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Overview of the interaction of helminth extracellular vesicles with the host and their potential functions and biological applications

Christian M. Sánchez-López [a,b], María Trelis [a,b], Dolores Bernal [c], Antonio Marcilla [a,b,*]

[a] Àrea de Parasitologia, Departament de Farmàcia i Tecnologia Farmacèutica i Parasitologia, Universitat de València, Av. V.A. Estellés, s/n, 46100, Burjassot, Valencia, Spain
[b] Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, Health Research Institute La Fe-Universitat de València, 46026 Valencia, Spain
[c] Departament de Bioquimica i Biologia Molecular, Facultat de Ciencies Biològiques, Universitat de València, C/ Dr. Moliner, 50, 46100 Burjassot, Valencia, Spain

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ABSTRACT

Helminth Extracellular Vesicles (EVs) have emerged as important mediators in host-parasite communications, participating in the parasite survival and its pathogenic effects. In the last decade, a growing amount of information reporting the isolation and characterization of EVs from different helminth species has appeared, but unfortunately, few reports have focused on functional studies of helminth EVs in different cell lines, organoids or animal models. We here review these *in vitro* and *in vivo* studies, which clearly demonstrate that helminths secrete EVs, which affect their environment. Helminth EVs are actively internalized by different cell lines, modulating cellular functions important for host-parasite communication. We discuss how these lines of investigation should provide potential new biomarkers of infection, and since helminth EVs can modulate the host immune response, we also discuss how they can provide a new landscape for the development of new vaccine tools against helminthiases as well as immunotherapy.

1. Introduction

Parasitic helminths affect nearly a third of the human population, and are highly prevalent in domesticated animals, such as livestock (Jourdan et al., 2018). The great economic losses caused by helminth infections in developing and developed countries, along with the emerging drug resistance, and the lack of effective vaccines make helminth parasites a global concern, which requires the development of new control programs, including research of potential treatments and vaccines (Hotez et al., 2008).

Despite helminths having significant differences in their biological life cycles and tissue tropism, they all establish long-term infections presumably by modulating the immune response in the host to promote their survival (Maizels and McSorley, 2016; Gazzanelli-Guimaraes and Nutman, 2018; Ryan et al., 2020). The manipulation of the host response is mostly due to the release of molecules at the host-parasite interface, commonly referred to as excretory/secretory products (ESP), including some specific proteins, lipids, metabolites, nucleic acids and extracellular vesicles (EVs) (Maizels et al., 2018).

Briefly, EVs are small membrane-enclosed vesicles classified according to their size and biogenesis as exosomes, microvesicles, or apoptotic bodies (Yáñez-Mó et al., 2015). As the current methods for EVs isolation generally provide a mixture of all three different subtypes, and most studies do not resolve between them, the term EVs is recommended (ISEV). EVs contain a variety of molecules, including proteins, nucleic acids, lipids and other metabolites (Doyle and Wang, 2019), and have rapidly emerged as powerful mediators of host-parasite communications, as well as an interesting source of potential biomarkers of parasitic diseases (Coakley et al., 2015; Eichenberger et al., 2018a; Tritten and Geary, 2018; Drurey et al., 2020). The finding that parasites produce and release EVs was first demonstrated in the trematodes *Fasciola hepatica* and *Echinostoma caproni,* with EVs isolated by differential centrifugation and characterized by Transmission Electron Microscopy (TEM) and proteomic analyses (Marcilla et al., 2012). Since then, EVs have been characterized from several species of parasitic helminths (Sotillo et al., 2020).

Advances in helminth EV genomics, transcriptomics and proteomics have revealed many molecules which may play an important role in immune regulation and host-parasite cross-talk (Cwiklinski et al., 2015; Mekonnen et al., 2018; Zakeri et al., 2018). However, in this review we

* Corresponding author at: Àrea de Parasitologia, Departament de Farmàcia i Tecnologia Farmacèutica i Parasitologia, Universitat de València, Av. V.A. Estellés, s/n, 46100 Burjassot, Valencia, Spain.
E-mail address: antonio.marcilla@uv.es (A. Marcilla).

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focus on the functional properties of EVs from helminths, discussing the existing studies describing the effect of EVs on different cell lines and/or animal models, highlighting the different roles of EVs depending not only on their origin, but also on the recipient cell (Fig. 1).

2. EV isolation from parasite helminths: general considerations

Helminths can be divided into 2 major groups known as nematodes (roundworms), and platyhelminthes (flatworms), which can further be divided into cestodes (tapeworms) and trematodes (flukes). The presence of EVs has been documented in both phyla (Coakley et al., 2015; Eichenberger et al., 2018a; Tritten and Geary, 2018; Sotillo et al., 2020), but differences in their external surface have a direct impact on the amount of vesicles produced. Platyhelminthes contain a lipid-rich membranous tegument, which plays an essential role in EV secretion, whereas nematodes have a rigid and flexible cuticular exoskeleton, formed mainly by collagen and matrix proteins (de la Torre-Escudero et al., 2016) (Fig. 1). Consequently, EV secretion in nematodes appear to be produced through the anterior and/or posterior openings of the parasite (Drurey et al., 2020), although reports also indicate the importance of the excretory-secretory pore in some nematodes like *Brugia malayi* (Harischandra et al., 2018). Therefore, as the surface area that can release EVs is proportionally higher in platyhelminthes, they produce higher amounts of EVs than nematodes, and accordingly, to obtain EVs from platyhelminthes fewer parasites and less time of incubation in culture are required. The time of incubation is also crucial for active secretion, since prolonged incubations lead to loss of worm viability in culture, a factor that is species-dependent.

In addition to the parasite from which the EVs are derived, the election of the EVs isolation method is also a crucial step, which may have drastic effects on the results obtained (Taylor and Shah, 2015). The most widely accepted method to isolate EVs is differential ultracentrifugation, which involves sequential centrifugations, increasing speed and time, to pellet particles decreasing in size (Konoshenko et al., 2018). Differential centrifugation has also been the most utilized method to isolate parasitic helminths EVs to date, however, it is time-consuming and operator sensitive, which limits the standardization and subsequent scaling up of the protocol (Davis et al., 2019). Moreover, growing evidence shows that centrifuging at high speeds may co-isolate contaminant proteins that may interfere in the functionality of EVs (Linares et al., 2015; Monguió-Tortajada et al., 2019). To overcome these limitations, other isolation methods such as size exclusion

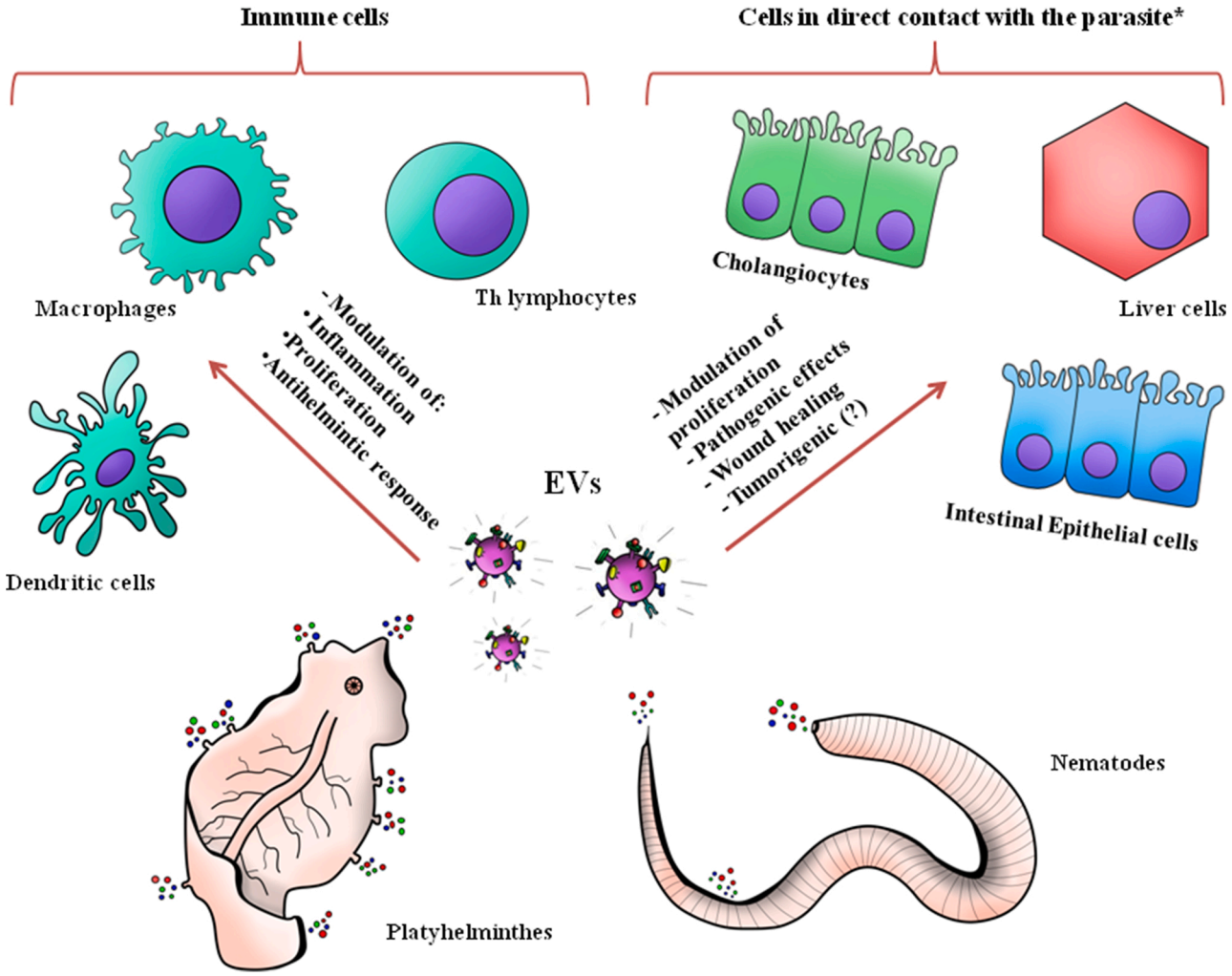

Fig. 1. Schematic representation of the effects of Helminth Extracellular Vesicles (EVs). EVs are secreted in nematodes mainly through the anterior and posterior ends of the worm due to the existence of a cuticular exoskeleton. In contrast, in platyhelminthes, the tegument plays an essential role in EV secretion, allowing them to produce higher amounts of EVs than nematodes. EVs released by helminths can interact with different host cells, including immune cells and cells close to the release of the EVs *in vivo*. Cell types (*) in primary contact with the parasite and EVs effects on those cells might differ depending on the parasite species. Effective modulation of proliferation and the immune response by helminth EVs has been reported, promoting or inhibiting proliferation and inflammation depending on the parasite and the cell type, and affecting the profile of cytokines released from host cells. In this context, EVs have been shown to be key players in the pathogenic effects produced by these parasites.

chromatography (SEC) (Davis et al., 2019; Hansen et al., 2019; Davis et al., 2020; Sánchez-López et al., 2020), density gradient ultracentrifugation (DGUC) (Sotillo et al., 2016; Samoil et al., 2018; Eichenberger et al., 2018b,c; Chaiyadet et al., 2019; Mekkonen et al., 2020; Meningher et al., 2020), and gravity flow methods (Murphy et al., 2020), have been also used to isolate EVs from parasitic helminths.

In the following sections, we summarize the existing literature describing the different effects observed in response to parasitic helminth EVs, both *in vitro* and *in vivo* (Tables 1 and 2), indicating the EVs produced by nematodes and by platyhelminthes. Different studies reporting the active internalization of helminths EVs *in vitro* by recipient cells are also summarized in Table 3.

3. Immune regulatory effects of helminth EVs on the mononuclear phagocyte system

There is growing evidence of the active uptake of nematode EVs by host innate immune cells (mainly macrophages). Live worms and their ESP may directly induce signalling pathways in host cells producing a regulatory immune phenotype (Gazzinelli-Guimaraes et al., 2018). However, the effects of purified and concentrated EVs on host cells may be different from the effects of the whole ESP of the parasites (Eichenberger et al., 2018a). More specifically, infective L_3 stages of the human filarial parasite *Brugia malayi* release EVs that are actively internalized *in vitro* by a murine macrophage cell line and generate significant increases in G-CSF, MCP-1, IL-6 and MIP-2 levels, eliciting M1 type macrophage activation (Zamanian et al., 2015). Also, EVs from the nematode *Trichinella spiralis* also exhibit immunomodulatory properties by increasing the production of IL-10 and IL-6 in peripheral blood mononuclear cells (PBMC), as well as decreasing IL-17a production (Kosanović et al., 2019).

In contrast, several reports show downregulation or immunosuppressive effects of nematode EVs *in vitro*. It has been described that the nematode *Heligmosomoides polygyrus* can suppress the activation of bone marrow derived macrophages (BMDM) by downregulating type 1 (IL-6 and TNF-α) and type 2 (Relm-α and Ym1), immune response-associated molecules, as well as inhibiting the expression of the IL-33 receptor subunit ST2 (Coakley et al., 2017). Moreover, recent reports showed that the internalization of EVs from the microfilarial stage of *B. malayi* by human monocyte derived dendritic cells (THP-1) downregulate the phosphorylation of mTOR. Additionally, the presence of miRNA (miR-100, miR-7 and miR-71) targeting mTOR within microfilarial-derived EVs may indicate one specific method used to downregulate the pathway, modulating the cellular metabolism of host

Table 1
Potential *in vitro* functions and effects described for helminth EVs.

	Helminth	Recipient cell	Function	Effect / Mechanism	Reference
Nematodes	*Brugia malayi*	Murine macrophages cell line J774A.1	M1 type macrophage activation	Significant increases in G-CSF, MCP-1, IL-6 and MIP-2 levels	Zamanian et al., 2015
	Brugia malayi	THP-1 monocites and THP-1 derived dendritic cells	Regulation of Immune Metabolism	Downregulation of mTOR phosphorilation	Ricciardi et al., 2021
	Heligmosomoides polygirus	BMDM (C57BL/6, BALB/c, and ST2–/– mice)	Supression of BMDM activation	Downregulation of type 1 (IL-6 and TNF-α), type 2 (Relmα and Ym1) molecules and IL-33R receptor subunit ST2	Coakley et al., 2017
	Heligmosomoides polygirus	Murine epithelial cell line MODE-K	Immunomodulatory properties	Downregulation of *Dusp1* and *IL-33R* genes	Buck et al., 2014
	Trichinella spiralis	human PBMC	Immunomodulatory properties	Significant increases in IL-10 and IL-6 levels, increased production of IL-17a	Kosanović et al., 2019
	Trichuris muris	Enteroid and caecaloid organoids (C57BL/6 N mice)	Immunomodulatory properties	Downregulation of genes involved in type-I IFN signaling	Duque-Correa et al., 2020
	Schistosoma japonicum	Murine macrophages cell line RAW264.7	Immunomodulatory properties	Increases in CD16/32, iNOS, TNF-α, IL-12, and Arg-1 production	Wang et al., 2015
	Schistosoma japonicum	Murine macrophages cell line RAW264.7	Immunomodulatory properties; proliferation promotion	Changes in RNA expression profile; increase in TNF-α production	Liu et al., 2019
	Schistosoma japonicum	Murine liver cell line NCTC clone 1469	Immunomodulatory properties	Downregulation of the expression of Gins4, Tysnd1 and Utp3 *via* their miRNAs	Zhu et al., 2016
	Schistosoma mansoni	Th cells (ICR mice)	Immunomodulatory properties	Promotion of differential gene expression *via* their miRNAs	Meningher et al., 2019
	Schistosoma mansoni	HUVECs and THP-1 monocytes	Vascular remodeling; Immunomodulatory properties	Promotion of differential gene expression	Kifle et al., 2020
Platyhelminthes	*Schistosoma mansoni*	Human monocyte-derived dendritic cells	Immunomodulatory properties	EVs glycoconjugates favor active internalization; increases in IL-6, IL-10, IL-12 and PD-L1 secretion	Kuipers et al., 2020
	Opisthorchis viverrini	human cholangiocytes cell line H69	Wound-healing; Immunomodulatory properties; tumorigenic	Cell proliferation promotion; differences in protein expression; IL-6 secretion	Chaiyadet et al., 2015
	Fasciola hepatica	BMDCs (BALB/c and OT-II mice)	Immunomodulatory properties	Increased TNF-α, CD80, CD86, CD40, OX40L expression; decreased ICAM-1 expression	Murphy et al., 2020
	Echinococcus multilocularis	Murine macrophages cell line RAW264.7	Immunomodulatory properties; decrease in NO production	Increased expression of genes involved in LPS/TLR4 pathway; supression of IL-1α, IL-1β and iNOS expression	Zheng et al., 2017
	Echinococcus granulosus	Murine BMDCs (CF-1 mice)	Immunomodulatory properties; BMDCs activation	Increase of CD86 expression; decrease in MHC class II molecules expression	Nicolao et al., 2019
	Echinococcus granulosus	Murine PBMCs (BALB-c mice)	Immunomodulatory properties; inhibition of lymphocyte proliferation	Decreased IL-10, IL-6, IL-17a and IFN-γ secretion; increased IL-2 and IL-4 secretion	Zhou et al., 2019
	Taenia pisiformis	Murine macrophages cell line RAW264.7	Immunomodulatory properties; polarization towards M2 phenotype	Increased Arg-1, IL-4, IL-6, IL-10 and IL-13 expression; decreased iNOS, IL-12 and IFN- γ expression	Wang et al., 2020

BMDCs, Bone marrow-derived dendritic cells; PBMC, peripheral blood mononuclear cells; alum, adjuvant; HUVECs, Human primary umbilical vein endothelial cells; BMDM, Bone marrow-derived macrophages.

Table 2
Potential *in vivo* functions and effects described for helminth EVs.

	Helminth	Recipient host	Administration	Effect	Reference
	Heligmosomoides polygirus	BALB/c mice	Intranasal administration of EVs	Suppression of eosinophilia and innate Type 2 responses; inhibition of IL-33R and DUSP1 production	Buck et al., 2014
	Heligmosomoides polygyrus	BALB/c mice and ST2−/− mice	Intraperitoneal vaccination with EVs + alum	Protective immunity only in wild type mice	Coakley et al., 2017
	Trichuris muris	C57BL/6 mice	Subcutaneous vaccination with EVs w/ o alum	Protective immunity	Shears et al., 2018
Nematodes	*Trichuris muris*	BALB/c mice (TNBS induced colitis)	Intraperitoneal injection of EVs	No protective effect on colitis	Eichenberger et al., 2018c
	Nippostrongylus brasiliensis	BALB/c mice (TNBS induced colitis)	Intraperitoneal injection of EVs	Amelioration of colitis; downregulation of 1 L-1β, IL-6, IL-17a, and IFNγ; upregulation of IL-10	Eichenberger et al., 2018c
	Trichinella spiralis	BALB/c mice (TNBS induced colitis)	Intraperitoneal injection of EVs	Amelioration of colitis; downregulation of 1L-1β, TNF-α, IL-17a, and IFNγ; upregulation of IL-10, IL-4, IL-13, and TGF-β; reduction of Th1 and Th17 cells, increase of Th2 cells	Yang et al., 2020
	Echinostoma caproni	BALB/c mice	Subcutaneous vaccination with EVs w/ o alum	Induction of Th2/Treg response; protective immunity; decrease of eggs output; increased IL-4, TGF-β, IFN-γ levels	Trelis et al., 2016
Platyhelminthes	*Opisthorchis viverrini*	Syrian golden hamsters	Intraperitoneal vaccination with EVs + alum	Protective immunity; decrease of worm burden and eggs output	Chaiyadet et al., 2019
	Fasciola hepatica	C57BL6 and RAG1 -/- mice (DSS induced colitis)	Subcutaneous injection of EVs	Amelioration of colitis; decreased TNF-α, IL-6 and IL-17a expression; suppression of MAPK/NF-κβ signaling pathways; reduced neutrophil infiltration	Roig et al., 2018
	Fasciola hepatica	BALB/c mice	Intraperitoneal injection of EVs + Alum	Induction of mixed Th1/Th2-cell response; increased levels of IFN-γ and IL-2	Murphy et al., 2020

TNBS, 2,4,6-trinitrobenzene sulfonic acid; DSS, Dextran sodium sulphate.

Table 3
In vitro studies reporting the active internalization of helminth EVs by recipient cells.

Phylum	Helminth	Recipient cell	Type of studies	References
	Trichuris muris; Nippostrongylus brasiliensis	Murine small intestinal organoids (from C57 Bl6/J mice)	Proteomics; Transcriptomics; *in vivo* studies	Eichenberger et al., 2018c
Nematodes	*Trichuris muris*	Murine colonic organoids (C57BL/6 N mice)	Proteomics; Transcriptomics	Eichenberger et al., 2018b
	Ascaris suum	Canine intestinal organoids	Characterization of organoids	Chandra et al., 2019
Platyhelminthes	*Echinostoma caproni*	Rat intestinal epithelial cell line IEC-18	Proteomics	Marcilla et al., 2012
	Taenia asiatica	Human colorectal cancer cell line LoVo	Proteomics; Transcriptomics	Liang et al., 2019

antigen presenting cells (Ricciadi et al., 2021).

As with nematodes, there is also increasing evidence of the active uptake of platyhelminthes EVs by host cells, including reports in both cestodes and trematodes. Like for the nematode *B. malayi*, different reports have shown that EVs from platyhelminthes also drive macrophages towards the M1 phenotype. In this context, the exposure of murine macrophages to EVs from the blood fluke *Schistosoma japonicum* significantly increased the expression of CD16/32, iNOS, TNF-α and IL-12, indicating a classical activation. However, there is also an increase of Arg-1 (marker of alternative activation), suggesting a more complex role for these EVs in macrophage polarization (Wang et al., 2015). Several experiments confirm the complex role of EVs from *S. japonicum in vitro*. It has been demonstrated that *S. japonicum* EVs are primarily taken up by host peripheral blood monocytes. Interestingly, vesicular miRNAs (miR-125b, miR-61, bantam and miR-277b) have been detected in murine macrophage RAW264.7 cells previously incubated with EVs from this parasite, promoting TNF-α production and their proliferation (Liu et al., 2019). A similar approach has been used in another blood fluke of the same genus, to demonstrate the active uptake of *Schistosoma mansoni* EVs containing miRNAs in murine T helper cells targeting the differentiation toward the Th2 pathway (Meningher et al., 2019).

Interestingly, recent studies have demonstrated that EVs released by *S. mansoni* contain glycoconjugates, which may mediate their internalization by monocyte-derived dendritic cells (moDCs) (Kuipers et al., 2020). This is the first evidence of EV molecules that could act as ligands for receptors on host cells. Kuipers et al. (2020) have also shown that EVs uptake increases IL-6, IL-10 and IL-12 secretion, as well as the secretion of co-stimulatory molecules, such as PD-L1.

Different immunoregulatory properties for EVs from other parasitic platyhelminthes have been described. EVs produced by the tapeworm *Echinococcus multilocularis* are internalized in RAW264.7 cells, suppressing pro-inflammatory cytokines (mainly IL-1α and IL-1β), but increasing the expression of key components involved in the LPS/TLR4 pathway (Zheng et al., 2017). Moreover, *E. multilocularis* EVs suppress nitric oxide production in murine macrophages, which is a common anti-helminth response (Zheng et al., 2017). With regard to *Echinococcus granulosus* EVs, their internalization by bone marrow dendritic cells has been demonstrated, where they induce an unconventional activation profile with increases in CD86, but a slight decrease in MHC class II molecules (Nicolao et al., 2019). In addition, it has been reported that protoscoleces-EVs from this parasite show immunomodulatory properties on mouse PBMCs, inhibiting lymphocyte proliferation and IL-10, IL-6, IL-17a and IFN-γ secretion in a dose-dependent manner, as well as promoting IL-2 and IL-4 secretion (Zhou et al., 2019). In addition, the immunomodulatory effects of EVs from *T. pisiformis* cysticercus have been described and been shown to induce polarization of RAW264.7 macrophages toward the M2 phenotype, increasing the production of Arg-1, IL-4, IL-6, IL-10 and IL-13, and decreasing the expression of iNOS, IL-12 and IFN- γ (Wang et al., 2020).

There are several studies showing that helminth infection could suppress the expression of co-stimulatory markers in dendritic cells (Walsh et al., 2009; Hamilton et al., 2009). In contrast, the stimulation of BMDCs with EVs from the liver fluke *Fasciola hepatica* induced the secretion of TNF-α and an enhanced expression of these co-stimulatory

markers (CD80, CD86, CD40, OX40 L), but generated a semi-mature DC phenotype characterized by the lack of ICAM-1 expression (Murphy et al., 2020). These results might be integrated by considering that it is the interplay of a wide range of different molecules, rather than one molecule alone, which ultimately leads to the strong Th2 and immunosuppressive response produced in *F. hepatica* infection.

4. Effects of Helminth EVs on non-immune cells and organoids

The complexity of the life cycles of helminth parasites each with a specific habitat reflects a tropism for a particular anatomical niche. The existing studies report the functionality of helminth EVs *in vitro*, not only on immune cells (such as macrophages and PBMCs), but also on cell types with the same characteristics as the cells, tissues or organs, that are typically in contact with the adults and the multiple developmental stages of the parasite *in vivo* (*i.e.* intestinal epithelial cells) (Fig. 1).

In this context, we showed that EVs from the trematode *E. caproni* were taken up by intestinal cells in culture, suggesting a functional role of worm-derived EVs on host cells (Marcilla et al., 2012). *S. japonicum* EVs are also taken up by mouse liver cells, downregulating the expression of the *Gins4*, *Tysnd1* and *Utp3* genes *via* the miRNAs transported in the EVs (Zhu et al., 2016). Within the same genus, EVs from *S. mansoni* have been shown to be also actively internalized in human umbilical vein endothelial cells (HUVECs) and THP-1 monocytes, promoting the differential expression of genes involved in coagulation or immune cell trafficking and signalling in HUVECs (Kifle et al., 2020). With regard to the study of cestodes, the isolation of EVs in the genus *Taenia*, and the active internalization of EVs from *T. asiatica* adults by colorectal cancer cells has been recently reported (Liang et al., 2019).

EVs from helminths not only modulate the immune response, but also can be responsible for pathogenic effects on the host. In this sense, Loukas and co-workers showed that the EVs from the liver fluke *Opisthorchis viverrini* are internalized by human cholangiocytes in culture, inducing cell proliferation, IL-6 secretion and stimulating wound-healing and tumorigenic pathways on recipient cells (Chaiyadet et al., 2015).

For the nematode *H. polygyrus*, other studies show that EVs are actively taken up by mouse epithelial cells, a cell type that is naturally in direct contact with this helminth *in vivo*, downregulating the genes *Dusp1* and IL-33. These genes are involved in dampening the type 1 pro-inflammatory reaction to Toll-like receptor (TLR) ligands and driving the early type 2 immune responsiveness, respectively (Buck et al., 2014).

Alternative studies have evaluated the uptake of EVs not only in cultured cells but also in organoids, where EVs are microinjected. It has been shown that the intestinal nematode parasites *Nippostrongylus brasiliensis* and *Trichuris muris*, both release EVs which are actively taken up by mouse small intestinal and colonic organoids, respectively (Eichenberger et al., 2018b,c; Duque-Correa et al., 2020). Although no effects on the organoids have been described for *Nippostrongylus brasiliensis*, *Trichuris muris* EVs have been shown to downregulate the expression of interferon-stimulated genes involved in type-I IFN signaling (Duque--Correa et al., 2020). A similar approach has been used recently to visualize the internalization of *Ascaris suum* EVs co-cultured with canine intestinal organoids (Chandra et al., 2019) (Table 3).

5. Immunomodulatory effects of Helminth EVs in animal models

The effects of the EVs from both nematodes and platyhelminthes on experimental animal models have also been analyzed. These models provide valuable information to assess the effects of the EVs over longer periods of time and environment. Hence, *in vivo* studies have been mainly aimed at evaluating the immunomodulatory role of EVs.

Different studies have been performed administering EVs from *H. polygyrus* to BALB/c mice, confirming their ability to suppress eosinophilia and innate Type 2 responses in the host through the inhibition of IL33R and DUSP1 production (Buck et al., 2014). In the case of platyhelminthes, studies on EVs from *S. japonicum* have shown their potential role in the regulation of host macrophages and highlighted EVs as key players in parasite survival (Liu et al., 2019). More specifically, these researchers showed that BALB/c mice treated with clodronate liposomes, which reduce the levels of both monocytes and TNF-α, presented a significant reduction of worm burden and egg deposition upon infection with *S. japonicum*. These data suggests that a decrease in monocytes and in the levels of TNF-α are detrimental for the establishment of the *S. japonicum* infection. Interestingly, after the uptake of *S. japonicum* EVs by peripheral blood monocytes, their cargo miRNA (miR-125b and bantam) can target host genes leading to increased levels of monocytes and TNF-α (Liu et al., 2019). Taken together, these data suggests that EVs may be modulating the host immune response in order to facilitate parasite survival (Liu et al., 2019).

6. Potential use of Helminth EVs as vaccination agents

Some research has been done to contribute to a better understanding of helminth EVs by characterizing their content and have identified some potential biomarkers and vaccine candidates for parasitic infections (for comprehensive reviews see Tritten and Geary, 2018; Drurey et al., 2020; Sotillo et al., 2020). These characterization studies have also contributed to assess specific proteins, lipids, and nucleic acids with immunomodulatory properties within helminth EVs cargo (Eichenberger et al., 2018a; Sotillo et al., 2020; Whitehead et al., 2020).

Several studies describe the potential use of platyhelminthes EVs as vaccines. We reported that immunization of BALB/c mice with EVs from *Echinostoma caproni* induced a balanced Th2/Treg response after challenge, increasing levels of IL-4, TGF-β, IL-10 and showing a therapeutic potential for alleviating clinical signs and mortality upon infection with metacercariae. However, EV injection did not affect the worm burden significantly, but favoured a decrease in the egg output (Trelis et al., 2016).

The liver fluke *Opisthorchis viverrini* is a platyhelminth that lives in the bile ducts of humans, where they cause hepatobiliary pathology, including cholangiocarcinoma. The vaccination of hamsters with EVs and recombinant tetraspanins derived from the EV surface from this trematode induced production of antibodies in serum and bile. Challenge of vaccinated hamsters with infective stage flukes markedly reduced the worm burden and egg output, affecting the body length of the infecting worms (Chaiyadet et al., 2019). This study provides proof-of-concept for the development of vaccines for a carcinogenic infection. Although parasite EVs may have an interesting potential as vaccines, these findings also suggest that, for some parasites, EVs may promote parasite survival instead of being effective vaccines, adapting the ecological niche of the host to facilitate the establishment of the parasite. Therefore, these approaches must be carefully evaluated.

In the case of nematodes, it has been shown that the immunization with EVs could provide protective immunity against *H. polygyrus* (Coakley et al., 2017). In a similar way, it has been demonstrated that EVs from *T. muris* can also induce protective immunity in C57BL/6 mice, even without administering any adjuvant (Shears et al., 2018).

Since helminth EV isolation and purification are time-consuming, and large-scale production is currently unavailable, some vaccination studies have been performed with specific EV-proteins instead of isolated EVs, and these studies have shown promising results (Mekonnen et al., 2018; Drurey et al., 2020). Future studies using artificial EVs loaded with combinations of recombinant parasite proteins/small RNAs would potentially circumvent this inconvenience.

7. Immunotherapeutic applications of Helminth EVs

There is growing evidence that helminths or their derived products may have a protective effect against allergic disorders and autoimmune diseases (Maizels, 2016; Maizels et al., 2018). A major challenge has been working with live worms/eggs in humans, with the possibility of

producing unwanted side effects of parasitosis (Bonovas et al., 2016; Pastille et al., 2017), together with the ethical problems derived from these kinds of treatments (Maruszewska-Cheruiyot et al., 2018). Therefore, researchers have investigated whether ESP, including EVs, could be used as a novel approach to treat these chronic inflammatory diseases. In this context, the treatment with EVs from the intestinal nematode *N. brasiliensis* showed efficacy in preventing colitis in a BALB/c mice model of acute colitis induced using 2,4,6-trinitrobenzene sulfonic acid (TNBS). Helminth EVs significantly reduced the levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-17a, and IFN-γ, as well as increasing the levels of the anti-inflammatory cytokine IL-10 (Eichenberger et al., 2018c).

Supporting the evidence for the role of EVs in protecting against TNBS-induced colitis in mice, recent studies have shown that EVs from *T. spiralis* ameliorate the induced colitis in mice dramatically reducing the expression of pro-inflammatory cytokines and neutrophil infiltration, while increasing levels of anti-inflammatory cytokines like TGF-β or IL-10 in colon tissue. *T. spiralis* EVs also influence T-cell population composition, reducing the number of Th1 and Th17 cells, and augmenting Th2 and Treg cells (Yang et al., 2020). Interestingly, these therapeutic effects seem to be parasite species-dependent, since EVs from the related nematode *T. muris* did not provide significant protection against this inflammatory disease in a mouse model (Eichenberger et al., 2018c).

As in the case of nematodes, the protective effect of flatworm EVs in animal models of autoimmune diseases has also been analysed. In this context, our group showed that EVs from the liver fluke *F. hepatica* were protective against Dextran Sodium Sulfate (DSS) induced-colitis in C57BL/6 mice (Roig et al., 2018). EVs from *F. hepatica* caused a significant downregulation of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-17a, suppressed MAPK/NF-κβ signalling pathways and reduced neutrophil infiltration of the damaged colon, ameliorating colitis symptoms. This protective effect was not dependent on lymphocytes, since similar results were obtained in RAG1$^{-/-}$ mice, which lack mature T and B lymphocytes (Roig et al., 2018). Interestingly, the effects were different depending on the mice strain immunized, with no major effects observed for the BALB/c strain (Marcilla et al., unpublished results), which may be correlated with the balanced Th2 response phenotype observed in this strain (Watanabe et al., 2004; Fransen et al., 2015). Supporting this notion, very recent studies by Dalton and co-workers have shown that immunization of BALB/c mice with EVs from *F. hepatica* requires the administration of adjuvant to induce a mixed Th1/Th2-cell response with increased levels of IFN-γ and IL-2, but no significant induction of IL-10 or IL-17 (Murphy et al., 2020).

Altogether, these findings demonstrate that helminth EV-mediated immunomodulatory effects could be very different depending on the parasite species and the host phenotype, and that helminth EVs effects may have an important beneficial role by skewing the inflammatory response characteristic of autoimmune diseases and allergic disorders.

8. Conclusions and future perspectives

Helminth parasites and their hosts have co-evolved over centuries, resulting in a very complex host-parasite relationship, where EVs have become an evident key player used by both, helminths and hosts, to influence each other and, ultimately, the outcome of the infection. During the last decade, studies investigating EVs from parasite helminths have grown gradually, describing their presence in an increasing number of species (Sotillo et al., 2020), and even considering them as "great balls of wonder" (Buck et al., 2020). In fact, in 2014, we suggested that helminth EVs could constitute major modulators of the immune response in mammals, with useful properties for controlling not only parasitic diseases, but also other diseases like autoimmune diseases (Montaner et al., 2014). The collection of studies reviewed here confirm that there is ample experimental data to support our proposal. EVs clearly have important immunomodulatory functions, which can vary depending on the helminth species, but they also participate in pathogenicity by influencing important parameters, like cell proliferation. Interestingly enough, EV effects can be different from those observed for the whole ESP. This whole set of molecules could be named "effectome" as suggested in plant pathogen infections (Arroyo-Velez et al., 2020).

Several studies have contributed to a better understanding of helminth EVs by characterizing their content and have identified some potential biomarkers and vaccine candidates for parasitic infections (for comprehensive reviews see Tritten and Geary, 2018; Drurey et al., 2020; Sotillo et al., 2020). These characterization studies have also contributed to assess specific proteins, lipids, and nucleic acids with immunomodulatory properties within helminth EVs cargo (Eichenberger et al., 2018a; Sotillo et al., 2020; Whitehead et al., 2020).

There are still some challenges inherent to helminth EVs, like the possible co-isolation of host molecules, and the difficulty to obtain sufficient amount of EVs for further studies. New ways of production of EVs, apart from the culture of parasites, should be explored (*i.e.* specific isolation from biofluids, organs, *etc.*). In relation to the *in vitro* culture, the possible effects on the parasite and EV cargo and functions remain unclear.

The study of helminth parasite EVs is still in its infancy, and the function of EVs in host-parasite interactions warrants further investigation.

Studies about EV functionality and composition are now shedding light on their crucial role in parasite survival and on the modulation of their environment. These lines of investigation should provide potential new biomarkers of infection, as well as potential new vaccine candidates. Furthermore, the reported protective effect of EVs from various helminth parasites on autoimmune diseases opens a new window towards their investigation for other clinical purposes, suggesting EVs could be "foe or friends".

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Declaration of Competing Interest

The authors report no declarations of interest.

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