

Review Article

Exosome-transported microRNAs of helminth origin: new tools for allergic and autoimmune diseases therapy?

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SUMMARY

Chronic diseases associated with inflammation show fast annual increase in their incidence. This has been associated with excessive hygiene habits that limit contacts between the immune system and helminth parasites. Helminthic infections induce regulation and expansion of regulatory T cells (Treg) leading to atypical Th2 type immune responses, with downregulation of the inflammatory component usually associated with these type of responses. Many cells, including those of the immune system, produce extracellular vesicles called exosomes which mediate either immune stimulation (DCs) or immune modulation (T cells). The transfer of miRNAs contained in T-cell exosomes has been shown to contribute to downregulate the production of inflammatory mediators. It has been recently described the delivery to the host–parasite interface of exosomes containing miRNAs by helminths and its internalization by host cells. In this sense, helminth microRNAs transported in exosomes and internalized by immune host cells exert an important role in the expansion of Treg cells, resulting in the control of inflammation. We here provide relevant information obtained in the field of exosomes, cell–cell communication and miRNAs, showing the high potential of helminth miRNAs delivered in exosomes to host cells as new therapeutic tools against diseases associated with exacerbated inflammatory responses.

Keywords diseases, exosomes, helminths, miRNAs, therapeutics

INTRODUCTION

The prevalence of immune-mediated diseases such as allergies and autoimmune diseases has increased in developed countries at the level of epidemics. It has been hypothesized that the origin of these disorders is mainly associated with an extreme hygiene and therefore a low exposure to infectious agents, including parasitic helminths (1), resulting in an imbalance of the immune system. There are two facts mainly associated with this imbalance, first the absence of pathogen-derived immunomodulatory stimuli soon and repeatedly after birth – when the immune system acts as a computer, processing and storing the beneficial stimuli – and second the decline in the biodiversity of species infecting humans, increasing the risk of developing specific chronic diseases (2, 3).

In this context, there are evidences pointing out that those uncontrolled inflammatory reactions can be balanced by helminthic infections (4). However, the use of live parasites might have secondary effects such as gastrointestinal adverse events, early toxicity and changes in serum protein levels and in hormonal microenvironment, besides an unguaranteed safety (5). There are many other factors influencing the overall effect of live parasitic infections on the treated patients to have into account, for example, parasite species, subsets of patients, efficacy and reproducibility, which makes their use not advisable (5, 6). Recently, it has been demonstrated that helminths produce and secrete extracellular vesicles called exosomes. This kind of vesicles has an essential role in cell-to-cell communication (7), and there are already some studies highlighting the role of pathogen-derived exosomes in the modulation of host inflammatory responses, including those from parasitic helminths (8, 9). Thus, specific molecules inside helminth exosomes could be delivered in artificial exosomes to host cells with the aim of regulating pathological inflammatory responses.

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Helminth exosome cargo has been characterized mainly at the protein level, although other molecules such as microRNAs (miRNAs) have been also identified inside exosomes (8, 10). MicroRNAs are a highly conserved group of small, noncoding RNA molecules that control gene expression, having critical relevance in nearly all developmental, physiological and pathological processes (11, 12). It has been shown that miRNAs in exosomes produced by immune cells can activate and modulate the innate immunity, resulting in important and broad effects over immune cells function and phenotype (13). Furthermore, miRNAs have a direct role in the regulation of inflammation (14). Thus, given the ability of helminths to regulate immunity, the host–parasite intercellular communication through miRNAs contained in exosomes during the infectious process and lately during chronicity might be essential in this context. Importantly, miRNAs identified inside helminth-derived exosomes have shown to be also delivered by Treg cells to control inflammation (10, 15). Several of these miRNAs (miR-125b or miR-31) seem to be homologous to others in humans having important roles in the function of T-cell subsets and controlling aspects of immune responses to infections, inflammation and autoimmunity.

HELMINTHIC INFECTIONS, INNATE IMMUNITY AND INFLAMMATION

Helminthic infections induce genetic, epigenetic, proteomic, hormonal and metabolic changes in their hosts, allowing parasites to productively infect them without triggering harmful immune responses (16). The infection drives the host immune system towards regulated immune responses and immunosuppression in chronic stages of infection, both in humans and animals (17). To be successful, helminths counteract the strictly regulated host innate response downregulating inflammatory responses that could be deleterious for the parasite.

Once infected with a helminth, the immune system of mammals develops a rapid polarization towards a Th2 type immune response, based on CD4⁺ T cells, eosinophils, mast cells and basophils, all of them involved in the regulation of inflammation (18). Importantly, this response lacks the inflammatory component usually associated with a typical Th2 response, primarily due to the upregulation through dendritic cells and alternatively activated macrophages of a specific cell subset called CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Treg) (19). The molecular mechanisms involved in this upregulation upon helminth infection are not well known, but it has been demonstrated that helminths and their products are a potent stimulus for the generation of this specific Treg subset, promoting the secretion of the anti-inflammatory cyto-

kines IL-10 and TGF- β , accompanied by a weakened Th17 response that results in the inhibition of IFN- γ and IL-17 production (18). These features distinguish them from the canonical Th2 response usually accompanied with a significant inflammatory component (20).

As mentioned, the infection with helminths or the treatment with some of their products promotes the expansion of Treg cells specifically expressing the transcription factor Foxp3 (CD4⁺ CD25⁺ Foxp3⁺) (21). *In vitro*, the expansion of CD4⁺ CD25⁺ Foxp3⁺ Treg cells is triggered after incubating dendritic and undifferentiated T cells with specific developmental stages of some helminths (22). Treg Foxp3⁺ cells are strictly regulated by the cytokines IL-6, IL-9, TGF- β and IL-4, and after immune stimulation by helminth parasites or their components, they exert their suppressor and immunomodulatory effects also against hyperinflammation in immune-mediated diseases (23–41; Table 1) in which CD4⁺ CD25⁺-Foxp3⁺ cells are dominant.

These experiments have demonstrated that autoimmune diseases could be prevented by the maintenance of the immune system homeostasis by triggering it with parasitic helminths. For this, it is essential the downstream stimulation of Treg Foxp3⁺ signals to activate defined immune cells surface receptors, including the AhR receptor and the Toll-like receptor – TLR – TIR8, resulting in the negative regulation of IL-1 production and eliciting the production of the anti-inflammatory cytokines TGF- β and IL-10. These signalling events are related with the inhibition of inflammatory responses (42). Importantly, the regulatory T-cell function and stability essential for preventing inflammatory diseases is maintained by specific miRNAs (43).

As mentioned, the inhibition of inflammatory responses propitiated by Treg Foxp3⁺ cells after helminth infection could be beneficial against several pathologies, including autoimmune and allergic processes (Table 1). Additionally, the pathophysiological process of inflammation is emerging as an essential event in other important diseases, such as cardiovascular disease and atherosclerosis, for which the helminth-derived anti-inflammatory effect is also regarded as an attractive alternative of treatment (44, 45). These diseases have a strong inflammatory component and are also associated with the expansion of CD4⁺ CD25⁺-Foxp3⁺ Treg cells. Foxp3⁺ cells expansion can be induced at least transiently through the activation of TLRs present in immunocompetent cells including T and B cells, highlighting the potential of activating TLRs expressed on these cells to directly and positively modulate the immune response (46). However, signalling through different TLRs can modulate T lymphocytes with different outcomes (46). In this context, helminths also activate TLRs and suppress key TLRs signalling pathways related with Th1 immune

Table 1 Some examples of the beneficial effects of helminths in diseases with an inflammatory component

Helminth	Components	Disease	References
<i>Trichinella spiralis</i>	Infection Excreted/secreted	Allergic colitis, asthma, autoimmune encephalomyelitis	23–25
<i>Heligmosomoides polygyrus</i>	Excreted/secreted Exosomes	Asthma, allergy, inflammatory bowel disease	8, 26, 27
<i>Necator americanus</i>	Infection	Celiac disease	28
<i>Fasciola hepatica</i>	Excreted/secreted	Autoimmune diabetes	29
<i>Strongyloides ratti</i>	Infection	Inhibition of mast cell degranulation	30
<i>Schistosoma japonicum</i>	Infection	Autoimmune arthritis, asthma	31, 32
<i>Schistosoma mansoni</i>	Somatic	Allergy, multiple sclerosis	33, 34
<i>Oesophagostomum dentatum</i>	Somatic	Allergy	35
<i>Acanthocheilonema viteae</i>	Excreted/secreted	Asthma	36
<i>Ascaris suum</i>	Galectin-9	Lung inflammation	37
<i>Taenia solium</i>	Infection	Neuroinflammation	38
<i>Clonorchis sinensis</i>	Somatic	Asthma	39
<i>Anisakis simplex</i>	As-MIF	Allergic colitis, allergy	40
<i>Litomosoides sigmodontis</i>	Somatic	Type 1 diabetes	41

responses potentially harmful for the parasite (47), pointing out the redundant therapeutic effects that could be induced by helminth parasites and the need of in-depth research regarding the effects of specific helminth molecules stimulating different immune regulatory mechanisms.

HELMINTH EXOSOMES AND THE REGULATION OF IMMUNE RESPONSES

Exosomes are intraluminal vesicles derived from late endosomes inside cells and are released constitutively by exocytosis of multivesicular bodies. Their size ranges from 20 to 120 nm and is present in the majority of the biological fluids – blood, saliva, urine, etc. (48).

Many cells produce exosomes, including those of the immune system such as CD4⁺ and CD8⁺ T cells, B cells and dendritic cells (DCs). Exosomes from these cells have been shown to mediate either immune stimulation (DCs) or immune modulation (T cells) (rev. in 15). Exosome cargo contains a variety of molecules carried to neighbouring or distant cell that internalize the exosomes in a specific manner (49). Thus, exosomes play an important role in the intercellular communication acting as shuttles for transferring proteins, lipids, miRNA, mRNA and cytokines from one cell to another (15). Exosomes have also been shown to be produced and delivered by different pathogens, including parasitic helminths (rev. in 8–10). The possibility of concentrating virulence/modulator factors in vesicles offers to pathogens a powerful mechanism for sending effectors to the host cells in a specific and protected manner. It has been already demonstrated that the helminth exosomes secreted at the host–parasite interphase are actively captured by host cells, resulting in the release

of their cargo inside the target cells (8–10). This vesicle-based system allows helminthic parasites to regulate the host immune responses (rev. in 8, 9), although the exosome cargo components responsible for such regulation have not been fully characterized (rev. in 9).

As mentioned, in addition to a variety of proteins, exosomes contain nucleic acids, including miRNAs, which have been also identified in helminth exosomes (8, 10). In eukaryotes, specific miRNAs have an active role in the regulation of immune response outcomes (rev. in 15). Recently, the transfer of miRNAs contained in Treg cell exosomes has been shown to affect the function of recipient antigen-presenting cells (APCs) by inhibiting translation of target mRNA molecules and contributing to downregulate the production of inflammatory mediators (50). Thus, transfer of defined miRNAs among immunocompetent cells is a key event for the final outcome of the immune response, by the regulation of gene expression mediated by miRNAs of important components of the innate and adaptive immune responses including cytokines, chemokines, adhesion molecules, anti-apoptotic factors and others (15, Figure 1). Recent studies have indicated a role for several miRNAs such as miR-146a, miR-125b, miR-155 and miR-9, among others, in regulating inflammatory responses downstream of TLR activation after recognition of pathogens (rev. in 15). One example is miR-125b, which is downregulated in the presence of LPS, a potent inducer of inflammation (51). Importantly, miR-125b has been described in schistosomes and has been found in nematode exosomes (8, 52).

MiRNAs are also ligands and activators of TLRs on immunocompetent cells, modifying signalling events during the innate immune response mediated by these

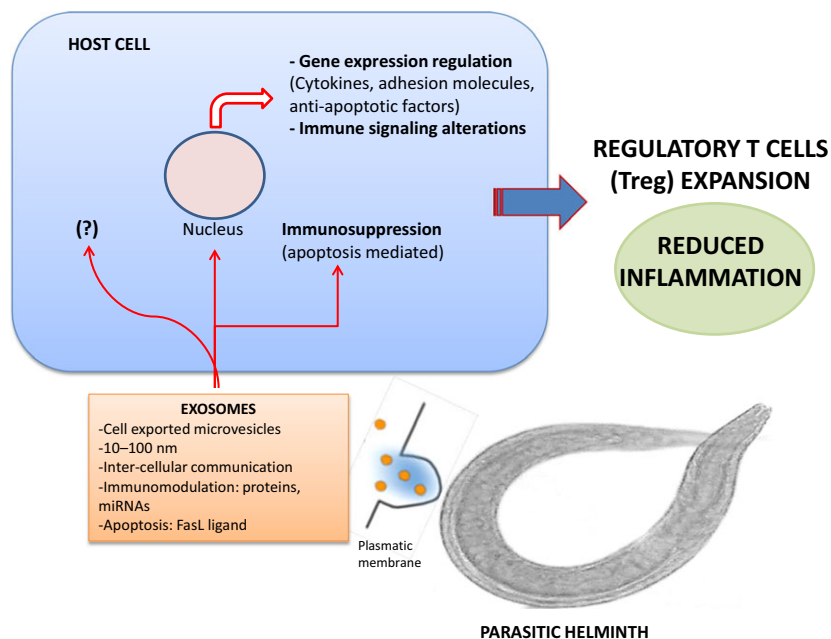


Figure 1 The hypothesis of the exosome effects upon helminth infections. Helminth exosomes could trigger the differentiation of T cells in a specific subset of T regulatory cells (FoxP3+ Treg cells). These promote the production of the anti-inflammatory cytokines IL10 and TGF- β and downregulate the Th17 cell population. This anti-inflammatory response could be triggered by specific components contained in exosomes delivered by the parasite by reaching immune cells.

pathogen recognition receptors (53). Thus, microRNAs have been postulated as master regulators of inflammation in allergies and autoimmune diseases (14).

Given the ability of helminths to regulate host immunity, the miRNAs transported in their secreted exosomes could play a critical role in such regulation, although this aspect has been scarcely investigated so far. Buck *et al.* (8) have recently shown that RNA-mediated communication occurs between the nematode *Heligmosomoides polygyrus* and its host, and that parasite small RNAs transported inside exosomes produced in the nematode intestine interfere with host cells gene expression. More importantly, these authors have demonstrated that local administration of the exosomes of *H. polygyrus* to mice suppresses the Th2 (inflammatory) innate responses against specific allergens (8).

In addition to the triggering of a modified Th2 response without inflammation by helminths, these can also give rise to immunosuppressive phenomena mediated by cell apoptosis (22). In this sense, key cells produce exosomes expressing in their surface the so-called Fas ligand – FasL – that can induce apoptosis in CD4(+) and CD8(+) T cells upon binding with the Fas receptor on the surface of target cells (54,55). This process has a pivotal role during intercellular communication among T cells (56). Whether helminth exosomes show the FasL in their surface for the induction of immune cell apoptosis is unknown to date.

Networking at the level of host immunity and the inflammatory response during persistent parasite infections are in the origin and progression of parasitic diseases. Understanding the influence of parasite-derived miRNAs transported in exosomes over these responses and processes is of great interest for animal and human health, highlighting the emerging importance and potential of the miRNA cargo inside helminth exosomes in the therapy of inflammatory diseases.

THERAPEUTIC POTENTIAL OF EXOSOMES

The interest of exosomes for its use as therapeutic tools is a relatively recent development. Exosomes are growingly considered a new transport system of therapeutic agents and a cell-free tool in regenerative medicine (57). The exosomal functional delivery of biological cargo can be exploited using exosomes as novel delivery platforms, as the technology offers promising exosome-based strategies to achieve efficient delivery of therapeutic compounds. The ability to alter the RNA composition of the exosomes either by transferring a specific miRNA to the parent cell or by directly delivering miRNAs into the vesicles enables exosomes to be used as carriers of therapeutic RNAs. The feasibility of this approach has been shown using tumour-derived extracellular vesicles to deliver a therapeutic small interfering RNA (rev. in 58).

Nevertheless, some difficulties related with delivery of miRNAs by exosome-like particles for therapy are still under investigation. These include, among others, the targeting of specific cells, the cargo stability, and the qualitative and quantitative effect of defined miRNAs in the gene expression of the target cells.

Regarding targeting specific cell populations, it has been shown that exosomes produced *in vitro* by miRNA-transfected cells can efficiently deliver miRNAs to specific target cells, by engineering the donor cells (e.g. dendritic cells) to express defined molecules, interacting with receptors at the surface of the target cells, fused with specific components of the exosome membrane (e.g. 59, 60; rev. in 61), in what it has been called hybrid exosomes. In some cases, engineering could be avoided, as defined exosome subpopulations have specific tropism for certain cells. One example is the existence of antigen-driven unidirectional transfer of miRNAs from the T cell to the APC, mediated by the delivery of CD63+ exosomes on immune synapse formation (62). However, targeting specific cells and achieving the desired effect on them need a detailed understanding of several aspects, including the tropism specificity within different exosome subpopulations, and also the efficiency of the exosome manipulation strategies, the integrity of the transfected miRNAs before being loaded inside exosomes by the donor cell. (63).

Transfection of cells with specific miRNAs can be substituted by the direct loading of isolated exosomes with miRNAs. Exosomes can then be used as 'gene delivery vectors' (GDV) and substitute viral particles avoiding side effects, as exosomes isolated from a patient (e.g. from plasma) can practically be used as a nonimmunogenic GDV for the same individual (64). MiRNA loading of exosomes can be achieved by chemical transfection and by electroporation, with a variety of kits offered by several companies. Electroporation is preliminary more efficient than transfection (64), although improved and/or alternative methods should be developed to load exosomes, as it has been shown that the exosome colloidal stability is affected following electroporation (65), and electroporation can induce siRNA precipitation (66), reducing the efficiency of siRNA loading into extracellular vesicles.

A main problem related with the use of natural exosomes is that cells secrete a heterogeneous group of nano-sized vesicles depending on the cell and activation state and procedures to isolate and collect them in a detectable way are complex; these complications impede their use in mass delivery of components to targeted cells. Very recently, it has been proposed a more efficient method that forces cells through hydrophilic microchannels to generate artificial nanovesicles (67). Additionally, the possibility of

artificially generate exosome-like particles has been explored by some authors. As the bilayer structure of the exosome membrane is reminiscent of a liposome, artificial liposome-based systems are obvious candidates as starting materials for the creation of exosome mimics. This could propitiate the efficient synthetic generation of exosome-like particles, and the low complexity and use of well characterized components will increase the acceptability of such systems. However, exosomal components that would be required for the assembly of functional and targeted exosome mimetics remain to be fully identified (rev. in 66).

Regarding miRNA cargo, some other obstacles need to be overcome before considering its application to humans. Due to the broad spectrum of potentially affected genes by single miRNAs, the refining of cell and gene targeting are among the main hindrances (68).

CONCLUSIONS

The currently available therapeutic options against diseases with an exacerbated inflammatory component are based on the systemic suppression of inflammation, but the adverse effects of these approaches and the lack of commonly used strategies highlight the need to develop innovative solutions.

Helminth-secreted exosomes have essential functions in the triggering of a modified, non inflammatory Th2 immune response and have shown to avoid inflammation elicited by exposure to allergens in experimental models. In this sense, the use as therapeutic tools of helminth products delivered in exosomes is a promising area of research that could help defining new treatment alternatives for these diseases.

The best characterized molecules in the helminth exosome cargo are mainly proteins, but these may be of low application for immunotherapy. Mammalian exosomes also transport miRNAs, which are capable of modulating the host immune system promoting the production of regulatory T cells, which are essential to give rise to anti-inflammatory environments. Assessing miRNA parasite expression and identifying those involved in immunoregulation, as well as defining their host gene targets controlling inflammation in chronic helminth infection, will require comprehensive approaches of the systems biology in several host–parasite models.

To further select the molecules implied in the desired protective action, *a priori in vitro* screening methods should be planned to corroborate their therapeutic potential and evaluate their stability and effects depending on their modifications and concentration. In this respect, there is still scarce information about miRNA stoichiometry, but due to their mechanism of action, similar to that

of hormones, low miRNA levels could already exert the desired effects (69, 70).

The translation of defined miRNAs in the helminth exosome cargo for its use in patients' therapy, either alone or in combination with other components to trigger the expansion of Treg cells, requires the development and testing of new-generation exosome-based therapeutic tools capable of targeting cells responsible of the regulation of inflammation and associated exacerbated responses. In this

sense, a better understanding of the host molecules needed for the efficient internalization of exosomes, as well as the longevity of their therapeutic effects and their implications controlling or promoting Treg Foxp3⁺ cell survival, is needed.

The definition of these variables will most probably provide new and safer therapeutic approaches against diseases with an inflammatory component.

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