bonnet, 2016). These vesicles are enriched in phosphatidylserine, which can be important for infection (Chen et al., 2015), as observed for classical enveloped viruses such as vaccinia, dengue, Ebola and pseudotype lentivirus (Amara and Mercer, 2015). The viruses inside exosomes or large autophagosome-derived vesicles were found to be more infective than the viruses released when the autophagosomes were lysed (Altan-Bonnet, 2016). It is believed that these vesicles can be disrupted once internalized by host cells in acidic compartments such as acidified endosomes, releasing the viral particle (Bird et al., 2014). A recent study reviewed the interaction of MERS, SARS-Cov, and SARS-CoV2 on autophagic processes. The literature data are very inconsistent

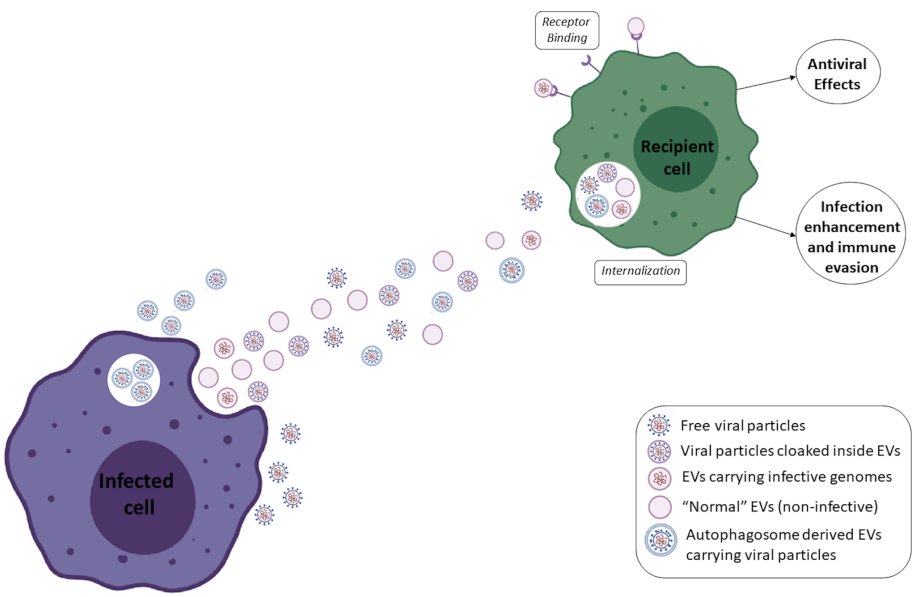

FIGURE 1 | Scheme depicting an interplay between extracellular vesicles, viruses and subpopulations of viral particles.

regarding the role of autophagy in coronavirus replication, with some studies suggesting that it is necessary, while others stating that replication is autophagy-independent, and some studies showing that the virus can inhibit the autophagy process. This ambiguity may indicate a nonclassical pathway of autophagy that may be related to the secretory form of autophagy described above (Yang & Shen, 2020).

EVs IN IMMUNE COMMUNICATION AND CYTOKINE RESPONSES DURING INFECTION

The infection process can drastically change the composition of host EVs, changing the proportions of host proteins and RNAs inside these structures (Hoen et al., 2016). During infection, EVs can amplify inflammation and deflagrate antiviral responses (Urbanelli et al., 2019) and can also mediate communication between immune cells and other cell types (Isola and Chen, 2017). The involvement of EVs in viral infection and/or host interactions in disease has already been described for several viruses, such as rabies (Wang et al., 2019b), coronaviruses (Maeda et al., 1999; Kuate et al., 2007; Börger et al., 2020; Deffune et al., 2020; Hassanpour et al., 2020; Inal, 2020a; Inal, 2020b; Kumar et al., 2020; O'Driscoll, 2020; Tsuchiya et al., 2020; Urciuoli and Peruzzi, 2020), HCV (Bartosch et al., 2003; Timpe et al., 2008; Dreux et al., 2012; Bukong et al., 2014), HBV (Jia et al., 2017; Li et al., 2019), HIV (Princen et al., 2004; Khatua et al., 2009; Xu et al., 2009; Lenassi et al., 2010; Bernard et al., 2014; Raymond et al., 2016; Sampey et al., 2016; Kodidela et al., 2018; Haque et al., 2020; Ranjit et al., 2020), HPV (Honegger et al., 2015; Guenat et al., 2017; Sadri Nahand et al., 2019; Chiantore et al., 2020), HSV (Temme et al., 2010; Han et al., 2016; Deschamps and Kalamvoki, 2018) dengue (Martins et al., 2018; Mishra et al., 2019; Sung et al., 2019), HTLV-1 (Pinto et al., 2019), Zika (Zhou W. et al., 2019; Martínez-Rojas et al., 2020), West Nile (Slonchak et al., 2019), Epstein Baar (Keryer-Bibens et al., 2006; Klibi et al., 2009; Zhao M. et al., 2019), influenza (Liu Y. et al., 2019; Maemura et al., 2020), and SFTS (Silvas et al., 2016).

EV secretion occurs in several body systems during homeostasis, and it represents an important communication pathway in the immune system (Isola and Chen, 2017). Vesicles transferred between immune cells can transmit signals that trigger an increase or decrease in cytokine production and transfer antigens, and some EVs are able to trigger direct antigen presentation (Lindenbergh and Stoorvogel, 2018). EVs carry cytokines and cytokine-related RNAs that can elicit the production of target molecules in recipient cells, having a role in the antiviral response (Urbanelli et al., 2019). EVs secreted by infected cells are able to activate other cells, as observed when vesicles secreted from U937 macrophages infected with DENV-2 activate endothelial cells (Velandia-Romero et al., 2020). Infection with West Nile virus changes the composition of host microRNAs, small noncoding RNAs and mRNAs in EVs, and the enriched RNAs are related to viral processing and host responses to infection (Slonchak et al., 2019). It was also observed that two strains of dengue virus with different virulence profiles induce the secretion of EVs with drastically different RNA compositions from monocyte-derived dendritic cells (Martins et al., 2018). When taken up by macrophages, vesicles from HIV-infected cells containing Nef can trigger the inflammasome, inducing the secretion of proinflammatory cytokines (Mukhamedova et al., 2019). EVs released by airway epithelial cells infected with RSV (respiratory syncytial virus) have increased expression of regulatory small RNAs and can stimulate chemokine production in monocytes without transferring infective particles (Chahar et al., 2018).

Sometimes high levels of proinflammatory cytokine production can contribute to disease severity, as seen for several infectious diseases, and EVs can mediate this process. EVs isolated from bronchoalveolar fluid of mice infected with a highly pathogenic avian influenza virus (H5N1) showed enrichment with miR-483-3p, which stimulates innate immune responses in pneumocytes (Maemura et al., 2018). This molecule was also enriched in the serum of infected mice, and pneumocyte-derived EVs enriched with this molecule increased the expression of proinflammatory cytokine genes in vascular endothelial cells, suggesting the involvement of EVs in the inflammatory pathogenesis of H5N1 (Maemura et al., 2020). A similar process occurs for dengue hemorrhagic fever, a severe disease in which massive secretion of cytokines and high vascular hyperpermeability can lead to shock syndrome, and extracellular vesicles were shown to be involved in this process (Mishra et al., 2019). A summary of the main findings associated to the EVs and viruses are described in **Table 1**.

EVs CAN ELICIT AND PROPAGATE ANTIVIRAL RESPONSES

The protective effect of EVs during infection can also involve classical antiviral pathways, such as the interferon response, because effector molecules, such as interferon stimulated genes (ISGs), can be carried to other cells (Li et al., 2013). The secretion of type I interferon is a potent and conserved antiviral response strategy. IFN protein is produced and then secreted into the extracellular space after pathogen-associated molecular patterns (PAMPs) are recognized by Toll-like receptors. After secretion, the produced IFN molecules can bind to surface receptors in other cells and trigger a protective response (Schneider et al., 2014). The translocation of NFKb to the nucleus induces the transcription of several ISGs, which are the true antiviral effectors of these pathways. The EV pathway is linked to the IFN response in several ways. First, viral components from infected cells can be transferred to other cells through EVs, where they will induce IFN production. An example of this is what happens with plasmacytoid dendritic cells (pDCs), that have an important role in innate immunity by recognizing viral nucleic acids through TLR7 and TLR9 (Gilliet et al., 2008), inducing their activation and production of IFN among other molecules. When transferred to pDCs, EVs from infected cells

TABLE 1 | Summary of the main findings associated to the EVs and viral infections.

EVS FAVORING VIRAL PROPAGATION	
Mechanism	**Virus**
EVs facilitate viral transmission	HSV-1 (Bello-Morales and López-Guerrero, 2020), KSHV (Chen et al., 2020)., NDV (Zhou C. et al., 2019), PRRSV (Wang et al., 2017), enterovirus 71 (Gu et al., 2020), HCV (Bukong et al., 2014), HIV (Kadiu et al., 2012), SFTS (Silvas et al., 2016)
Viral RNAs/proteins inside EVs	Coronavirus (Maeda et al., 1999), EBV (Keryer-Bibens et al., 2006), HCV (Kouwaki et al., 2017)., HTLV-1, (Jaworski et al., 2014), RVFV (Ahsan et al., 2016), ZIKV (Zhou W. et al., 2019; Martínez-Rojas et al., 2020)
Infectious virus-like particles/cloaked virions inside EVs	DENV (Reyes-Ruiz et al., 2019), enterovirus 71 (Gu et al., 2020), HCV (Bartosch et al., 2003; Timpe et al., 2008)
Transfer of infective RNA through EVs withouth complete viral particles	HCV (Longatti et al., 2015), FMDV (Zhang et al., 2019),
EVs turn cells more permissive to infection, membrane/receptor modulation	HIV (Arenaccio et al., 2014; Dubrovsky et al., 2020), Rhinovirus (Miura, 2019)
Host molecules in EVs facilitate viral stability and replication in recipient cells	HBV (Li et al., 2019), HCV (Bukong et al., 2014; Altan-Bonnet, 2016), HIV (Arenaccio et al., 2014; Ranjit et al., 2020)
Amplification of EV production	ZIKV (Zhou W. et al., 2019)
EVs from uninfected cells can activate latent viruses	HIV (Barclay et al., 2020)
EVS RELATED TO IMMUNE RESPONSES	
Mechanism	**Virus**
EVs from infected cells are able to activate other cells	DENV (Velandia-Romero et al., 2020; Mishra et al., 2019)
RNAs inside EVs related to host responses to infection	DENV (Martins et al., 2018a), H5N1 (Maemura et al., 2018; Maemura et al., 2020), HBV (Zhao X. et al., 2019), HIV (Bernard et al., 2014), HSV-1 (Han et al., 2016; Huang et al., 2019), influenza (Liu Y. et al., 2019), Rabies (Wang et al., 2019a), RSV (Chahar et al., 2018), West Nile (Slonchak et al., 2019)
EVs from infected cells can trigger the secretion of proinflammatory molecules in other cells	HIV (Sampey et al., 2016; Mukhamedova et al., 2019), H5N1 (Maemura et al., 2018; Maemura et al., 2020), HBV (Zhao X. et al., 2019), RSV (Chahar et al., 2018)
EVs involved in IFN-mediated responses	DENV (Martins et al., 2018), HBV (Yao et al., 2019; Zhao X. et al., 2019), HCV (Dreux et al., 2012; Okamoto et al., 2014), HIV-1 (Khatua et al., 2009), HSV-1 (Huang et al., 2019), influenza (Liu et al., 2019)
EVs that can restrict viral replication	Rabies (Wang et al., 2019a), HBV (Zhao X. et al., 2019), HIV (Ouattara et al., 2018),
Induction of massive inflammatory responses/vascular permeability	DENV (Sung et al., 2019)
EVs can block/impair viral propagation	Enterovirus (Chen et al., 2015), Influenza (Liu Y. et al., 2019), HIV-1 (Khatua et al., 2009), HSV-1 (Han et al., 2016; Deschamps and Kalamvoki, 2018; Huang et al., 2019), Rabies (Wang et al., 2019a)
EVs can induce antibody production	SARS (Kuate et al., 2007)
EVS CAN HELP VIRUSES BLOCK ANTIVIRAL RESPONSES	
Mechanism	**Virus**
EVs reduce IFN-mediated antiviral protection in recipient cells	enterovirus 71 (Wang et al., 2018), HBV (Shi et al., 2019), HCV (Florentin et al., 2012)
EVs carry host RNAs related to antiviral response blocking	enterovirus 71 (Wang et al., 2018)., NDV (Zhou C. et al., 2019)
More cytopatic effect in recipient cells	NDV (Zhou C. et al., 2019)
EVs turn recipient cells more permissive to infection	rhinovirus (Zhou et al., 2017).
EVs impair other antiviral mechanisms/promote immune evasion	EBV (Klibi et al., 2009), HCV (Ashraf Malik et al., 2019), HIV (Schaefer et al., 2008; Xu et al., 2009; Lenassi et al., 2010; De Carvalho et al., 2014), KSHV (McNamara et al., 2019)
EVS SECRETED DURING INFECTION CAN TRIGGER SECONDARY DISEASES	
Mechanism	**Virus**
Oncogenig effect	gamma-herpes virus (Zheng et al., 2019), human papillomavirus (Honegger et al., 2015; Ambrosio et al., 2019; Chiantore et al., 2020), HIV (Sharma, 2019), MVP (Teng et al., 2017)
Accumulation of beta amyloid plaques	HIV (Fulop et al., 2019).
Trigger inflammation	human papillomavirus (Sadri Nahand et al., 2019)
Contribute to tissue fybrosis	HCV (Kim et al., 2019).
Mediate chemoresistance	HBV (Liu D. et al., 2019)
Mediate autoimmunity/transplant rejection	respiratory viruses (Gunasekaran et al., 2020).
EVs involved in viral latency/persistant infections	HIV (Olivetta et al., 2019; Barclay et al., 2020), HCV (Ashraf Malik et al., 2019),
Thrombosis induction	SARS CoV-2 (Inal, 2020a), Nomura et al., 2020

can be internalized and their viral RNA can activate TLR7 (Assil et al., 2015). The activation of IFN response in pDCs by extracellular vesicles may be more powerful than the one induced by only mature virions, since EVs from HCV infected cells can induce a strong IFN response in pDCs (Dreux et al., 2012), while conventional HCV particles may block TLR7

induced signaling as an immune evasion strategy (Florentin et al., 2012). A recently explored area gaining attention involves the transfer of ISGs through EVs. The mRNAs for ISGs can be transferred to bystander cells or over long distances, and the recipient cells can translate these mRNAs (Li et al., 2013). Complete ISG proteins can also be transferred (Borghesan et al., 2019; Yao et al., 2019). The viral entry machinery can also be used by the cells to transfer antiviral protection since the same receptors that some viruses use to bind the cells can also bind EVs. This is exemplified by macrophage-derived exosomes that depend on T-cell immunoglobulin and mucin receptor 1 (TIM-1), a receptor used by Hepatitis A Virus (HAV) (Yao et al., 2018). EVs secreted by THP-1 macrophages treated with IFN-alpha are enriched with proteins related to the "defense response to virus" and "type I IFN signaling pathway". Some of the proteins for ISGs found in this work were upregulated both in macrophages treated with IFN and in the EVs secreted by them (IFI44L, IFIT1, ISG15, EIF2AK2, MX2, IFIT3, MX1, STAT2, OAS3, IFI16, OAS2, STAT1 and IFIT2), while one ISG was found upregulated only in EVs (SAMHD1) (Yao et al., 2019). These vesicles also present potent antiviral activity when delivered to hepatocytes infected with hepatitis B, suggesting that effector antiviral molecules induced by IFN can be transferred through exosomes (Yao et al., 2019).

The presence of HBV-miR-3 in EVs secreted by HBV-infected cells can induce macrophage polarization to an M1 phenotype, increase IFN production, activate the Jak/STAT signaling pathway and induce IL-6 secretion. These actions may restrict HBV replication and suppress the acute liver cell injury caused by HBV (Zhao X. et al., 2019). Hepatocytes infected with HCV can produce EVs loaded with infective HCV RNA that, when internalized by pDCs, can trigger type I IFN production upon TLR7 binding. This effect is attenuated when ESCRT-I and ESCRT-III are depleted from the infected hepatocytes, suggesting a correlation with the EV pathway (Dreux et al., 2012). Similarly, EVs secreted by cells with HCV replicons induced the TLR3-mediated production of IFN I and III by delivering viral RNAs to DCs (Okamoto et al., 2014) (Kouwaki et al., 2017).

Influenza-infected cells secrete EVs containing miR-1975 that induce interferon expression in recipient cells (Liu Y. et al., 2019). Cells infected with herpes simplex virus (HSV-1) produce EVs packed with miR-H28 and miR-H29, which are able to restrict viral transmission to uninfected cells through the induction of IFN-gamma production (Huang et al., 2019). Other mechanisms of EV-mediated viral inhibition can also occur, as observed for MRC-5 cells infected with rabies, that show increased production of EVs containing miR-423-5p, which inhibits RABV replication in neighboring recipient cells (Wang et al., 2019a). Exosomes from the microenvironment and biofluids can also modulate viral infection, as shown for seminal EVs, which seems to have a protective effect against HIV infection. They can directly inhibit HIV-1 cellular entry, prevent transmission of HIV from vaginal epithelial cells to monocytes, T lymphocytes and PBMCs. They can also inhibit replication after internalization by blocking reverse transcriptase activity and inhibiting binding of transcription factors to the HIV1 promoter (Ouattara et al., 2018). This helps to explain the low infection rates of people exposed to the virus (Ouattara et al., 2018; Welch et al., 2019).

VIRUSES CAN EXPLOIT EV MACHINERY TO BLOCK ANTIVIRAL RESPONSES AND ALLOW THEIR OWN PROPAGATION

EVs have an important role in transferring antiviral molecules, facilitating propagation of responses such as interferon activity. However, several viruses are known to disrupt the defense pathway mediated by IFN, thus evading the immune response (**Table 1**). For example, EVs loaded with IFITM2 delivered to dendritic cells reduce the capacity of the recipient cells to produce IFN-alpha and thus counteract HBV infection (Shi et al., 2019). EVs secreted from cells infected with NDV carry miR-1273f, miR-1184 and miR-198, which are able to block IFN-beta antiviral responses and increase the virus-induced cytopathic effect in recipient cells (Zhou C. et al., 2019). Similarly, EVs secreted by human epithelial cells infected with enterovirus 71 (EV71) can transfer miR-30a to macrophages to target the MyD88 gene, suppressing type I IFN production (Wang et al., 2018).

Viruses can also use the EV pathway to make neighboring cells more permissive to infection. Monocytes treated with conditioned media from rhinovirus-infected epithelial cells exhibited increased secretion of proinflammatory cytokines and ICAM1, which makes the monocytes more permissive to infection and viral replication (Zhou et al., 2017). Usually, TCD4 + T cells are able to secrete EVs with surface CD4 molecules. These receptors act as decoys, binding the HIV1 virus and inhibiting the infection of new cells. However, in addition to reducing CD4 on cell surfaces, the HIV protein Nef can also reduce the expression of CD4 on EVs, blocking this mechanism to allow viral propagation (De Carvalho et al., 2014). When transferred to B cells through EVs, the HIV Nef protein can impair the production of IgG and IgA antibodies (Xu et al., 2009). Nef can induce cell death when delivered to bystander TCD4+ cells (Lenassi et al., 2010) and the degradation of the viral receptors CD4 and MHC-1 (an important molecule that presents viral antigens to the immune system) through the action of Nef-interacting protein B-cop (Schaefer et al., 2008). In addition, EVs secreted by uninfected cells can activate the transcription of latent viruses in HIV-1-infected cells through cellular SRC-1 and the PI3K/AKT/mTOR pathway (Barclay et al., 2020).

EVs SECRETED DURING VIRAL INFECTIONS CAN CAUSE SECONDARY DISEASE

EVs secreted during the course of a viral infection can have effects in many parts of the body, triggering secondary diseases

(**Table 1**). For example, gamma-herpes virus can induce an oncogenic effect (Zheng et al., 2019), among several others. There is also evidence that EVs contribute to the beta-amyloid plaque accumulation that occurs in the brain of HIV patients, probably contributing to cognitive decline (Fulop et al., 2019). EVs secreted during human papillomavirus infection can carry miRNAs that induce cervical inflammation (Sadri Nahand et al., 2019) and are related to the development of squamous cell carcinoma (Ambrosio et al., 2019). EVs secreted by HCV-infected hepatocytes contain miR-192 and, when transferred to hepatic stellate cells (HSCs), induce TGF-β1 upregulation, triggering differentiation into myofibroblasts. This process contributes to the liver fibrosis induced by HCV (Kim et al., 2019). HIV-1-infected cells secrete EVs containing TAR RNAs that have pro-growth and pro-survival effects on cancer cells, having the potential to induce tumor progression and malignancy (Sharma, 2019). Kaposi's sarcoma-associated herpesvirus (KSHV), a tumor-associated virus, can induce the proliferation, migration and transcriptional changes of uninfected endothelial cells through EVs, thereby evading the pathogen recognition surveillance system (McNamara et al., 2019). It was also shown that salivary EVs from HIV patients carried *tar*, *tat* and *nef* RNAs but not TAT or Nef proteins. Treatment with these EVs increased the KSHV infection rate of oral epithelial cells through the EFG receptor (EGFR), and this effect was blocked by cetuximab, a drug that targets EGFR. This facilitation of KSHV infection caused by HIV EVs can explain the high rates of Kaposi sarcoma in HIV patients and indicates that this virus can break the epithelial barrier to spread through the body (Chen et al., 2020). HBV-associated liver cancer presents more chemoresistance than non-HBV tumors. EVs from HBV-infected cancer cells were able to downregulate the apoptosis of recipient cells upon drug treatment, modulate cell death through the CMA pathway and upregulate Lamp2A, suggesting that the EVs induced by infection can mediate chemoresistance through chaperone-mediated autophagy (Liu D. et al., 2019). Patients with respiratory viral infections after lung transplantation had circulating EVs containing lung self-antigens, the 20S proteasome and viral antigens that can trigger a rejection response against the transplanted lung and lead to allograft dysfunction (Gunasekaran et al., 2020). EVs in this context can also facilitate the establishment of persistent viral infections. HIV can persist in latent reservoirs that are not recognized by the immune system, leading to a rebound infection after discontinuation of antiretroviral therapy, and it is believed that EVs are crucial for the preservation of these reservoirs (Olivetta et al., 2019). For HCV, CD81+ EVs loaded with viral particles allow the virus to escape immune surveillance, helping to establish persistent infections (Ashraf Malik et al., 2019). Additionally, systemic hemorrhagic diseases that involve vascular permeability, such as dengue hemorrhagic fever, show the involvement of EVs during pathogenesis. Besides being important in immune cell communication, like EVs secreted by mdDCs that carry RNAs related to antiviral response and inflammatory cytokines (Martins et al., 2018), DENV infection can induce the secretion of platelet EVs that cause massive inflammatory responses by activation of CLEC5A and TLR2 on macrophages and neutrophils (Sung et al., 2019), and induce the formation of Neutrophil Extracellular Traps (Mishra et al., 2019). Additionally, it was already discussed that extracellular vesicles can be involved in thrombosis events observed after infection by several types of viruses (Nomura et al., 2020).

CONCLUDING REMARKS

Despite experimental difficulties, the field of extracellular vesicles in viral infections is growing and has tremendous potential to solve healthcare problems. The EVs can carry infective viral particles, they also influence the response of surrounding cells and turning them more susceptible to infection. On the contrary the EVs can also help the host cell to fight the infection, by triggering antiviral responses and cytokine secretion. As stated, the EVs can either facilitate or impair the antiviral response, and sometimes both mechanisms are observed in infections by the same virus. Since those pathways are intrinsically interlinked, understand the role of EVs during viral infections is crucial to comprehend viral mechanisms and respond better to emerging viral diseases. In summary, several mechanisms of virus and EV biogenesis are shared, and knowledge in one field can help to advance the prospects of the other. Understanding the interplay between viruses and extracellular vesicles can also help to develop mechanisms to respond better to public health threats caused by viral pathogens.

AUTHOR CONTRIBUTIONS

SM and LA wrote and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Extracellular Vesicles in Trypanosomatids: Host Cell Communication

Ana Claudia Torrecilhas[1], Rodrigo Pedro Soares[2], Sergio Schenkman[3], Christopher Fernández-Prada[4] and Martin Olivier[5]**

[1] Departamento de Ciências Farmacêuticas, Federal University of Sao Paulo (UNIFESP), Diadema, Brazil, [2] Instituto René Rachou, FIOCRUZ—MG, Belo Horizonte, Brazil, [3] Departamento de Microbiologia, Imunologia e Parasitologia, UNIFESP, São Paulo, Brazil, [4] Faculty of Veterinary Medicine, Université de Montréal, St-Hyacinthe, QC, Canada, [5] The Research Institute of the McGill University Health Centre, McGill University, Montréal, QC, Canada

Trypanosoma cruzi, Trypanosoma brucei and *Leishmania* (Trypanosomatidae: Kinetoplastida) are parasitic protozoan causing Chagas disease, African Trypanosomiasis and Leishmaniases worldwide. They are vector borne diseases transmitted by triatomine bugs, Tsetse fly, and sand flies, respectively. Those diseases cause enormous economic losses and morbidity affecting not only rural and poverty areas but are also spreading to urban areas. During the parasite-host interaction, those organisms release extracellular vesicles (EVs) that are crucial for the immunomodulatory events triggered by the parasites. EVs are involved in cell-cell communication and can act as important pro-inflammatory mediators. Therefore, interface between EVs and host immune responses are crucial for the immunopathological events that those diseases exhibit. Additionally, EVs from these organisms have a role in the invertebrate hosts digestive tracts prior to parasite transmission. This review summarizes the available data on how EVs from those medically important trypanosomatids affect their interaction with vertebrate and invertebrate hosts.

Keywords: ***Trypanosoma cruzi*, *Leishmania*, inflammation, insect vector, innate immunity, skin pathology, *Trypanosoma brucei*, extracellular vesicles**

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****Correspondence:***
Ana Claudia Torrecilhas
ana.torrecilhas@unifesp.br
Martin Olivier
martin.olivier@mcgill.ca

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INTRODUCTION

Trypanosoma cruzi and Chagas' Disease

American trypanosomiasis, popularly known as Chagas' disease (CD), whose etiologic agent is the flagellated protozoan *Trypanosoma cruzi* was first described by Carlos Chagas in 1909. CD is a neglected disease among the 17 tropical diseases. It is estimated that 8 million people are infected with *T. cruzi* in the world, the majority located in Latin America (WHO, 2019). In addition, 100 million people are at risk of infection and 2,000 deaths each year, circumstances that make CD a serious public health problem. In several Latin American countries, CD is controlled in blood banks and by elimination of vector to prevent transmission (Dias et al., 2002; Cavalcanti et al., 2009). Prevention in some countries like Bolivia is deficient (Coura and Vinas, 2010) and prevalence of the infection in Bolivia has been estimated at 6.8% (Luna et al., 2017) and (Bern, 2015). Nevertheless, oral transmission by ingestion of contaminated food, mainly açai fruit and sugar cane remains a source of new cases everywhere and congenital transmission

(Brutus et al., 2008; Dias et al., 2008; Nóbrega et al., 2009; Bastos et al., 2010; Shikanai-Yasuda and Carvalho, 2012).

Despite being an endemic disease in Latin America, cases of CD have been reported in non-endemic regions such as North America (5,500 in Canada and 300,000 infected people in the United States), Europe (80,000 cases), Japan (3,000) and Australia (1,500) (Coura and Vinas, 2010; Perez et al., 2015). The occurrence of CD in these non-endemic regions creates a new epidemiological reality, which is due to population mobility, mainly due to migratory activities, as well as the lack of control in blood banks the source of infection in most of these cases (Coura and Vinas, 2010; Cura et al., 2013; Ries et al., 2016).

The invertebrate host is infected by ingesting trypomastigote forms present in the bloodstream of the mammalian host during hematophagy. In the vector gut, the parasite differentiates in epimastigotes, which subsequently multiply by binary division in the posterior intestine. Along the intestinal tract epimastigotes differentiate into metacyclic-trypomastigotes prior to release with urine and feces during a new blood meal. Such released metacyclic-trypomastigotes are capable of infecting the mammalian host through mucosa or injured regions on the skin. Metacyclic-trypomastigotes enter several cell types forming a parasitophorous vacuole and differentiate into amastigotes (de Carvalho and de Souza, 1989). Cytosolic amastigotes multiply by binary division and after seizing the host cell, differentiate into trypomastigotes, which erupts to the extracellular environment and reaches the bloodstream to continue the life cycle.

Trypanosoma brucei and Human African Trypanosomiasis

Trypanosoma brucei is the causative agent of Sleeping sickness or Human African Trypanosomiasis (HAT) and Nagana in cattle. It affects millions of people in countries at sub-Saharan Africa (Buscher et al., 2017). There are three subspecies group; *T. brucei brucei* (the agent of Nagana), *T. brucei gambiense* and *T. brucei rhodesiense*, which cause respectively the chronic and acute forms of the disease. These parasites are transmitted by Tsetse flies (*Glossina* spp.). The insect injects parasites into skin during the blood meal. From the bite, parasites enter the lymphatic system and then pass into the bloodstream. The bloodstream forms persist in blood causing anemia, and reach the central nervous system causing neurological disorders including encephalopathy that can lead to death if untreated (Buscher et al., 2017). Differently from *T. cruzi*, *T. brucei* is an extracellular parasite and divides in the mammalian bloodstream, in the insects' gut and salivary glands. The bloodstream trypomastigotes surface is covered by a variant surface glycoprotein (VSG) that forms a coat around the parasite that protects it against host immune defenses (Vickerman and Luckins, 1969). There are about 2500 VSG different genes (Cross et al., 2014). Only one of them is expressed at each time and it is replaced allowing the parasite survival upon the establishment of a robust humoral responses (Mugnier et al., 2016; Aresta-Branco et al., 2019). Once the host establish an immune response, another VSG is expressed allowing the parasite to escape leading to chronic infection. In long-term infections, the parasite crosses the blood brain barrier and causes encephalopathy (Schwede and Carrington, 2010; Rudenko, 2011). In addition, parasite-derived molecules including surface metalloproteases (MSPs), phospholipase-C (PLC) and transferrin receptor (TfR) have been shown to play key roles in the interaction (Ponte-Sucre, 2016).

Leishmania and Leishmaniasis

Leishmaniases are a spectrum of diseases widely distributed in 98 countries in the world where approximately 350 million people are at risk of infection. It is considered be the World Health Organization (WHO) as the 6th tropical disease. Its control involves basically the treatment of patients with Amphotericin B and Sb-based drugs depending on the country. However, the appearance of side-effects, discontinuation and drug resistance have been increasingly reported hindering chemotherapeutic control in several regions of the world (Kaye and Scott, 2011). Leishmaniasis may exhibit three distinct forms with variations: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). VL can be lethal if not treated in 90% of the cases, whereas CL lesions are of benign course but can be worsened by secondary infection. Although not lethal MCL is the most morbid type of infection destroying the tissues surrounding naso-oro-pharyngeal areas leading to disfiguration of the face. Main viscerotropic species include *Leishmania donovani* and *Leishmania infantum*, in the Old and New World, respectively. Dermotropic species are represented by *Leishmania major/Leishmania tropica* (Old World) and *Leishmania braziliensis*, *Leishmania guyanensis*, and *Leishmania amazonensis* (New World) (Herwaldt, 1999).

Leishmania parasites are transmitted through the bite of sand fly vector included in two main genera: *Lutzomyia* and *Phlebotomus* in the New and Old World, respectively (Elnaiem et al., 1994; Vexenat et al., 1994). Sand flies are pool feeder insects, inserting their mouth parts in the skin creating a pool where they take a bloodmeal and parasites are released. During their life cycle *Leishmania* parasites undergo several morpho-physiological modifications from an intracellular amastigote form to extracellular promastigotes in the midgut of the insect vector (Assis et al., 2012). In the sand fly midgut, during a process called metacyclogenesis, parasites differentiate into metacyclic promastigotes that are the infective forms injected in the vertebrate host's skin. In this proinflammatory milieu formed by sand fly-derived factors including saliva, exosomes, promastigote secretory gel (PSG), and bacteria, several cell types including macrophages and neutrophils are attracted to phagocyte the parasites. Inside those cells, metacyclic promastigotes lose their flagellum and become round-shaped forms so called amastigotes. Those divide until cell rupture and infection of the surrounding cells. During a next blood meal, the sand fly ingests macrophage containing amastigotes and the cycle continues (Peters et al., 2008; Atayde et al., 2015; Atayde et al., 2016; Dey et al., 2018).

EXTRACELLULAR VESICLES

EVs are particles formed by a lipid bilayer containing proteins and nucleic acids, which are derived and released by many types of cells (Yáñez-Mó et al., 2015; Devhare and Ray, 2018; Théry et al., 2018; Kao and Papoutsakis, 2019). Therefore, EVs can act as mediators in intercellular communication, either in prokaryotes or eukaryotes

organisms (Abels and Breakefield, 2016). These particles modulate short- and long-range events, allowing cells to communicate even at long distances. EVs regulate physiological processes, such as blood coagulation, cell differentiation and inflammation, as well as pathological processes caused cancer, neurological, cardiovascular, and infectious diseases (Raposo and Stoorvogel, 2013; Yáñez-Mó et al., 2015; Kao and Papoutsakis, 2019). EVs are present in several biological fluids, such as: bile, feces, cerebrospinal fluid, nasal, synovial, uterine fluid, breast milk, amniotic fluid, saliva, blood, semen, and urine, as reviewed by Yanez-Mó et al. (2015).

According to "Minimal Information for Studies of Extracellular Vesicles" (MISEV2018), proposed by the International Society for Extracellular Vesicles (ISEV), the term "extracellular vesicles" is referred to all sub-populations of EVs, so it is recommended to use it collectively and universally (Théry et al., 2018). Among the various subtypes of extracellular vesicles, the particles can be defined as exosomes, microvesicles and apoptotic bodies, according to their origin, size, and constituents (Kandasamy et al., 2010; Yáñez-Mó, et al., 2015; Théry et al., 2018).

Exosomes are 50–100 nm in diameter released by eukaryotic cells during differentiation, stimulation and stresses (Mathivanan et al., 2010). They are formed in endosomes as multivesicular bodies and are released after exocytosis through fusion with the plasma membrane (Raposo and Stoorvogel, 2013; Hessvik and Llorente, 2018; Skotland et al., 2019). Exosomes released by B lymphocytes, mast cells, immature dendritic cells, platelets and cytotoxic T lymphocytes act in different physiological and pathological conditions (Théry et al., 2002; Kandasamy et al., 2010; Mathivanan et al., 2010; Raposo and Stoorvogel, 2013; Record, 2014; Record et al., 2014). They promote antigenic presentation (Andre et al., 2001; Wolfers et al., 2001; Théry et al., 2002; Chaput, Andre, et al., 2003a; Chaput, Schartz, et al., 2003b; Théry et al., 2009), signaling in cancer by promoting angiogenesis the immune response and remodeling of the surrounding parenchymal tissues to favor tumor progression (Becker et al., 2016). Exosomes are also involved in the formation of the pre-metastatic niche (Mathivanan et al., 2010; Rak, 2010; Araldi et al., 2012; Raposo and Stoorvogel, 2013; Ichikawa, 2015).

Microvesicles range in size from 50 to 1,000 nm in diameter and are generated from the outer buds of the cell's plasma membrane. The formation of these particles depends on the redistribution of phosphatidylserine in the membrane bilayer and on the reorganization of the cytoskeleton, mainly through the participation of actin-myosin (Akers et al., 2013). Previous treatment activated platelets with cytochalasin D inhibits the formation of microvesicles by reduction of actin polymerization (Crespin et al., 2009; Li et al., 2013). Microvesicles are involved in several functions. For example, they modulate coagulation and fetomaternal communication (Théry et al., 2018). Apoptotic bodies also formed by external buds from the plasma membrane but have a diameter between 50 and 5,000 nm. They are released during the process of cell death, for example during apoptosis.

These different EV populations contain common sets of proteins, both of cytosolic origin, as heat shock proteins (Hsp 70 and 90) and membrane bound, as CD9, CD37, CD53, CD63, CD81, CD82, tetraspanins, and major histocompatibility complex I (MHC class I) and II (MHC class II). Tetraspanins are a family of proteins composed of four transmembrane domains (Théry et al., 2009; Li et al., 2013; Théry et al., 2018) and considered an exosomal marker, since they were first identified in exosomes derived from B lymphocytes. However, further studies have shown that tetraspanins are also found in several subpopulations of EVs (Zöller, 2009).

EVs are relevant for the communication between pathogens and host cells (Marcilla et al., 2014; Campos et al., 2015; Cwiklinski et al., 2015; Kim et al., 2015; Ancarola et al., 2017; Evans-Osses et al., 2017; Soares et al., 2017). Several protozoan parasites are capable of releasing EVs (Torrecilhas et al., 2012; Marcilla et al., 2014) including *Trichomonas vaginalis, Plasmodium* spp., *T. brucei*, and *T. cruzi* (da Silveira et al., 1979; Gonçalves et al., 1991; Trocoli Torrecilhas et al., 2009; Martin-Jaular et al., 2011; Cestari et al., 2012; Regev-Rudzki et al., 2013; Szempruch et al., 2016a; Szempruch et al., 2016b; Evans-Osses et al., 2017; Ofir-Birin et al., 2017; Ramirez et al., 2017; Ofir-Birin and Regev-Rudzki, 2019; Rossi et al., 2019; Cronemberger-Andrade et al., 2020), *Leishmania* spp. (Olivier et al., 2012; Hassani and Olivier, 2013; Hassani et al., 2014; Atayde et al., 2015; Atayde et al., 2016; Atayde et al., 2019a; Atayde et al., 2019b), and *Toxoplasma gondii* (Silva et al., 2018). These EVs have immunomodulating functions and affects the fate of the parasite in the host body (Torrecilhas et al., 2012; Campos et al., 2015; Borges et al., 2016).

T. CRUZI EVs

EVs are produced by the different life stages of *T. cruzi* (da Silveira et al., 1979; Gonçalves et al., 1991; Cestari et al., 2012; Bayer-Santos et al., 2013; Nogueira et al., 2015; Ramirez et al., 2017; Caeiro et al., 2018; Ribeiro et al., 2018; Paranaiba et al., 2019; Rossi et al., 2019) and participate in parasite host interactions (Marcilla et al., 2014; De Pablos et al., 2016; Diaz Lozano et al., 2017; Ramirez et al., 2017; Wyllie and Ramirez, 2017; de Pablos Torro et al., 2018; Retana Moreira et al., 2019; Cronemberger-Andrade et al., 2020). **Figure 1** illustrates EVs in different *T. cruzi* life stages. The lack of studies of amastigote EVs is expected since it's intracellular location and the secreted material as it is often contaminated with host cell constituents. In both epimastigotes and trypomastigotes, EVs contain several surface components of the parasite involved in the parasite adhesion, invasion, and migration in the vector's gut (Nogueira et al., 2015; Ribeiro et al., 2018; Paranaiba et al., 2019; Cronemberger-Andrade et al., 2020). Trypomastigote EVs modulates *T. cruzi* infection and affect the protozoan ability to enter and escape the parasitophorous vacuole. Several signaling cascades are activated by EV components and modulate the cell responses (Burleigh and Andrews, 1995a; Burleigh and Andrews, 1995b; Burleigh et al., 1997; Burleigh and Andrews, 1998; Caler et al., 1998; Morty et al., 1999; Kima et al., 2000). For example, EVs affects host cell actin filaments, which allows migration of lysosomes and formation of the parasitophorous vacuole required for parasite internalization (Schenkman et al., 1992; Schenkman and Eichinger, 1993; Schenkman et al., 1993; Schenkman et al., 1994; Fernandes et al., 2011a; Fernandes et al., 2011b; Barrias et al., 2019;

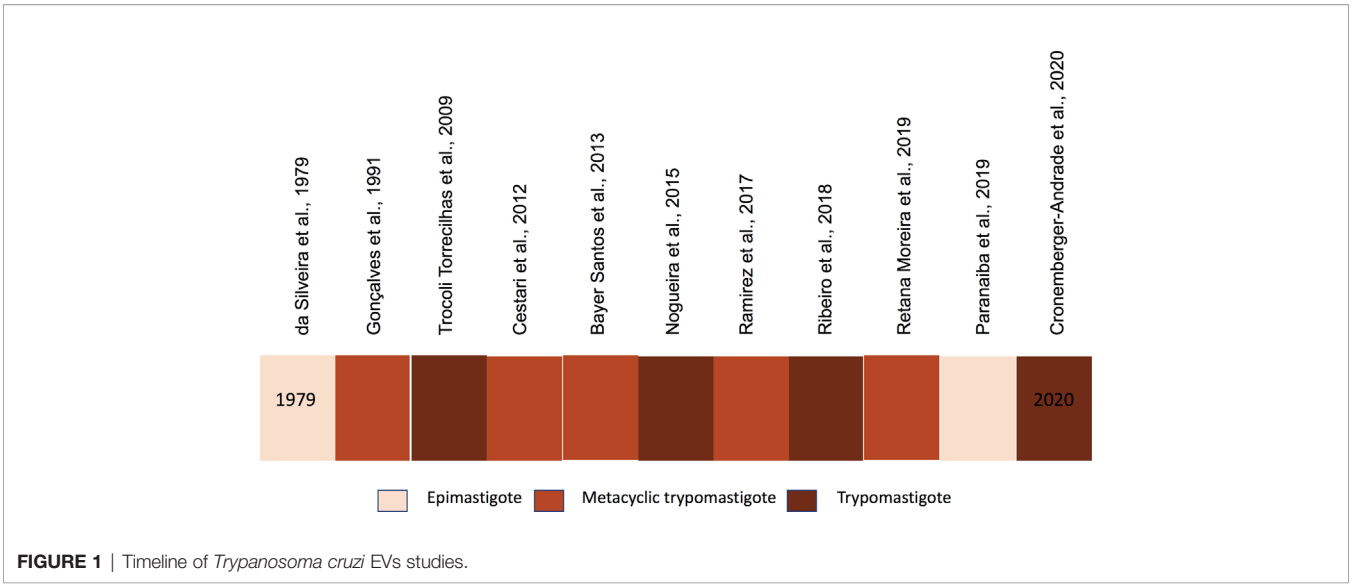

FIGURE 1 | Timeline of *Trypanosoma cruzi* EVs studies.

Reignault et al., 2019). EVs also modulate the invasion of metacyclic-trypomastigotes by inducing host cell signaling through tyrosine phosphorylation and nucleation of actin filaments (Yoshida and Cortez, 2008).

Proteomic Analysis of *T. cruzi* EVs

EVs isolated from *T. cruzi* trypomastigotes derived from mammalian cells contain most of the parasite cell-surface proteins, including major surface glycoproteins that resemble to mucins (Ribeiro et al., 2018) (**Table 1**). These glycoproteins are encoded by more than a thousand genes many of them containing heterogenous sequences rich in proline and threonine with N-acetyl glucosamine *O*-linked oligosaccharides that can be either sialylated by the parasite trans-sialidase (TS), or modified by α-galactose (McConville et al., 1990; Almeida et al., 2000; Soares et al., 2012). These carbohydrate moieties are involved in the interaction of the parasite with mammalian host cells (Buscaglia et al., 1998; Almeida et al., 2000; Almeida and Gazzinelli, 2001; Buscaglia et al., 2004; Lantos et al., 2016).

Trypomastigote EVs also contain N-linked glycoproteins, which are encoded by hundreds of different genes (TS/GP85 superfamily) (Ribeiro et al., 2018) (**Table 1**). The family encodes mostly proteins known as Tc85, GP85, complement-regulatory protein (CRP), flagellum-associated protein, 85 kDa surface antigen, c71 surface protein (Freitas et al., 2011). It is proposed that their lectin like domains in the C-terminal domain can interact with different types of surface molecules of host cells. For example, some of these glycoproteins interact with cytokeratins exposed in the mammalian surface. The superfamily includes a minor population of proteins with trans-sialidase (TS) activity, some of them with an additional 12 amino acid repeats in the C-terminus named shed-acute-phase-antigen (SAPA). There are

TABLE 1 | Extracellular vesicles isolated from Trypanosomatid: features, cargoes, effects on host parasite interaction.

Trypanosomatid	Cargoes/molecules	Effect on host	References
T. cruzi	Trans-sialidase Mucin Cruzipain GP85 RNA* GP82** Lipids*** SAPA MASP	i) Induce innate immune response *via* TLR2. ii) Increase Invasion of the parasite iii) Parasites escaping the complement attack modulate TGF-β-bearing EVs released from host cells. iv) Immunomodulation, cell attachment and regulation of host complement system.	(Cestari et al., 2012; Campos et al., 2015; Nogueira et al., 2015; Ramirez et al., 2017; Ribeiro et al., 2018)
T. brucei	VSG Protease MSPs PLC	i) Evasion from the host immune system and increase invasion BBB.	(Szempruch et al., 2016)
L. donovani *L. infantum* *L. mexicana*	GP63 LPG HSP	i) Parasite evasion by altering complement mediated lysis and promoting parasite phagocytosis.	(Silverman et al. 2010a; Hassani et al., 2011)

MSPs, Major surface proteases; PLC, phospholipase-C; BBB, Blood Brain Barrier; VSG, Variant Surface glycoprotein; LPG, lipophosphoglycan.
**RNA; ** GP82 and ***Lipid, are unpublished data.*
SAPA, Shed-Acute-phase-antigen; MASP, Mucin-Associated Surface Protein.

two distinct populations of *trans*-sialidase differentiated by the substitution of a tyrosine residue by a histidine in the active site of the sialidase domain. This substitution abrogates the hydrolytic activity, but the protein is still able to bind sialylated residues. One of the main targets of these inactive and active trans-sialidase is the CD43, a sialoglycoprotein in hemopoietic cells (Freire-de-Lima et al., 2010), which particularly relevant in the myocarditis development in experimental CD (Alisson-Silva et al., 2019). Mucins and TS/GP85 glycoproteins are associated to the surface of *T. cruzi* through glycosylphosphatidylinositol (GPI) anchors (McConville et al., 1990; Pontes de Carvalho et al., 1993; Schenkman et al., 1993; Schneider et al., 1993; Izquierdo et al., 2015). The GPI moiety is recognized by Toll 2 (TLR2) and 4 (TLR4) receptors in immune cells (Camargo et al., 1997; Almeida et al., 1999; Almeida et al., 2000; Almeida and Gazzinelli, 2001; Campos et al., 2001; Procópio et al., 2002; Campos et al., 2004; Monteiro et al., 2006). EVs, which carry these GPI-containing molecules are able to induce inflammatory responses in murine macrophages *via* TLR2, triggering the synthesis of pro-inflammatory cytokines, such as TNF-α and IL-12, in addition to the production of nitric oxide (NO) (Nogueira et al., 2015; Cronemberger-Andrade et al., 2020). Consistent with these observations, EVs from Y strain also triggered the production of those cytokines and lipid body formation *via* Prostaglandin E2 (PGE2) (Lovo-Martins et al., 2018). Furthermore, other constituents such as the *T. cruzi* Trypomastigote Alanine Valine and Serine rich proteins (TcTASV-C) might also induce EVs signaling as it is largely present in EVs from trypomastigotes from different DTUs (I, II, and VI) (de Pablos Torro et al., 2018).

Cysteine-proteases such as cruzipain and the surface metalloproteases, named GP63 that modulate several processes in the host cells are also present in trypomastigotes EVs. These proteases are involved in several process affecting the infection, including the process of parasite internalization and survival (Scharfstein and Lima, 2008; Alvarez et al., 2012; Watanabe Costa et al., 2016). In addition, cruzipain is also involved in the activation of the host's immune system (Duschak and Couto, 2009; Acosta et al., 2012; Soprano et al., 2018). Therefore, these peptidases can act independently of the presence of the parasite on the host through circulating EVs. Proteins from cytoskeletal proteins, such as tubulin, heat shock proteins, and other soluble proteins, were also detected in the total EV fractions (Ribeiro et al., 2018).

Another class of proteins found in some proteomic analysis of EVs are cytoskeleton-related proteins, such α- and ß-tubulin, kinesins, and myosins. These cytoskeleton proteins are usually of exosomes from eukaryotic cells. We also identified other proteins related to exosomes and vacuoles, such as heat-shock protein 85 (HSP85), elongation factor 1-alpha, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), vacuolar ATP synthase subunit B, and tetratric-peptide-repeat (TPR) protein (Théry et al., 2018; Doyle and Wang, 2019).

EVs of metacyclic-trypomastigotes also contain major surface components of the parasite, including the gp35/50 mucin-like glycoproteins and members of trans-sialidase family of glycoproteins (Bayer-Santos et al., 2013). These surface components participate in the cell adhesion and invasion of the metacyclic-trypomastigotes (Schenkman et al., 1993; Santori et al., 1996; Neira et al., 2002; Neira et al., 2003; Eickhoff et al., 2010; Cortez et al., 2012; Cortez et al., 2014; Cordero et al., 2019). Gp82 glycoprotein, a metacyclic specific member of the gp85/trans-sialidase family activates mTOR signaling cascades that allow lysosome migration and fusion with the parasitophorous vacuole producing acidification and parasite escape to the cytosol (Cortez et al., 2012; Cortez et al., 2014; Cordero et al., 2019). It also binds to LAMP-2 receptor favoring parasite internalization (Andrade and Andrews, 2005; Albertti et al., 2010; Andrade, 2019). However, previous incubation with EVs released by metacyclic-trypomastigotes decrease invasion of HeLa cells mediated by gp90, another member of the family (Yoshida et al., 1990) and inhibits cell invasion (Rodrigues et al., 2017). These proteins are also released in the soluble form by the endogenous activity of a GPI phospholipase C activity (Schenkman et al., 1988). Therefore, EVs in this stage provide signals that can either increase up to10 times cell invasion. One possibility is that this combination provides specific responses among different host cells and tissues. EVs from epimastigote and metacyclic-trypomastigote forms also contain mucin-like surface glycoproteins GP35/50, typical of the insect stages of the parasite (Schenkman et al., 1993; Bayer-Santos et al., 2013). These molecules act as adhesion molecule in the interaction of the parasite with the host (Ruiz et al., 1993). Therefore, GP35/50 in EVs could modulate their interaction with host cells. Another protein in the epimastigote EVs is the flagellum calcium binding protein (FCaBP), which is linked to internal membrane leaf of the parasite and modulates several proteins through Ca^{2+} signaling (Buchanan et al., 2005). In fact, microvesicles isolated from metacyclic-trypomastigotes forms enhance communication between *T. cruzi* and host cell promoting invasion (Ramirez et al., 2017). These vesicles inhibit complement-mediated lysis and enhance invasion of Vero cells.

EVs Cargoes Lipids and Nucleic Acids

Studies of non-protein components in EVs are still in their infancy and a limited number of publications is available for trypanosomatids. This provides a promising field to be explored by researchers. Purified EVs of different *T. cruzi* isolates contains phospholipids including cardiolipin, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Also, they possess an unidentified non-polar component, as detected after extraction and thin-layer chromatography (unpublished). Several fatty acids were detected after extraction of total lipids by gas chromatography and mass spectroscopy (GC-MS) with a large variation (25 to 8) according to the strain. The four common compounds species were: 5-octadecene, 1-octadecene, 1-hexadecene, and 3-heptadecene with 1-Docosene, But-2-enoic acid, amide, 3-methyl-N-metallyl-, heneicosane, tritetracontane found in the Y strain but not in the YuYu (unpublished). As the fatty acids are critical to induce pro-inflammatory responses, these EV components may be important for TLR activation. We also detected RNA, but not DNA in EVs isolated from trypomastigote but their nature and composition should be further investigated. Epimastigotes were also found to release fragments of tRNA that could modify host cells behavior (Garcia-Silva et al., 2014).

Internalization of EVs Into the Target Cells

Initially, our group showed that *T. cruzi* EVs could be rapidly internalized *in vitro* by cells suggesting that they could also activate

intracellular pathways in the host (Torrecilhas et al., 2012). Further, we showed that those EVs could also act as TLR2 agonists suggesting that perhaps both mechanisms could occur (Nogueira et al., 2015). Recently, *T. cruzi* EVs incubated with Toll-like-receptor 2 (TLR2)-transfected CHO cells increased invasion by the parasite. In parallel, they elicit the translocation of NF-κB and gene expression of proinflammatory cytokines (TNF-α, IL-6 and IL-1β), and STAT-1 and STAT-3 signaling pathways (Cronemberger-Andrade et al., 2020). Since GPI-anchored proteins in EVs are TLR2 ligands, this activation is probably one of the mechanisms triggered by EVs.

Role of *T. cruzi* EVs on Host Responses

Incubation of *T. cruzi* EVs, or parasite infection of host cells induce several responses, including the increased secretion of EVs by host cells (Ramirez et al., 2017; Choudhuri and Garg, 2020; Cronemberger-Andrade et al., 2020). The host EVs contain some parasite antigens and nucleic acids that possible elicit activation of intracellular Toll receptors and increase the inflammatory response. Interestingly, the presence of the remaining parasite DNA processed by Poly(ADP-ribose) polymerase 1 (PARP1) and cyclic GMP-AMP synthase (cGAS) amplified the inflammatory signal.

The interaction of trypomastigote EVs with host cells with epithelial cells or macrophages occurs rapidly and after 15 min parasite proteins are detected by specific antibodies and increase in cytosolic Ca^{2+} (unpublished). This finding is related to increased invasion by the parasite after incubation with the parasite EVs (Cronemberger-Andrade et al., 2020) in agreement with the requirement for Ca^{2+} mobilization during cell invasion (Burleigh and Andrews, 1998). The EVs could also affect the host cell surface cytoskeleton and that might facilitate parasite internalization.

In Vivo Studies

Mice that received EVs prior to infection developed severe heart pathology, with intense inflammatory reaction and higher number of intracellular amastigotes (Trocoli Torrecilhas et al., 2009). The inflammatory infiltrates contain CD4+ and CD8+ T lymphocytes and macrophages and decrease of iNOS spots expression. Also, IL-4 and IL-10 mRNAs in the heart from animals treated with EVs isolated from parasites. The molecules present on EVs act as primers of the host immune system, facilitating the establishment of the infection and inflammation in the animal model. The modulation of prostaglandin is also modulated by pre-injection of parasite EVs (Lovo-Martins et al., 2018).

T. cruzi EVs derived from epimastigotes were evaluated during interaction of the parasite with triatomine bugs *Rhodnius prolixus* and *Triatoma infestans*. EVs were artificially offered to the insects prior to infection with epimastigotes. Pre-feeding with EVs delayed early parasite migration to the rectum in *R. prolixus*, but not in *T. infestans*, affecting parasite-host interaction during the initial events of infection in the invertebrate's gut (Paranaiba et al., 2019). However, the mechanisms underlying parasite retainment remain to be explored.

Therefore, the release of EVs reflects a strategy developed by the parasite to secrete its main surface components that act, for example, facilitating processes such as adhesion and invasion to the host cell (Fernandes, Andrade, et al., 2011a; Fernandes, Cortez, et al., 2011b; Cronemberger-Andrade et al., 2020), as well as evading the host immune system (Cestari et al., 2012). EVs modify the host allowing a more favorable environment for parasite survival and, consequently, to promote infection and possible disease progression (Trocoli Torrecilhas et al., 2009; Cestari et al., 2012; Torrecilhas et al., 2012). It is relevant that different parasite isolates of *T. cruzi* showed surface variations, and most likely different EVs population, that influence the differential activation of the immune response in the host. In fact, EVs of two different strains of the parasite (Y and YuYu) have qualitative and quantitative differences in content, which directly interfere with virulence and the rate of infectivity in different cell types (Ribeiro et al., 2018).

Our finding that several glycoconjugates and other virulence factors are released as main components of EVs, indicates that these vesicles could play a major role in the interaction with host cells. Those key elements may represent novel targets for chemotherapy and vaccines.

Trypanosoma brucei EVs

T. brucei bloodstream forms nanotubes originated from the flagellar pocket that breakdown into EVs that contain molecules that are present in the parasite surface (Geiger et al., 2010). These EVs contain mostly VSGs but are particularly enriched in flagellar proteins and proteins involved in the parasite virulence factors (Szempruch et al., 2016a; Szempruch et al., 2016b). These EVs are in the range of 50 to 100 nm in diameter, which correspond to exosomes size. The proteins identified in the secretome of *T. b. rhodesiensis* are also related to the parasite survival strategies as they contain the serum resistance-associated protein (SRA) that allow the parasite to survive in human blood (Geiger et al., 2010). It has been shown that EVs derived from *T. brucei* are highly fusogenic and transfer lipids and VSG glycoproteins to erythrocytes. This alteration in erythrocytes membrane results in its phagocytosis by macrophages in the liver and spleen leading to anemia (Szempruch et al., 2016a; Szempruch et al., 2016b). The EVs released by the parasite may provide additional cues for the parasite scape from the host. For example, VSGs and parasite DNA containing CpG, present in EVs, are recognized by SRA and Toll-like receptor 9, respectively. These interactions could potentiate a strong immune response and modulate macrophage and dendritic cells to activate TNF-α, IL-6, and IL-12 (Stijlemans et al., 2016). *T. brucei* EVs are also proposed to modulate changes in the vascular endothelium (Varikuti et al., 2020).

Additionally, microvesicles (MVs) isolated from *T. brucei* infected hosts induce a progression meningo-encephalitic late stage (S2), inflammatory processes and modulation in astrocytes that resembled the one produced by IFN-γ, a central mechanism in HAT pathogenesis (Dozio et al., 2019).

T. brucei has at least two types of EVs. One, that is continuously released by the parasite, and the other that occurs upon parasite stress, as those affecting RNA transcription and processing. In these parasites, pre-mRNAs are transcribed through long polycistronic gene arrays and further processed by trans-splicing with a spliced leader (SL) exon and by polyadenylation (Clayton, 2019). The spliced leader (SL) RNA is processed in the cytosol and upon trans-splicing inhibition is incorporated into multivesicular bodies through the endosomal sorting complexes required for

transport (ESCRT), in a similar way as microRNA is secreted in exosomes of mammalian cells. These exosomes have been shown to affect the motility pattern of the parasite itself (Eliaz et al., 2017). These EVs are internalized by insect form (procyclics of *T. brucei* in the insect host and are key components in the parasite-parasite communication. Taken together, the release of *T. brucei* EVs plays a role in cell-cell communication with the host and among themselves and open new insights to development new potential therapeutic targets or diagnostic markers.

BIOGENESIS OF EVs IN TRYPANOSOMES

Very little is known how the EVs are formed and biogenesis whether they are released in special situations, for example during parasite differentiation. Recently, it has been proposed that the parasite surface resembles a quilt with different types of surface proteins distributed in separate patches (Mucci et al., 2017). The GP85/Trans-sialidase proteins form surface aggregates exclude the mucin-like glycoproteins and each patch might be enriched in different groups of EVs either as a consequence of their secretory pathway or biophysical properties (Niyogi et al., 2014). Therefore, it is expected that different EVs might coexist and affect differently the host cells. Many orthologs of the secretory machinery of eukaryote cells (ESCRT machinery) have been described in *T. brucei* (Silverman et al., 2013) and it was shown that inhibiting Vsp36 (an ESCRT component) compromises EVs secretion, but not of nanotube derived EVs (Eliaz et al., 2017). Therefore, different mechanisms of secretion may occur for these distinct types of particles that are also biochemically distinct and functions. How and whether the release of EVs from the membrane is regulated is still a matter of investigation.

Leishmania EVs

As well covered in the previous sections, parasites of the *Leishmania* genus -being also trypanosomatids are known to secrete proteins *via* their endoplasmic reticulum and Golgi apparatus (McConville et al., 2002; Corrales et al., 2010) and flagellar pocket (Field et al., 2007). In this context, several important virulence factors of *Leishmania* (e.g. Zinc-metalloprotease GP63 and LPG) can be released within the insect vector's gut and inoculated to the mammalian host during the sand fly's blood meal (Yao et al., 2003; Joshi et al., 2005). Markedly, as previously discussed for *T. cruzi* and *T. brucei*, *Leishmania* parasites require a non-conventional protein secretion system to release proteins lacking a signal peptide. In this way, exosomes have been found to be a selective and efficient pathway for proteins to leave *Leishmania* (Théry et al., 2009; Atayde et al., 2015). In fact, the great majority of *Leishmania* species studied to date revealed that only 5-9% of exosomal proteins have a signal peptide (Silverman et al., 2008; Atyame Nten et al., 2010; Geiger et al., 2010).

Since 2008, several studies reported the release of exosomes by various species of *Leishmania* when cultured *in vitro*, as well as during their replication and development into metacyclic *Leishmania* promastigotes. Notably, in both cases, *in vitro* or *in vivo* produced *Leishmania* exosomes/EVs strongly contributed to enrich the parasite population with major virulence factors such as GP63. Moreover, exosomes have shown to alter myeloid cell signaling and microbicidal functions as strongly as whole promastigotes (Silverman et al., 2010a; Silverman et al., 2010b; Hassani et al., 2014; Atayde et al., 2015). The growing knowledge on leishmanial exosomes highlights the critical role of these extracellular vesicles not only in the infectious process but also in the progression of the parasites within the mammalian host leading to the various leishmaniasis-related pathologies.

Olivier's initial observation of GP63 clustered within vesicles being transferred to the macrophage led us to study whether *Leishmania* may produce EVs, thus including exosomes (Gomez et al., 2009; Gomez and Olivier, 2010). Markedly, studying the temperature-induced exoproteome of *Leishmania mexicana*, we obtained our first clear evidence demonstrating that *Leishmania* parasites can release small vesicles (Hassani et al., 2011). However, Reiner's laboratory was the first one to provide a clear demonstration of *Leishmania* parasites secreting well characterized exosomes (Silverman et al., 2010a).

During Olivier's lab first study, they found that by emulating the transition of the parasite from vector to the mammalian host, the temperature shift was inducing an increase of proteins secretion *via* exosome-like vesicles on the surface of the parasite. Proteomic analysis revealed that almost all *Leishmania* exosomal proteins lacking a signal peptide were secreted in a non-conventional manner (Hassani et al., 2011). Of utmost interest, it was found that those EVs showed similar capacities as whole promastigotes to hijack host macrophage signaling and functions, in part by inducing host phosphor-tyrosine protein (PTP) negative regulatory mechanisms. Using GP63-deficient *Leishmania major* parasites, we demonstrated that the absence of this virulence factor, which is greatly enriched in wild-type *L. major* exosomes, was almost completely abrogating their capacity to modulate host immune response comparatively to its wild-type counterpart (Hassani et al., 2014). This is different from *L. infantum* LPG1 -/- mutants, where the lack of this glycoconjugate did not affect NO and cytokine induction by its EVs (Nogueira et al., 2020). This further demonstrates the critical role played by *Leishmania* virulence factors enriched in leishmanial exosomes. Previous works reported by Silverman and colleagues were seminal to strengthen the role of *Leishmania* EVs as macrophage immunomodulators (Silverman et al., 2010a; Silverman et al., 2010b) such as for instance, to modify IFNγ-induced cytokines secretion by human monocytes cultured in the presence of *L. donovani* vesicles. Moreover, in an *in vivo* context, $CD4^+$ lymphocytes from mice inoculated with leishmanial exosomes were more prone to produce immunosuppressive cytokines (i.e. IL-10, IL-4) and exacerbate pathology (Silverman et al., 2010b). More recently, EVs from New-World, dermotropic *L. amazonensis* were found to be more pro-inflammatory, triggering the production of NO, IL-6 and TNF-α in a TLR4/TLR2/NF-κB-dependent fashion (Nogueira et al., 2020). On the other hand, in the same study, EVs from *L. infantum* and *L. braziliensis* were not able to induce significant levels of those cytokines (Nogueira et al., 2020) but induced IL-10 (Castelli et al., 2019). Although this study was *in vitro*, *in vivo* studies with *L. amazonensis* EVs in B-1 cells and *L. infantum* confirmed their pro-inflammatory activity (Barbosa et al., 2018; Perez-Cabezas et al.,

2019; Toledo et al., 2020). Pre-injection with EVs from those species induced cytokine production and increased parasite burden. Regarding *L. infantum*, proteomic analysis has suggested several molecules responsible for their functional activities (Santarem et al., 2013). However, a more detailed proteomic analysis was provided comparing procyclic and metacyclic-like forms (Forrest et al., 2020). Those analysis found approximately 50 virulence factors and some differentially expressed proteins in each stage. Most of the studies have focused on qualitative characterization of exoproteomes especially in *L. infantum* (Marshall et al., 2018). However, comparative exoproteome analysis from different *Leishmania* species are still scarce. For example, *L. infantum* and *L. mexicana* proteomics have been reported under the same conditions (Lynn et al., 2013). There is still a need for comparisons including a wider panel of *Leishmania* species. Those will provide information on how polymorphisms in the EVs protein could affect parasite-host interplay in different clinical forms. Collectively those findings strongly support the paramount role of exosomes released by various *Leishmania* spp. to trigger major immunomodulatory actions favorable for guaranteeing the infection and survival of the parasites in their hosts.

In a parallel study, exosomes released by macrophages infected with *L. mexicana* were found to be solely enriched in GP63 in terms of *Leishmania* proteins, whereas, as expected, the rest of the proteins corresponded to those of the macrophage (Hassani and Olivier, 2013). Macrophage exosomes released in response to LPS and *Leishmania* stimulation were found to induce macrophage inflammatory genes slightly differently in comparison to exosomes derived from naïve macrophages, reinforcing that EVs released during infectious context could be more prone to induce inflammatory reaction in the host.

Leishmania Exosomes and Sand Fly Midgut

One critical matter in the field of EVs, is to realize that the great majority of the studies performed in various fields (i.e. immunology, cancer, stem cells) were conducted with vesicles isolates from biological fluids or from cell cultures. However, Olivier's team finally reported a pioneering finding showing that *Leishmania* EVs were being formed and freed within sand fly midgut and were delivered by *Leishmania* metacyclic promastigotes to the mammalian host during the blood feeding of the vector (Atayde et al., 2015). This co-transmission was revealed to be highly inflammatory in Balb/c mice and concurring to the development of hyper skin ulceration under a Th17-type response. Markedly, this study is the first to demonstrate *Leishmania* EVs enriched with GP63 are key sand fly-egested virulence factors, revealing *Leishmania* exosomes as critical infectious instruments for the proper development of leishmaniasis.

While *L. major* exosomes induce an IL-17a-mediated immune response (Atayde et al., 2015), Reiner and colleagues (Silverman et al., 2010a; Silverman et al., 2010b) found that IL-4 was a cardinal cytokine ruling over IL-17a in the exacerbation of skin inflammation. Of note, while in Reiner's study, this phenomenon was observed in a context where exosomes were used for vaccination first prior to the challenge with promastigotes, Olivier's lab experiments tried to mimic the physiological setup of the sand fly when taking a blood-meal and both exosomes and parasites are simultaneously transferred. Moreover, and according to previous reports, IL-17a is a very powerful signal to recruit neutrophil and to favor cutaneous leishmaniasis development in mouse and human skin pathology (Maurer et al., 2006; Boaventura et al., 2010; Griewank et al., 2014; Dietze-Schwonberg et al., 2019). Markedly, a study from Olivier's laboratory strongly supports that *Leishmania* EVs concur to neutrophil migration toward the inoculation site (Hassani et al., 2014). Finally, several derived-insect components and parasite/bacteria were shown to be important during the sand fly bite (Dey et al., 2018). Those induced IL-1β induction *via* inflammasome by neutrophils and were important for *L. donovani* visceralization. Although a mixture of different components is found in the inoculum, the solely role of EVs in this process is an open field to be explored.

Hijack of Leishmania Exosomes by LRV1 Endovirus

Over the last 10 years, there have been several reports showing that certain *Leishmania* species belonging to the *Viannia* subgenus, which are lower eukaryotic organisms, can be infected by viruses. For instance. *L. (V.) guyanensis* is known to be infected by the endovirus *Leishmania* RNA Virus 1 (LRV1) (Guilbride et al., 1992; Stuart et al., 1992). Of note, this viral culprit within *Leishmania* was found to exacerbate the development of mucocutaneous leishmaniasis due to its capacity to trigger a strong TLR3/TLR7-dependent inflammatory response, which directly correlates with an enhancement in *Leishmania* metastatic behavior that is characteristic of mucocutaneous leishmaniasis (Ives et al., 2011). Consequently, understanding how this virus is transmitted is a critical step towards a better comprehension of not only *Leishmania* pathogenesis but also virus evolution and adaptation mechanisms.

Of interest, it is believed that LRV1 virus can propagate within *L.* (*V.*) *guyanensis* during their division, as well as to influence the host immune cells while dead parasites release the non-enveloped form of the virus (Olivier, 2011). While this remains a potential mechanism of viral propagation, some other pathways could be involved in such complex process. Olivier's team recent observations using both *in vitro* cultures and *in vivo* sand fly vector approaches, showed that *Leishmania* promastigotes can actively produce and release exosomes in natural condition. Based on this discovery, they further investigated whether LRV1 could exploit *Leishmania* exosomal pathway to safely exit the promastigotes. Of utmost interest, they found that 30% of the exosomes released by *L.* (*V.*) *guyanensis* contained the whole LRV1 endovirus, conferring the virus protection against the different adverse conditions that it will encounter in the external milieu (Atayde et al., 2019a; Olivier and Fernandez-Prada, 2019). Additionally, LRV1 "exosomal shield" favored the virus to efficiently infect naïve *Leishmania* (*V.*) spp., which naturally fuse to and integrate EVs content to potentially gain information, as reported for *T. brucei* (Eliaz et al., 2017). However, in this case, naïve parasites rapidly become virally infected and see their level of infectivity modified, as previously discussed. In addition, LRV1 contained within *Leishmania* exosomes were found to be

responsible for the TLR3-dependent induction of inflammasome and exacerbation of *L. (V.) guyanensis* infection (Barrias et al., 2019; Olivier and Zamboni, 2020).

Of note, although viral particles and exosomes are very alike, they share several physical and structural similitudes. A variety of viruses infecting humans have been found to use host-cell exosomes as vessels for transporting their nuclear materials and capsids, also impacting the overall viral pathogenesis and virus-induced subsequent pathologies (Meckes and Raab-Traub, 2011; Alenquer and Amorim, 2015). However, LRV1 using *Leishmania* exosomes is the first demonstration of exosomes harboring a whole virion. Overall, it is clear that *Leishmania* parasites of the *Viannia* subgenus and their LRV1 endovirus represent an impressive mutualistic relationship in which both entities are interconnected by exosomes.

Leishmania Exosomes in Drug Resistant Parasites

Control of leishmaniasis is based on a very short list of chemotherapeutic agents headed by pentavalent antimonials, followed by miltefosine and amphotericin B. These drugs are far from ideal due to host toxicity, elevated cost, limited access, and high rates of drug resistance (Leprohon et al., 2015; Olivier and Fernandez-Prada, 2019; Saha et al., 2020). As leishmaniasis is a vector-transmitted disease, the spread of drug-resistant parasites is ultimately dependent on their transmission potential for which within- and between-host ecology plays a key role. Markedly, as discussed through all this review, EVs play a major role in all these transmission/interaction events. Considering that the molecular content of eukaryotic EVs is a fingerprint of the origin cell reflecting its physiological/functional status, Fernandez-Prada's laboratory recently explored the composition of leishmanial EVs in the context of drug resistance, in collaboration with Olivier's group (Douanne et al., 2020). This was the first study describing *L. infantum* EVs' core proteome, as well as all those proteins specifically enriched in EVs released by antimony-, miltefosine- and amphotericin-resistant parasites. Fernandez-Prada's work showed for the first time that drug-resistance mechanisms can induce changes in the morphology, size, and distribution of EVs in *Leishmania*, with drug-resistant parasites releasing larger vesicles when compared to the wild-type counterpart (especially amphotericin B-resistant parasites with vesicles larger than 200 nm). Of note, several virulence factors (i.e. GP63), putative transcription factors (i.e. CBF/NF-Y), as well as proteins encoded by drug-resistance genes (i.e. antimony drug-resistance gene *mrpA* (Douanne et al., 2020) were identified among drug-specific enriched proteins. In relation to these last, MDR transporters were shown to transmitted from drug-resistant to drug-sensitive tumor cells by exosomes *in vivo* and *in vitro* (Lopes-Rodrigues et al., 2016), which points to a possible similar mechanism of drug-resistance transmission in *Leishmania* parasites. Currently ongoing and future studies will bring new knowledge on how EVs released by drug-resistant parasites contribute to drug resistance and, in a more general context, to the survival of *Leishmania* parasites through all the stressful conditions encountered during their life cycle.

FUTURE APPLICATIONS AND THERAPEUTIC USE OF HOST AND TRYPANOSOMATID EVs

One major challenge for the near future is the development of new and improved therapies for the treatment and prevention of diseases caused by trypanosomes. The EVs from these parasites can offer new potential developments for diagnosis, follow-up of treatment responses, monitoring disease progression and determining the prognosis. EVs can also be helpful in devising new vaccine targets. For example, we have found that immunization mice with *T. cruzi* EVs obtained from trypomastigotes can be induce protection against experimental Chagas disease (unpublished). Nevertheless, the molecules found in EVs responsible for this protection are yet to be determined and will in turn, represent novel biomarkers. In addition, EVs isolated from patients can be a biomarker during early clinical stage, acute and chronic phase. These EVs can be associated with clinical stage or allow the follow up in clinical trials new drugs. Further investigation of these EVs regarding the micro-RNA and long coding RNA, for example, could provide relevant information about Chagas's disease progression in humans. We have started to compare the circulating EVs isolated from plasma from patients with Chronic Chagas Disease (CCD). The plasma from CCD released less EVs with differences in their ability to induce cytokine production. CCD patients EVs were able to induce a differential production of IFN-γ and IL-17 in relation to controls, with differences being more evident in earlier/less severe stages of the disease (Madeira et al., 2020 submitted).

CONCLUSIONS

EVs studies increase every year, since they represent valuable biological markers (**Table 1**) with a powerful diagnostic and therapy function. Several works published in the literature show that the EVs released by trypanosomatids might play a fundamental role in the pathogenesis of CD, HAT and several *Leishmania* species and in the host's immune response to the parasite (Trocoli Torrecilhas et al., 2009; Cestari et al., 2012; Torrecilhas et al., 2012; Santarem et al., 2013; Hassani et al., 2014; Atayde et al., 2015; Nogueira et al., 2015; Barbosa et al., 2018; Ribeiro et al., 2018; Toledo et al., 2020). These major effects are depicted in **Figure 2**. However, little is known about the mechanisms involved in the EV release process and whether this occurs as a result of damage to the parasite. In addition, the understanding of the relationship between different strains of *T. cruzi* (with different degrees of virulence) in the infectious process and in the production of EVs is still scarce. The same is valid for the different species of *Leishmania* that cause different type of diseases. Therefore, more studies are needed, to understand the EVs role in the pathogenesis of these parasitic endemic diseases, as well as in the mechanisms of pathogen-host interaction. This may be the starting point for the development of new preventive and therapeutic strategies with more efficient pharmacological targets against these parasitic which continues difficult to prevent, and treat and, whose current available drugs are toxic and not completely effective. The papers published in the

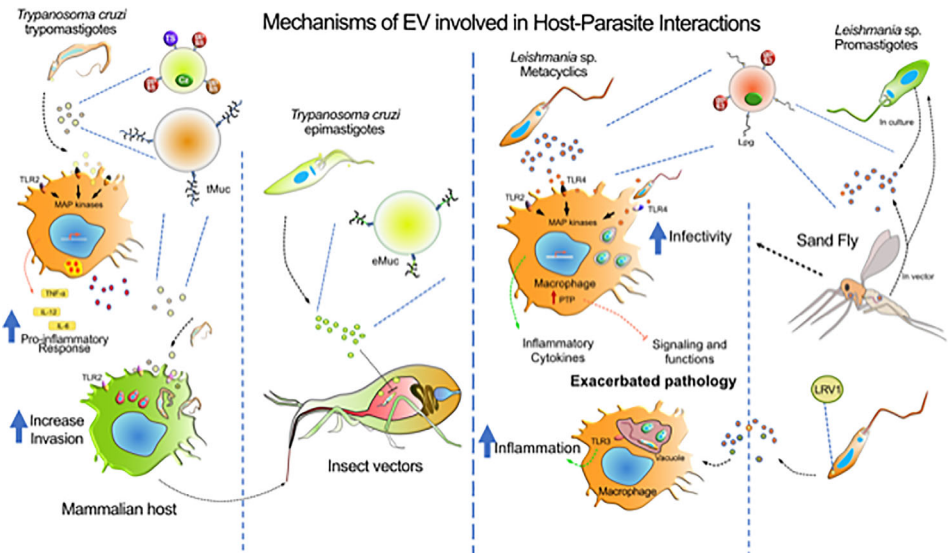

FIGURE 2 | Mechanisms of EV involved in host-parasite interactions. The diagram represents the major contributions of EVs from *T. cruzi, Leishmania* sp. and host cells during the infection by these parasites. *T. cruzi* release heterogeneous populations of EVs containing surface mucin-like glycoproteins (tMuc and eMuc), GPI anchored proteins (GP63 protease, GP85, and TS superfamily members) and soluble proteins (cruzipain, Cz), which interact with macrophages through TLR2 receptors which activate MAP kinases and induce a pro-inflammatory response (TNF-α, IL-12, and IL-6). Macrophages also release EVs that might affect the host immune system. The TLR2—*T. cruzi* EVs interaction enhances further parasite invasion. In the insect vector, the parasite epimastigote EVs delays translocation of parasites through intestine. *Leishmania* metacyclics release EVs enriched with GP63 and LPG, that could interact with macrophages *via* TLRs and, depending on the species, to induce pro- and anti-inflammatory responses. GP63-enriched EVs were found to induce host protein tyrosine phosphatases concurring to alter macrophage signaling and microbicidal functions. However, receptors involved in EVs recognition concurring to augment infection of phagocytes is still not identify. *Leishmania* virus (LRV1) incorporate within Leishmania exosomes was found to induce myeloid cells (macrophage, neutrophil) inflammasome in a TLR-dependent fashion concurring to exacerbate skin pathology. *Leishmania* exosomes are released in the gut of the sand Fly vector and have been found to be co-inoculated with promastigotes during blood meal. Revealing that this co-inoculation can exacerbate myeloid cells infection and skin pathology development.

literature, so far, show the important role that these vesicles play in the infectivity and in the modulation of the host's immune response by the parasite, however the study in this field still has some gaps to be filled, mainly in functional biological aspects that allow a better understanding of the mechanisms and conditions for the release of these vesicles. Therefore, more studies are needed, as one of the fundamental points for the control of endemic parasitic diseases is the understanding of mechanisms involving the pathogen-host interaction.

AUTHOR CONTRIBUTIONS

All authors have been equally involved in writing and editing. Figures have been done by SS and AT, and edited in addition by MO. All authors contributed to the article and approved the submitted version.

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Multiparameter Flow Cytometry Analysis of the Human Spleen Applied to Studies of Plasma-Derived EVs From *Plasmodium vivax* Patients

Melisa Gualdrón-López[1,2‡], Míriam Díaz-Varela[1†‡], Haruka Toda[1], Iris Aparici-Herraiz[1], Laura Pedró-Cos[2], Ricardo Lauzurica[3], Marcus V. G. Lacerda[4,5], Marco Antonio Fernández-Sanmartín[2], Carmen Fernandez-Becerra[1,2] and Hernando A. del Portillo[1,2,6*]*

[1] ISGlobal, Hospital Clinic - Universitat de Barcelona, Barcelona, Spain, [2] IGTP: Germans Trias i Pujol Research Institute, Barcelona, Spain, [3] Nephrology Service, Germans Trias i Pujol University Hospital, Badalona, Spain, [4] Fundaçao de Medicina Tropical Dr. Heitor Vieira Dourado, Manaus, Brazil, [5] Instituto Leônidas & Maria Deane (ILMD), Fiocruz, Manaus, Brazil, [6] ICREA: Catalan Institution for Research and Advanced Studies, Barcelona, Spain

The spleen is a secondary lymphoid organ with multiple functions including the removal of senescent red blood cells and the coordination of immune responses against blood-borne pathogens, such as malaria parasites. Despite the major role of the spleen, the study of its function in humans is limited by ethical implications to access human tissues. Here, we employed multiparameter flow cytometry combined with cell purification techniques to determine human spleen cell populations from transplantation donors. Spleen immunophenotyping showed that $CD45^+$ cells included B (30%), $CD4^+$ T (16%), $CD8^+$ T (10%), NK (6%) and NKT (2%) lymphocytes. Myeloid cells comprised neutrophils (16%), monocytes (2%) and DCs (0.3%). Erythrocytes represented 70%, reticulocytes 0.7% and hematopoietic stem cells 0.02%. Extracellular vesicles (EVs) are membrane-bound nanoparticles involved in intercellular communication and secreted by almost all cell types. EVs play several roles in malaria that range from modulation of immune responses to vascular alterations. To investigate interactions of plasma-derived EVs from Plasmodium vivax infected patients (PvEVs) with human spleen cells, we used size-exclusion chromatography (SEC) to separate EVs from the bulk of soluble plasma proteins and stained isolated EVs with fluorescent lipophilic dyes. The integrated cellular analysis of the human spleen and the methodology employed here allowed in vitro interaction studies of human spleen cells and EVs that showed an increased proportion of T cells (CD4+ 3 fold and CD8+ 4 fold), monocytes (1.51 fold), B cells (2.3 fold) and erythrocytes (3 fold) interacting with PvEVs as compared to plasma-derived EVs from healthy volunteers (hEVs). Future functional studies of these interactions can contribute to unveil pathophysiological processes involving the spleen in vivax malaria.

Keywords: ***Plasmodium vivax*, human spleen, extracellular vesicles, multiparameter flow cytometry, interaction**

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Tuan M. Tran,
Indiana University Bloomington, United States
Ziv Porat,
Weizmann Institute of Science, Israel

***Correspondence:**
Melisa Gualdrón-López
melisa.gualdron@isglobal.org
Hernando A. del Portillo
hernandoa.delportillo@isglobal.org

†Present address:
Míriam Díaz-Varela, Department of Biochemistry, WHO Collaborative Center for Research and Training in Immunology, University of Lausanne, Epalinges, Switzerland

‡*These authors have contributed equally to this work*

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INTRODUCTION

The spleen is a secondary lymphoid organ with multiple functions in physiology and immunity, including the removal of senescent red blood cells (RBCs) from circulation, the recycling of iron and the coordination of innate and adaptive immune responses against blood-borne pathogens (Bowdler, 2002; Mebius and Kraal, 2005). Functionally, the spleen is organized in two distinct compartments: i) the white pulp, which is responsible for initiation of adaptive immune responses against blood pathogens and is composed by B cell and T cell zones; ii) the red pulp, which contains neutrophils, monocytes, dendritic cells (DCs), γδ T cells and macrophages, and its functions include the monitoring of aged, dead or opsonized RBCs as well as pathogen surveillance (Lewis et al., 2019). The spleen is involved in the control of bacterial, viral, fungal and parasitic infections, including malaria (Bronte and Pittet, 2013), a world threatening infectious disease caused by several species of the genus *Plasmodium* spp. In 2018, this disease registered 228 million cases and 405,000 deaths globally (World Health Organization, 2019).

Despite the fundamental role of the spleen, most of our current understanding of its structure and function in humans comes from extrapolations from rodent species. Animal models enable more convenient access to tissue samples and have allowed to investigate the spleen using techniques such as multiparameter flow cytometry in a great variety of physiological and pathological conditions (Steiniger, 2015). Comparisons between mice and human spleen architecture have shown similarities between species but also have revealed important structural differences, suggesting functional divergence requiring further investigation in the human spleen (Steiniger, 2015). The technical and ethical implications to perform such studies using human tissue have hampered our advance in this regard. Human spleen studies have been mostly performed in postmortem samples or biopsies of particular tissue sites using non-specific cellular architecture staining methods and single antigen characterization by immunohistochemistry (Lewis et al., 2019). Several studies of the immunological function of particular splenic cell types in humans have been reported (Buffet et al., 2006; Langeveld et al., 2006; Velásquez-Lopera et al., 2008; Petvises et al., 2016; Meinderts et al., 2017; Nagelkerke et al., 2018); however, to the best of our knowledge, no approaches have addressed the description of the whole spectrum of cells in the human spleen. A recent study using a large cohort of organ transplantation donors as source of lymphoid organs, including the spleen, has shown the most complete characterization of human spleen immune cells including myeloid populations as monocytes and neutrophils as well as lymphoid cells at different activation states (Carpenter et al., 2018a). These studies highlighted the relevance of organ transplantation donors as a source of physiological human tissue to conduct immunological studies.

Extracellular vesicles (EVs) are heterogeneous double membrane particles that have emerged as relevant mediators of intercellular communication and can be secreted by virtually every cell type (Théry et al., 2002b; Gho and Lee, 2017). EVs can be classified into two main categories, exosomes and microvesicles (MVs), based on their size, biogenesis and composition. Exosomes are 30–100 nm vesicles of endocytic origin that are released after the fusion of multivesicular bodies (MVBs) with the plasma membrane. MVs, also sometimes referred to as microparticles (MPs), have a more heterogeneous shape, can be bigger in diametrical size (1 μm) and are shed directly from the plasma membrane. Notably, exosomes and microvesicles have been reported to play several roles during malaria infections, including modulation of immune responses, promotion of development of sexual stages responsible for transmission, and alteration of vascular endothelium, among others (Regev-Rudzki et al., 2013; Marcilla et al., 2014; Mantel et al., 2016; Sampaio et al., 2017; Sisquella et al., 2017). Indirect associations of EV release with malaria pathology were originally observed in infections caused by *P. falciparum* and *P. vivax*, the two malaria species responsible for most of the burden, morbidity and mortality associated to human malaria (Campos et al., 2010; Nantakomol et al., 2011). Most studies of EVs in malaria, however, have been performed using EVs isolated from *in vitro* culture systems or experimental rodent infections. Thus, the physiological role of EVs in human malaria infections remains to be determined. Remarkably, using EVs obtained from circulating blood of *P. vivax* patients, we recently showed that they contain parasite proteins and are taken up by human spleen fibroblasts inducing expression of ICAM-1 *via* NF-kB and facilitating cytoadherence of *P. vivax*-infected reticulocytes obtained from patients (Toda et al., 2020).

Here, we report the first integrated characterization of human spleen cells using multiparameter flow cytometry describing subpopulations of splenic leukocytes and RBCs. We employed this methodology combined with cell purification techniques to address the interaction of plasma-derived EVs from *P. vivax* patients as opposed to healthy human volunteers with different spleen cell subpopulations.

METHODS

Malaria Patients and Healthy Donors

Plasma samples from *P. vivax*-infected patients (PV) were collected at the Hospital of the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (Manaus, Amazonas, Brazil). The local ethical committee of FMT-HVD approved these studies. Clinical data of patients participating of this study has been recently published (Toda et al., 2020). Plasma from healthy donors (HD) was obtained at the Hospital Germans Trias i Pujol (Badalona, Barcelona, Spain) after expressed consent from the donors.

Human Spleen Donors

Human spleens used in this study were retrieved from deceased transplantation donors at the Hospital Germans Trias i Pujol (Badalona, Barcelona, Spain). Donors comprised 57% of men and 43% women aged 25–66 years old. Cause of death of transplantation donors is described in **Supplementary Data Sheet 1**. Donation of these organs for biomedical research received written consent from family members and was in accordance with the protocol approved by the Ethics Committee for Clinical Research of the Hospital Germans Trias i Pujol.

Blood Collection and Plasma Processing for Extracellular Vesicles Isolation

Three mL of peripheral blood were collected by venipuncture in citrate pre-treated tubes. Samples were centrifuged at 400 xg for 10 min at RT. Plasma was collected and centrifuged at 2,000 xg for 10 min at 4°C. Supernatant was recovered, aliquoted and frozen at -80°C. Frozen plasmas were shipped from malaria endemic regions to IGTP (Badalona, Spain). Plasma collected from HD was similarly processed.

Extracellular Vesicles Purification by Size-Exclusion Chromatography

EVs were isolated from plasma samples of either *P. vivax*-infected patients or healthy donors by size-exclusion chromatography as previously described (de Menezes-Neto et al., 2015). Briefly, plasma was defrosted on ice, centrifuged twice at 2,000 xg for 10 min at 4°C to pellet debris. 1 mL of supernatant was loaded on the top of 10 mL-sepharose CL-2B (Sigma) that had been packed in a syringe and pre-equilibrated with 1X PBS. 15 fractions of 500 µL were collected immediately after loading of plasma, aliquoted and frozen at —80°C until use. Protein concentration of chromatographic fractions was measured by BCA assay (Thermo Scientific). The whole purification procedure was performed in sterile conditions.

Bead-Based Flow Cytometry Analysis of Extracellular Vesicles

EV-enriched SEC fractions were identified and molecularly characterized by bead-based flow cytometry (Théry et al., 2006). 45 µL of SEC fractions were coupled to 5 µL of 1:10 pre-diluted solution of 4 µm-aldehyde/sulfate-latex beads (Invitrogen). PBS was added to the negative-control tubes. Coupling incubation was performed for 15min at RT. Beads were then resuspended in 1 mL of bead-coupling buffer (BCB: PBS with 0.1% BSA and 0.01% NaN_3) and incubated O/N at RT on rotation. EV-coated beads were then centrifuged at 2,000 xg for 10 min at RT and washed once with BCB prior incubation with primary antibodies [CD71 (Ab08436), CD5L (ab45408) and CD63 (Hybridoma supernatant Clone TEA 3/10.1)] for 30 min at 4°C. After washing with BCB, EV-coated beads were incubated for 30 min at 4°C with secondary antibodies Anti-rabbit Alexa 488 (Invitrogen A11008) or Anti-mouse Alexa488 (Southern Biotec 1032-02). Negative controls included sample-coated beads only incubated with secondary antibodies. Labeled EV-beads were washed twice with BCB before being finally resuspended in PBS and subjected to flow cytometry analysis (FACS Verse, BD). FlowJo software was used to compare median fluorescence intensity (MFI) of EV-coated beads.

Processing of Human Spleens for Cell Isolation

At the time of organs removal from the donors, whole peripheral blood was removed by perfusion with University of Wisconsin solution (Viaspan). Collected spleens were kept in this solution for their overnight storage at 4°C before processing. Typically, 5–10g of spleen were cut in small pieces (approximately 2 mm^2) and tissue was disrupted mechanically in the presence of complete Dulbecco's modified eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin solution (Gibco). Tissue suspension was passed twice through a 70 µm cell strainer washing with complete DMEM medium to obtain a spleen single-cell suspension (SCS). The SCS was let sit for 30 min at RT to sediment aggregated particles and released nucleic acids. The supernatant was then collected avoiding the precipitated components. The cleared SCS was passed once more through 70 µm cell strainer immediately before storage, separation of cell populations, red blood cell lysis and/or analysis by flow cytometry. Viability of cells was assessed by trypan blue (Sigma) exclusion. Total spleen cells were either frozen in freezing solution [FBS supplemented with 10% dimethyl sulfoxide (Sigma)] in liquid nitrogen until use, or used immediately for cell separation and EVs interaction studies. All assays shown were performed with fresh specimens unless stated otherwise.

Separation of Spleen Cell Populations

Human SCSs were processed to separate T lymphocytes, myeloid cells and RBCs. First, cell suspensions were diluted up to 5×10^7 cells/mL with complete RPMI before layering over Ficoll-Histopaque 1077 (Sigma) to isolate spleen mononuclear cells by density centrifugation. Two-thirds of the splenocytes suspensions were processed by conventional Ficoll density centrifugation and one-third was depleted of T lymphocytes using RosetteSep™ CD3 Depletion Cocktail (Stem cell Technologies) combined to Ficoll density centrifugation following manufacturer's instructions. After centrifugation, whole mononuclear cells and T lymphocyte-depleted mononuclear cells were collected from the interphase and washed separately with complete RPMI medium. T cells were isolated from 1×10^8 whole mononuclear cells by negative selection with Pan-T Cell Isolation Kit (Miltenyi Biotec) or with CD3+ positive selection magnetic beads (Miltenyi Biotec). T lymphocyte-depleted mononuclear cells were pooled with the cells obtained from the CD3 negative fraction when CD3 MACS separation was performed. T lymphocyte-depleted mononuclear cells were enriched in DCs using EasySep™ Pan-DC Pre-Enrichment Kit (Stem cell Technologies). RBCs pelleted after the conventional Ficoll density centrifugation were collected and washed with complete RPMI. RBCs were depleted from any remaining CD45+ cells with CD45 magnetic beads (Miltenyi Biotec). Counting of viable cells was assessed throughout the whole process by trypan blue (Sigma) exclusion. Enriched T-lymphocytes, DCs and mature RBCs were immediately used in EVs interaction assays.

Multiparameter Flow Cytometry Analysis

SCS were processed both with and without red blood cell lysis by BD Pharm Lyse buffer following manufacturer's instructions prior to staining. Briefly, SCS and enriched cell populations were washed with PBS before staining with Fixable Viability Stain 575V (BD Biosciences) at 1:1,000 dilution for 15 min at RT. Then, cells were washed with PBS -1% FBS (Gibco) and incubated for 15 min at RT with antibodies against surface

markers (**Supplementary Data Sheet 2**) in Cell separation buffer [(1X MACS separation buffer (Miltenyi Biotec) supplemented with 0.5% BSA (Sigma)]. Cells were further washed with Cell separation buffer prior acquisition in LSR Fortessa flow cytometer (BD). Instrument settings are shown in **Supplementary Data Sheet 3**. For each sample, a minimum of 10^5 cells was acquired. Controls included unstained samples and single fluorochrome compensation beads. Results were analyzed using FlowJo software 10.6.2.

Extracellular Vesicles Fluorescent Labeling

Pools of plasma-derived EVs from 10 P. *vivax* patients (*Pv*EVs) and from 10 healthy donors (*h*EVs) were labelled with PKH67 or PKH26 labeling mini kit (Sigma). EV staining was conducted as following our own standard methodology. Briefly, up to 50 μg of EVs diluted up to 1mL with Diluent C were gently mixed with 4 μL of dye in 1 mL of Diluent C and incubated for 5 min at RT. Labeled EVs were then washed 5 times using Amicon® Ultra-15 100-kDa filters units (Millipore) to remove the excess of dye. The washes were performed as follows: samples were centrifuged at 4,000 xg for 10 min, washed twice with 1 mL of 1X PBS and washed three times more with 100 μL of 1X PBS. As a control, 1X PBS was labeled with the fluorescent probes and washed in the same manner as EVs. Protein concentration of labeled-EVs was quantified by BCA assay (Thermo Scientific). Labeled-PBS control was diluted in an equivalent manner.

Interaction Assays of *Pv*EVs and *h*EVs With Human Splenocytes

Total spleen cells and enriched spleen cell populations were seeded in 24-well plate at 1×10^6 cells/well and in 96-well plates at 5×10^5 cells/well, respectively, using complete DMEM medium (Sigma) supplemented with EV-depleted 10% FBS (Gibco) and 50 U/mL penicillin–50 μg/mL streptomycin (Gibco). EV-depleted medium was prepared by ultracentrifugation at 100,000 xg for 16h at 4°C. Three microgram per milliliter of protein of PKH26 or PKH67-labelled *Pv*EVs or *h*EVs were added and incubated at 37°C for 3h. In parallel, labeled-PBS was incubated with the cells as a staining background control. After 3h-incubation, total spleen cells and enriched populations were washed in PBS before staining and analyzed by flow cytometry as described above. It is important to mention that due to the ethical and technical restrictions in obtaining human spleen tissue and plasma of *P. vivax* patients from endemic regions, we could only asses EVs-interactions with purified spleens cells from two transplantation donors.

Confocal Microscopy

Three microgram per milliliter of PKH67 stained *Pv*EVs and *h*EVs were incubated with 0.5 x 10^6 RBCs in 200 μL of DMEM supplemented with 10% EVs-depleted FBS using μ-Slide 8 Well (Ibidi) at 37°C, 5% CO_2 for 3 h. Cells were washed at 400 xg for 5 min and resuspended with 200 μL of incomplete DMEM. Confocal images were acquired on a Zeiss LSM 710 Confocal Module coupled to the Zeiss Axio Observer Z1 microscope with a 20x/NA0.40 immersion objective. The intensity of all the channels was standardized through all the experiments. Images of ten different fields were randomly captured and Fiji (ImageJ distribution) software was used for processing images. Total and fluorescent cells were counted manually and percentage of fluorescence cells per field were compared between the conditions (n = 10).

Statistical Analysis

All statistical tests were performed using GraphPad Prism version 8 (GraphPad Software, CA, USA). Statistical significance was determined using Student's *t*-test. *P*-values <0.05 were considered significant. Comparisons of RBCs-EVs interaction by confocal microscopy was performed using the non-parametric, unpaired and two-sided Mann-Whitney test to calculate p-values. *P*-values <0.05 were considered significant.

RESULTS

Immunophenotyping of Human Splenocytes

To gather understanding on the diversity of cell populations that form the human spleen, we performed a multiparameter flow cytometry approach to immunophenotype splenocytes obtained from organ transplantation donors. The methodology involved mechanical tissue disruption followed by exhaustive filtration of the spleen tissue. Importantly, we refrained from using enzymatic digestions in order to avoid alterations of cell surface markers necessary for cell phenotyping. By using a combination of antibodies (**Supplementary Data Sheet 2**) and the gating strategy shown in **Figure 1A**, we characterized the main leukocyte populations as well as mature and immature RBCs (**Figure 1B**). As expected, even after perfusion, erythrocytes ($CD45^-CD235a^+CD71^-$) comprise the majority of cells in the human spleen accounting for approximately 70% of total cells (**Figure 1B**). Noticeably, we consistently detected a minor population (0.7%) of reticulocytes ($CD45^-CD235a^+CD71^+$) (**Figure 1B**). Regarding human spleen leukocyte populations ($CD45^+$), B cells ($CD45^+CD19^+$) were the most abundant (30%) while $CD4^+$ T cells ($CD45^+CD56^-CD3^+CD4^+$) and $CD8^+$ T cells ($CD45^+CD56^-CD3^+CD8^+$) accounted for 16 and 10%, respectively. Innate lymphocytes such as NK cells ($CD45^+CD3^-CD56^+$) and NKT cells ($CD45^+CD3^+CD56^+$) were also found (6 and 2%, respectively) (**Figure 1C**). Innate cells from the myeloid lineage, including neutrophils ($CD45^+CD15^+$), represented an abundant population in the human spleen (16% of total leukocytes), although a high variability was observed between different donors (**Figure 1C**). Myeloid phagocytic cells like monocytes ($CD45^+CD19^-CD14^+CD11c^{+/-}$) and DCs ($CD45^+CD19^-CD14^-CD11c^+$) were also present (2 and 0.3%, respectively). Unexpectedly, macrophages defined as ($CD45^+CD19^-CD14^{med}CD163^+$), were scarce (0.1%) (**Figure 1B**). We also estimated the resident population of hematopoietic stem cells

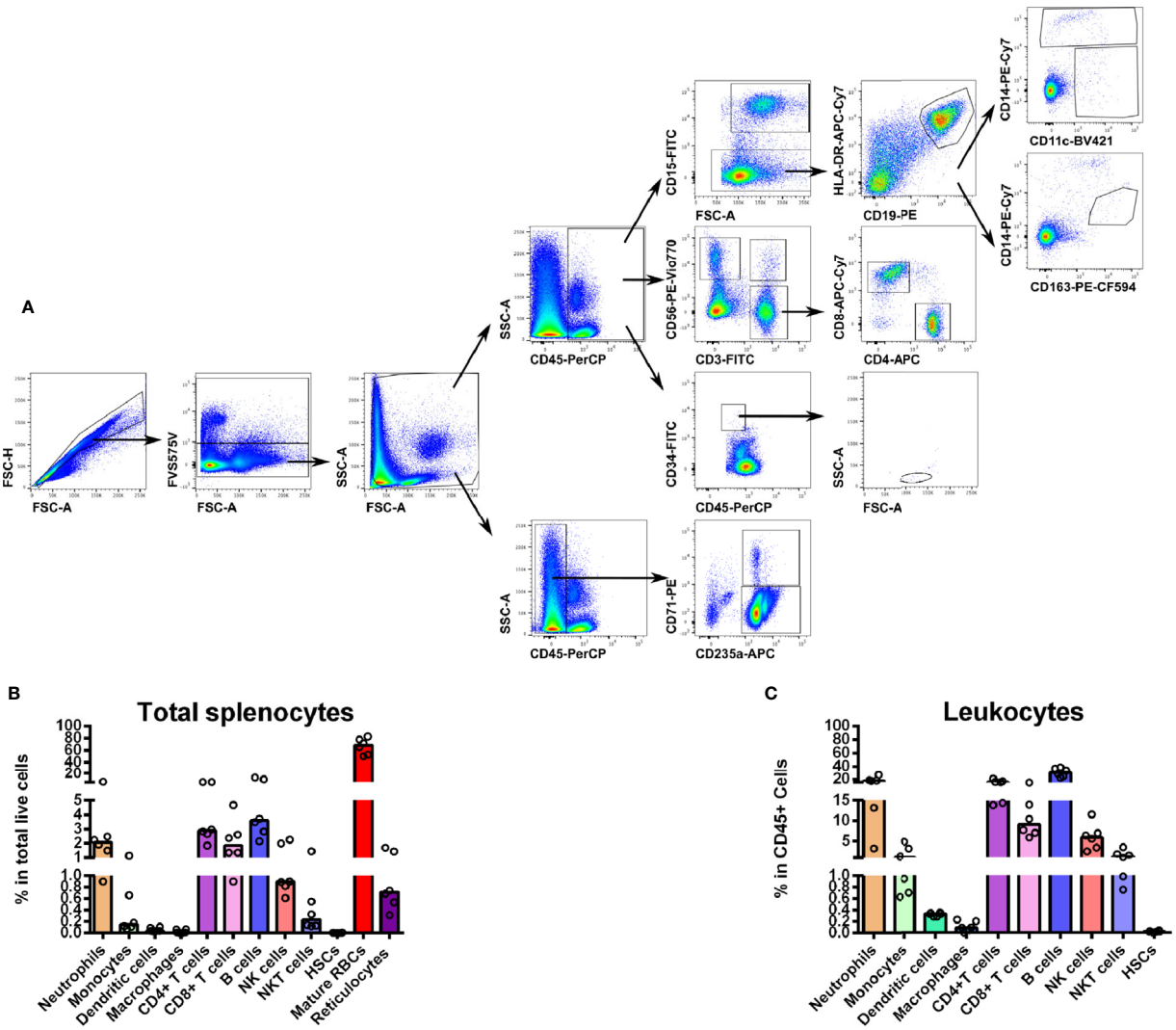

FIGURE 1 | **(A)** Hierarchical multiparameter flow cytometry gate strategy for human spleen cells immunophenotyping. Total spleen cells obtained in suspension after mechanical tissue disruption were assessed for viability and phenotyped using surface markers for several populations. First, singlets were selected by gating events in the diagonal of FSC-H vs. FSC-A plots. Live cells were gated out from the positively stained population with FVS575V viability marker. This total live cell population was then divided in $CD45^+$ and $CD45^-$ to differentiate whole leukocytes and erythroid cells, respectively. Reticulocytes ($CD235a^+CD71^+$) and mature RBCs ($CD235a^+CD71^-$) were gated in $CD45^-$ cells. From the $CD45^+$ population we gated neutrophils ($CD15^+$), B cells ($CD15^-CD19^+HLA\text{-}DR^+$), monocytes ($CD15^-CD19^-HLA\text{-}DR^{+/-}CD14^+CD11c^{+/-}$), total DCs ($CD15^-CD19^-HLA\text{-}DR^{+/-}CD14^-CD11c^+$), macrophages ($CD15^-CD19^-HLA\text{-}DR^{+/-}CD14^{med}CD163^+$), T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$), $CD4^+$ T cells ($CD3^+CD56^-CD4^+CD8^-$) and $CD8^+$ T cells ($CD3^+CD56^-CD4^-CD8^+$). We gated hematopoietic stem cells as a $CD45^{med}CD34^+$population with a low SSC and medium FSC. **(B)** Distribution of total spleen cells. Frequencies of the different spleen populations, defined as described above were quantified. **(C)** Distribution of total spleen leukocytes. Plots represent the median of six different human spleens analyzed independently.

(HSCs) ($CD45^{med}CD34^+$) finding that 0.02% of cells showed the typical HSC cell surface markers and morphology (**Figure 1C**).

Isolation and Characterization of Extracellular Vesicles From P*lasmodium vivax* Patients and Healthy Donors

In order to demonstrate the value of this integrated approach to study different human spleen cell subpopulations, we isolated EVs from plasma of ten infected *P. vivax* patients and ten healthy donors using the single-step technology of size exclusion chromatography (de Menezes-Neto et al., 2015). We performed bead-based flow cytometry over the different chromatographic fractions to assess the expression of the classical EV marker tetraspanin CD63, the plasma-derived EV marker CD5L and the reticulocyte marker CD71 to identify the EV-enriched SEC fractions. As previously shown (de Menezes-Neto et al., 2015; Gualdrón-López et al., 2018), EVs were efficiently separated from the bulk of soluble plasma proteins as inferred from the low protein concentration in the EV-enriched fractions with the highest MFI signal of the studied

molecular markers (**Supplementary Data Sheet 4A**). CD71 is the major component of reticulocyte-derived exosomes (Harding Stahl, 1983; Pan et al., 1985; Díaz-Varela et al., 2018) and it is a reticulocyte-specific receptor for *P. vivax* (Gruszczyk et al., 2018). Importantly we have recently shown by mass spectrometry-based proteomics and molecular profiling that CD71 is a component of circulating EVs from *P. vivax* patients (Toda et al., 2020). Therefore, we used it as a surrogate molecular marker of EVs derived from *P. vivax*-infected reticulocytes for selection of SEC fractions from individual patients (**Supplementary Data Sheet 4A**) and healthy donors (**Supplementary Data Sheet 4B**) to compose a pool of vesicles for spleen cells interaction experiments.

Interaction of *Pv*EVs and *h*EVs With Total Spleen Cells

Initially, we explored the capacity of total spleen cells to interact *in vitro* with PKH labeled CD71^{high} *Pv*Evs and *h*EVs (**Supplementary Data Sheet 5A–C**). After incubation of total splenocytes with *Pv*EVs and *h*EVs, we compared the proportion of different cell populations positively stained with PKH26 and PKH67 as a measurement of EVs-cells interaction. Except for CD45$^+$CD14$^-$CD11c$^+$ (**Supplementary Data Sheet 5B**) and CD45$^-$CD235a$^+$CD71$^-$ cells (**Supplementary Data Sheet 5C**) which showed a slightly significant higher proportion of PKH$^+$ cells interacting with *Pv*EVs, neither T lymphocytes, NK/NKT cells (**Supplementary Data Sheet 5A**), myeloid cells, nor immature RBCs showed statistically significant difference with EVs from infection compared to control EVs.

Interaction of *Pv*EVs and *h*EVs With Isolated Spleen Cell Populations

In order to address the interaction of *Pv*EVs and *h*EVs with particular human spleen cells of relevance for malaria infections, we have settled a pipeline of cell separation steps involving density centrifugations followed by immunomagnetic cell separation using specific cell surface markers (**Figure 2**) to enrich spleen T-lymphocytes, DCs, and mature RBCs. The rationale for studying EVs interaction with isolated cells is based on the clustered distribution of cells in the human spleen architecture. Therefore, enrichment of immune cells enabled us to assess their interaction with EVs in a more physiological context. Our purification procedure allowed obtaining 70% enrichment of CD3$^+$ cells, composed by 56% of CD4$^+$ T cells and 37% of CD8$^+$ T cells (**Figure 3A**), a proportion previously reported for human spleen T cells (Carpenter et al., 2018b). Similar purification techniques have been previously used achieving 95% pure T cells from peripheral blood (Finney et al., 2004). The differences in purity observed in our experiments could be attributed to the different proportion of T cells in peripheral blood compared to the spleen and the

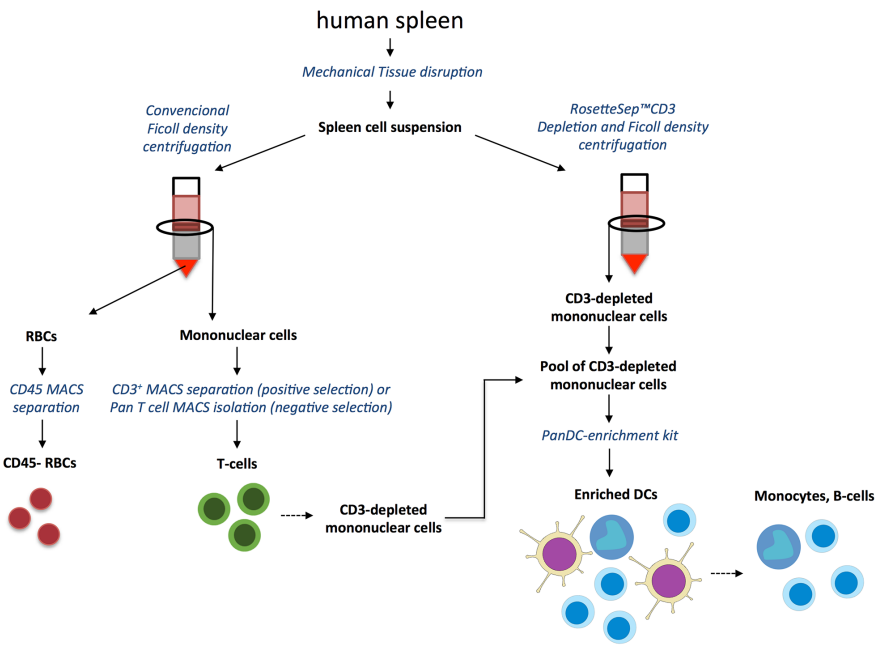

FIGURE 2 | Human spleen T cells, dendritic cells and red blood cells purification strategy. Human spleen was processed to produce a single cell suspension by mechanical tissue disruption and exhaustive filtration. Cell suspensions were processed by conventional Ficoll density centrifugation to isolate mononuclear cells from which T cells were enriched by positive selection with anti-CD3-magnetic beads or by negative selection using Pan-T Cell Isolation Kit. Post-Ficoll sedimented RBCs were further purified by negative selection using anti-CD45 magnetic microbeads to deplete leukocytes. In parallel, DCs were enriched by a first step of T cells depletion using RosetteSep^{TM} CD3 Depletion Cocktail followed by Ficoll density gradient centrifugation. By mixing the CD3- depleted mononuclear fraction obtained by Rossette sep^{TM} and CD3$^-$ depleted cells from the flow-through of CD3-positive selection described above we composed a CD3$^-$ mononuclear cells pool. DCs were then enriched from the CD3$^-$ pool by negative selection using the EasySep^{TM} Pan-DC Pre-Enrichment Kit. The retained fraction rich in monocytes and B cells was also included in the EVs *in vitro* interaction characterization.

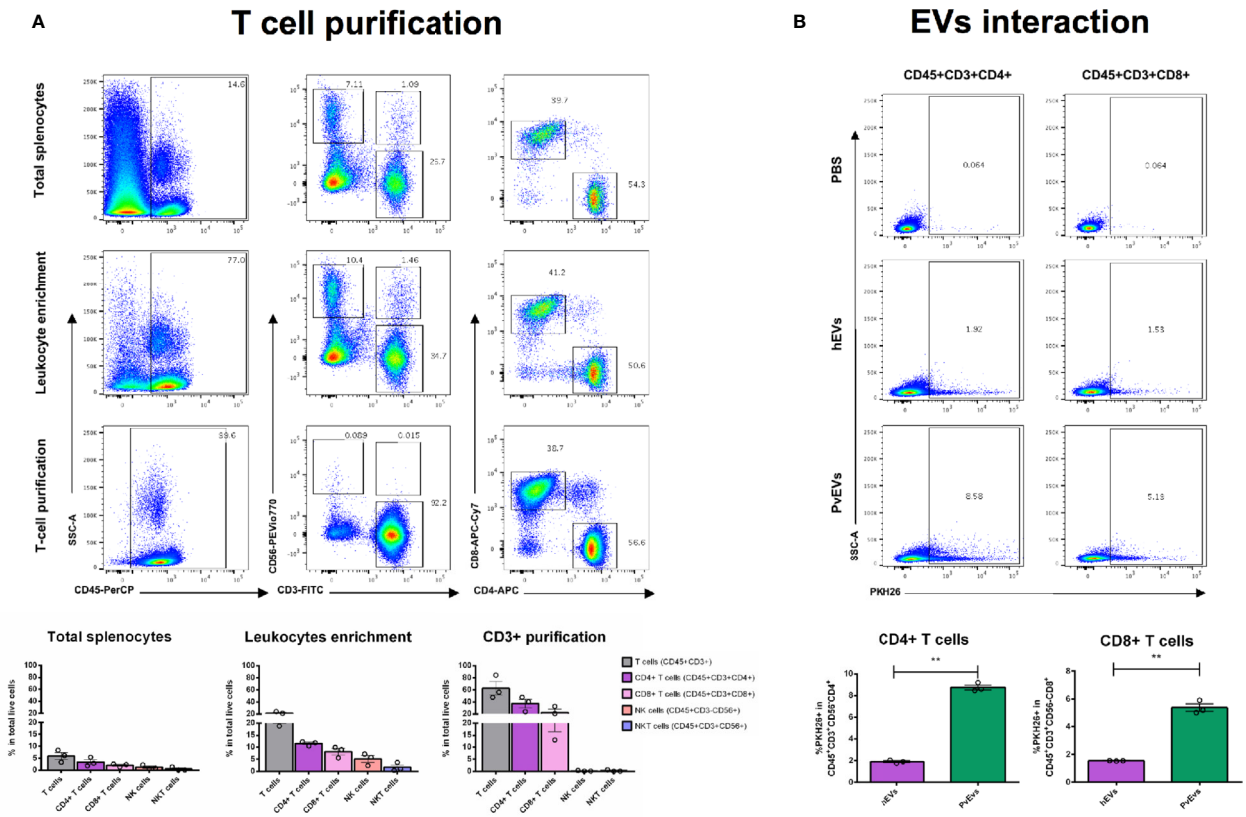

FIGURE 3 | *Pv*EVs and *h*EVs *in vitro* interaction with spleen T cells. **(A)** T cell purification. T cells were purified from spleen cell suspensions by a two-step procedure as shown in **Figure 2**. Cells from all purification steps were stained with fluorescent-conjugated antibodies against surface markers and analyzed by flow cytometry. Representative images show plots of the gating strategy to follow T cell purification. Enrichment quantification shown corresponds to the mean of two purifications from two spleen donors processed independently by negative selection. **(B)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen T cells. Flow cytometry plots showing frequencies of $CD4^+$ and $CD8^+$ T cells positively labeled with PKH26. PKH26 staining was gated according to cells incubated with PKH26-stained PBS. Frequencies quantification of three technical replicates is shown. Data is representative of two independent experiments performed with two spleen donors. Statistical significance ($P<0.05$) was assessed using a Student's *t*-test, $^{**}p < 0.001$.

optimization of commercial kits with peripheral blood mononuclear cells (PBMCs) samples and not lymphoid tissue. When enriched T cells were incubated with EVs, we observed a 3-4 fold increased proportion of $CD4^+$ T cells and $CD8^+$ T cells, respectively, interacting with PKH-labeled *Pv*EVs when compared to *h*EVs (**Figure 3B**). The same result was obtained using positively isolated T cells with anti-CD3 magnetic beads from different spleen donors (**Supplementary Data Sheet 6** and **8A**), suggesting this interaction might be independent of the T cell activation state. Given the T cell suspension contained 30% contaminating cells, we cannot rule out that the increased *Pv*EVs interaction observed with T cells could be due to an indirect effect produced by the response of those contaminant cells to *Pv*EVs.

We conducted interactions assays of the PKH67-labelled *Pv*EVs and *h*EVs with enriched DCs fraction, as well as with monocytes and B cells retained in the DCs-negative fraction of the negative selection step (**Supplementary Data Sheet 7A**). We found that these populations showed interactions above the background level with both types of EVs (**Supplementary Data Sheet 7B**). Interestingly, we found an increased proportion of monocytes ($CD45^+$Neutrophils$^-$$CD19^-$$CD14^+$$CD11c^{+/-}$) (1.51 fold) and B cells ($CD45^+$ Neutrophils$^-$$CD19^+$) (2.3 fold) interacting with *Pv*EVs when compared to *h*EVs. This pattern was also observed in the contaminant cells of the DCs enriched fraction (data not shown). Besides the tendency to an increased frequency of DCs ($CD45^+$Neutrophils$^-$$CD19^-$$CD14^-$$CD11c^+$) interacting with *Pv*EVs when compared to *h*EVs, we could not perform a statistical comparison due to the presence of only two technical replicates due to the low number of cells obtained after the enrichment procedure. Similar results were observed in an independent experiment using a different spleen donor (**Supplementary Data Sheet 8B**).

In addition, we also explored whether mature spleen RBCs ($CD45^-$$CD235a^+$$CD71^-$) could interact with *Pv*EVs and *h*EVs. To consistently test this interaction, we depleted contaminant $CD45^+$ leukocytes in a two-step purification procedure achieving 99% purity of RBCs (**Figure 4A**). Remarkably, *in vitro* interaction

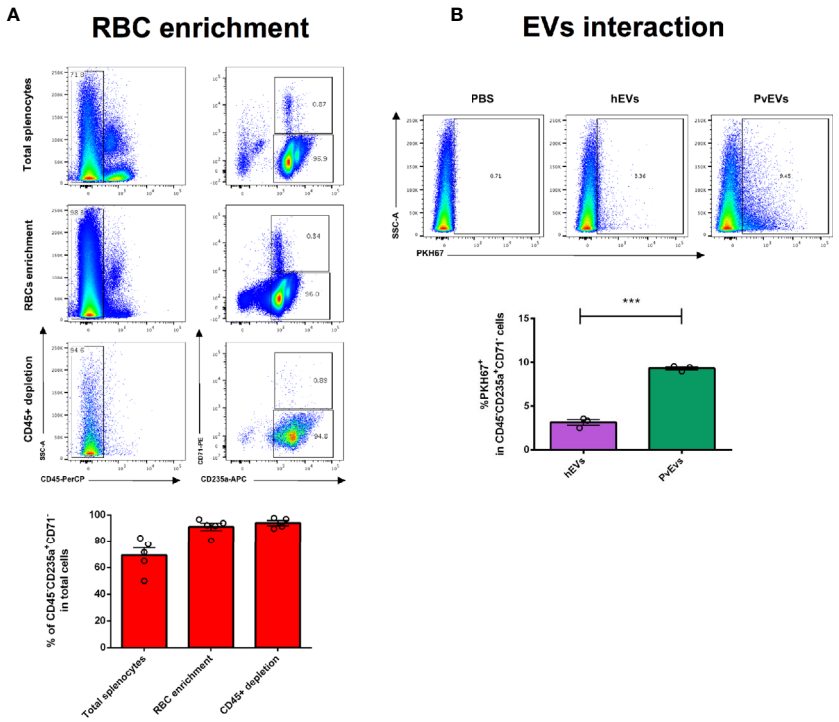

FIGURE 4 | *Pv*EVs and *h*EVs *in vitro* interaction with spleen mature red blood cells by flow cytometry analysis. **(A)** Red blood cells purification. RBCs were purified from spleen cell suspensions by a two-step procedure as shown in **Figure 2**. Enriched RBCs were stained with fluorescent conjugated antibodies against surface markers and analyzed by flow cytometry. Representative images show plots of the RBCs purification procedure. Data shown correspond to the mean and standard deviation of independent purifications from five spleen donors. **(B)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen RBCs cells. Flow cytometry plots showing frequencies of mature RBCs positively stained with PKH67. PKH67 staining was gated according to cells incubated with PKH67-stained PBS. Frequencies quantification of three technical replicates is shown. Data is representative of two independent experiments performed with two spleen donors. Statistical significance (P<0.05) was assessed using a Student's *t*-test, $^{***}p < 0.0001$.

assays showed that purified mature spleen RBCs strongly interact with *Pv*EVs as a three-fold higher proportion of cells stained with PKH67-labelled *Pv*EVs were observed when compared to *h*EVs (**Figure 4B**). Interestingly, an increased number of green fluorescent dots was observed in the plasma membrane of spleen RBCs incubated with PKH67-labelled *Pv*EVs as compared with *h*EVs (**Figure 5**). A similar increased interaction of RBC with *Pv*EVs was observed in an independent experiment using a different spleen donor (**Supplementary Data Sheet 8C**).

DISCUSSION

In this study we have performed an integrated characterization of human spleen cells using multiparameter flow cytometry, a powerful technique that has been systematically applied in immunophenotyping in clinical and research settings (O'Donnell et al., 2013). In our approach, we have employed spleens from transplantation donors as a source of healthy splenic tissue followed by mechanical tissue disruption to prepare viable spleen single cell suspensions with intact cell surface markers. Our work allowed the quantification of several immune cells including neutrophils, monocytes, DCs, T and B lymphocytes, NK and NKT cells as well as resident reticulocytes, HSCs and mature RBCs (**Figure 1**). In spite of the limitations faced in human spleen studies due to ethical and practical constraints, diverse studies of human spleen specific cell populations have provided a great understanding of the function and phenotype of resident splenic immune cells (Colovai et al., 2004; Langeveld et al., 2006; Velásquez-Lopera et al., 2008; Meinderts et al., 2017; Nagelkerke et al., 2018) and stromal cells such as fibroblasts and endothelial cells (Briard et al., 2002; Rosti et al., 2013) under physiological and pathological conditions. However, few studies applied a comprehensive approach that addresses the cellular diversity of the spleen, taking into account not only leukocytes and stromal cells, but also immature and mature RBCs. The immunophenotyping performed in this work demonstrated that the frequencies of T cells, B cells, monocytes and neutrophils in the spleen are in agreement with a previous report where a similar holistic approach was conducted to quantify leukocytes from the spleen and other human lymphoid organs across several clinical conditions (Carpenter et al., 2018a). Innate lymphoid cells such as NK and NKT cells showed similar proportions to

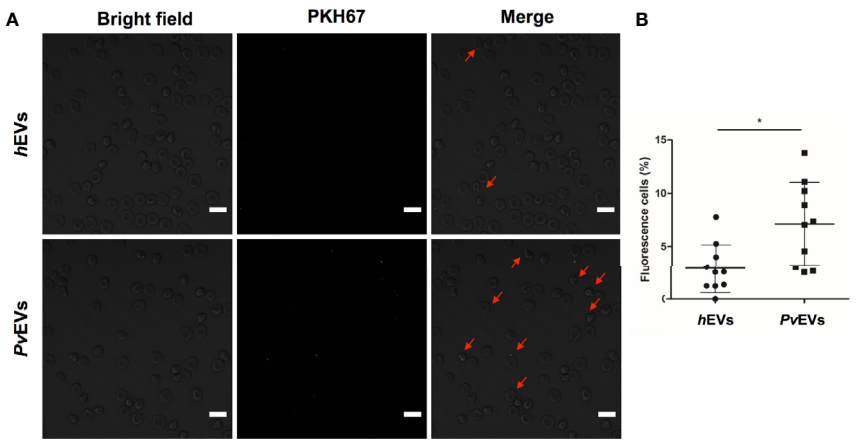

FIGURE 5 | *Pv*EVs and *h*EVs *in vitro* interaction with spleen mature red blood cells by confocal microscopy. **(A)** Purified RBCs were incubated with PKH67-labelled *Pv*EVs and *h*EVs. Confocal microscopy analysis shows RBCs with green fluorescent dots in the membrane (red arrows). Scale bar: 10 µm. **(B)** Quantification of RBCs with positive staining for PKH67. Data represent the mean and standard deviation of the percentage of cells with fluorescent green dots per field (n=10) in each condition. Statistical significance (P<0.05) was assessed using unpaired and two-sided, Mann-Whitney test, $*p < 0.05$.

those previously reported in the human spleen (Colovai et al., 2004; Langeveld et al., 2006). Prior studies have shown that macrophages and DCs in the human spleen represent a 9 and 0.7% of mononuclear cells, respectively (Mcilroy et al., 2001). The reduced amount of these two populations in our study is likely due to the absence of collagenase treatment during tissue disaggregation, which might be necessary to release these cells from parenchyma. As expected, mature RBCs account for the great majority of splenocytes reflecting the primordial function of this organ in blood filtration. Our immunophenotyping also revealed a small proportion of young $CD71^+$ reticulocytes, classically believed to be restricted to the human bone marrow. Importantly, $CD71^+$ reticulocytes are the host cell of *P. vivax* (Kitchen, 1937), and our previous studies have indicated that *P. vivax* parasite can be found in the spleen in human infections (Machado Siqueira et al., 2012; Elizalde-Torrent et al., 2018). Of note, spleens in this study were removed during transplantation surgery after whole-body perfusion. This implies that spleen tissue used for our experiments is largely depleted from circulating cells. Whether the presence of these reticulocytes is used by the parasite to establish spleen infections remains to be determined.

Given the importance of the spleen in parasite removal and immune response during malaria (Engwerda et al., 2005; del Portillo et al., 2012) and the role of secreted extracellular vesicles in cell communication processes involved in pathogenesis of this infectious disease (Babatunde et al., 2020), we explored the *in vitro* interaction of circulating EVs from *P. vivax* patients with total spleen cells by a flow cytometry approach. Overall, our results showed that with the exception of DCs and mature RBCs, no other cell populations differentially interacted with *Pv*EVs as compared to *h*EVs (**Supplementary Data Sheet 2**). Despite the statistical significance of this result, the low number of PKH67 positively stained cells in all populations studied may be a limitation in the interpretation of these interactions. However, the increased interaction observed in DCs when whole splenocytes were in contact with PKH-labeled *Pv*EVs can reflect its specific phagocytosis. Indeed, we have previously demonstrated that human reticulocyte-derived exosomes are specifically taken up by DCs in a Siglec-1-dependent manner (Díaz-Varela et al., 2018) and circulating EVs in patients with acute *P. vivax* infection contain parasite proteins (Toda et al., 2020). In the absence of supportive evidence, this remains to be determined.

A clear limitation of the total splenocytes-EVs approach in delineating physiological relevant interactions is the impossibility of recapitulating the structural features derived from the spleen microcirculation (Mebius and Kraal, 2005; Ferrer et al., 2014). We expect that blood circulating EVs are restricted to encounter parenchymal and immune cells enriched in particular spleen compartments. Such site-specific interactions with the distinct spleen cell populations could trigger differential physiological responses and alter local signaling at autocrine and paracrine levels. In order to overcome this limitation, we explored *Pv*EVs interactions with enriched human spleen cell populations of physiological relevance for malaria immune response and mechanism of anemia, two processes in which the spleen is a major player (del Portillo et al., 2012). We designed a multistep cell purification methodology to enrich T cells, DCs and mature RBCs using density centrifugation and sequential magnetic immunocapture (**Figure 2**). Our results showed that T cells enrichment, both, by negative (**Figure 3A**) and positive selection (**Supplementary Data Sheet 6A**) resulted in around 56–70% pure and viable $CD45^+CD3^+$ cells indicating effective cell separation. The enrichment method for DCs, however, showed limitations since a very low yield and compromised purity was observed (**Supplementary Data Sheet 7A**). Notably,

the DC enrichment commercial kit used has been standardized using PBMCs in which the proportion of leukocytes differs from lymphoid tissue such as the splenic one. We can attribute the high contamination of B cells in our DC enriched sample to this fact. In contrast, the method for RBCs purification was highly efficient at the initial density centrifugation step as we managed to obtain 99% pure RBCs. Importantly, the subsequent $CD45^+$ cells depletion allowed to eliminate the totality of contaminating leukocytes (**Figure 4A**).

Following this spleen-cell population separation approach we demonstrated an increased interaction of monocytes, B cells and T cells from the human spleen with circulating EVs from *P. vivax* infected patients compared to EVs from healthy donors (**Supplementary Data Sheet 7B** and **Figure 3B**). EVs are now well-known mediators of the regulation of immune responses under physiological and pathological conditions such as cancer and infection (Robbins and Morelli, 2014). Given that the spleen is a key organ to generate immune responses, components potentially present on EVs during *P. vivax* infection (e.g., parasite antigens) could contribute to the initiation and/or the promotion of such responses. Indeed, it is widely demonstrated that EVs derived from malaria infected cells contain parasite proteins (Martin-Jaular et al., 2011; Mantel et al., 2013; Antwi-Baffour et al., 2016) and that they can induce proinflammatory responses in innate immune cells such as macrophages, neutrophils and monocytes (Couper et al., 2010; Mantel et al., 2013; Sisquella et al., 2017). One potential mechanism for the *Pv*EVs increased interaction with splenic phagocytic cells (monocytes and B cells) could be the recognition by Fc receptors of immune complexes formed by host antibodies and parasite antigens in EVs. Indeed, VIR proteins are associated to *Pv*EVs (Toda et al., 2020) and anti-VIR IgGs have been detected in the serum of *P. vivax* patients (Oliveira et al., 2006). Although there are no published reports that have demonstrated the formation of immune complexes with EVs from malaria patients, EV-derived circulating immune complexes have been described in chronic Chagas disease (Díaz Lozano et al., 2017), reinforcing this hypothetical interaction mechanism.

We previously demonstrated that immunization with exosomes derived from reticulocytes infected with *P. yoelii* 17X, a rodent malaria strain that preferentially invades reticulocytes and often used as a model for *P. vivax* malaria, elicited humoral responses (Martin-Jaular et al., 2011) and induced non-exhausted effector memory T cells that conferred a spleen-dependent long-lasting protection (Martín-Jaular et al., 2016). The induction of efficient T cell responses by EVs is one of the most extensively studied EV-mediated immune mechanisms (Zitvogel et al., 1998; Théry et al., 2002a; Segura et al., 2005; Menningher et al., 2020). Its relevance for immunointerventions was initially investigated in cancer (Wolfers et al., 2001) and later in models of infection (Aline et al., 2004; Beauvillain et al., 2007; Schnitzer et al., 2010; Cheng and Schorey, 2013). T cell responses are also relevant for the control of *P. vivax* infection, where a protective role of $CD8^+$ T cells against blood-stage *P.* vivax parasites has been suggested (Burel et al., 2016; Junqueira et al., 2018). Importantly, HLA class I molecules have been identified in human reticulocyte-derived exosomes (Díaz-Varela et al., 2018).

One of the homeostatic functions of the spleen is the destruction of senescent and damaged RBCs in a process called erythrophagocytosis. This is mediated by red pulp macrophages and neutrophils and promoted by IgG opsonization (Meinderts et al., 2017). Interestingly, we observed a three-fold increase of spleen isolated RBCs interacting with *Pv*EVs compared with its *h*EVs counterparts (**Figure 4B**). In addition, confocal microscopy showed that this interaction occurs at the membrane of RBCs (**Figure 5**). This observation can have important implications for the pathophysiological mechanism of anemia in malaria. EVs derived from malaria infected RBCs that contain parasite proteins and physically interact with mature healthy RBCs in the spleen, could induce its opsonization and destruction by phagocytes leading to severe anemia. A similar mechanism has been proved for *Trypanosoma brucei* secreted EVs in the induction of anemia in sleeping sickness patients (Szempruch et al., 2016). If such EV-mediated opsonization of healthy RBCs is involved in severe anemia in vivax malaria needs further investigation.

In summary, to the best of our knowledge, our data show the first integrated analysis of the human spleen at cellular level including HSCs, lymphoid, myeloid and erythroid lineages. We implemented a methodology for the sequential enrichment of specific human spleen cell populations to study their *in vitro* interaction with plasma-derived EVs from *P. vivax* infected patients. These studies show that monocytes, T and B lymphocytes as well as mature RBCs interact with circulating EVs from patients. Further exploration of the functional relevance of such interactions can contribute to not only our understanding of important pathophysiological processes involving the spleen in vivax malaria, but also to rational vaccine development.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved. 1. Plasma samples from *P. vivax*-infected patients (PV) were collected at the Hospital of the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (Manaus, Amazonas, Brazil). 2. Plasma from healthy donors (HD) was obtained at the Hospital Germans Trias i Pujol (Badalona, Barcelona, Spain) after expressed consent from the donors. 3. Human spleen donations for biomedical research received written consent from family members and was in accordance with the protocol approved by the Ethics Committee for Clinical Research of the Hospital Germans Trias i Pujol. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MG-L, MD-V, HT, IA-H, and LP-C performed experiments of spleen immunophenotyping and cell purification. MG-L, MD-V, and HT performed experiments EVs purification and EVs–splenocytes interaction. MG-L, MD-V, and MF-S analyzed the data. MG-L, MD-V, CF-B, and HP suggested the experiments. RL and ML contributed materials. MG-L, MD-V, and HP drafted the manuscript. HP conceived this study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.596104/full#supplementary-material

Supplementary Data Sheet 1 | Summary of spleen tissue processed for immunophenotyping and EVs interaction experiments.

Supplementary Data Sheet 2 | Fluorescent conjugated antibodies used for immunophenotyping of spleen cells.

Supplementary Data Sheet 3 | LSR Fortessa flow cytometer (BD) configuration indicating laser intensities and acquisition channels.

Supplementary Data Sheet 4 | Molecular characterization of plasma-derived EVs from healthy donors and *P. vivax* infected patients purified by size exclusion chromatography (SEC) and analyzed by flow cytometry bead-based assay. **(A)** Distribution of CD5L, CD71 and CD63 markers in SEC fractions from plasma of *P. vivax* patients. Negative Control (Cont) refers to a mix of all fractions incubated with anti-rabbit Alexa 488- 2^{ary} antibodies. Dashed rectangles refer to fractions selected to constitute EVs pools for PKH staining and *in vitro* interaction studies. **(B)** Distribution of CD5L and CD71 EVs markers in the SEC fractions from plasma of healthy donors. Negative Control (Cont) refers to Fractions F8 incubated with anti-rabbit Alexa 488- 2^{ary} antibodies.

Supplementary Data Sheet 5 | *In vitro* interaction of *Pv*EVs and *h*EVs with total human spleen cells. Total spleen cells were obtained and frozen as described in the Methods section. Splenocytes were thawed and incubated with PKH26 labelled *Pv*EVs, *h*EVs and PBS for 3h. Cells were washed and then stained with surface markers. Positive PKH26 staining was gated according to unlabeled cells. **(A)** Flow cytometry plots showing frequencies of NK, NKT, total T lymphocytes, $CD4^+$ T cells and $CD8^+$ T cells positive for PKH26. Frequencies quantification of three technical replicates is shown. **(B)** Flow cytometry plots of spleen monocytes and DCs showing frequencies of positive PKH26 cells. Frequencies quantification of three technical replicates is shown. **(C)** Flow cytometry plots of spleen reticulocytes and mature RBC showing frequencies of positive PKH26 cells. Frequencies quantification of three technical replicates is shown. Data shows a representative experiment of two different spleen donors analyzed independently. Statistical significance ($P<0.05$) was assessed using a Student's *t*-test, *$p<0.05$.

Supplementary Data Sheet 6 | *Pv*EVs and *h*EVs *in vitro* interaction with spleen T cells isolated by positive selection. **(A)** T cell purification. T cells were purified from spleen cell suspensions by a two-step procedure involving density gradient centrifugation and $CD3^+$ immunomagnetic cell isolation. Cells from all purification steps were stained with fluorescent-conjugated antibodies against CD4 and CD8 surface markers and analyzed by flow cytometry. Plot corresponds to a representative image showing T cell enrichment quantification of two independent experiments. **(B)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen T cells. Frequencies of $CD4^+$ and $CD8^+$ T cells positively labeled with PKH26. PKH26 staining was gated according to cells incubated with PKH26-stained PBS. Frequencies quantification of three technical replicates is shown. Data is representative of two independent experiments performed with two spleen donors. Statistical significance ($P<0.05$) was assessed using a Student's *t*-test, * $p<0.05$.

Supplementary Data Sheet 7 | *Pv*EVs and *h*EVs *in vitro* interaction with spleen phagocytic cells. **(A)** Dendritic cells enrichment. DCs were enriched from spleen cell suspensions by a two-step procedure as shown in **Figure 2**. Enriched cells were stained with fluorescent-conjugated antibodies against surface markers and analyzed by flow cytometry. Images show flow cytometry plots of the gating strategy to follow DC enrichment through the purification procedure. Enrichment quantification shown corresponds to one purification from one spleen donor. **(B)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen phagocytic cells. Flow cytometry plots showing frequencies of DCs, monocytes and B cells positively labeled with PKH67. PKH67 staining was gated according to cells incubated with PKH67-stained PBS. Frequencies quantification of three technical replicates is shown. Data represents mean and standard deviation of technical replicates of one experiment performed with one spleen donor. Statistical significance ($P<0.05$) was assessed using a Student's t-test, * p<0,001.

Supplementary Data Sheet 8 | Summary of *Pv*EVs and *h*EVs *in vitro* interaction with spleen cells from biological sample 2. **(A)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen T cells isolated by positive selection. T cells were purified from spleen cell suspensions by a two-step procedure involving density gradient

centrifugation and CD3$^+$ immunomagnetic cell isolation. Enriched cells were tested for its *in vitro* interaction with PKH67 labeled *Pv*EVs and *h*EVs. Plots show frequencies of CD4$^+$ and CD8$^+$ T cells positively labeled with PKH26. **(B)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen phagocytic cells. Phagocytic cells were enriched by depletion of CD3$^+$ cells. Cells were tested for its *in vitro* interaction with PKH67 labeled *Pv*EVs and *h*EVs. Plots shows frequencies of DCs (CD45$^+$Neutrophils$^-$CD19$^-$CD14$^-$CD11c$^+$), Monocytes (CD45$^+$ Neutrophils$^-$CD19$^-$ CD14$^+$CD11c$^-$) and B cells (CD45$^+$ Neutrophils$^-$CD19$^-$) positively stained with PKH67. **(C)** *Pv*EVs and *h*EVs *in vitro* interaction with spleen mature red blood cells (RBCs). RBCs were purified from spleen cell suspensions by a two-step procedure as shown in **Figure 2**. Enriched cells were tested for its *in vitro* interaction with PKH26 labeled *Pv*EVs and *h*EVs. Plots shows frequencies of mature RBCs (CD45$^-$CD71$^-$) positively stained with PKH67. PKH67 was gated according to cells incubated with PKH67-stained PBS. All plots shows frequencies of three technical replicates. Statistical significance (P<0.05) was assessed using a Student's *t*-test, **p<0,01, **p<0,001.*

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REVIEW
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Extracellular Vesicles: Schistosomal Long-Range Precise Weapon to Manipulate the Immune Response

Dror Avni[1,2,3]* *and Orly Avni*[4]*

[1] Laboratory of Molecular Cell Biology, Sheba Medical Center, Tel Hashomer, Israel, [2] Laboratory for the Study of Tropical Diseases, Sheba Medical Center, Tel Hashomer, Israel, [3] Department of Medicine C, Sheba Medical Center, Tel Hashomer, Israel, [4] The Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel

Schistosomiasis (Bilharziasis), a neglected tropical disease that affects more than 240 million people around the world, is caused by infection with the helminth parasite *Schistosoma*. As part of their secretome, schistosomes release extracellular vesicles (EVs) that modulate the host immune response. The EV-harbored miRNAs upregulate the innate immune response of the M1 pathway and downregulate the differentiation toward the adaptive Th2 immunity. A schistosomal egg-derived miRNA increases the percentage of regulatory T cells. This schistosomal-inducible immunoediting process generates ultimately a parasitic friendly environment that is applied carefully as restrained Th2 response is crucial for the host survival and successful excretion of the eggs. Evidence indicates a selective targeting of schistosomal EVs, however, the underlying mechanisms are unclear yet. The effects of the schistosomes on the host immune system is in accordance with the hygiene hypothesis, attributing the dramatic increase in recent decades in allergy and other diseases associated with imbalanced immune response, to the reduced exposure to infectious agents that co-evolved with humans during evolution. Deciphering the bioactive cargo, function, and selective targeting of the parasite-secreted EVs may facilitate the development of novel tools for diagnostics and delivered therapy to schistosomiasis, as well as to immune-associated disorders.

Keywords: extracellular vesicles, *Schistosoma*, Th2 immunity, miRNAs, M1 pathway

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***Correspondence:**
Dror Avni
droravni@msn.com
Orly Avni
orly.avni@biu.ac.il

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INTRODUCTION

Schistosomiasis is caused by the trematode helminth of the genus *Schistosoma*. The three main species that infect humans, the definite hosts, are *S. mansoni* (Africa, South America, Caribbean, and Middle East), *S. japonicum* (China and South East Asia), and *S. haematobium* (Africa and Middle East) (McManus et al., 2018). Humans are infected in freshwater bodies, where the schistosomal cercariae penetrate the skin, transform into schistosomula that migrate to the lungs, and then to the liver. In the liver, the male and female copulate and mature into adult worms that further migrate together to their final destination–either urogenital venules for *S. haematobium* or mesenteric venules for *S. mansoni* and *S. japonicum* (Gryseels, 2012). In the venules, the female produces eggs that reach the water through either urine or feces. The miracidia larvae that hatch from these eggs invade specific aquatic snails, the intermediate hosts, in which asexual reproduction

yields thousands of infective cercariae (Dunne and Cooke, 2005). Symptoms of schistosomiasis are caused mostly by the immune response to eggs that failed to be delivered out of the body and become trapped in tissues, where they may induce intestinal, hepato-splenic or urogenital diseases (McManus et al., 2018).

The infection course of schistosomiasis and the immune response of the host evolved in concert during continuing coevolutionary relationship that exerts selective pressures on the parasite for survival and reproduction without excessively harming its host, and on the host to expel the invasive parasite with a minimum collateral damage to its own tissues (Allen and Maizels, 2011). Parasites release biologically active molecules, and it is clear now that some of them are packed inside extracellular vesicles (EVs) (Coakley et al., 2015; Siles-Lucas et al., 2015; Eichenberger et al., 2018; Zakeri et al., 2018; Ofir-Birin and Regev-Rudzki, 2019; Bischofsberger et al., 2020). EVs are cell-derived membrane-enclosed particles that varied by their size, content, and intra-cellular origin. It was demonstrated that both *S. japonicum* and *S. mansoni* adult worms, as well as *S. mansoni* schistosomula and *S. japonicum* eggs, secrete EVs (Wang et al., 2004; Nowacki et al., 2015; Sotillo et al., 2016; Zhu et al., 2016a; Zhu et al., 2016b; Samoil et al., 2018; Kuipers et al., 2020; Meningher et al., 2020). This review is focused on the current knowledge on the way by which schistosomal EVs modulate the immune response, mostly as was learned from the murine model. However, these findings are applicable to humans, as schistosomal microRNAs (miRNAs) have been found in blood-derived EVs of schistosomal infected humans (Meningher et al., 2017). Deeper dissection of these communicable particles may facilitate the development of novel approaches to treat schistosomiasis and retuning immune disorders.

Schistosomal EVs Promote the Innate M1 Immune Response

It was found that adult *S. japonicum*-derived EVs increase the polarization of macrophages *in vitro* into the classic M1 subtype (Wang et al., 2015). M1 macrophages possess pro-inflammatory activity such as phagocytosis and secretion of pro-inflammatory cytokines, whereas the alternatively activated macrophages (M2 macrophages) regulate mostly the resolution phase of inflammation including the repair of the damaged tissues. MiRNAs play a major role in transducing the functional program harbored by EVs (Rayner and Hennessy, 2013; Xu et al., 2013; Arora et al., 2017). They are a class of small non-coding RNAs that negatively regulate gene expression by complementary binding to the 3′ untranslated region (3′ UTR) of their mRNA targets, and either silence translation or decrease mRNA levels. MiRNAs are involved in the regulation of development, differentiation and activation of immune cells (Baumjohann and Ansel, 2013; Montagner et al., 2014; Mehta and Baltimore, 2016). It was shown that uptake of the schistosomal enclosed miRNAs miR-125b and bantam by macrophages expand their proliferation and the production of the M1 cytokine TNF-α (Liu et al., 2019). Decreasing either the number of host monocytes or the TNF-α levels in the infected mice alleviate the worm and egg burden and consequently the pathology. Secreted-EVs from *S. mansoni* schistosomula are also internalized by human monocyte-derived dendritic cells (DCs) and increase their expression of IL-12 (Kuipers et al., 2020), another hallmark cytokine of the M1 response, and a powerful inducer of the Th1 pathway (see below). These data altogether indicate that schistosomal EVs skew the innate immune response toward the M1 pathway, and this deviation is beneficial for the parasite survival.

Schistosomal EVs Down-Modulate the Adaptive Th2 Immune Response

Pathologically, the acute stage of schistosomiasis begins 1-2 weeks following the cercarial penetration and continues until the adult parasites are set at the blood vessels. The chronic stage starts with the egg deposition and can last for years. The acute infection initiates a strong Th1 reaction, which in mice persists for ~ 5 weeks (Fallon et al., 1998; Pearce et al., 2004), whereas the chronic stage promotes the Th2 response (Fairfax et al., 2012; Maizels et al., 2012).

T helper (Th; $CD4^+$) cells have a fundamental role in orchestrating the immune response. After the first encountering of naïve Th cell with the appropriate antigen – as a pathogen-derived peptide that is presented by antigen presenting cells (APCs; initially DCs and subsequently other APCs such as macrophages) – it can differentiate toward effector or regulatory lineages (Avni and Rao, 2000; Ansel et al., 2006; Lee et al., 2006; Wilson et al., 2009; Zhou and Littman, 2009; Sallusto, 2016). Each lineage is characterized by the expression of a distinct set of cytokines that eventually instruct the strategy of the immune response. IFN-γ is the signature cytokine of Th1 cells, IL-4, IL-5, and IL-13 (the 'Th2 cytokines') of Th2 cells, IL-17 of Th17 cells, and TGF-β and IL-10 of T regulatory (Treg) cells. IL-10 is also expressed by other immune cells such as the M2 macrophages and Th2 cells. IFNγ exerts protective functions during intracellular infections, IL-17 contributes to the host defense against extracellular infections, Th2 cytokines play a major role in response to extracellular parasites, and Treg derived cytokines prevent potential self-reactivity and dampen hyper-immune response (Shamriz et al., 2016; Avni and Koren, 2018). A poorly understood aspect of schistosomiasis is the decline in the Th2 response at the chronic stage after the initial peak at approximately week 8 of infection in humans (Pearce and MacDonald, 2002; Dunne and Cooke, 2005). This decay is intriguing because it occurs even though, without treatment, the parasitic worms live for years and continue to produce eggs (Fairfax et al., 2012; Maizels et al., 2012; Nutman, 2015); There are evidence of infected immigrants that carry schistosomal eggs in their feces decades after leaving the endemic areas (Warren et al., 1974; Hornstein et al., 1990).

Macrophages and DCs incorporate EVs *via* phagocytic or endocytic processes. However, in our quest to understand the decline in the Th2 response during the chronic stage of schistosomiasis, we found that adult *S. mansoni*-secreted-EVs are internalized by Th cells, and downregulate their differentiation toward the Th2 lineage, in an APC-independent manner (Meningher et al., 2020). Inside the Th cells, the schistosomal EV-enclosed miRNAs modulate restrictedly the Th2 transcriptional program, most prominently reduce the expression

of the Th2-lineage specifying transcription factor *Gata3* and of the Th2 cytokines *Il-4*, *Il-13*, and *Il-5*. This decrease is not accompanied by alteration in the expression levels of either Th1, Th17 or Treg specifying transcription factors or their hallmark cytokines. *In-vivo*, the schistosomal miRNAs are found in Th cells that are isolated from Peyer's patches and mesenteric lymph nodes of infected mice. Mechanistically, the schistosomal-enclosed miRNA miR-10 targets MAP3K7 and consequently down-modulates the activity of NF-kB, a critical transcription factor for Th2 cell differentiation and function (Meningher et al., 2020). This is probably only one example, out of many unexplored yet, of anti-Th2 function that is mediated by the EV-derived miRNAs.

The Th2 response, in which Th2 cells are the major players, was evolved during evolution as the most appropriate reaction to multicellular pathogens and tissue injury (Allen and Maizels, 2011; Harris and Loke, 2017). Th2 cells promote macrophage differentiation toward the M2 pathway, and these alternative macrophages can directly bind opsonized parasite larvae through complement components or antibodies to reduce larvae motility (Burke et al., 2009; Maizels et al., 2009; Allen and Maizels, 2011; Neill and McKenzie, 2011; Fairfax et al., 2012; Maizels et al., 2012; Ariyaratne and Finney, 2019). Th2 cytokines recruit also eosinophils that can damage schistosomula through antibody-dependent cell-mediated cytotoxicity (ADCC), which eventually employ degranulation and parasite killing. The humoral profile of Th2 type immunity is associated with elevation levels of the IgG1, IgE and IgG4 isotype antibodies. IgE functions largely through its ability to bind eosinophils and mast cells, whereas IgG4 is more associated with the resolution phase. Therefore, increasing the innate immune response of the M1 pathway and downregulation of the adaptive immune response of the Th2 pathway, reflect the schistosomal efforts to evade hostile environment. However, the relationship between the parasite and its host is more complicated.

Schistosomal Eggs and the Th2 Double Edged Sword

During the chronic stage, a fraction of the produced eggs is not excreted successfully and become permanently lodged in organs such as the intestine, liver (*S. mansoni* and *S. japonicum)*, bladder, and urogenital system (*S. haematobium)*. These tissue-trapped eggs are actually the major cause to the severe pathology of schistosomiasis (Burke et al., 2009; Fairfax et al., 2012; Kamdem et al., 2018; Ariyaratne and Finney, 2019; Giorgio et al., 2020). Egg antigens, which are taken up by dendritic cells, promote an APC-dependent Th cell differentiation toward the Th2 pathway (Pearce, 2005). Accumulation of the Th2 cells around the trapped eggs increases the infiltration and differentiation of M2 macrophages, eosinophils, and additional immune cells that mediate tissue regeneration and repair and assembly of the Th2-type granulomas. Granulomas are well-defined clusters of inflammatory cells embedded in a collagen-rich extracellular matrix around the parasite eggs to wall off the eggs and their toxic secreted products. Generation of granuloma is Th2 cell-dependent, as T cell-deficient mice or either of the IL-4 or the α-chain of the IL-4R are being unable to mount granulomatous response against schistosomes and are dying earlier than immunologically competent infected mice (Brunet et al., 1997; Fallon et al., 2000). Also, the lack of M2 macrophages reduces the size of the egg-associated granulomas and significantly exacerbated the disease (Herbert et al., 2004; Nascimento et al., 2014). However, continuous repair in chronic Th2 setting results in excessive extracellular matrix deposition, hepatic fibrosis and increased portal hypertension as well as other symptoms that drive the disease morbidity and mortality (Pearce, 2005; Burke et al., 2009; Fairfax et al., 2012; Kamdem et al., 2018; Ariyaratne and Finney, 2019; Mickael and Graham, 2019; Giorgio et al., 2020). The granuloma formation therefore seems to be an evolutionary chosen compromise that allows the host to live with the infection for many years (Hams et al., 2013).

It was shown that in addition to egg-antigens, the *S. japonicum* eggs release EVs that can transfer parasitic miRNA cargo into hepatocytes in the infected mice (Zhu et al., 2016b). The transferred schistosomal miRNA miR-71a attenuates the pathological progression and liver fibrosis (Zhu et al., 2016b), *via* inhibition of the TGF-beta1/SMAD and IL-13/STAT6 pathways by direct targeting of semaphorin 4D (Wang et al., 2020). In addition, treatment of *S. japonicum* infected mice with miR-71a increases the percentage of Treg cells and reduces of the effector Th1/Th2/Th17 cells in the liver. Hence, trapped egg-derived EVs can balance the immune response and tune the fibrosis extent.

Granulomatous response is beneficial to schistosomes by maintaining the host health, but granulomas are also obligatory for the schistosomal reproduction. Intestinal granulomas, which are more organized, aid the transport of the parasite eggs from the mesenteric vasculature to the intestinal lumen and excretion in the feces (Costain et al., 2018; Schwartz and Fallon, 2018; Ariyaratne and Finney, 2019; Giorgio et al., 2020). Although the underlying mechanisms are unclear yet, in mice infected with *S. mansoni* and deficient in the IL-4 expression, almost no eggs were found in the feces (Fallon et al., 2000). The absence of macrophage-specific IL-4Rα signaling, also results in impaired egg expulsion (Herbert et al., 2004). These findings indicate that the Th2-associated granulomas facilitate a non-inflammatory evacuation of the eggs, which is probably the best solution for both humans and schistosomes. It is unclear yet whether the excreted and trapped eggs secrete EVs with alternative cargos.

The Harbored miRNAs Execute the EV-Functions

MiRNAs can contribute to quantitative regulation of gene expression, mostly of dosage-sensitive genes for which minor fluctuations in protein expression levels may significantly affect the functional input (Zhu et al., 2014; Arora et al., 2017). In the current miR database (version 21), 79 mature miRNAs of *S. japonicum* and 225 mature miRNAs of *S. mansoni* have been reported (Wang et al., 2010). However, until now, very little is known about the role of the miRNAs in helminthic infections (Zhu et al., 2014; Arora et al., 2017). Since the miRNAs play a major role in the transmission of the EV-instructions, we compared the profiles of EV-harbored miRNAs from different schistosomal stages, according to several recent published studies (**Figure 1**). The ability to perform a reliable quantitative

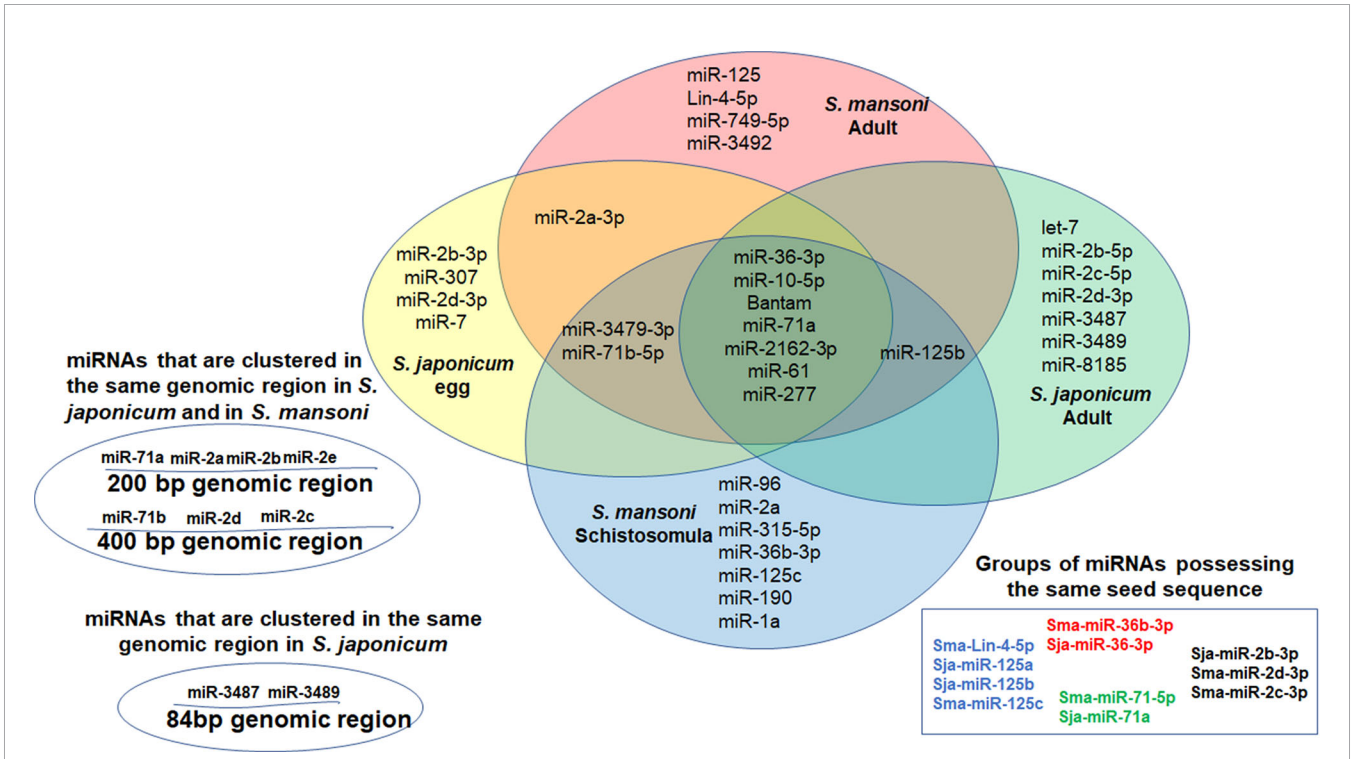

FIGURE 1 | Venn Diagram presenting miRNAs that were found in EVs derived from *S. japonicum* eggs (Zhu et al., 2016b), *S. mansoni* schistosomula (Nowacki et al., 2015) and both *S. japonicum* adult (Zhu et al., 2016a) and *S. mansoni* adult (Samoil et al., 2018). Only miRNAs that are listed in the miRBase and have more than 100 reads are included.

sequencing comparison of EV-contained miRNAs between independent studies is limited, especially due to the absent of standard EV gene. Hence, we have chosen to include only miRNAs that are listed in the miRBase and have more than 100 reads in either *S. japonicum* eggs (Zhu et al., 2016b), *S. mansoni schistosomula* (Nowacki et al., 2015), adult *S. japonicum* (Zhu et al., 2016a) or adult *S. mansoni* (Samoil et al., 2018) (**Figure 1**). The miRNA sequences are presented in **Table 1**. Most of the miRNAs are found in more than one developmental stage. Six of the miRNAs are clustered in two conserved genomic regions in both *S. japonicum* and *S. mansoni*; miR-71a, miR-2a, and miR-2b are clustered in one region and miR-71b, miR-2c, and miR-2d are clustered in another genomic region. MiR-3487 and miR-3489 are clustered in a third genomic region in *S. japonicum* (as shown in **Figure 1**). This clustering may suggest the existence of a local EV-packaging signal (Zhang et al., 2015), although there is no evidence for that in schistosomes. Several miRNAs that are expressed through all stages possess the same seed sequence (**Figure 1**) and therefore putatively regulate the same targets.

Although very little is known about the functions of EV-associated miRNAs (Samoil et al., 2018; Bischofsberger et al., 2020), it is very likely that many of them are involved in the immune evasion. MiR-36-3p, miR-10-5p, miR-71a, miR-2162-3p and miR-61, were found in EVs from all stages of schistosomal development and have a known involvement in immune regulation. MiR-36-3p regulates ERK1/2-induced EMT in pancreatic ductal adenocarcinoma (Hu et al., 2018). ERK1 is also important for Th2 differentiation and development in experimental model of asthma (Goplen et al., 2012). MiR-10-5p downregulates NF-kB, which is critical for Th2 differentiation (Meningher et al., 2020). Bantam induces the differentiation toward the M1 macrophages (Liu et al., 2019), and additional 39 potential human targets (Samoil et al., 2018). MiR-71a attenuates the pathological progression of liver fibrosis (Wang et al., 2020). MiR-2162-3p, a schistosome-specific microRNA that is consistently present in the hepatic stellate cells of *S. japonicum* infected mice, promotes hepatic fibrosis by regulating TGFβ receptor III, a negative regulator of TGF-β signaling (He et al., 2020). MiR-61 targets Vav-1, (Yoo and Greenwald, 2005; Jannot and Simard, 2006), a critical signaling molecules of the immune cells (Katzav, 2015). The miR-125 family appears in EVs from all stages except eggs, and it was shown that miR-125b promotes the M1 pathway (Liu et al., 2019). This initial analysis support the idea that the EV-enclosed miRNAs have a fundamental role in executing the immune modulation by the schistosomes.

Schistosomal EV-Guidance

In the murine model, the adult *S. mansoni* worms are located at the mesenteric and small intestine venules, which are drained by the gut-associated lymph nodes. The schistosomal miRNAs miR-10 and Bantam were found selectively in the gut-associated lymph nodes, the Peyer's patches and mesenteric lymph nodes, and not in the inguinal lymph node or spleen (Meningher et al., 2020).

TABLE 1 | Sequences of miRNAs from schistosomal EVs that were taken from the miRBase.

Table 1a: miRNAs that were found in all EVs		
miRNA	Accession	Sequence
sja-miR-36-3P	MIMAT0016258	5′-c**caccggg**uagacauucauucgc-3′
sja-miR-10-5p	MIMAT0016253	5′-a**acccugu**agacccgaquuugg-3′
Sja-Bantam	MIMAT0010177	5′-u**gagaucg**cgauuaaagcuggu-3′
sja-mir-71a	MIMAT0010176	5′-u**gaaagac**gaugguagugaga-3′
miR-2162-3p	MIMAT0016273	5′-u**auuaugc**aacquuucacucu-3′
sja-miR-61	MIMAT0016259	5′-u**gacuaga**aagugcacucacuu-3′
Sja-miR-277	MI0015296	5′-u**aaaugca**uuuucuggcccg-3
Table 1b: EVs derived miRNAs with the same seed sequence		
sma-lin-4-5p	MIMAT0003956	5′-u**cccugag**accuucgacugugu-3′
sja-miR-125a	MIMAT0010178	5′-u**cccugag**acccuuugauuguc-3′
sja-miR-125b	MIMAT0010179	5′-u**cccugag**acugauaauugcuc-3′
sma-miR-125c	MIMAT0033510	5′ -u**cccugag**accuuagaguuguc-3′
sma-miR-36b-3p	MIMAT0033515	5′-c**caccggg**uagacauucauccgc-3′
sja-miR-36-3p	MIMAT0016258	5′-c**caccggg**uagacauucauucgc-3′
sma-miR-71b-5p	MIMAT0025043	5′-u**gaaagac**uugaguagugagacg-3′
sja-mir-71a	MIMAT0010176	5′-u**gaaagac**gaugguagugaga-3′
sja-miR-2b-3p	MIMAT0016248	5′-u**aucacag**cccugcuugggacaca-3′
sma-miR-2d-3p	MIMAT0025035	5′-u**aucacag**uccugcuuagguga-3′
sma-miR-2c-3p	MIMAT0025034	5′-u**aucacag**ccgugcuuaagggc-3′
Table 1c: EVs derived miRNAs that were found in part of the schistosomal stages		
miRNA	Accession	Sequence
sja-miR-3479-3p	MIMAT0016275	5′-u**auugcac**uuaccuucgccuug-3′
sja-miR-3487	MIMAT0016296	5′-u**ccucgaa**cuguuguggcca-3′
sja-miR-3489	MIMAT0016298	5′-g**ccacaac**aguucgaggacg-3′
sme-miR-315-5p	MIMAT0011282	5′-u**uuugauu**guugcucugagaguu-3′
sma-miR-190-5p	MIMAT0025027	5′-u**gauaugu**augggguacuuggug-3′
sma-miR-1a-5p	MIMAT0033630	5′-u**ggaaugu**ggcgaaguaugg-3′
sja-miR-8185	MIMAT0032784	5′-a**ggaucga**ugaacggagcauu-3′
sja-miR-3492	MIMAT0016301	5′-a**uccgug**cugagauuuogucu-3′
sma-miR-96-5p	MIMAT0033635	5′-c**uuggcac**uuuggaauugucac-3′
sme-miR-749	MIMAT0004016	5′-g**cugggau**gagccucgguggu-3′
sja-miR-307	MIMAT0016270	5′-u**cacaacc**uacuugauugag-3′
sja-miR-7-5p	MIMAT0016249	5′-u**ggaagac**uggugauauguuguu-3′
sja-let-7	MIMAT0010175	5′-g**gagguag**uucguuguguggu-3′
sma-miR-2c-5p	MIMAT0025033	5′-u**ccuugu**ucgacugugaugug-3′
sma-miR-2d-5p	MIMAT0032135	5′-g**ucauccu**uggauugugauu-3′
sja-miR-2a-5p	MIMAT0016245	5′-c**agucaau**auuggcugauggca-3'
sja-miR-2b-5p	MIMIAT0016247	5′-c**gucucaa**aggacugugagcca-3′
sja-miR-2a-3p	MIMAT0016246	5′-u**cacagcc**aguauugaugaacg-3′

Bold marks the seed sequences.

These findings suggest a targeted long-distance delivery of the schistosomal EVs through the lymphatic system, rather than a systemic distribution through the blood.

The specificity is probably imposed by membrane associated proteins or other cell surface molecules. It was shown that the uptake of the S. *mansoni* schistosomula-derived EVs by monocyte-derived DCs is mainly mediated *via* the C-type lectin receptor DC-SIGN (CD209) (Kuipers et al., 2020). DC-SIGN is an adhesion molecule that recognizes and binds high-mannose-containing glycoproteins on pathogens, and is present on the surface of both macrophages and dendritic cells.

Studies performed proteomic analysis of schistosome-derived EVs (Nowacki et al., 2015; Sotillo et al., 2016; Zhu et al., 2016a; Samoil et al., 2018; Meningher et al., 2020) are presented in **Table 2**. EV-associated proteins of adult schistosomes include well-described exosomal markers designated in ExoCarta such as heat sock proteins (HSP70), energy-generating enzymes (enolase, pyruvate kinase, GAPDH, phosphoglycerate kinase 1), cytoskeletal proteins (actin, tubulin, fimbrin), and tetraspanins. All of these proteins have already described as the most frequently secreted proteins from *S. japonicum* and *S. mansoni* (Samoil et al., 2018). These proteomic analyses were performed mainly to confirm the characterization of schistosomal vehicle as EV, according to the manually updated online database of EV-proteins, -RNAs and -lipids (Pathan et al., 2019) listed in the Vesiclepedia (http://microvesicles.org/extracellular_vesicle_markers). However, functional studies to explore their function in the targeted delivering and immune modulation are still missing.

Summary and Perspective

Although there are only few studies that characterize the schistosome-secreted EVs, it is possible to conclude that schistosomes from all the developmental stages, including the eggs, secrete EVs. These EVs promote a more permissive immune response to the parasite. The EV-derived miRNAs promote the M1 pathway of the innate immunity and divert the adaptive immunity away from the Th2 pathway (**Figure 2**).

TABLE 2 | Proteomic analyses of schistosome-derived EVs.

	Mammalian Gene Symbol	Protein name	International Journal for Parasitology 2015 (Javier Sotillo et al.)	Scientific Reports 2016 (Lihui Zhu et al.)	Journal of Extracellular Vesicles 2015 (Fanny C. Nowacki et al.)	EMBO reports 2020 (Tal Meningher et al.)
1	PDCD61P	programmed cell death 6 interacting protein	√		√	√
2	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	√	√	√	√
3	HSPA8	heat shock 70 kDa protein 8	√	√	√	√
4	ACTB	beta Actin,	√	√	√	√
5	ANXA2	annexin A2	√	√	√	√
6	YWHAZ (14-3-3)	tyrosine 3-monooxygenaseltryptophan 5-monooxygenase activation protein,		√	√	√
7	PKM	pyruvate kinase		√		√
8	ENO1	enolase 1,(alpha)		√	√	
9	HSP90AA1	heat shock protein 90kDa alpha		√		
10	tetraspanin	CD9 (SM23), CD63(TSP-2), CD81	(TSP-1,TSP-2, TSP-4, TSP-18)	TSP-1, TSP-(CD63)	TSP-2(CD63)	TSP-2(CD63), SM23
11	EEF1A1	translation elongation factor 1-alpha		√	√	√
12	PGK1	phosphoglycerate kinase		√		√
13	CLTC	clathrin, heavy chain				
14	ALDOA	aldolase	√	√	√	√
15	EEF2	eukaryotic translation elongation	√	√		

However, since the Th2 response is crucial for the host survival and eggs departure, the regulation of the Th2 response by the schistosomes is carefully balanced and targeted, as EV-contained miRNAs from adult schistosomes reduce the differentiation of the Th2 cells selectively in the mesenteric lymph nodes, whereas, miRNA derived from EVs of trapped eggs increases the percentage of Treg cells in the liver and reduces of the effector Th1/Th2/T17 cells. Further studies are required to assess precisely the content, function and targeting of EVs from different developmental stages and physiological circumstances.

From what we have learned so far, the modified immune response by the parasite is in accordance with the 'hygiene hypothesis' (Stiemsma et al., 2015; Versini et al., 2015; Alexandre-Silva et al., 2018; Bach, 2018), associating the dramatic increase in autoimmune and allergic diseases, observed in recent decades in industrialized countries, with the reduced exposure to diverse infectious agents. Indeed, epidemiological evidence supports the fact that schistosomiasis can protect against allergy in an endemic population (Medeiros et al., 2003; Oliveira et al., 2014). Therefore, exploring the schistosomal 'strategy' may facilitate the development

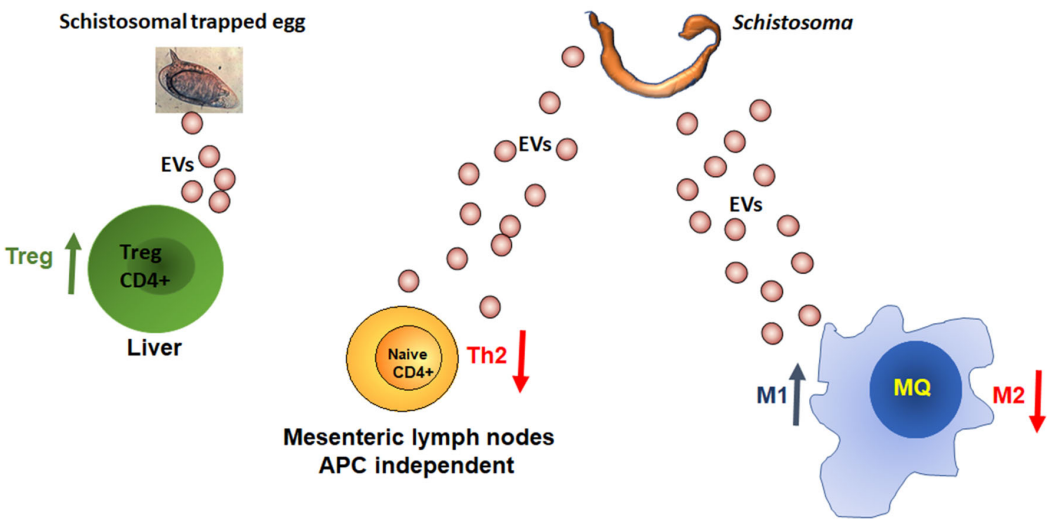

FIGURE 2 | MiRNAs from schistosomal EVs manipulate the host immune response. More details in the text.

of novel diagnostic tools (Meningher et al., 2017; Weerakoon et al., 2018; Silva-Moraes et al., 2019), vaccines (Sotillo et al., 2016; Mekonnen et al., 2020) and therapeutic approaches for human schistosomiasis and immune dysregulation (Siles-Lucas et al., 2015; Ayelign et al., 2020).

AUTHOR CONTRIBUTIONS

These authors have contributed equally to this work and share senior authorship. All authors contributed to the article and approved the submitted version.

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