Filarial nematode exosome-like vesicles (ELVs): Functional significance and control relevance

by

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A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics and Genomics

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

Dedication

To my father Sanath Hettiarachchi, mother Priyani Hettiarachchi, brother Kanchana Hettiarachchi and husband Dilshan Harischandra for your love and support throughout my PhD, without which this would not have been a reality.

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my major professor Dr. Michael Kimber for providing me the opportunity to join his lab, for his guidance and encouragement throughout my graduate studies. Your constructive comments and advice has made me a better scientist. Thank you for your understanding and support throughout my PhD training and for allowing me to explore avenues on my own. I would also like to thank my committee members, Drs. Clark Coffman, Heather Greenlee, Karen Scheel and Tim Day for their guidance throughout my graduate studies.

I would also like to thank Dr. Mostafa Zamanian for initiating the exosome project and his mentorship during my time in the Kimber lab and also Dr. Prince Nii Agbedanu for his mentorship throughout my time in the Kimber lab and for being a senior who I could always count on. I would also like to thank my fellow colleagues Dr. Nicholas Wheeler for his input during discussions and for his help in the analysis of proteomic data, Dr. Wang Yuan for his support during the final stretch of my training and Sreemoyee Acharya, Edmund Norris and Matthew Lindquist for their pleasant collegiality and making the lab a welcoming place.

I want to also gratefully acknowledge the support from Biomedical Sciences staff, Cathy Martens, Amy Brucker, Kim Adams, William B. Robertson, Linda Erickson and Shelly Loonan. I also want to acknowledge the National Institutes of Health, and College of Veterinary medicine for funding my research.

Lastly, my deepest gratitude goes to my parents for their unconditional love and for going above and beyond as usual in supporting me during my final year, my husband Dilshan Harischandra for always believing in me and the constant encouragement, especially during the

tough times and my little brother for constantly nagging me about deadlines and for the emotional support. This would not have been possible without the love and support of all four of you. I’m also thankful for my in-laws for supporting my career choice.

ABSTRACT

Lymphatic filariasis (LF) is a neglected tropical disease that affects over 120 million people worldwide and is caused by three filarial nematodes including *Brugia malayi*. Despite mass drug administration (MDA) to populations at risk for over 17 years, LF still remains a global health concern. Parasitism depends on specific interactions between the parasite and host. Our efforts of understanding these intricate interactions revealed a novel mechanism through which *Brugia* actively delivers small regulatory RNA and proteins to the host tissues via exosomes-like vesicles (ELV). Proteomics reveal stage and gender specific cargo, including proteins with potential immunomodulatory capacity. This suggests not only stage specific modulation of the host but also potential sexual dimorphism, and supports the hypothesis that these vesicles are released by the parasite to cater to its survival within the host. We have shown that these ELVs are functional and elicit a stage specific response in host macrophages, further supporting the above hypothesis. Further exploration of the possibility of exploiting these ELVs in disease control efforts revealed that exosomal release in these parasites is inhibited by current anthelmintics used to treat LF including ivermectin (IVM). Previous studies suggest a host component in parasite clearance by IVM and the fact that ELV release is inhibited by IVM not only supports this hypothesis but also provides evidence for how such a mechanism might take place. If our hypothesis that ELVs are released by these parasites to immunomodulate the host to aid in survival within the host is true, then inhibition of these vesicle by IVM leaves the parasites vulnerable to host defense mechanisms, thereby allowing effective parasite clearance. The use of ELV release inhibition as a platform for screening novel drugs resulted in the identification of a panel of L-type calcium channel inhibitors that block exosomal release in these parasites more potently than IVM. Current diagnostic methods for LF are suboptimal and here we demonstrate

proof of principle of using miRNA as biomarkers for more accurate diagnosis. Collectively our results reveal a novel mechanism in *Brugia* and demonstrate the potential of exploiting it in a wide spectrum of control efforts.

CHAPTER 1: GENERAL INTRODUCTION

Neglected Tropical Diseases and Lymphatic Filariasis

The World Health Organization (WHO) classifies seventeen diseases as Neglected Tropical Diseases (NTDs): Buruli ulcer, Chagas disease, Dengue and Chikunguya, Dracunculiasis, Echinococcosis, Foodborne trematodiases, Human African trypanosomiasis, Leishmaniasis, Leprosy, Lymphatic filariasis, Onchocerciasis, Rabies, Schistosomiasis, Soil-transmitted helminthiases, Taeniasis, Trachoma and Yaws. These are coined NTDs because they occur in low-income populations in tropical countries and disproportionately few research dollars are allocated to investigate these diseases relative to their health and socioeconomic impact. NTDs are caused by a wide array of infectious agents ranging from viruses to bacteria to protozoa to parasitic helminths [3] that can be transmitted via a range of mechanisms, including vectors such as sandflies, blackflies, mosquitoes or snails [3]. Of these NTDs, Lymphatic filariasis, Onchocerciasis, Schistosomiasis, Soil-transmitted helminthiases, Trachoma and Dracunculiasis are known as "tool-ready" NTDs because they can potentially be controlled or eliminated via tools that are already in place such as mass drug administration (MDA) or other effective interventions [4].

Lymphatic filariasis (LF) is an NTD caused by filarial nematodes which puts 947 million people at risk worldwide in 73 countries where LF is considered endemic [5]. LF is caused by three filarial nematode parasites *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori.* These three species have different geographic distributions and vector mosquito species. Each parasite has its own genera of vector mosquitoes; *Anopheles*, *Culex* and *Aedes* for *W. bancrofti*, and mainly *Mansonia* and sometimes *Anopheles* for *B. malayi* while *Anopheles* is the primary vector for *B.*

timori. The vector species for *W. bancrofti* can be found in tropical areas across the world, whereas those for *B. malayi* are found only in Asia and those for *B. timori* only in Indonesia. While Bancrotian filariasis is the most common form of LF, *B. malayi* is the etiological agent of most of the remaining cases. These parasitic nematodes have a cyclodevelopmental life cycle, where they develop in a vector and become infective to a vertebrate host. When the infected female mosquito takes a blood meal, the infective third stage larvae (L3) are deposited on the skin within a droplet of saliva and once the mosquito withdraws they enter the body through the puncture wound. The L3 worms migrate to the lymphatic system where they develop into fourth stage larvae (L4) and then into adults after a subsequent molt. These adults mate and the females produce millions of microfilaria (mf) larvae. The mf circulate in the blood stream and are taken up by a mosquito during the blood meal. The mf rapidly penetrate the mosquito gut wall and migrate to the flight muscles where they develop into the first larval L1 stage then following molts, to the second L2 stage and L3 larval stages. The L3s then leave the flight muscles and migrate to the proboscis of the mosquito where they can exit the mosquito during a blood meal and the cycle continues.

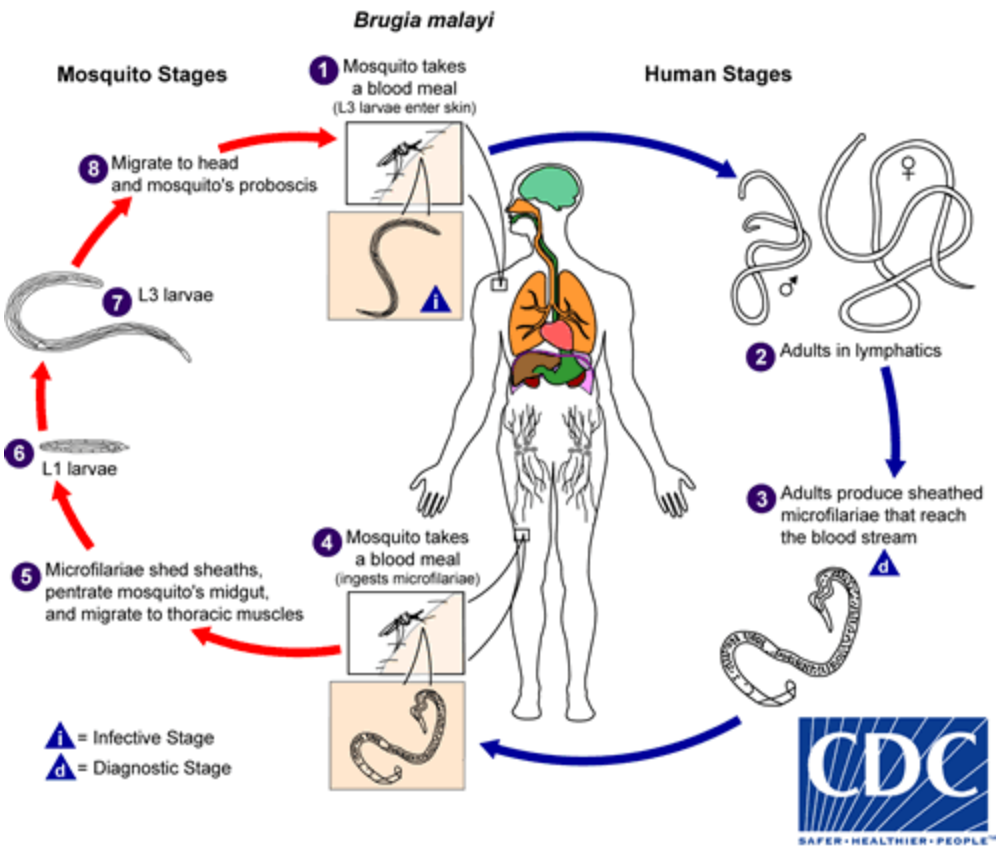

Figure 1. The life cycle of *Brugia malayi* from Centers for Disease Control and Prevention (CDC), www.cdc.gov [1]

There are five clinical sub-categories of LF in endemic areas: (1) the percentage of the population who are not infected despite being continuously exposed (known as endemic normals, EN) [6], (2) infected but clinically asymptomatic, (3) those with acute infection [7], (4) those chronically infected [7] and (5) those with tropical pulmonary eosinophilia caused by hyperresponsiveness to the filarial parasites [8]. The majority of infected individuals are asymptomatic, however, the most noticeable symptom of LF results from chronic infection and is characterized by the gross swelling of extremities (lymphedema) and thickening of the skin and tissue of limbs. This condition is more commonly known as elephantitis and it affects 36 million people [4]. Elephantiasis develops only in about 30-40% of those infected with lymphatic filariasis

[9]. Development of elephantiasis is a two-step process. LF parasites release mediators that cause structural rearrangement of lymphatic vasculature, resulting in dilatation and tortuosity of lymph vessels [10-12]. This results in malfunctional lymph circulation and lymph build-up, which predisposes infected individuals to secondary bacterial infections and accelerates the progression of pathology [13]. Another notion is that the proinflammatory immune responses against dying or dead adult parasites cause lymph buildup in the malfunctioning vessels and cause clinical manifestations [14, 15].

Of important note is that even though individuals who do not have elephantiasis appear visually asymptomatic, there may still be considerable damage to the kidneys, immune system and lymphatic system. Immune responses to the parasite can lead to acute adenolymphangitis (ADL) episodes of LF. Furthermore, a comprised immune response due to underlying lymphatic damage can cause secondary bacterial infections leading to inflammation of skin, lymph nodes and lymphatic vessels and eosinophilia. These acute attacks may last for weeks and sometimes lead to chronic LF.

Prevention and treatment

Transmission of the disease relies on many factors; the number of infected people or prevalence, the number of vector mosquitoes, and the frequency of human-vector contact, and each of these are being targeted individually to disrupt transmission. In 2000, the World Health Organization (WHO) launched the Global Program for Elimination of Lymphatic Filariasis (GPELF) to reduce the number of infected patients with LF. The goals of GPELF are two-fold. The first is to stop the spread of the infection through Mass Drug Administration (MDA), reducing infection below a threshold to prevent further transmission and demonstrate sustained reduction of

infection four years after stopping MDA. The second is morbidity management to alleviate the suffering of affected populations.

Three MDA regimens are currently in use to treat LF: 6 mg/kg diethylcarbamazine citrate (DEC) + 400 mg albendazole or 150 µg/kg ivermectin + 400 mg albendazole in those areas that are co-endemic with Onchocerciasis [16]; or 400 mg albendazole in areas also co-endemic with Loiasis (African eyeworm) [17]. These regimens need to be administered for at least five rounds to be effective. The presence of these co-endemic diseases complicates drug regimens, which are discussed in detail below.

(I) Diethylcarbamazine citrate (DEC)

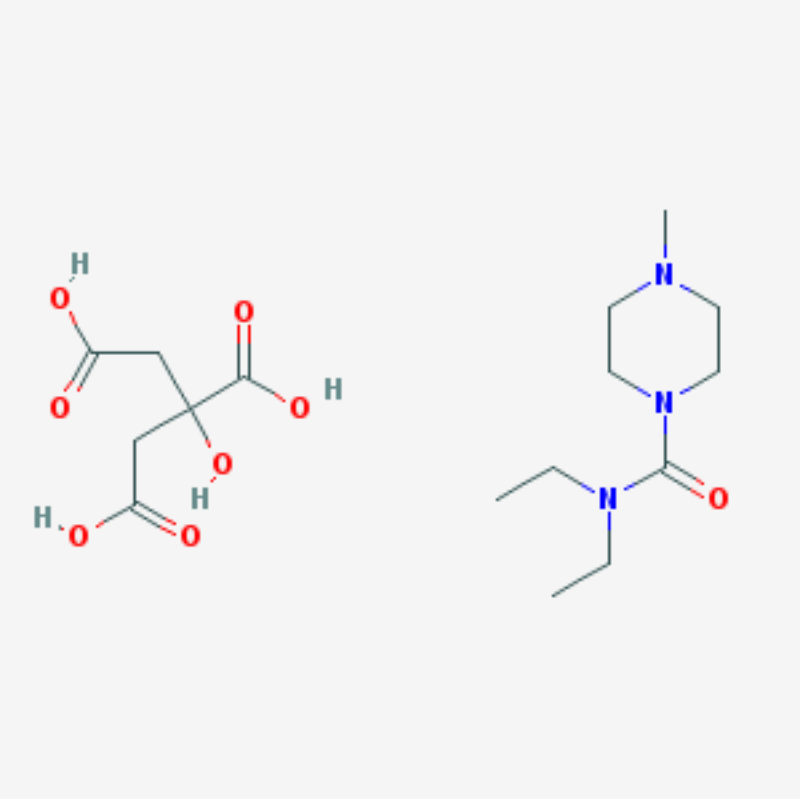

Figure 2. Chemical structure of Diethylcarbamazine from NIH PubChem Compound Data base; CID15432 [2]

Diethylcarbamazine (DEC) is an anthelmintic drug that is primarily used to treat diseases caused by filarial nematodes. Although its use is widespread, the exact mode of action is unclear. However, several mechanisms have been proposed. One is that it alters the arachidonic acid metabolism in both microfilaria (mf) and host endothelial cells, which can lead to the constriction of blood vessels and increased endothelial adhesions. This results in immobilization of mfs and allows host platelets and granulocytes to better adhere and exert cytotoxic activity against the parasites [18]. Another proposed mode of action of DEC is by inhibiting prostanoid production [19]. Filarial nematode parasites are known to constitutively release prostaglandins [20-22] which in the host are known to inhibit platelet aggregation [23], promote vasodilation of blood vessels [24] and increase immune suppression. DEC has been shown to inhibit prostanoid production, although it is not well understood whether it is the worm or host production that is affected by the drug. DEC has also been shown to increase nitrogen oxide induced mf clearance [21] but again, how it achieves this is not well understood. Nitrogen oxide prevents platelet aggregation and it is speculated that this might be one route of mf clearance. The observation that it has a rapid response *in vivo* where mf counts drop dramatically within minutes of treatment, yet there is no significant activity *in vitro* at comparable concentrations, suggests that some host factors influence the action of DEC [18].

Even though DEC has been widely used since 1947 to treat LF and is generally considered to be a safe drug, it has its limitations. It cannot be used in areas co-infected with *Onchocerca* due to the possibility of severe adverse immune response to *O. volvulus* mf in the eye.

(II) Ivermectin (IVM)

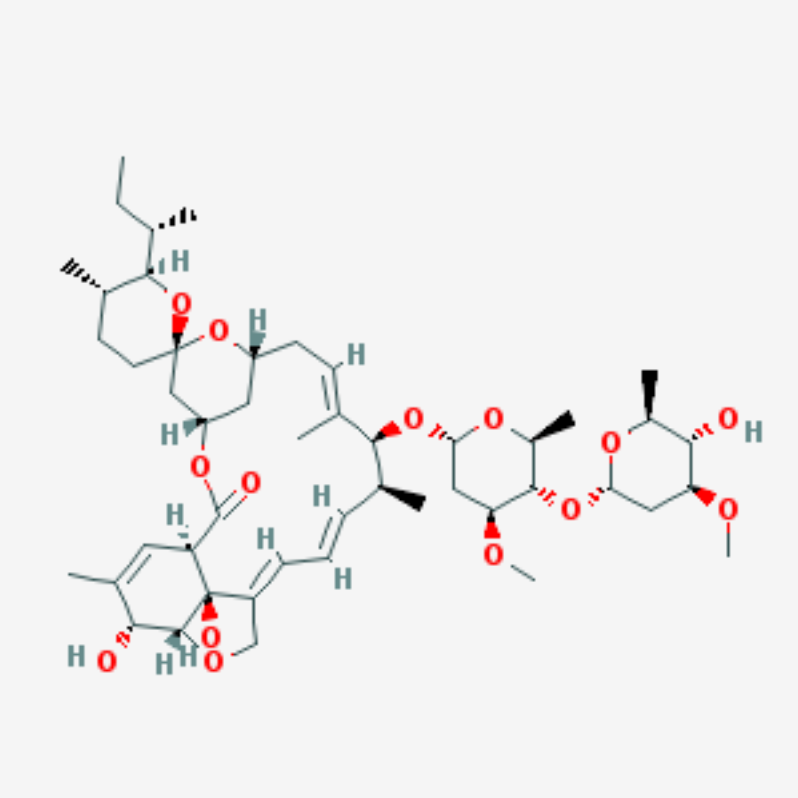

Figure 3. Chemical structure of Ivermectin from NIH PubChem Compound. Data base; CID 6321424 *[25]*

Ivermectin (IVM) is a member of the macrocyclic lactone compound family and in nematodes acts as a glutamate gated chloride channel ($GluCl^-$) potentiator at low levels, which results in a small transient hyperpolarization of the membrane, or at high levels opens the $GluCl^-$ channel directly [26]. The most well described physiological effect of IVM on nematodes is an inhibition of pharyngeal pumping [27] caused by hyperpolarization of an inhibitory motor neuron in the pharynx. However, IVM also acts on somatic muscle and inhibits the neurotransmitter gamma-aminobutyric acid (GABA) mediated activated channels [28]. These direct effects of IVM are specific to the parasite since vertebrates do not have $GluCl^-$s [26].

IVM is a highly effective filarial nematode microfilaricide *in vivo*. However, studies performed on *Onchocerciasis. volvulus, Dirofilaria. immitis, Acanthocheilonema viteae, B. pahangi* and *W. bancrofti* have shown that it is barely efficacious *in vitro* at therapeutic concentrations [29-32]. This has led to speculation that the effect of IVM is not simply by its direct action on the nematode but also through a synergistic effect of the drug and the host immune system, resulting in rapid microfilarial clearance *in vivo* [33, 34]. Another hypothesis is that IVM could acting directly but its mode of action is not to produce gross neuromuscular or motor defects but rather more subtle phenotypes such as inhibition of mf release from females. However, studies on *D. immitis* and *O. volvulus* have shown that females exposed to IVM treatment shed mf at the same rate as untreated females [34], thus nullifying this speculation.

IVM is highly effective against *W. bancrofti* microfilariae but lack of prolonged suppression of microfilarial production by *W. bancrofti* females [35] dampens its effectiveness in interrupting disease transmission. It is also effective against *O. volvulus* microfilariae which cause Onchocerciasis and *D. immitis* (canine heartworm) L3, L4 and juvenile adults although not mature adults, hence making it a valuable candidate for targeting multiple filarial nematode diseases. IVM also clears coinfection with a number of soil-transmitted helminths, including *Ascaris lumbricoides* and *Strongyloides stercoralis*, and some ectoparasites such as *Sarcoptes scabies,* posing an inter-phyla applicability. However, there are some limitations to the use of IVM. It cannot be used in areas co-infected with *Loa loa* since it results in severe adverse reactions such as coma and even death from the massive death of *L. loa* microfilariae. LF and Loiasis are co-infected in 10 African countries and therefore, MDA cannot be initiated in these areas. Moreover, there is concern that IVM can penetrate the immature blood-brain barrier and cause neurotoxicity to children below five years of age or who weigh less than 15kg [36] and therefore there is some

reservation in administering IVM to this population demographic. As a result approximately 15% of the population are excluded from IVM treatment [37] and potentially serve as reservoirs for LF, contributing to continued transmission of the disease. Further studies investigating the safety of the drug can be helpful in ensuring maximum coverage is achieved.

(III) Albendazole (Alb)

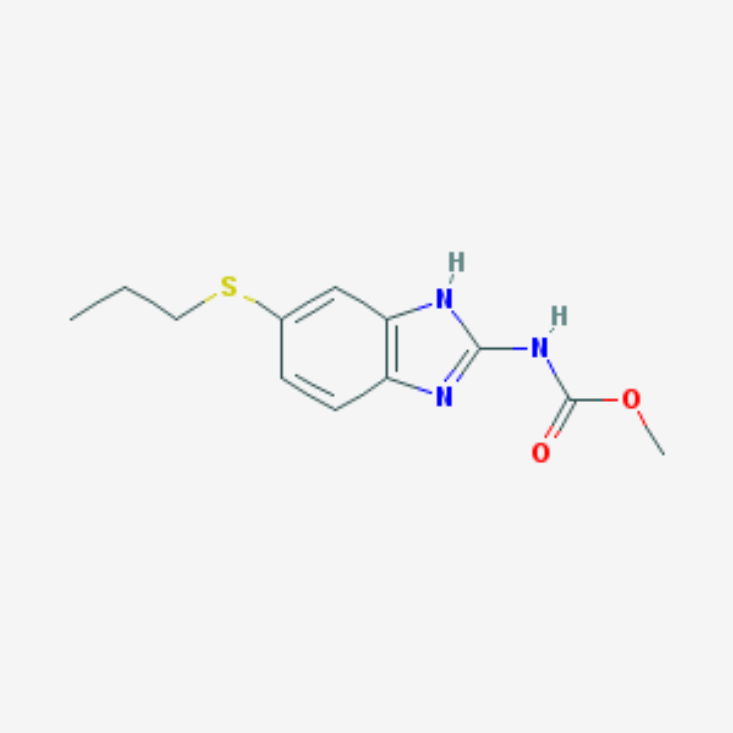

Figure 4. Chemical structure of Albendazole from NIH PubChem Compound Data base; CID 2082 *[38]*

Benzimadoles exert their anthelmintic activity by inhibiting the formation of β-tubulin, which is an essential component of microtubules. Tubulin needs to polymerize at the positive pole and depolarize at the negative pole in order for microtubules to form. Albendazole binds to the colchicine sensitive site of the positive pole of β-tubulin, thereby inhibiting the formation of microtubules [39]. In physiological terms this results in impaired glucose uptake and leads to

reduced parasite viability. Benzamioles have also been reported to inhibit egg production and muscle movement of parasite [40]. The higher affinity of benzimadoles to parasitic β-tubulin relative to mammalian β-tubulin results in drug selectivity for parasitic worms and effectiveness as an anthelmintic. [41]

The coverage goals for mass drug administration targeting lymphatic filariasis is >65% of those in areas endemic for the disease [42]. Per the weekly epidemiological report published in September 2016 by the World Health Organization [5], a 58.8% coverage of the total population requiring MDA had been achieved by 2015. MDA was ongoing in 55 out of the 80 countries declared endemic at the beginning of GPELF in 2000. 18 countries including Malawi, Togo, Egypt, Yemen, Thailand, American Samoa, Kirirbati, Marshall Islands, Palau, Tonga, Vietnam and Wallis and Futuna had finished Phase one and were under post-MDA surveillance to validate elimination. 100% geographical coverage had been achieved in 13 countries by 2015, which scaled rapidly up to 23 in 2016. LF has been eliminated as a public health problem from Maldives, Sri Lanka, Cambodia, Cook Island, Niue, Vanautu while Gambia was reclassified as non-endemic. Among the 10 countries yet to start MDA in 2015 Central African Republic, Liberia and Brunei Darussalam were waiting to be confirmed for MDA implementation.

Although MDA programs have been efficacious in reducing prevalence by approximately 40% since their inception, there are rising concerns due to the limited applicability of current drugs and a concern of the emergence of resistance as with any other drugs that are so widely used [43]. Moreover, pregnant women in infected areas are excluded from treatment due to the absence of safety information on these drugs during pregnancy, serving as potential human reservoirs of the parasite [16].

Alternative strategies for LF Control

Of the factors influencing LF transmission, the frequency of host-vector contact is the most influential and controllable and can be reduced by using simple measures such as mosquito nets and repellents. Gleave *et al*. [44] have shown that host-mosquito convergence depends on the stage of larvae harbored by the mosquito. They demonstrated that mosquitoes harboring larvae younger than the infective L3 stage are five times less likely to converge with a host, whereas the likelihood of mosquitoes harboring infective stage L3s converging with a host is above average. This suggests both a suppressive and enhancing effect exerted by the larvae on the mosquito depending on the maturity of the infection [44]. There is also a considerable interest in implementing vector control to reduce the number of vector mosquitoes. In fact, several studies have been conducted to investigate this aspect [45-48]. Trial experiments to eradicate Bancroftian filariasis solely by vector control were carried out in Nagate village in Japan [45]. Residual spraying was done once a year for ten years starting from 1962 in combination with the application of larvicide once a week to potential breeding grounds during the breeding season of vector mosquitoes. After eight years of implementation, the microfilaremia positive population had decreased from 14% to 0.5% and the remaining carriers tested negative in 1971. They also demonstrated that the vector population could be reduced markedly by destroying breeding grounds where the population of *Culex pipiens pallens* vector mosquitoes diminished from 71.2 in 1961 to 0.6 in 1994 as a result of changing the open ditches collecting household waste water to underdrains. Moreover, mineral oils [48], polystyrene beads [47] and Spherix, a *Bacillus sphaericus* based biocide, have been successfully used as larvicides in open water areas serving as potential breeding sites. However, mosquitoes developed resistance against Spherix after 20-25 uses within a year reducing its value as an effective larvicide. Elimination of filariasis transmitted by *Anopheles* mosquitoes in Solomon

Islands and Togo by indoor spraying of dichlorodiphenyltrichloroethane (DDT) has also been reported and shows promise for this approach [46]. Another attractive extension of this application is an integrated approach to vector control, where control for vector mosquitoes are in place where LF is co-endemic with other mosquito-borne diseases such as malaria [49] or dengue [50]. Studies have been conducted to genetically modify mosquitoes such that they are refractory or carry a lethal gene. Paratransgenesis of symbiotic bacteria is also being considered as a method of vector control [51, 52].

Another interesting and promising control strategy is targeting the *Wolbachia* symbiont. The *Wolbachia* bacteria is an obligatory symbiont present in all human filarial nematodes except *Loa loa* [54] and has been shown to be important for parasite fertility, development and survival [55]. It is present in all life stages with abundance increasing as the parasite is transferred from the vector to the host and also as it develops into adulthood. It has been shown that antibiotic treatment to reduce *Wolbachia* population decreases adult lifespan, embryogenesis and microfilariae release [56]. *Wolbachia* are also present in insect vectors of these nematodes. In fact, *Wolbachia* cause four types of reproductive alterations in mosquitoes, namely cytoplasmic incompatibility resulting in sperm-egg incompatibility skewing the mosquito population to females infected with *Wolbachia*, feminization of genetic males, male-killing or killing male progeny of infected females and pathogenic induction resulting in unfertilized eggs [57]. The presence of Wolbachia in both the parasitic nematode and the vector present the possibility of being used as dual-use control measures. Doxycycline, an antibiotic that targets the *Wolbachia* symbiont, can be used as an alternative therapy. In fact, it is widely used in veterinary medicine to treat canine heartworm disease. However, this cannot be given to children under 9 years of age or pregnant and lactating mothers so all of them are excluded from MDA. This area is considered a promising field for novel

antifilarial drugs and efforts to identify anti-*Wolbachia* compounds continue. The Anti-*Wolbachia* Consortium (AWOL) is an international consortium funded by the Gates Foundation to identify such compounds that are suitable for MDA with a focus on repurposing existing antibiotics and some effective options are currently in development [58].

Current methods of diagnosis

The rationale behind diagnosis for LF deviates somewhat from the norm in that it is not used to identify individuals who need treatment. Rather, it is used to assess the success of MDA programs and for post-treatment surveillance to ensure the disease does not resurface after treatment regimens have been concluded. Many methods have been used historically for LF detection with the most frequently used method being detection of microfilariae in a smear of finger-prick blood. Mf have nocturnal periodicity where they live in the lungs during the day and circulate in the peripheral blood at night [59]. Due to this nocturnal periodicity of mf, blood for the finger-prick method needs to be collected at night and therefore is inconvenient. Additionally, it introduces a degree of uncertainty or unreliability because absence of mf in the blood samples could be because the mfs had not yet entered the peripheral blood. In areas where blood cannot be collected at night, 2mg/kg diethylcarbamazine (DEC) can be administered which results in increased circulating mf in the peripheral blood within 30 – 45 minutes of administration. Another microscope-based detection method is by concentrating microfilariae from 1-2 ml of intravenous blood on to a 3 μm membrane filter and examining it on a microscope slide. Yet another method is by detection of microfilaria using a microhematocrit tube coated with acridine orange. This method allows for observation of mfs and their morphology. One major drawback of this method

is the need of a fluorescence microscope [60]. Sensitivity of microfilarial-based detection methods is limited because microfilariae are undetectable in pre-patent, light and post-patent infections.

Circulating filarial antigen (CFA) can be detected in all microfilaraemic individuals and a proportion of amicrofilaraemic individuals with chronic manifestations of LF [61]. These tests detect antigens released by adult filarial worms, so they may be positive even in amicrofilaremic individuals [62]. The sensitivity of this test is such that it has been able to detect circulating antigen in individuals without clinical presentation who have been cleared of microfilariae after DEC administration post testing. CFA based tests are available in the form of enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic card tests. These tests are highly sensitive and specific, are easy to produce and there is no need to draw night blood [63]. A major drawback of this method is that it is only available for detecting *Wuchereria bancrofti* antigens and not Brugian filariasis.

Other detection methods augment the microscopic- and antigen-based approaches. Elevated eosinophil levels are often associated with filarial nematode infections and a marked IgE elevation can be supportive of a primary diagnostic method. However, it is difficult to develop an accurate relationship between eosinophilia and filarisis due to the frequent coexistence of other infections among individuals in endemic areas [64]. Visualization of the parasites themselves or the pathologies they cause can confirm a diagnosis. The rapid movement of adults also known as the “filarial dance” can be detected by ultrasonography of the scrotal lymphatics in men and the breast and retro-peritoneal lymphatics in women while X-rays of infected individuals show characteristic interstitial thickening and diffused nodular molting. Studies are continuously being conducted to improve LF detection methods. The emergence of molecular tools for disease

diagnostics may be translated to LF detection. A recent study introduces qPCR as an assay sensitive enough to detect mf burden as low as 18mf/ml in a pool of 20 individuals [65]

Brugia malayi as a Model for Studying Lymphatic Filariasis Agents

The great majority of LF infections are caused by *Wuchereria bancrofti* but this parasite is not laboratory adapted whereas the ability of *B. malayi* to successfully infect monkeys and cats translates to the ability to maintain the *B. malayi* life cycle in a laboratory setting and therefore *B. malayi* is the primary LF model. In these terms, *B.* malayi is widely considered a good model for Lymphatic filariasis. Previous studies have shown that there is significant overlap in the antigens generated by the two species [66] and also that antibodies generated against *W. bancrofti* antigens in infected and immune individuals show significant cross reactivity with *B. malayi* [67]. This validates the use of *B. malayi* to study various aspects of these filarial nematodes, disease pathobiology and potential novel treatment and diagnostic avenues.

The use of *B.* malayi as the primary LF model was reinforced through the identification of several experimental hosts for *B. malayi* that allow economical large scale production of parasite materials. L3s injected subcutaneously in jirds (rodent Meriones spp) develop into L4s within about a week and into adults within about a month. The adults can survive up to about seven months. L3s injected subcutaneously into *Mastomys* rodents develop into adulthood and can produce mf; *Mastomys* rodents are, however, resistant to inter-peritoneal injections. L3 infections in macaques have been known to be able to produce mfs with the infection lasting to about one year. This availability of a vast array of experimental hosts allow for experimental modulation and further supports the use of *B. malayi* to study LF.

Host parasite interface in the context of *Brugia malayi* infection

An immune response ranges in a spectrum between Th1 and Th2 depending on the type of T cell activation. Activated T cells produce cytokines of which IFN-γ is the main Th1 cytokine while IL-4, IL-5, IL-10 and IL-13 are produced in a Th2 response [68]. A Th1 response is proinflammatory and aids in the clearance of foreign particles. However, prolonged Th1 responses cause tissue damage and can be destructive to the host and therefore an anti-inflammatory Th2 response that down regulates the Th1 response follows. Many changes in the immune system occur during a LF infection. An adaptive Th2 immune response with increased levels of IL-4, IL-5, IL-9, IL-10 and IL-13 cytokines [68] and antibody isotypes IgG1, IgG4 is prototypical, along with expanded populations of eosinophils, basophils, mast cells and alternatively activated macrophages [69]. Infected individuals show elevated levels of IL-4. However, there does not seem to be a difference in the IL-4 levels between the different clinical groups in an endemic area (i.e asymptomatic, acute and chronic) [70]. The burst of IL-4 transcription within 24 hours of infection might explain the above observation because individuals in endemic areas are constantly exposed to L3 stage parasites [70]. Adult worms, especially females have also been shown to induce IL-4 production in mice [71]. In contrast, mfs induce proliferative and IFN-γ responses (which are more Th1-like than Th2) at the early stage followed later by increased IL-4 and IL-5 production [71]. The fact that proliferative and IFN-γ responses were restored in infected individuals following ivermectin treatment, which clears mfs but not adults [72], further supports the role of mfs in proliferation suppression, although the mechanism of this Th1-Th2 switch remains to be elucidated. Studies done with *Litomosoides sigmodontis*, a mouse filarial parasite closely related to *B. malayi* have shown that increased IFN-γ and IL-5 are important for encapsulation and killing of adult parasites [73, 74] and could explain the importance of

maintaining elevated IL-4 levels (from the parasite's perspective) for immunosuppressive containment of the parasite. Implantation of *B. malayi* adults in mice result in an increase in alternatively activated macrophages that suppress proliferative activity of lymphocytes upon contact [75]. The fact that this phenotype is not observed when dead parasites are implanted provides evidence that the immunosuppressive effect observed is actively exerted by these parasites [76]. A study investigating the role of filarial parasite in the induction of lymphangiogenesis and lymphatic differentiation reported endothelial rearrangement in response to antigens from adult *B. malayi* parasites and sera from infected patients [77]. This suggests that these parasites influence multiple host processes and systems for their benefit.

The fact that parasites modulate the host to create an environment favorable for them is apparent. However, the mechanistic basis for this host manipulation is not clear. Some studies have speculated a role for secreted parasite proteins in host modulation [78-80]. A study done by Hewitson *et al*. (2008) revealed that Galectin 1 and 2 are the most abundant proteins secreted by *Brugia* [79]. Interestingly, they potentially have a role in immune evasion since mammalian galectins have been associated with downregulation of immune responses [81] and can suppress regulatory phenotypes of T cells [82]. They also identified leucyl aminopeptidase (Lap), the *B. malayi* homologue of ES-62 of a related rodent filarial nematode *Acanthocheilonema viteae,* which is known to modulate the immune response towards a Th2 response through interactions with B and T lymphocytes, dendritic cells, macrophages and mast cells [83]. Antioxidant proteins can detoxify damaging reactive oxygen and nitrogen intermediates produced by immune cells. Superoxide dismutase (SOD), glutathione peroxidase (gp29) and thioredoxin peroxidase 2 were 3 such proteins that were detected in the *Brugia* secretome in this study. CPI-2, a cysteine-protease inhibitor, which potentially can dampen the immune response [84] was also identified in this study.

Lastly, *B. malayi* macrophage migration inhibitory factor, BmMIF-1 which has been demonstrated to be a chemotactic for human cells [85] was detected in this study. Another study published on the secretome the same year identified many of the above proteins as well [80]. Among them were MIF-1, galectin 1 and 2, CPI-2 and LAP. In addition to these proteins, they also identified another isoform of human MIF, MIF-2 in the adult stages. Yet another study on the *B. malayi* secretome was published the following year [78] which identified BmMIF-1 and galectins in the secretome of adult females and mfs. ES-62 and BmMIF-2 were also identified in the excretory-secretory (E/S) products of these stages and in L3s molting to L4s. Two antioxidant proteins identified previously, glutathione peroxidase and superoxide dismutase were also found in each life stage at different abundance levels. Peptides matching to LL20, an immunologically important antigen were found to be secreted in abundance by the molting L3s, adult females and microfilariae. A protease inhibitor-like protein, phosphatidyl-ethanolamine binding protein and a serine protease inhibitor were found as well in addition to the previously identified CPI-2 protease inhibitor.

These proteomic studies have identified a number of proteins secreted by the parasite that have potential host modulatory functions. However, what is still unclear is how these effector proteins are released and the mechanisms by which they interact with host tissues. For example, are these proteins freely secreted into the host environment or are they somehow packaged for release and to prevent degradation? Is protein interaction with host cells non-specific or are these parasite-derived molecules targeted or trafficked to specific host cells? Recently there has been an explosion of studies revealing the role of exosomes in cell-to-cell communication and in disease pathogenesis. Exosomes represent a potential mechanism by which parasite proteins could be released into the host environment and delivered to target cells or tissues.

Extracellular Vesicles and Exosomes

Intercellular communication is an essential phenomenon conserved throughout eukaryotes to regulate normal biological functions. Until recently, researchers had limited understanding of these communication pathways and often believed this to occur through cell-to-cell contact or interchange of specific secreted molecules, such as lipids, proteins or nucleotides. However, the recent discovery of extracellular vesicles (EVs) harboring bioactive molecules has advanced a new paradigm of cell-to-cell communication. EV were first identified in the 1940s as one of the pro-coagulant components in blood [86]. Since then, EVs have been identified in various biological fluids including semen [87], urine [88], saliva [89] and breast milk [90]. EVs are a heterogenous population and generally include exosomes, microvesicles and apoptotic bodies, specific EV subtypes categorized by their size, biogenesis and cargo. Despite their different characteristics, these vesicles play a key role in cell-to-cell communication exerting their effects locally as well as over greater distances. Apoptotic bodies are the largest EVs (50-5000 nm) and are released when plasma membrane blebbing occurs during programmed cell death. In contrast, microvesicles and exosomes are much smaller in size and are generated by budding from the plasma membrane or derived from the endosomal pathway respectively. The current consensus on exosome particle size range is between 50 -150 nm in diameter, a size distribution that overlaps with that of microvesicles (50-2000 nm). Therefore when studying the function of exosomes and other EVs it is important to evaluate not only the size but also protein markers commonly enriched in these EV subtypes in order to differentiate exosomes from other similar vesicles.

Membrane-derived vesicles are secreted across all kingdoms [91, 92]. Exosomes, or exosome-like vesicles, are secreted by eukaryotic organisms ranging from single cellular protists, to fungi, multicellular plants and most phyla of animals [93, 94]. Exosomes were first discovered

by Harding *et al*. in 1983 [95]. Briefly, in a study performed to investigate the mechanism of transferrin receptor turnover, the investigators discovered that the internalized colloidal gold-conjugated transferrin (AuTf) particles were rarely present in the structures speculated to be lysosomes. Instead, AuTf particles were found predominantly in small vesicles within multivesicular bodies. They also observed that these multivesicular bodies fused with the plasma membrane and that the AuTf-labeled vesicles, which are now known as exosomes, were exocytosed. A paper by Pan and Johnstone was published around the same time complementing this discovery where they reported that sheep reticulocytes released vesicles of a uniform size containing transferrin receptors [96]. The term exosome was coined by Jonhstone *et al*. in 1987 [97].

Exosome biogenesis

Biomolecules including proteins, lipids as well as immunomodulatory molecules such as antigens are taken up by cells via endocytosis. Following internalization these vesicles fuse to form early endosomes (EE) [98]. EEs are characterized by the presence of early endosomal antigen 1 (EEA1) [99], Rab4 [100], -5 [101] and -14 [102] as well as transferrin receptor (TfR) [103] and have a mildly acidic pH (between 6.0 – 6.5) [104]. Since EEs lack degradative enzymes, the cargo in these vesicles can be recycled back to the cell surface when the tubular endosome buds off during membrane recycling. The internalized proteins are also sorted with some fated for recycling while others are transported to late endosomes (LE) for degradation via the lysosomal pathway. Switching of Rab5 for Rab7 leads to the transitioning of EE to LE [105]. LEs are circular and have a more acidic environment compared to early endosomes (pH = 4.9 - 6.0). In this environment recycling receptors are lost and the vesicular cargo is exclusively targeted for degradation. Acid

hydrolases can begin protein degradation although the high pH allows only a small percentage of degradation to occur in the LEs. When LE formation is initialized, they begin moving from the peripheral cytoplasm to the perinuclear area. This movement is initiated by dynein-dynactin via the Rab7 interacting lysosomal protein (RILP) [106]. Through bi-directional shuttling with the trans golgi network, lysosomal components are added to the LE while endosomal components such as Rab5 and other proteins associated with EEs are removed [107]. An important aspect in the maturation of endosomes is the formation of intraluminal vesicles (ILV's). The Endosomal sorting complex required for transport (ESCRT), Alix and Vps4 among other protein complexes are required for the formation of ILV's. A cluster of ILVs within the endosome forms what is known as a multivesicular body (MVB).

Besides transporting the endosomal cargo to the lysosome for degradation, MVBs can also fuse with the cell membrane and release the ILVs outside the cell. These exocytosed ILVs are called exosomes. The mechanism by which exosomes are released has only become clear in the past few years. Switching of specific Rab proteins is thought to determine the fate of ILVs in the MVBs; Rab27b on the membrane of MVBs leads to the lysosomal degradation pathway [108]. Exosome secretion involves the presence of various Rab GTPases such as Rab27, Rab35 and Rab11. Importantly, switching of Rab27b to Rab27a is required for the release of exosomes in the intercellular environment [109]. This was shown by Ostrowski *et al*. (2010) who demonstrated that knockdown of Rab27a and Rab27b reduced exosome release while exogenous addition of Rab27a and Rab27b lead to greater numbers of peripheral vesicles or perinuclear endosomes respectively [109, 110]. A general scheme of exosome release involves the concerted action of the following mechanisms: 1) Inward budding of LEs to form ILVs containing cytosolic, ubiquinated proteins. 2) A number of budding incidences leading to a number of ILVs in what is now called

an MVB. 3) Recruitment of the ESCRT proteins ESCRT I (recognizes the ubiquinated cargo in developing MVB's), II (involved in membrane budding of the LE's to form ILV's in the MVB) and III (involved in cleavage of MVB to release their cargo) as well as Alix and Vps4 among other proteins during the formation of MVBs. 4) For most cell types recruitment of Rab11 and Alix as well as flotillin and glycosphingolipids or lipid rafts leads to the docking and fusion of the MVB to the inner membrane of the cell and finally release of the ILV's now - termed exosomes, outside the cell. [88, 111-113] The above steps are an over simplified version of exosome formation and release, indeed studies carried out in different cell types report different sets of proteins involved in exosome biogenesis and release. However, in general the proteins from the ESCRT and retromer complexes and their associated effector molecules are the main components that drive this phenomenon. While the above-mentioned phenomenon can be considered constitutive, exosome release can also be caused by stress stimuli including lipopolysaccharide (LPS) stimulation, heat-shock and hypoxia [114-116].

Exosomes in host-parasite interactions

While serving as communicators between cells within an organism, there are also reports suggesting that these structures have the capacity to serve as communication between species, specifically between parasites and their hosts. An emerging body of evidence suggests a role of exosomes (exosome-like vesicle, ELVs would be a better suited term for these vesicles since the biogenesis pathways have not yet been described) in pathogenesis of various helminth parasites [117-123]. Studies have shown that these parasite-secreted vesicles are being taken up by host cells, demonstrating proof of principle that these vesicles can serve as a mode of communication between the two species [118, 119]. Moreover, the discovery that microRNA (miRNA) and protein

molecules contained within these vesicles have the potential of manipulating host processes adds further support to this hypothesis. We recently demonstrated that ELVs secreted by *B. malayi* L3 larval stage parasites are internalized by murine macrophages and that they are capable of triggering an inflammatory activation phenotype in these cells [119]. We also identified miRNAs and proteins with the potential of modulating host processes. The discovery of let-7 and cathepsin L-like proteins in these ELVs are prime examples of such molecules. The report by Buck *et al*. (2014), the only other report to date of ELVs secreted by nematodes demonstrated that the intestinal parasitic nematode *Heligmosomoides polygyrus* secretes exosomes containing miRNAs and worm Argonaute protein (WAGO) that are internalized by mouse small intestine epithelial cells and can suppress Type 2 innate responses in mice [118].

miRNA Mediated Gene Regulation

In eukaryotes, the default state of gene expression is “off” [124]. Gene silencing occurs at many levels: transcriptional repression by mechanisms such as acetylation, methylation, RNAi of chromatin; post-transcriptional regulation via small RNAs, riboswitches, alternative splicing; and translational repression of the protein via histone modification, phosphorylation of protein synthesis initiation factors. Post-transcriptional regulation via small RNAs is present in all eukaryotes except yeast. Bacteria and archae have the argonaute genes which are key components in small RNA pathways but there is no evidence yet that they have other small RNA machinery. There are three types of endogenous non-coding small RNAs that induce gene silencing; microRNA (miRNA), small interference RNA (siRNA) and piwi-interacting RNA (piRNA) [125]. miRNA induced gene silencing occurs mainly via repression with the regulatory small RNA binding to interaction sites on the target mRNA and therefore doesn’t require a 100% sequence

identity between the miRNA and target [126]. siRNA and piRNA induce gene silencing by enzyme-directed cleavage and therefore require exact or near exact sequence match with the target RNA [126, 127]. In mammals, small RNA regulation is post-transcriptional but in plants and yeast it can affect DNA by converting the chromatin to heterochromatin such that it isn't easily accessible by the transcription machinery. Argonaute proteins interact with miRNA and siRNA for cytoplasmic post-transcriptional gene silencing while Piwi proteins interact with piRNA to silence transposable elements in the germline [128].

miRNA structure and biogenesis

miRNA genes are transcribed into primary miRNA, also known as pri-miRNA. This was initially thought to be transcribed by RNA pol II [129] but a more recent report states that some genes are transcribed by RNA pol III [130]. The pri-miRNA is an imperfectly paired double stranded region containing a hairpin loop. The 5' and 3' ends of the pri-miRNA are then cleaved by the microprocessor complex which comprises of the DROSHA nuclease and the RNA-binding protein DGCR8 [131]. The resultant product is a 70-90nt region with a hairpin loop and is known as the pre-microRNA (pre-miRNA). This pre-miRNA is then translocated from the nucleus into the cytoplasm by a complex comprised of Exportin-5, Ran and GTP. Here regions of the pre-miRNA including the loop are cleaved by a complex containing DICER 1 and TARBP2 or PRKRA and results in a double stranded region of 21-23nts with protruding single-stranded 3' ends of 2-3 nt. The cleaved product is mounted on to a RNA-Induced Silencing Complex (RISC) with an argonaute protein. Here the passenger strand is removed and degraded; the other strand, known as the guide strand, moves on to exert its gene modulatory effects.

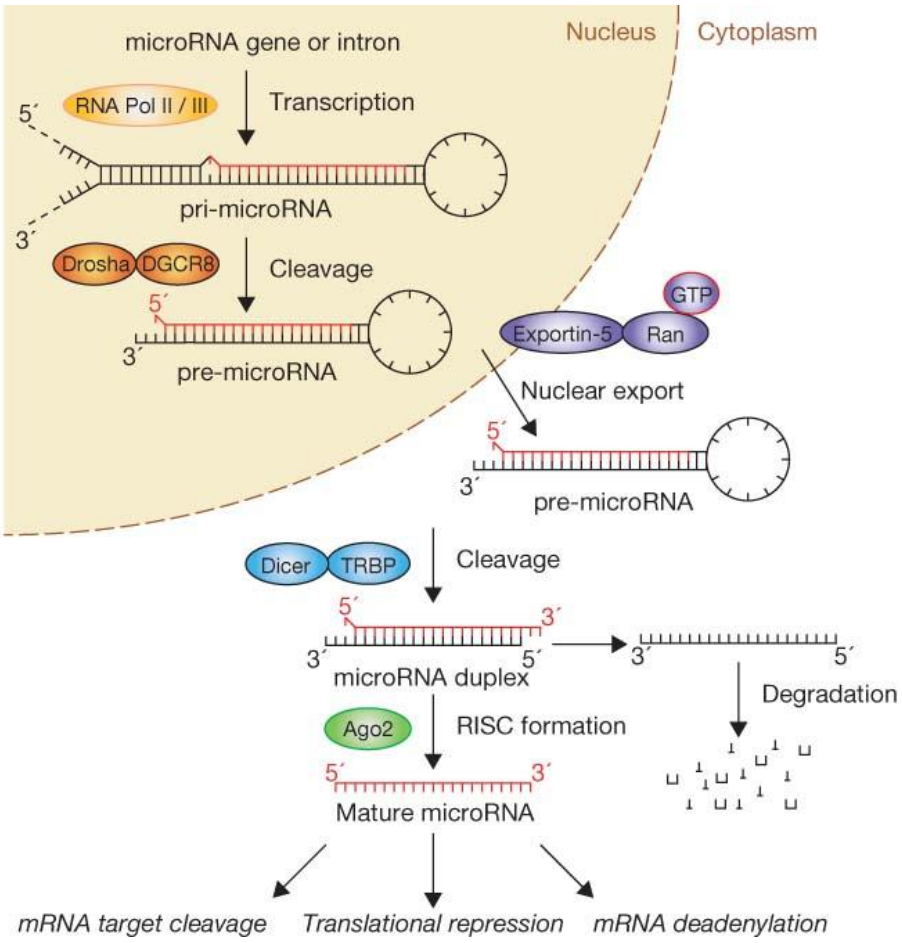

Figure 5. Pictorial depiction of the miRNA Biogenesis pathway reproduced with permission from Winter J. *et al*, 2009 *[132]*.

The role of parasite-derived miRNA at the host parasite interface

Studies have shown that like most eukaryotic organisms, *B. malayi* also expresses miRNA [119, 133, 134]. These miRNAs represent a powerful mechanism of regulation of endogenous gene expression in *Brugia* [134, 135] and it is possible that they also influence gene expression at the host-parasite interface. Indeed, miRNA contained within exosomes in other biological systems such as breast cancer [136] and neurodegenerative diseases [137], have been shown to regulate gene expression in target cells. The only two reports on parasitic nematode exosome-like vesicles (ELVs), document miRNA contained within these vesicles [118, 119]. In our report [119], we

identified miRNA belonging to the let-7 class with potential immunomodulatory capacity. Let-7 is constitutively present and functions as a molecular brake on cytokine release from macrophages [138]. Following infection, let-7 expression is downregulated allowing for the release of specific cytokines by macrophages. The discovery that *B. malayi* ELVs contain let-7 with direct homology to human let-7 allows us to speculate as to its function in filarial infection. It is possible that parasite-derived let-7 compensates for the diminished levels of endogenous let-7 thereby modulataing cytokine release by macrophages, allowing the parasite to establish itself within the host. Buck *et al*. (2014) also report a collection of miRNA contained within *H. polygyrus* exosomes [118]. They demonstrated that parasitic let-7 and mir-200 downregulated host *Dusp1* which is a key regulator associated with dampening the proinflammatory response of the host. They also found that *Il1rl1*, a subunit of IL-33 a key alarmin cytokine associated with protection against multicellular parasite was downregulated by *H. polygyrus* exosomes. *H. polygyrus* exosomes also contain the worm Argonaute protein, suggesting that possibly the parasite is secreting these vesicles fully equipped to induce necessary gene manipulations. These examples indeed provide support that the hypothesis that parasitic nematodes may secrete exosome-like vesicles containing effector molecules including miRNA as a method of modulating the host to their advantage plausible.

Leveraging parasite miRNA release in the context of lymphatic filariasis

We have shown that miRNAs are packaged within ELVs and secreted into the host environment by filarial nematodes. It may be possible to exploit this mechanism to provide novel approaches in LF diagnostics and control efforts. Current methods of LF diagnosis are imperfect. Detection of mf in a smear of finger-prick blood is the main method of detection but is

inconvenient and poses unreliability issues due to the need to draw blood during the night due to nocturnal periodicity of mfs [59]. Another method is the detection of circulating filarial antigen [62] and even though this test is very sensitive, it is only available for detection of Bancroftian filariasis and not Brugian filariasis. Detection of the "filarial dance" by ultrasonography of the scrotal lymphatics in men [139] and the breast [140] and retro-peritoneal lymphatics in women [141] is yet another method of confirming an LF infection. Although accurate, a patent infection needs to have been established for this method of detection by which the window of prevention or administration of effective treatment has passed. miRNAs have been proposed as a diagnostic tool for a wide variety of diseases ranging from neurodegenerative diseases to cancers [137, 142-144]. For example, a study on manganese-induced neurodegenerative diseases demonstrated that forty-three miRNAs were differentially expressed [137]. Of them twelve were significantly increased, some even as high as 15-fold, in Mn-induced exosomes, making them potential biomarkers for Mn-induced neurodegenerative diseases. Another study demonstrated that microRNA-126 and microRNA-182 can potentially be used to as biomarkers to identify urinary bladder cancer [144]. Likewise, identification of secreted miRNAs unique to each stage of *B. malayi* would enable the development of a non-invasive accurate detection platform, a liquid biopsy if you will, which not only detects the infection but also the maturity of it. We demonstrate the feasibility of this in chapter III of this thesis.

In this thesis we demonstrate that the filarial parasitic nematode *B. malayi* secretes exosome-like vesicles (ELVs) throughout its life cycle. Further we demonstrate that these vesicles harbor different cargo implying a stage and sex specific role of these ELVs. We show that ELVs of all life stages are being internalized by host cells, suggesting the potential of ELV-mediated host modulation by the parasite. We also show that ELV release decreases significantly in worms

treated with current anthelmintics, possibly uncovering one of the mechanisms of parasite clearance by these drugs. Further we identify compounds that inhibit ELV release more potently than current anthelmintics, suggesting the potential of employing ELV relsease as a screening platform for novel drug compounds. We also demonstrate proof-of-principal of using miRNA secreted by *Brugia* as biomarkers for identifying infection and disease maturity.

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CHAPTER 2. INVESTIGATION OF EXOSOME-LIKE VESICLE (ELV) RELEASE ACROSS THE *BRUGIA MALAYI* LIFE CYCLE

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Abstract

Lymphatic filariasis (LF) is a mosquito borne Neglected Tropical Disease caused by filarial nematodes including *Brugia malayi*. The interaction between host and parasite is critical for establishing and maintaining infection but is poorly understood. We have previously reported that exosome-like vesicles (ELVs) secreted by infective stage (L3) *B. malayi* parasites contain small RNA and protein effector molecules with the potential to manipulate the host immune system. Here we expand on this observation to report ELV secretion across the *B. malayi* life cycle and phenotypically profile ELV bioactivity. Nanoparticle tracking analysis and transmission electron microscopy describe ELV secretion by all parasite life stages examined. Further analysis by confocal microscopy and flow cytometry show ELVs are internalized by murine macrophages via phagocytosis. Proteomic profiles of ELVs released by the adult parasites contain canonical exosomal markers. Comparison of proteomic profiles reveal stage and gender specific cargo, suggesting targeted delivery of effector molecules catered to each life stage. Collectively our data

suggest a novel mechanism by which *B. malayi* may manipulate the host immune system. Targeting this interface in disease control efforts may potentiate disruption of infection and transmission of LF.

Introduction

Diseases caused by parasitic nematodes continue to have enormous global impact on the health of humans and animals. Lymphatic filariasis (LF) is a mosquito-borne Neglected Tropical Disease caused by the filarial nematodes *Wuchereria bancrofti, B. timori* and *Brugia malayi,* that puts over 947 million people in 55 countries where LF is considered endemic at risk [1]. The most recognizable symptom of LF is the gross swelling of extremities known as elephantiasis, caused by parasite obstruction of lymphatic vasculature; at least 36 million people suffer from this socially stigmatizing condition [2], making LF the second leading cause of permanent disability after leprosy [3]. Efforts to control LF rely heavily on Mass Drug Administration (MDA) programs in coordination with widespread surveillance of disease transmission. LF continues to be a global health concern despite sixteen years of MDA programs, and a sub-optimal drug portfolio is acknowledged to be a contributing factor [4]. There is a recognized need for novel therapeutics and control measures for LF and other parasitic nematode diseases but progress toward this goal is hindered by the limited understanding of general parasite biology.

Successful parasitism depends on specific interactions between the parasite and host. Longevity of adult *B. malayi* parasites within the human host is remarkable and wouldn't be possible without manipulation of the host to some extent by the parasite. Seminal work done by Buck *et al*. (2014) describe exosomes from *Heligmosomoides polygyrus* as a mechanism for host

manipulation by parasitic nematodes. Exosomes are a subtype of extracellular vesicles defined by their biogenesis pathway; inward budding of the late endosomes result in multivesicular bodies (MVB) [5]. The fate of these MVBs is two-fold, degradation by lysosomes or release of contents to the extracellular matrix by fusing with the cell membrane. Vesicles released in this manner are termed exosomes. They range between 30 – 120 nm and have a deflated-ball/ doughnut morphology when negatively stained. With the discovery that these specialized vesicles contain potently bioactive effector molecules and have been shown to have a role in cell-to-cell communication, there is a renewed interest in these extracellular vesicles and their cargo. In a recent study, we demonstrated that invasive *Brugia* L3 stage parasites secrete exosome-like vesicles (ELVs) containing small RNAs and proteins that have the capacity to regulate host processes [6].

Here we demonstrate pan-lifecycle secretion of ELVs by *Brugia* and show that they are being internalized by host macrophages which are early mediators of LF infection. We also report the site of ELV release in mf and the proteomic profile of ELVs from adult female and male stage parasites. Analysis of the proteomic profiles revealed canonical exosomal markers, adding confidence that the isolated vesicle population are *B. malayi* exosomes. Also of interest was the stage specific cargo contained within these vesicles with many immunomodulatory proteins identified in adult female ELVs. This stage and gender specificity suggests vesicle release is a regulated process via which the parasite releases effector molecules for targeted delivery within the host.

Results

Exosome-like vesicles (ELVs) are released throughout the *Brugia* life cycle.

In a previous study, we described the highly abundant release of a discrete ELV population from infective stage (L3) *B. malayi* [6]. ELV release from adult male and female nematodes, in contrast, was not definitive and led to the hypothesis that ELV secretion is primarily a strategy used by infective *B. malayi* larvae to facilitate host invasion and establishment of infection. To test this hypothesis we examined ELV release from all other intra-mammalian life stages of *B. malayi*. L4, adult male, adult female and microfilariae (mf) worms were incubated in culture media and secreted ELVs isolated, purified and quantified by nanoparticle tracking analysis using previously published protocols [6]. All life stages examined release extracellular vesicles of a size consistent with exosomes. The size distribution of the ELV fraction was homogenous with the mean size ranging from 50nm – 130nm (Figure 1A-E). The morphology of the parasite-derived vesicles also examined using electron microscopy and, consistent with our observations of *B. malayi* L3 ELVs, vesicles secreted by all life stages exhibited the accepted "cup-shaped" or "donut" morphology of exosomes [7-9] (Figure 1F). As with previous studies, we use the term exosome-like vesicles (ELVs) to describe these structures because although they present with the size and morphology of exosomes, uncertainty of vesicle biogenesis precludes unequivocal designation as exosomes.

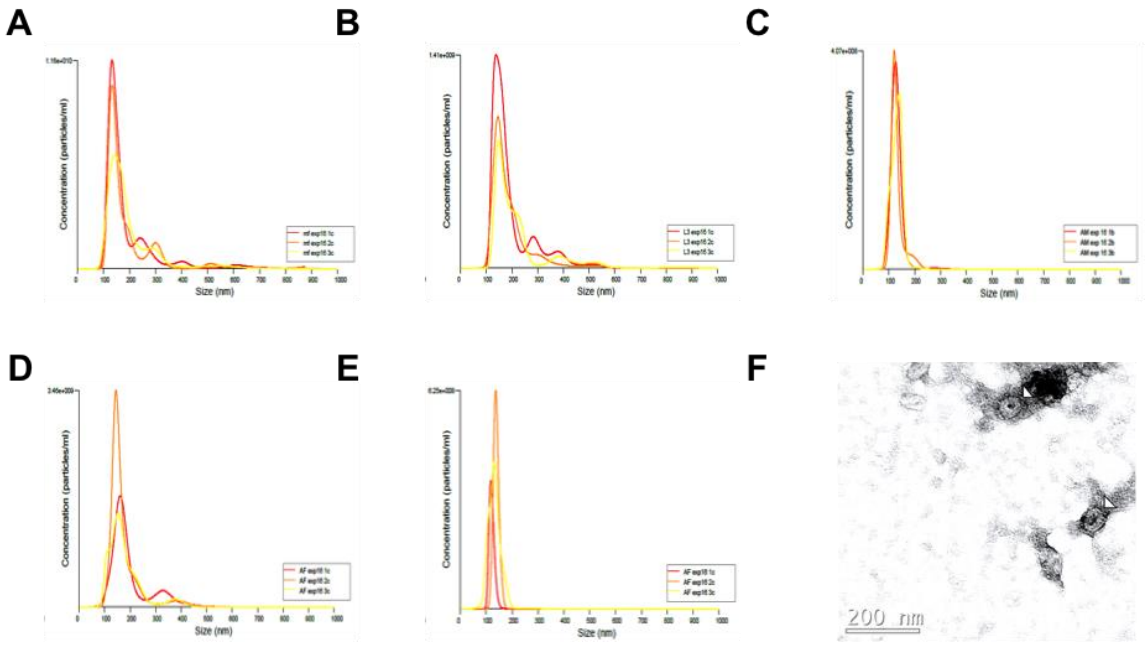

Figure 1. Vesicles of a size and morphology consistent with the exosome literature are released by all life stages of *Brugia malayi.* Nanoparticle tracking analysis shows discrete populations of vesicle within the exosome size range are secreted by microfilaria (A), larval L3 (B), L4 (C), adult female (D) and adult male (E). Transmission electron microscopy reveals parasite-secreted vesicles within the expected size range.

ELVs are released from microfilariae via the excretory-secretory pore.

The mechanism by which exosomes are released by parasitic nematodes is unclear. To identify a site for ELV secretion by *B. malayi* we employed whole-mount indirect immunocytochemistry (ICC) interfaced with confocal scanning laser microscopy using primary anti-Alix antibodies. ALG-2-interacting protein X (Alix) is an ESCRT associated protein found in many exosomes and is widely accepted as an exosomal marker [10, 11]. Strong anti-Alix immunoreactivity was observed in *B. malayi* microfilariae preparations but not in L3, adult male or adult female preparations. Immunoreactivity was highly focused around the Excretory-Secretory (ES) pore (Figure 2) suggesting this structure may be the site of ELV release in

microfilariae. The ES pore is the terminus of the parasite excretory system that may be considered functionally equivalent to the vertebrate renal system, and vesicle release via this system may be expected given its partial glandular arrangement [12, 13]. A lack of anti-Alix immunoreactivity in the L3 and adult preparations does not mean, however, that ELV secretion in these stages is not via the ES pore. It may be that ELVs released by these stages do not express Alix, or that a technical obstacle in whole mount ICC in the larger worm stages proved obstructive. Knowledge on the L3 and adult ELV protein content allows us to perform a more specified IHC to show ELV release. Custom antibodies against selected proteins we know are contained within these ELVs as revealed by the proteomic analysis can be generated and used to perform IHC to detect the site of ELV release by the L3 and adults. In case the IHC fails, running a western on ELV preparations (if there are some to spare) using these antibodies will help confirm whether it was a technical issue related to IHC and *Brugia*. IHC with antibodies raised against CD63 (considered a universal exosome marker) were also unsuccessful suggesting a shortcoming in IHC, rather than variance in exosomal marker expression, underpinning the lack of immunoreactivity in L3 through adult stages. If the IHCs on these stages failed due to technical problems related to IHC and *Brugia* as anecdotally evidenced, in situ hybridization can be an alternative technique used. The RNA content of the L3 life stage ELVs is currently available via work previously published by our lab. RNA probes can be generated against a selected few of these RNA targets and in situ hybridization can be done to visualize ELV release.

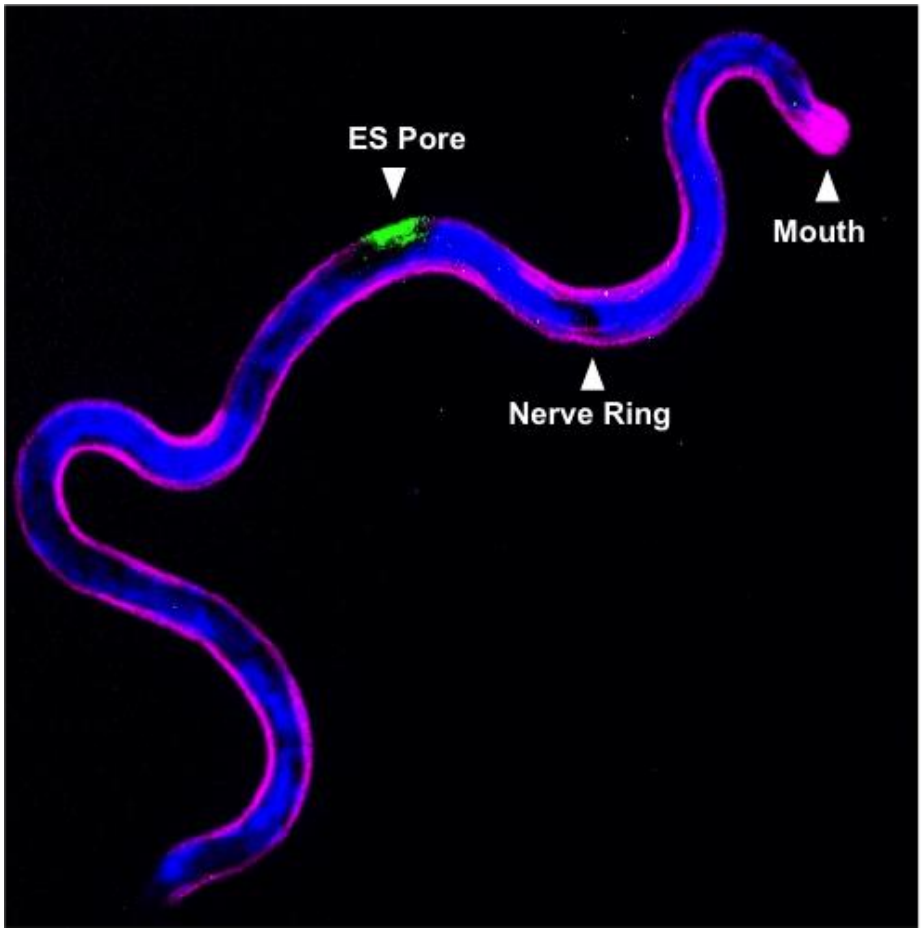

Figure 2. *Brugia* microfilaria release ELVs via the Excretory/Secretory pore. Confocal scanning laser micrograph of whole mount *B. malayi* microfilaria showing immunoreactivity against Alix (green), a canonical exosome marker, is focused around the excretory/secretory (E/S) pore. The preparation is counterstained with Hoechst 33342 (blue) and Phalloidin 647 (purple) to help identify major anatomical features.

Host macrophages internalize *Brugia* ELVs by phagocytosis.

Exosomes deliver their contents to target cells via different mechanisms; endocytosis by recipient cells, receptor mediated processes, and the fusion of vesicle and recipient cell plasma membranes [14-17]. Previously we reported that ELVs released by *B. malayi* L3 parasites are internalized by host macrophages [6]. Here we wanted to test whether all life stage ELVs (mf, L3,

L4, adult male and adult female) are internalized by host macrophages and identify a mechanistic basis for this internalization.

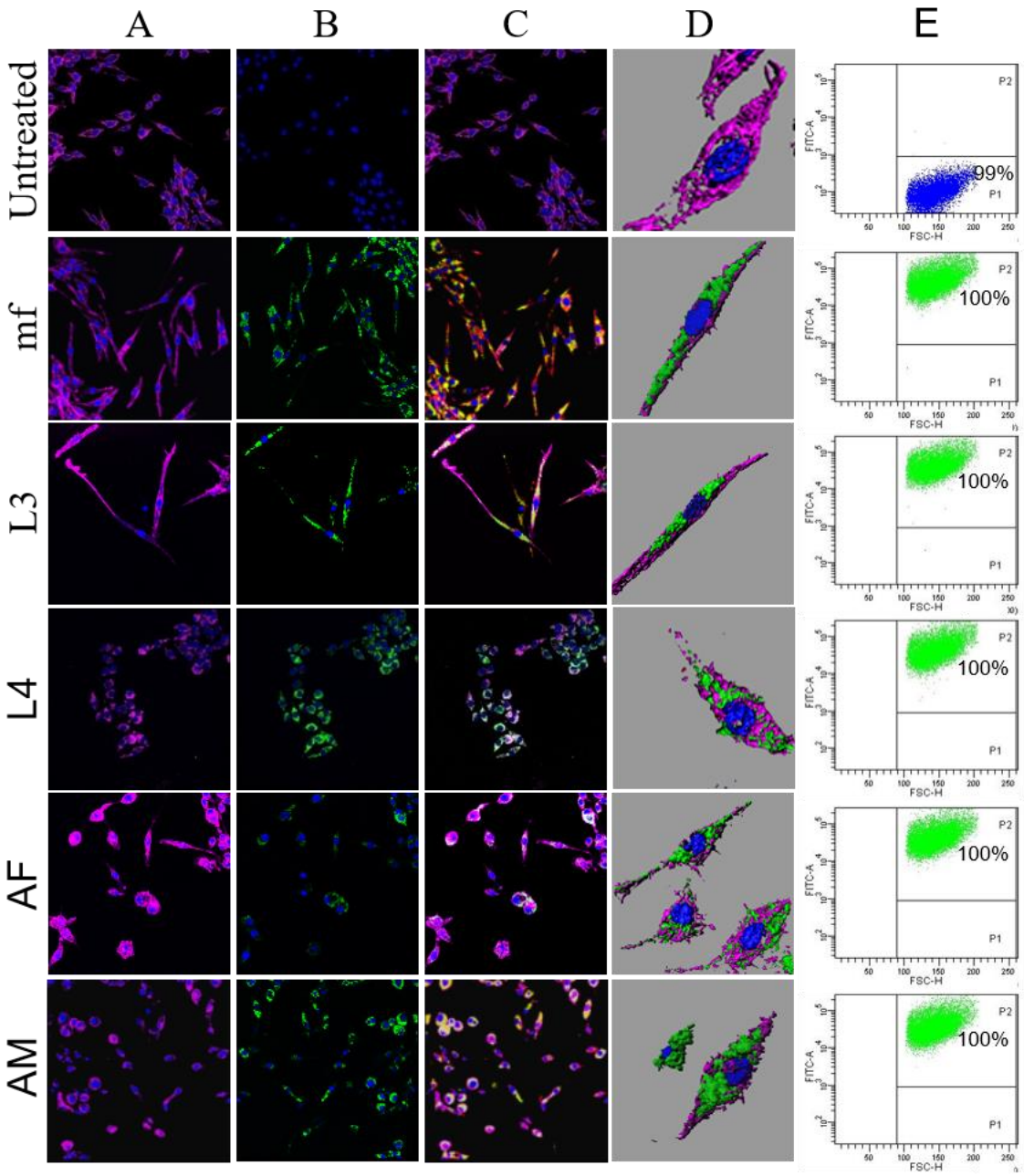

Figure 3. ELVs from all life stages are internalized by J774A.1 murine macrophages as seen by confocal microscopy (A-D) and flow cytometry (E). Cells were stained with Phalloidin 647 (purple) to visualize the actin skeleton and counter-stained with Hoescht 33342 (blue) to visualize nuclei (A)ELVs were stained with PKH67 (green) before adding to the cells (B). Overlays of these channels demonstrate internalization of ELVs from all the life stages by the macrophages (C) as confirmed via flow cytometry (E). A 3-dimentional cartoon image magnified 5X is shown for clarity (D).

The interaction of fluorescently labeled *B. malayi* ELVs with J774A.1 murine macrophages was visualized using confocal microscopy and quantified with flow cytometry. ELVs released by all parasite life stages are taken up by J774A.1 macrophages (Fig. 3). All macrophages took up parasite ELVs to some extent (Fig. 3E) and there was no significant difference in the uptake of ELVs from the different life stages.

In an effort to characterize the mechanisms of this ELV internalization, J774A.1 macrophages were pretreated with chemical inhibitors of discrete endocytic pathways before incubation with fluorescently labeled parasite ELVs. Pretreatment with Dynasore, a dynamin inhibitor and therefore inhibitor of dynamin-dependent uptake pathways [18], completely blocked ELV internalization (Fig. 4B-E) strongly suggesting that ELV uptake occurs via dynamin-dependent processes, that is to say, via either phagocytosis, clathrin-mediated endocytosis or caveoli-mediated endocytosis, or a combination thereof. ELV uptake was not inhibited in macrophages pretreated with Chlropromazine, a clathrin-mediated endocytosis blocker [19, 20] (Fig.4G-J), or by Genistein, an inhibitor of caveoli-mediated endocytosis [21] (Fig. 4M-P), suggesting that ELV uptake does not occur by these pathways. This leads to the conclusion that *B. malayi* ELVs are internalized by J774A.1 macrophages mainly via phagocytosis.

Our ability to selectively inhibit chosen endocytic pathways using these compounds in this experimental model was confirmed by measuring their ability to inhibit the internalization of explicit controls for each endocytic pathway i.e. a microsphere phagocytic tracer for phagocytosis (Fig. 4A, B), labeled transferrin for clathrin-mediated endocytosis (Fig. 4F, G) and labeled cholera toxin b for caveoli-mediated endocytosis (Fig. 4L, M).

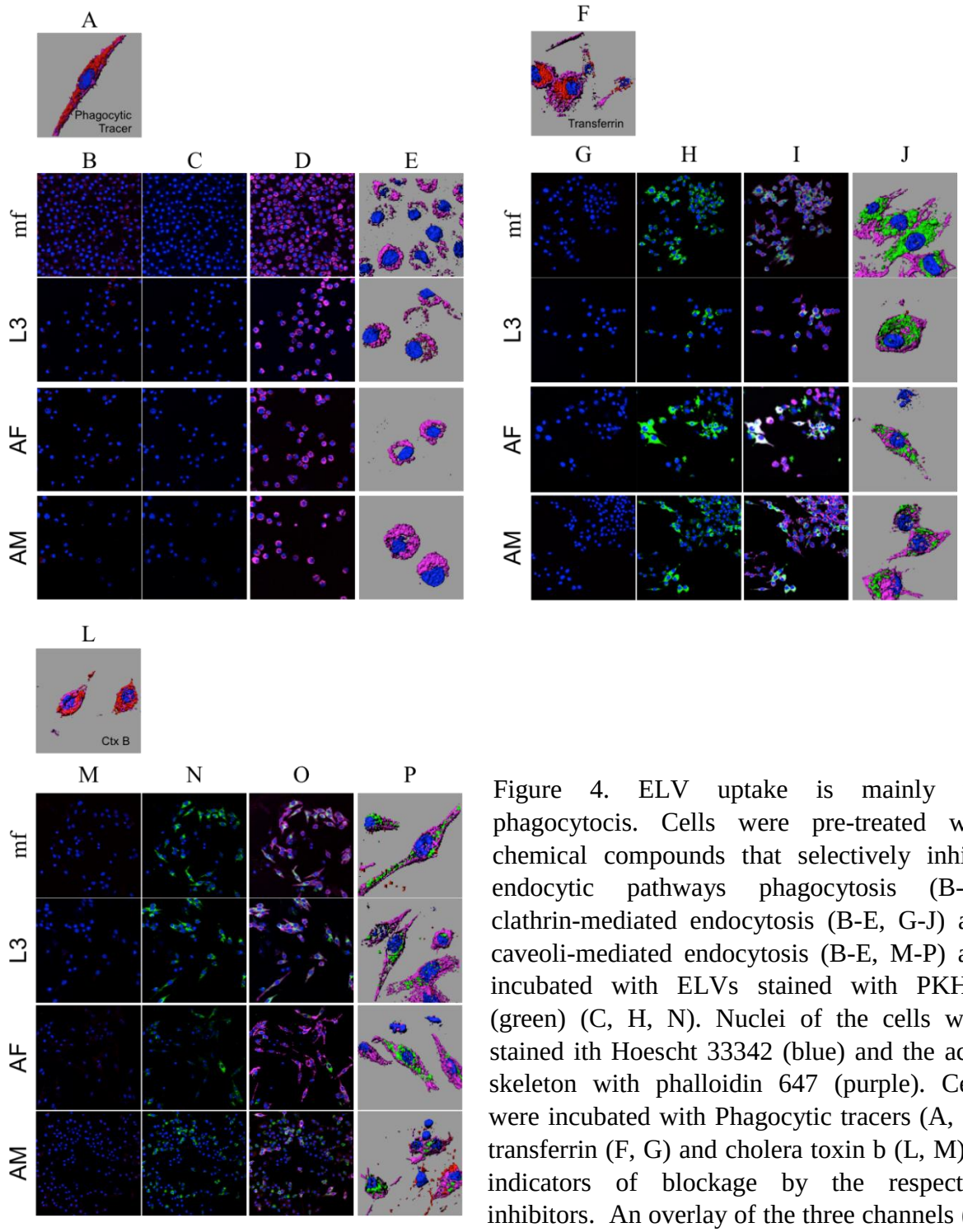

Figure 4. ELV uptake is mainly via phagocytocis. Cells were pre-treated with chemical compounds that selectively inhibit endocytic pathways phagocytosis (B-E), clathrin-mediated endocytosis (B-E, G-J) and caveoli-mediated endocytosis (B-E, M-P) and incubated with ELVs stained with PKH67 (green) (C, H, N). Nuclei of the cells were stained ith Hoescht 33342 (blue) and the actin skeleton with phalloidin 647 (purple). Cells were incubated with Phagocytic tracers (A, B), transferrin (F, G) and cholera toxin b (L, M) as indicators of blockage by the respective inhibitors. An overlay of the three channels (D, I, O) and 3D depiction of the merged channel magnified 5X (E, J, P) show varying degrees of internalization when treated with these chemical inhibitors.

Adult ELV proteomics reveals proteins involved in exosome biogenesis and immunomodulation.

Our overarching hypothesis is that ELVs are a mechanism of host immunomodulation employed by the parasitic nematode *B. malayi*. In support of this, we have shown that ELVs are being released by all parasitic life stages and that these ELVs are being internalized by host immune cells. In an attempt to delineate the function of these ELVs, we examined the protein content of adult male and female ELVs. Vesicles were purified from culture media as described, and following protein isolation and tryptic digestion, samples were subjected to nano-scale LC-MS/MS. Proteins were identified using Mascot to search *Brugia, Ascaris* and *Caenorhabditis* protein datasets, then parsed into Scaffold to generate a non-redundant list of proteins with host contaminant proteins subtracted. A total of 84 distinct proteins were identified in the adult female ELV preparation (Table 2) and 21 in the adult male vesicles (Table 1). The combined protein profile of adult ELVs revealed proteins commonly associated with exosomes and proteins considered canonical exosome markers; Rab GTPases such as Rab 11, Rab 27, Rab 35 and Rab 7 are involved in membrane transport and fusion [22-24] and the presence of all four of these proteins in the vesicle population provides support that these vesicles are indeed exosomes. Other proteins commonly found in exosomes, for example cytoskeleton proteins such as actin, ERM (ezrin, radixin and moesin) proteins and tubulin [22], lipid rafts such as GPI proteins (DAF-21 also known as CD55) [25] and molecular chaperones such as heatshock protein HSP-1 (also known as HSP70) [26, 27] were also present in the vesicle population. These proteins are commonly found in exosomes across a broad species range and are considered typical of this specific extracellular vesicle subtype. These data strongly support our hypothesis that *Brugia* secrete exosomes and

provide confidence that downstream identification of vesicle function can be attributed to this vesicles subtype rather than, for example, plasma membrane blebs.

Amongst the most abundant proteins identified in the adult female profile were proteins with known immunomodulatory properties. Galectins are a 15-member family that have been shown to be involved in many functions related to immunomodulation. Galectin-1 and 2 were identified in the adult female ELV proteome and have previously been shown to have immunomodulatory roles such as phagocytic recognition [28], inhibition of proinflammatoy cytokines [29] and shift T cells towards a Th2 paradigm [30]. Another interesting protein identified is annexin. This is a protein family of diverse functions ranging from membrane transport and fusion [31] to exerting anti-inflammatory effects [32, 33] which, depending on the annexin identified, suggests a function in exosome biogenesis or the canonical Th2 response associated with LF respectively. Macrophage migration inhibitory factor-1 (MIF-1) is another interesting protein that was identified. MIF-1 is the *Brugia* homolog of human MIF-1, the first cytokine to be identified [34]. It was one of the most abundant exosome proteins suggesting an active role of the molecule. MIF has been found in *Brugia* secretions and shown to promote differentiation of macrophages towards alternative activation (immunosuppressive phenotype) in the presence of IL-4 [35]. HSP-70, while serving as an exosomal marker has also been shown to have immunomodulatory capacity in tumor cells where soluble HSP-70 activates monocytes to secrete proinflammatory cytokines, while the membrane-bound form has been reported to be a target of cytosolic attack by natural killer cells [36].

Table 1. The proteomic profile of adult female ELVs reveal proteins with immunomodulatory capacity.

Identified Proteins	NSAF(AF)
Galectin-2	0.0605
BMA-ACT-5	0.0596
Annexin	0.0569
Galectin-1	0.0462
Triosephosphate isomerase	0.0379
Macrophage migration inhibitory factor homolog	0.0379
BMA-ERM-1	0.0348
BMA-UBQ-2	0.0335
BMA-HSP-1	0.0309
BMA-RAB-8	0.0261
Uncharacterized protein	0.0255
BMA-RAB-1	0.0247
Uncharacterized protein	0.0244
Bm3210	0.0228
thioredoxin peroxidase	0.0223
Glyceraldehyde-3-phosphate dehydrogenase	0.0221
BMA-RAP-1	0.0217
BMA-RAB-11.1	0.0213
BMA-DAF-21	0.0206
L-lactate dehydrogenase	0.0195
BMA-AQP-7	0.0190
Tubulin beta chain	0.0182

Table 1. Continued

BMA-RAB-35	0.0166
Galectin	0.0160
Sugar transporter family protein	0.0160
Malate dehydrogenase	0.0126
BMA-GPA-16	0.0125
Tubulin alpha chain	0.0123
GTP-binding nuclear protein Ran	0.0119
Bm5356	0.0116
Uncharacterized protein	0.0114
Histone H4	0.0103
Mechanosensory protein 2 isoform a	0.0089
Bm13965	0.0085
Uncharacterized protein	0.0075
Pyruvate kinase	0.0075
Bm13659	0.0074
ras-related protein Rab-7	0.0071
Fructose-bisphosphate aldolase	0.0071
Bm2511	0.0071
Bm9132	0.0065
BMA-UMPS-1 isoform a	0.0064
BMA-GPB-1 isoform a	0.0062
Elongation factor 1-alpha	0.0056
Bm3648	0.0055
BMA-RME-1, isoform a (Receptor Mediated Endocytosis)	0.0054
BMA-ARF-1.2	0.0054
Bm6727	0.0052

Table 1. Continued

BMA-ATGP-1	0.0048
Ras related Rab-27	0.0047
Nucleoside transporter family protein	0.0047
BMA-GST-1	0.0047
Annexin	0.0047
Bm8928	0.0044
Transketolase	0.0042
BMA-MCA-2, isoform d (calcium ATPase)	0.0041
BMA-EGL-30	0.0040
Transaldolase	0.0032
Bm795	0.0030
macroglobulin complement component family protein	0.0027
Sodium/potassium-transporting ATPase subunit alpha	0.0025
Bm1994, isoform a	0.0025
Adenosylhomocysteinase	0.0024
Bm13661	0.0023
cytochrome b superfamily	0.0022
BMA-LGC-21 ligand gated ion channel	0.0022
Protein kinase C	0.0021
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	0.0020
Transmembrane amino acid transporter protein	0.0019
Bm5460, isoform b	0.0018
Bm15985	0.0013
Bm Neprilysin	0.0012

Table 1. Continued

AP complex subunit beta	0.0010
Apolipophorin	0.0005

Of particular interest in the proteomic data was the comparison between proteins identified in the male and female preparations. 84 proteins were identified in the female sample whereas only 21 were identified in the male sample. Further, whilst the female sample included exosome markers as well as proteins with potential immunomodulatory properties, these immunomodulatory proteins were largely absent from the male profile. Two interpretations can be drawn from this. One is that perhaps the depth of sequencing of the male sample was not as good as the female preparation and therefore this analysis doesn't reflect the comprehensive profile of proteins contained within adult male ELVs. However, the male profile was repeated twice with similar vesicle numbers to the female sample, and protein extractions generated protein yields that were not only comparable to the female preparation but also more than exceeded protein requirements for nano-scale LC-MS/MS. Moreover, the vesicles were pooled from multiple worm incubations and therefore represent good biological coverage. The other interpretation would be that this is a representative analysis of male ELVs and that they contain limited functional cargo relative to female exosomes. Whilst these findings must be investigated further to validate, the lack of immunomodulatory proteins in male ELVs suggests exosome release may serve sexually dimorphic functions; female ELVs may be more effective at host immunomodulation than male vesicles. Differential pathobiology for male and female *Brugia* has not been reported in the literature but may also have not been explicitly investigated. It is not clear why male and female parasites would have discrepant functional roles in maintaining infection but a loss of immunomodulation in males may simply be a result of redundancy. Male and female worms

associate closely in infected lymphatic vasculature and it might be expected that males would benefit from host immunomodulation driven by female

Table 2. The proteomic profile of adult male ELVs reveal mainly structural proteins.

Identified Proteins	NSAF
Histone3	0.4710
BMA-ACT-5	0.4261
Bm4628	0.1656
ATP synthase subunit alpha	0.1029
BMA-RAB-11.1	0.0885
BMA-RAP-1	0.0867
BMA-HSP-1	0.0694
BMA-RAB-1	0.0660
Tubulin beta chain	0.0546
BMA-ARF-1.2	0.0434
Bm13659	0.0396
Tubulin alpha chain	0.0364
BMA-GPA-16	0.0222
Bm9583, isoform b	0.0209
Bm5196, isoform a	0.0194
Uncharacterized protein	0.0190
BMA-GPB-1, isoform a	0.0164
Adenosylhomocysteinase	0.0126
BMA-DAF-21	0.0110
Bm1994, isoform a	0.0067

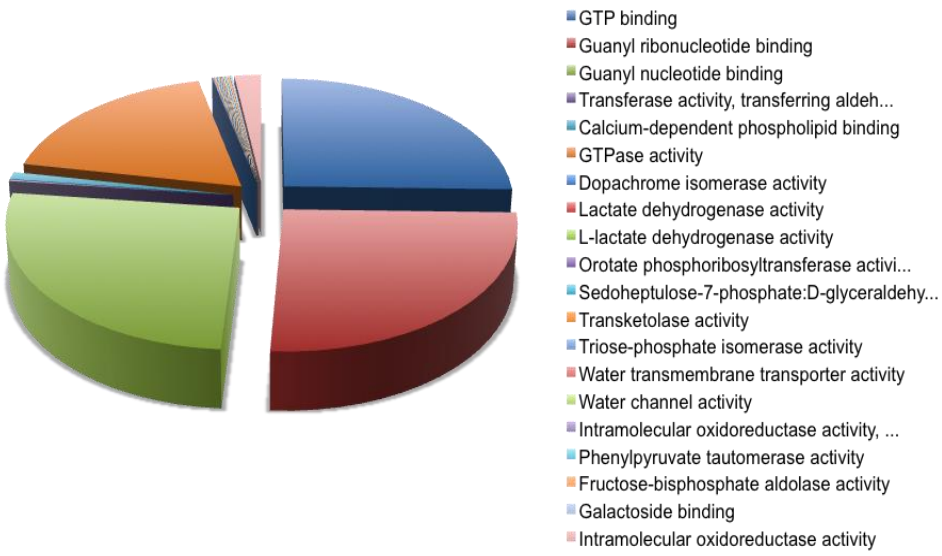

Figure 5. GO annotation of molecular function of adult female ELV proteins.

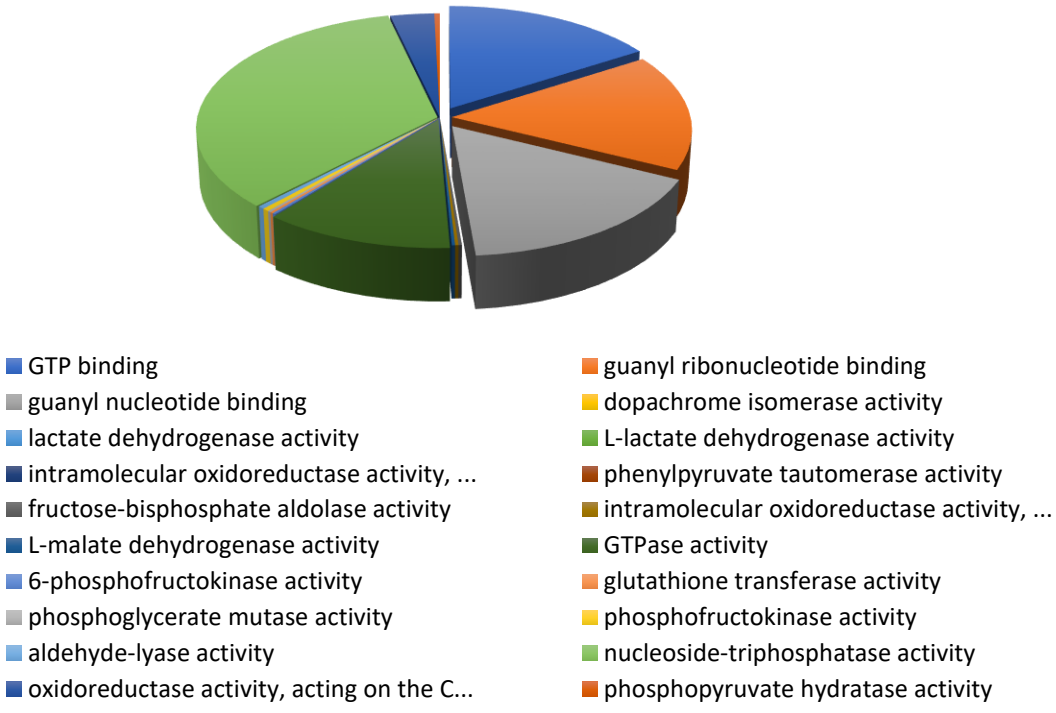

Figure 6. GO analysis of adult male proteomics based on molecular function

Discussion

Exosomes are a subtype of extracellular vesicle secreted by eukaryotic organisms ranging from single-celled protists to fungi to multicellular plants and most phyla of animals [37, 38]. Initially thought to be a mechanism of waste disposal, exosomes have gained renewed interest as mediators of cell-to-cell communication with the discovery that their cargo can contain potently bioactive nucleotide and protein effectors [31, 39-43]. There is an emerging body of evidence that parasitic worms, including nematodes and platyhelminths, release exosomes into the host milieu and that these exosomes contain potential effector molecules that may function at the host parasite interface [44-50]. We have previously shown that larval L3 stage *B. malayi*, a filarial nematode and etiological agent of Lymphatic filariasis (LF), release exosome-like vesicles (ELVs) containing known effector proteins and microRNAs (miRNA) with host homology [6]. Our overarching hypothesis is that filarial nematodes, including *B. malayi*, use ELVs to release effector molecules that facilitate the establishment or maintenance of parasitemia, specifically by actively directing the host response to infection. Here we provide evidence to support this hypothesis.

We previously described ELV release from the infectious L3 larval stage of *B. malayi* but here we show that ELV release is a conserved phenomenon across the entire mammalian host phase of the parasite life cycle. ELVs are secreted by mf, L3, L4 adult male and adult female worms with a size range between 50 -130 nm in diameter. This ELV size distribution falls within the range of 30 – 180 nm released from discrete life stages of other parasitic helminth species [44-52] and is consistent with the broader exosome literature. If parasite derived ELVs operate at the host-pathogen interface, the consistent pattern of ELV release reported here would suggest it is a conserved strategy adopted by all stages. It would be expected, however, that although the broad strategy is conserved, the specific modulatory properties of ELVs would change depending on

parasite life stage and host. ELVs released by L3 *B. malayi*, for example, in the mosquito host would likely have properties distinct from ELVs released by adult worms in human lymphatic vasculature since the host processes requiring modulation and potential host cues and stimuli are different. The variance in ELV size reported here points to some morphological differences in *Brugia* stage-specific ELVs but functional variance is more likely to arise from differences in structural components and cargo and such developmental regulation of vesicles has been suggested in parasitic flatworms [53]. Therefore, a trans-life cycle characterization of the molecular content of ELVs (including proteins and small RNAs) and the array of host phenotypic changes in response to them is important to fully understand the role of ELVs in parasitemia.

The mechanisms of ELV release by parasitic helminths, as separate from their cellular biogenetic pathways, remain undefined. In parasitic flatworms, a model of vesicle release from the apical membrane at the tegumental surface is supported by transmission electron microscopy [46, 47, 53, 54]; but may not be the only route of secretion [55]. In contrast to the flatworm tegument, the nematode cuticle is a relatively impervious interface. Composed primarily of collagens and cuticulins overlaid by a lipid- and glycoprotein-rich coat [56], the filarial cuticle is capable of small molecule transport [57, 58] but not dynamic vesicle release, turning the focus to other structures. Buck *et al*. (2014) note that extracellular vesicles secreted by *Heligmosomoides polygyrus* are enriched with intestinal proteins suggesting secretion from the digestive tract and in the same study observed vesicles associated with intestinal tissue preparations. Other routes may be relevant and our identification that the excretory-secretory (ES) pore is highly immunoreactive to exosomal markers in microfilariae suggests at least one other avenue. The fine structure of the ES system in filarial nematode larvae has been variously described [59-62]. Excretory and glandular cells empty into an ampulla structure, which connects to the ES pore via a channel, with close association of

neuronal elements suggesting regulated release via a valve-like structure. Microfilarial ELV release by this valve-like structure suggests that it is a regulated process, and not mere uncoordinated expulsion of vesicles to the environment, hence a process of biological importance to the parasite. Anatomical differences in this general apparatus between life stages suggest increased importance of the structure in larval parasites [63], which may account for our observed exosomal marker immunoreactivity in mf but not later stages. If *Brugia* mf release ELVs via the ES pore it provides a basis to speculate a link between ELVs and ivermectin (IVM) mode of action. The microfilaricidal activity of IVM is unclear and it has been suggested that IVM may work at therapeutic concentrations by disrupting the parasite's ability to regulate the host immune system [64, 65]. Moreno *et al*. (2010) showed that ivermectin-sensitive glutamate-gated chloride channels were expressed around the ES pore of *Brugia* mf and that IVM treatment reduced secretion from this structure. Consistent with our overarching hypothesis, IVM may inhibit ELV release from the mf ES pore, depriving the parasite of the host immunomodulatory property of the vesicles and allowing effective clearance. This poses an important avenue for filariasis control where parasitic load can potentially be reduced by targeting a mechanism such as ELV release which is universal across the *Brugia* life cycle. Analysis of the effect of IVM on ELV release in *Brugia* will be required to support this hypothesis.

Internalization of exosomes from various cell types have been shown to occur through different endocytic pathways [14, 66-68]. Even though uptake of parasitic helminth ELVs by various mammalian cell lines have been shown before [44, 46, 49-51], the mechanistic basis of the internalization pathway of these ELVs has, until now, not been elucidated. Here we show that ELVs from across the *Brugia* life cycle are internalized via phagocytocis. Internalization by phagocytocis requires recognition via specific opsinin receptors, scavenger receptors or toll-like

receptors (TLRs) [69], suggesting that *B. malayi* ELVs may contain special surface recognition molecules to ensure internalization. This again suggests that ELVs are released to serve a specific purpose within the host.

Profiling of adult male and female ELV protein components, added to our previous proteomic analysis of L3 ELVs [10] allows us to build a picture of the diversity of ELV structure and cargo across the *Brugia* life cycle. A cohort of proteins is shared across all ELV preparations and these molecules tended to be structural or markers of ELV biosynthesis, for example, actin and β tubulin, HSP-70 and elongation factor-1. The identification that classical exosomal markers are found in the ELV profile of L3, adult male and female preparations is important since it supports our designation of the vesicles under investigation as exosomes. Other subtypes of extracellular vesicle may be released by these parasites such as microvesicles and membrane blebs or apoptotic bodies, and the size profile of these vesicles may often overlap such that reliance on morphology to discriminate is unreliable. The presence of exosomal markers provides another layer of identification and provides a platform for downstream functional characterization. To this end, adult female ELVs were enriched with proteins that have previously been described as immunomodulatory whereas adult male ELVs lacked such proteins. A Cathepsin L-like protease was the only protein with known immunomodulatory capacity that was identified in the L3 ELV proteome [6]. These findings support our hypothesis that *B. malayi* release exosomes to facilitate the establishment or maintenance of parasitemia, specifically by actively directing the host response to infection. Our data focus the functional role of host immunomodulation of adult females since these ELVs contain candidate effector proteins whereas the male ELVs do not. This is not to say male ELVs do not contain any potential immunomodulatory molecules since we did

not examine small RNA cargo in this study. Previously we described the miRNA complement of L3 ELVs and identified miRNA with host homology that may affect host immune cell gene expression [10]. Further we noted that there were global differences in the small RNA cargo between L3, adult female and adult male ELVs. It Is possible that host immunomodulatory small RNAs are carried by male ELVs with the goal of manipulating host gene expression. Small RNA-seq analysis will be required to gain further insight into the small RNA cargo of stage- and sex-specific ELVs. However, the current findings do suggest sexual dimorphism in ELV structure and function and further emphasize the diversity and functional plasticity of parasite exosomes.

Comparison of the ELV proteome with previous *Brugia* secretome data [70-72] yielded some interesting observations. Just eight proteins identified in the present study overlapped with the consensus secretome, however this group was among the most abundant in the ELV proteome. For example, Hewitson *et al.* (2008) found that galectin-1 and 2 were among the most abundant proteins in the *Brugia* secretome [32] and galectin -2 was the most abundant protein in our study followed closely by galectin-1. Conversely, around 20 proteins identified in our study were absent in the consensus secretome. One reason for this disparity between identified proteins could merely reflect a difference in abundance. It could be that due to the sheer abundance of freely secreted protein, those packaged in ELVs are masked during whole secretome analysis. This is not to say that ELV proteins are of less importance. Rather, it could be a directed mechanism mounted by the parasite to exert specific effects. While freely secreted proteins are more easily subjected to degradation, those contained in ELVs are protected by the surrounding membrane. Moreover, packaging proteins within ELVs allows the parasite to send them to targetted destinations, increasing the probability of it achieving the desired function. Additionally, a difference in protein extraction and preparation methods could also partially explain this difference.

In summary, we have showed that ELVs are released across the *Brugia* life cycle, that they are internalized by host macrophages, that the protein cargo in these ELVs are stage- and sex-specific and that they indicate host immunomodulatory function. These data collectively support our overarching hypothesis that filarial nematodes, including *B. malayi*, use ELVs to release effector molecules that facilitate the establishment or maintenance of parasitemia, specifically by actively directing the host response to infection. We add to the emerging body of evidence that exosomes/ELVs are a mode of communication not only between cells of an organism but also between species.

Materials and Methods

Exosome-like vesicle (ELV) isolation

All *B. malayi* stages used in this study were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, USA. 600 L4 and 60 adult male and 60 adult female stage parasites were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham MA) containing 10 g/L glucose (Thermo Fisher Scientific) and pen-strep (penicillin 0.4 units/mL, streptomycin 0.4 mcg/mL; Thermo Fisher Scientific) at 37°C, 5% CO_2. Microfilariae (mf) were purified using established protocols and cultured in identical media at room temperature. Culture media was collected and replaced at 24 hr intervals for ELV purification for as long as the worms maintained the same level of activity as when they were received (i.e. approximately 3-5 days for adult females and 10 days for adult males). The culture media in which the L3s (600) were received in, was processed for ELV isolation and no further incubations were performed since we previously showed that the homogeneity of ELVs secreted by this life stage rapidly

deteriorates in culture. ELVs were purified via differential centrifugation. Culture media aliquots were centrifuged at 12,000 x g for 45 minutes at 4°C and filtered through 0.22 μm filters to remove debris then ultracentrifuged at 120,000 x g for 1.5 hours to pellet the ELVs. The ELV were washed with cold 1X dPBS (Thermo Fisher Scientific) and consolidated into 1.5 ml ultracentrifuge tubes (Beckman Coulter, Brea, CA) and centrifuged for 55,000 rpm for 2 hours at 4°C. All but 25 – 50 μl media were removed and the purified ELV preparations were quantified by Nanoparticle Tracking Analysis using NanoSight LM14 (Malvern Instruments, Malvern, UK). ELV samples were stored at -80°C.

Electron microscopy of ELVs

An aliquot of the ELV purification was mixed with uranyl acetate 2% (w/v), incubated for 5 min at room temperature and applied to carbon-coated copper grids. Images were taken at 80kV using a JEOL 2100 200-kV scanning and transmission electron microscope (STEM) with a Thermo Fisher Noran System 6 elemental analysis system.

Localization of ELV secretion

Microfilariae immunohistochemistry was performed as previously described. In brief, microfilariae were collected by centrifugation at 1000 X g for 5 minutes and washed in RT 1X dPBS. The worms were immersed in 4% PFA (Electron Microscopy Sciences, Hatfield, PA) in dPBS and freeze-cracked by immersing the tube in liquid Nitrogen for 2-3 minutes, thawing in a 37°C water bath and repeating the process twice more. They were then fixed for 4 hours at 4°C. The fixed worms were collected by centrifugation, washed with PBST (0.1% Triton-X 100 in PBS)

and permeablized overnight in fresh 2-mercaptanol solution (5% 2-mercaptoethnanol, 1% Triton, 120mM Tris, pH 7) at 37°C. Following thorough washes in PBST, the worms were incubated in antibody diluent (AbD; 0.1% BSA, 0.1% NaN_3 in PBST) overnight. Next, they were incubated with anti-Alix rabbit primary antibody (diluted 1:1000 in AbD) (Millipore) at 4°C for 2 days, washed in AbD three times and incubated in AbD overnight. The same procedure was followed for the incubation in donkey anti-rabbit secondary antibody (Invitrogen) at 1:1000 dilution in AbD. Following an overnight wash in AbD, the worms were incubated in 1:100 Phalloidin 647 (Life Technologies, Carlsbad, CA) and 1:100 Hoechst 33342 (Invitrogen, Carlsbad, CA) at 4°C overnight. The worms were washed in AbD the next day and mounted using Flouromount aqueous mounting medium (Sigma-Aldrich, St Louis, MO) and visualized by a Leica SP5 X MP confocal/multiphoton microscope system.

ELV uptake assay

5 x 10^5 J774A.1 cells were grown for 24 hrs on coverslips commercially coated with Poly D-lysine (Fisher). ELV preparations were stained with the green lipophilic dye, PKH67 (Sigma-Aldrich) according to manufacturer's instructions. In short, the ELV pellet was homogenized in 125µl of diluent C. Next 0.5µl of the dye was added to 125µl of diluent C and pipetted several times to mix well. This mixture was then added to the ELVs homogenized in diluent C and incubated for 5 minutes. The reaction was inactivated by adding 250 µl of heat inactivated FBS for 1 minute. 0.5-1ml 1X PBS was added and ultracentrifuged for 1 hour at 55,000 rpm to pellet the ELVs. 1 x 10^7 of stained ELVs of each life stage were added to cells grown on coverslips and incubated at 37°C for an additional 24 hrs. The internalization pathway of these ELVs was explored using endocytic inhibitors to selectively block each pathway. Cells were incubated in the

presence of 200 µM Dynasore (Sigma-Aldrich), 30 µM Chlorpromazine (Sigma-Aldrich) or 300 µM Genistein (Sigma-Aldrich) 1-2 hours prior to the addition of PKH labelled ELVs along with 0.1µm Fluoresbrite Carboxy NYO microspheres (PolySciences Inc., Warrington, PA) transferrin from human serum (Molecular probes), Alexa Fluor 555 conjugate (Life Technologies) and Cholera toxin subunit B, Alexa Fluor 555 conjugate CtxB (Life Technologies) with respective treatments to control for inhibition.

Cells were prepared for Immunocytochemisry 24 hours post-treatment as previously described. Briefly, media were removed and cells were washed with 1X dPBS heated to 37°C, followed by a 30 minute incubation at room temperature in 4% PFA heated to 37°C. All washes and incubations from this point onwards were done on a rocker at room temperature. Cells were washed for 10 minutes with 1X dPBS, followed by two more 10 minute washes. Cells were then incubated in 1:200 Phalloidin 647 (Life Technologies,) for 30 minutes followed by three 10 minute washes with 1X dPBS. 1:500 Hoechst 33342 was added to the cells and incubated for 10 minutes and washed twice in 1X dPBS. Coverslips were mounted on slides using Flouromount aqueous mounting media (Sigma-Aldrich) and visualized by a Leica SP5 X MP confocal/multiphoton microscope system.

Proteomic analysis

Protein was extracted from adult female and microfilarial stage exosome-like vesicles (System Biosciences). Samples were heated at 100°C for 15 minutes, lysate clarified by centrifugation and protein concentration determined by Qubit fluorometry (Invitrogen). 8µg of extracted protein from each sample was processed by SDS-PAGE using 10% Bis Tris NuPage

mini-gel (Invitrogen) in the MES buffer system. In-gel digestion with trypsin was done at 37°C for 4 hrs using a ProGest robot (DigiLab). The processed sample was analyzed by nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS. Data were searched against a copy of the *B. malayi* UniProt database using a locally running copy of MASCOT. The search was restricted using the following parameters; maximum missed cleavages = 2, fixed modifications = carbamidomethyl (C), variable modifications = Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q) and Deamidation (N, Q), a peptide mass tolerance of 10 ppm, and a fragment mass tolerance of 0.02 Da. Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a nonredundant list per sample. Data were filtered using at 1% protein and peptide FDR and requiring at least two unique peptides per protein. Adult female and male proteomic profiles were subjected to gene ontology (GO) analysis. GO terms from all the exosome proteins were compared to the distribution of GO terms from the entire *B. malayi* proteome. GO terms that occur in the exosome dataset more often than expected (as tested by the Fisher exact test) are said to be “enriched.”

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CHAPTER 3. INVESTIGATING THE ROLE OF *BRUGIA MALAYI* EXOSOME-LIKE VESICLES (ELV) AT THE HOST PARASITE INTERFACE

Introduction

Lymphatic filariasis (LF) is a debilitating, socially stigmatizing disease that is a major cause of socioeconomic burden in developing countries [1-3]. Of the three etiological agents of LF, *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, *W. bancrofti* is responsible for 90% of cases and *B. malayi* for most of the remainder [4]. Although different filarial nematode species, these parasites share a common life cycle differing only in the vector and definitive host range. The infective third larval stage (L3) of these parasites enters the human host via the puncture wound left behind by the mosquito following a blood meal. The L3s encounter a diversity of host cell types during their migratory journey from the puncture wound to the lymphatic vasculature where they develop into L4 stage parasites and then mature adults. The adults mate and the females produce microfilariae, which enter the blood stream to be taken up by a mosquito during a subsequent blood meal. For the duration of infection, the parasite is constantly exposed to host immune system components. Given the diverse range of barriers, sensors and response mechanisms of clearance present, it is remarkable that parasitic nematodes can still successfully evade these defense mechanisms and not only enter the host but also reside within it.

An immunosuppressive Th2 response is prototypical of LF infection [5, 6]. A few studies have focused on identifying how this immune response is generated with a focus on monocytes and macrophages since these cells are critical coordinators of the early immune response to filarial infection. Studies exposing monocytes to live microfilariae [7] and microfilarial lysate [8] have shown that these parasitic materials induce an alternative activation phenotype characterized by

elevated levels of IL-10 and ARG-1 among others. Interestingly, the study using microfilarial lysate reported that stimulation impairs the differentiation process of macrophages and their ability to produce cytokines. This is an important observation because impaired macrophages dampen the immune responses mounted against the parasites. Another study showed that macrophages diverted the Th1 immune response toward Th2 in the face of filarial infection by regulation of $CD4^+$ T cells [9]. Yet another study investigating the changes in macrophages during LF infection observed that cytokine production is not inhibited although cellular proliferation is [10]. It is apparent that the phenotype and function of macrophages are affected during LF infection, however, how the mechanisms mediating this modulation are unclear. One effort to characterize parasite-directed host modulation has focused on the molecules secreted by the parasites, revealing proteins that may play a role. Secretion of various molecules such as macrophage inhibitory factor [11], ES-62 [12] and cystatin [13] that have the capacity of modulating antigen presenting cells has been reported but a mechanism for bridging how these proteins exert their effects is not well understood. An explosion of recent studies has described the role of exosomes in cell-to-cell communication [14-16] and in disease pathogenesis, including in helminth infection. These extracellular vesicles represent an intriguing possible mechanism by which *Brugia* may package, secrete and deliver effector molecules to target host cells. Here, we present a novel potential immunomodulatory mechanism employed by the filarial nematode *B. malayi*. We hypothesize that *B. malayi* has adopted ELVs as a mechanism to fight and evade immune responses mounted by the host, ensuring its survival within the host.

Results and Discussion

Host macrophage phenotypes elicited by *Brugia* ELVs are stage-specific and reveal immunomodulatory capability.

Macrophages are early mediators of the immune response to LF infection [17]. Previously we noted differences in the cargo of vesicles secreted by L3, adult male and adult female worms (Chapter II and [18]). Such variation in cargo may be functionally relevant, reflecting that the host parasite interface is stage specific and would similarly require stage specific effector molecules. Therefore, we hypothesized that ELVs of different *B. malayi* life stages would elicit qualitative or quantitative differences in macrophage phenotype.

Macrophage activation spans a spectrum between classical activation (M1, CAMΦ) and alternative activation (M2, AAMΦ) phenotypes [19]. The classical activation phenotype is well defined and includes the release of proinflammatory cytokines, such as IL-6 [20], and increased transcription of *iNOS*, the enzyme necessary for digesting arginine into NO and citrulline [21]. Our previous studies suggest *B. malayi* L3 ELVs elicit an activation profile that is orientated towards the classical end of the macrophage activation spectrum [18]. Therefore we exposed murine J774A.1 macrophages to *Brugia* mf, L3, L4, adult male and adult female ELVs and examined the phenotypes of the macrophages within the context of classical activation. Macrophage incubation with L3 stage ELVs produced a strong increase in IL-6 production (mean value 852pg/ml; mean fold change over control 17.1) that was comparable in magnitude to the lipopolysaccharide (LPS; 100 ng/mL) positive control (mean value 1077pg/ml; mean fold change over control 20.3) (Fig. 1A). No other life stage ELV preparation stimulated IL-6 production suggesting host macrophage classical activation is specific to the L3 stage even though all life

stage ELVs are internalized by macrophages (chapter II). In broad terms this pattern of L3 specific activation was also seen when a Greiss assay was used to quantify nitrite production (Fig. 1B) and qPCR to quantify changes in *iNOS* transcription (Fig. 1C) in response to ELV exposure. L3 ELVs produced significant increases in nitrite levels (mean value 17.66mM) and *iNOS* transcription (mean fold change 14.6 over control) but no effect was observed with other life stage ELVs. J774A.1 activation requires internalization of ELVs since pretreatment of the macrophages with Dynasore (200 μM) abrogated the observed response in each assay by over 80% (Fig. 1D). Pretreatment with 5-(N-Ethyl-N-isopropyl) amiloride (EIPA; 200 μM), an inhibitor of phagocytocis and macropincytosis [22] also reduced nitrite production at a similar efficacy, supporting our conclusion that internalization occurs primarily by phagocytosis. Differential stimulation of macrophages by ELVs across the *Brugia* life cycle, despite the same level of internalization (Chapter II) suggests stage-specific stimulation via differential cargo or ELV surface proteins, further emphasizing their specific roles within the host.

Contrary to the immunosuppressive response typically associated with helminth infection *in vivo* [5, 6], infective stage L3 *Brugia* ELVs elicit a response more suggestive of a classical activation phenotype in J774A.1 macrophages. Wang *et al.* (2015) demonstrated that exosomes from adult *Schistosoma japonicum* worms drive a classical activation phenotype in macrophages. Similarly, exosomes derived from cancerous MDA-MB-231 and MCF7 cells also induce proinflammatory cytokines including IL-6, GCSF and TNF-α, whereas exosomes from non-malignant MCF10A cells do not [23], suggesting that an inflammatory response is not necessarily generated by exosomes of all origin. A proinflammatory response results in high traffic of cellular components to and from the site of infection, and parasites have been known to utilize

this for dissemination away from the infection site [24]. This leads us to speculate whether ELVs from the L3 stage elicit a classical phenotype to aid in transport of the parasite within the host.

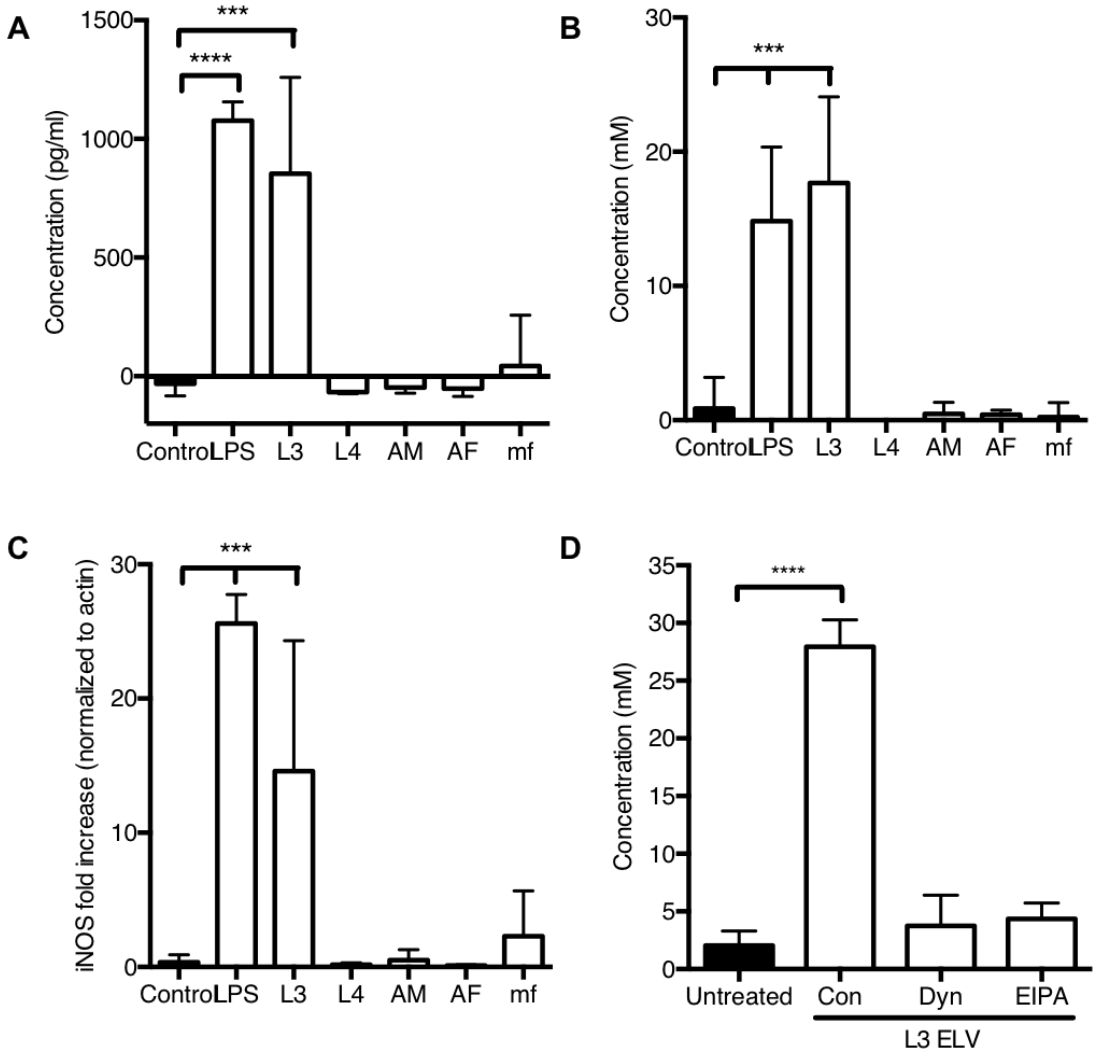

Figure 1. *B. malayi* ELVs induce stage specific activation profiles in host macrophages. Macrophages were incubated with ELVs from different life stages. Increases in IL-6 production through ELISA (A), NO through Griess assay (B) and iNOS trhough qPCR (C) indicate classical activation of macrophages in response to L3 stage ELVs but not those secreted by other stages. The proinflammatory effects are diminished when cells are pretreated with phagocytic inhibitors dynasore (Dyn) and N-Ethyl-N-isopropyl) amiloride (EIPA) (D).

Phenotypic plasticity of macrophages is well established in that M1 or M2 activated cells can revert to the contrary phenotype (i.e. from M1 to M2 or from M2 to M1) in response to appropriate immunomodulatory stimuli [25, 26]. We employed this characteristic to investigate the hypothesis that *B. malayi* ELVs have immunomodulatory properties. J774A.1 macrophages were driven towards an M2 activation phenotype using IL-4 before exposure to L3 ELVs. The ensuing macrophage response was profiled via Griess assay. L3 ELVs were not only able to drive macrophages from an M2 to M1 phenotype, demonstrating their immunomodulatory properties but also the magnitude of the response was significantly higher than that of undifferentiated macrophages to L3 ELVs. This demonstrates an active modulatory effort by the L3 ELVs. The significance of this lies in that the macrophages encountered by these parasites *in vivo* are more likely to be activated than naïve and therefore the parasites need to be equipped to revert an already established macrophage phenotype in their favor in order to establish and maintain infection.

***Brugia* ELVs elicit a similar activation profile in RAW 264.7 murine macrophages.**

Thus far we have established that *B. malayi* ELVs modulate J774A.1 murine macrophages and elicit an M1-like phenotype in a stage-dependent manner. It may be possible that these effects are specific to the J774A.1 cell line and are not representative of the broader parasite-macrophage interaction. To test this hypothesis, we repeated select experiments using RAW 264.7 macrophages, another murine macrophage line. In general, the responses of RAW 264.7 macrophages to *B. malayi* ELVs mirror those of J774A.1. RAW 264.7 cells internalize all *B. malayi* life stage ELVs examined (mf, L3, L4, adult male and adult female; Figure 3). The activation profile was also similar; treatment with L3 ELVs elicited potent increases in IL-6 production with a fold increase of approximately 3000 over control, nitrite levels with a fold

increase of approximately 30 over control and a fold increase over control of over 600 of *iNOS* transcription, all indicators of a classical or M1 type phenotype (Figure 4A-C). Other life stage ELVs did not stimulate this response. These data corroborate our findings with J774A.1 macrophages and suggest a stage-specific interaction of filarial nematode ELVs with host macrophages in the murine model.

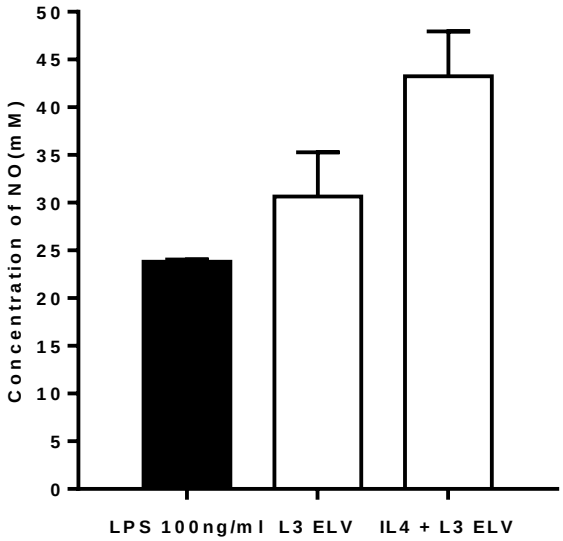

Figure 2. *B. malayi* L3 ELVs demonstrate immunomodulatory capacity. *B. malayi* L3 ELVs were added to J774A.1 cells and the response was measured using a griess assay. J774A.1 macrophages were classically activated upon exposure to *B. malayi* L3 ELVs. LPS, a compound known for driving a classical activation phenotype in macrophages was used a s a positive control. A subset of J774A.1 macrophages were pretreated with IL-4 to drive them towards an alternative activation phenotype prior to adding L3 ELVS. Interestingly, these alternatively activated macrophages were driven towards a classical activation phenotype upon exposure to L3 ELVs, demonstrating immunomodulatory capacity of L3 ELVs.

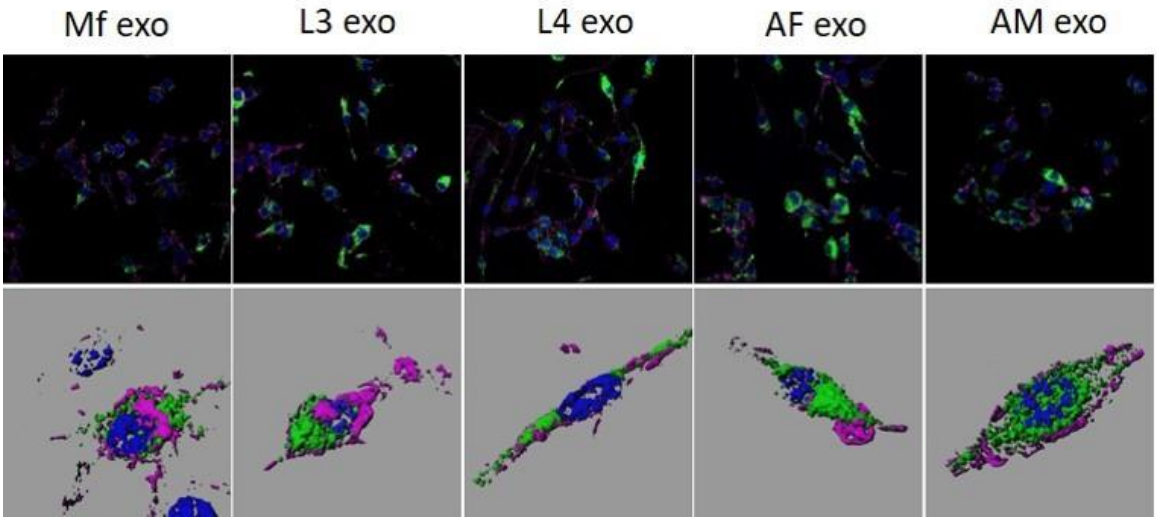

Figure 3: *B. malayi* ELVs are internalized by RAW 264.7 macrophages as seen by confocal microscopy. RAW 264.7 macrophages were incubated with ELVs from different life stages of *B. malayi* to visualize uptake by these cells. Cells were stained with Phalloidin 647 (purple) to visualize the actin skeleton and counter-stained with Hoescht 33342 (blue) to visualize nuclei. ELVs were stained with PKH67 (green) before adding to the cells. Overlays of these channels demonstrate internalization of ELVs from all the life stages by the macrophages (top panel). A 3-dimentional cartoon image magnified 5X is shown for clarity (bottom panel).

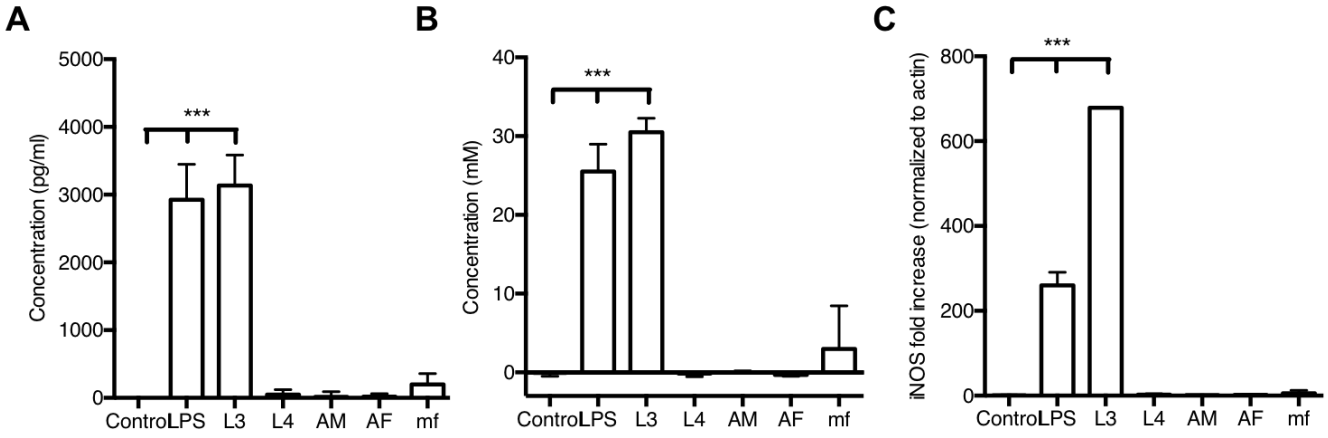

Figure 4: *B. malayi* ELVs trigger activation patterns similar to J774A.1 cells in RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with ELVs from different life stages of *B. malayi* and the response was measured via (A) IL-6 ELISA, (B) Griess assay and (C) qRTPCR of *iNOS* transcription, markers of classical activation. All showed marked increased in the face of L3 ELVs but not ELVs from other life stages. The magnitude of response was comparable to that of LPS, a known classical activator.

***Brugia* ELVs are internalized by Human Lymphatic Endothelial Cells (HLEC)**

The L3 larval stage parasites migrate to the lymphatics where they molt into the L4 larval stage and subsequently into adults. On par with our overarching hypothesis that ELVs are a mechanism of host immunomodulation by *B. malayi*, we hypothesized that ELVs from all life stages (mf, L3, L4, adult male and adult female) will interact with human lymphatic endothelial cells (HLEC). To test this hypothesis, the interaction of fluorescently labeled *B. malayi* ELVs with HLECs was visualized using confocal microscopy. Due to limited availability of sample only the internalization of L3, AF and AM ELVs were tested. ELVs released by all three life stages were internalized by HLECs (Fig. 4A-D). This internalization profile by HLECs of ELVs is expected given that all parasitic life stages are likely to encounter these cells in the natural host. In fact, a body of evidence suggests this interaction is not simply passive and that marked lymphatic rearrangement occurs during *Brugia* infection [27, 28]. Bennuru and Nutman (2009) reported that lymphangiogenesis can be observed in response to *B. malayi* antigens in an antigen specific manner [27]. Babu *et al*. (2012) further demonstrate a Toll-like receptor mediated increase of vascular endothelial growth factor [28]. Our observation that ELVs from these life stages are being internalized by HLECs might provide a mechanistic basis for the lymphatic rearrangement observed during LF. Further development of functional assays will help inform whether *Brugia* ELVs play a role in lymphangiogenesis and of other functions of these ELVs in the context of HLECs.

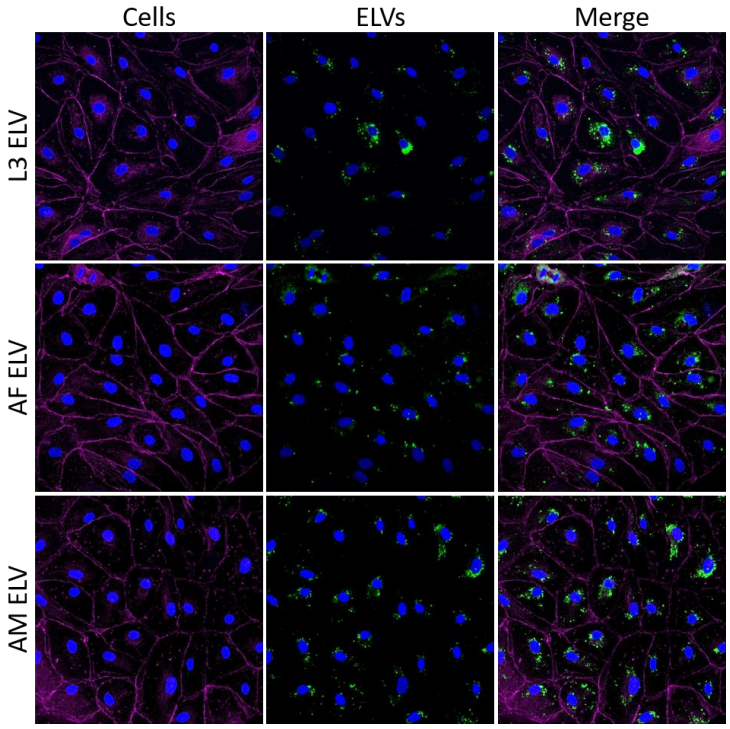

Figure 4: L3 and adult *B. malayi* ELVs are internalized by human lymphatic endothelial cells (HLEC). HLECs were exposed to L3, adult female and adult male ELVs and visualize via confocal microscopy. Cells were stained with Phalloidin 647 (purple) to visualize the actin skeleton and counter-stained with Hoescht 33342 (blue) to visualize nuclei, ELVs were stained with PKH67 (green) before adding to the cells. Overlays of these channels demonstrate internalization of ELVs from these life stages by the macrophages.

In conclusion, we demonstrate that *B. malayi* ELVs are internalized by macrophages and HLECs which are all cells these parasites encounter *en route* to the lymphatic vasculature. Further, we show that there is a clear difference between host macrophage responses to larval and adult ELV. We also demonstrate that infective stage ELVs are pro-inflammatory in nature and have immunomodulatory capacities. These data imply parasite-derived ELVs are potential bioactive agents with a specific purpose tailored to benefit the life stage from which they are released and its survival within the host. Description of this mode of communication between the parasitic nematode *B. malayi* and the human host also open interesting avenues for drug targeting and filariasis control.

Materials and Methods

ELV uptake assay

5 x 10^5 J774A.1, RAW264.7 and human lymphatic endothelial (HLEC) cells were grown for 24 hrs on coverslips commercially coated with Poly D-lysine (Fisherbrand). ELV preparations were stained with the green lipophilic dye, PKH67 (Sigma-Aldrich) according to manufacturer's instructions. In short, the ELV pellet was homogenized in 125µl of diluent C. Next 0.5µl of the dye was added to 125µl of diluent C and pipetted several times to mix well. This mixture was then added to the ELVs homogenized in diluent C and incubated for 5 minutes. The reaction was inactivated by adding 250 µl of heat inactivated FBS for 1 minute. 0.5-1ml 1X PBS was added and ultracentrifuged for 1 hour at 55,000 rpm to pellet the ELVs. 1 x 10^7 of stained ELVs of each life stage were added to cells grown on coverslips and incubated at 37°C for an additional 24 hrs. The internalization pathway of these ELVs was explored using endocytic inhibitors to selectively block each pathway. Cells were incubated in the presence of 200 µM Dynasore (Sigma-Aldrich), 30 µM Chlorpromazine (Sigma-Aldrich) or 300 µM Genistein (Sigma-Aldrich) 1-2 hours prior to the addition of PKH labelled ELVs along with 0.1µm Fluoresbrite Carboxy NYO microspheres (PolySciences Inc., Warrington, PA) transferrin from human serum (Molecular probes), Alexa Fluor 555 conjugate (Life Technologies) and Cholera toxin subunit B, Alexa Fluor 555 conjugate CtxB (Life Technologies) with respective treatments to control for inhibition. Cells were analyzed via flow cytometry and confocal microscopy 24 hours post-treatment.

Cells were prepared for flow cytometry as follows. Cells were washed with FACS buffer (0.1% BSA, 0.1% NaN_3 in PBS) heated to 37°C, scraped and resuspended in 1% PFA. Samples were analyzed by the BD Biosciences FACSCanto machine.

Cells were prepared for Immunocytochemisry 24 hours post-treatment as previously described. Briefly, media were removed and cells were washed with 1X dPBS heated to 37°C, followed by a 30 minute incubation at room temperature in 4% PFA heated to 37°C. All washes and incubations from this point onwards were done at room temperature on a rocker. Cells were washed for 10 minutes with 1X dPBS, followed by two more 10 minute washes. Cells were then incubated in 1:200 Phalloidin 647 (Life Technologies,) for 30 minutes followed by three 10 minute washes with 1X dPBS. 1:500 Hoechst 33342 was added to the cells and incubated for 10 minutes and washed twice in 1X dPBS. Coverslips were mounted on slides using Flouromount aqueous mounting media (Sigma-Aldrich) and visualized by a Leica SP5 X MP confocal/multiphoton microscope system.

Macrophage activation assays:

Changes in nitrite production, *iNOS* transcripttion and IL-6 cytokine release were measured to determine the activation profile of J774A.1 and RAW264.7 macrophages in response to ELVs. 5 x 10^5 cells were grown overnight and were subjected to different treatments for an additional 24 hours; Cells were treated with 100 ng/ml LPS (EMD Millipore, Billerica, MA) and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) as positive controls for classical and alternative activation respectively as well as 1 x 10^7 ELV preparations from different life stages with or without endocytic inhibitors 200 µM Dynaore and 200 µM EIPA (Tocris Bioscience, Bristol, UK). Nitrite levels were measured using Griess reagent (Sigma-Aldrich) as follows. Standards were prepared by adding Nitrite (2 µl of 0.1M sodium nitrite to 1ml of media) in 5 µl increments to media with each standard totaling 100 µls. 100 µl of Griess reagent was added to 100 µls of standards and samples (cell culture supernatants) and incubated on a shaker for 10 minutes for the color to

develop. Air bubbles were removed and the plate was read on a SoftMaxPro plate reader at 540 nm wavelength. Urea assays (BioAssay Systems, Hayward, CA) were also performed according to manufacturer's instructions on media collected. RNA lysates for *iNOS* and *Arginase I* qPCR were prepared and converted to cDNA using the Cells-to-cT kit (Thermo Fisher Scientific). The qPCR was performed in a GeneAmp 5700 machine for 95°C for 10 mins followed by 40 cycles of 30 seconds at 95°C and 45 seconds at 60°C using 5' GCCACCAACAATGGCAACA 3' forward and 5' CGTACCGGATGAGCTGTGAATT 3' reverse primers to amplify the *iNOS* mRNA transcript. β-actin served as an internal control (Forward primer: 5' GGCTGTATTCCCCTCCATCG 3' and reverse primer 5' CCAGTTGGTAACAATGCCATGT 3'). Changes in the levels of IL-6 were measured using the Mouse IL-6 ELISA Ready-SET-Go! Kit (Affymetrix, Santa Clara, CA).

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CHAPTER IV. FILARIAL NEMATODE ELVS: EFFECT OF ANTHELMINTICS ON VESICLE RELEASE AND THEIR USE AS BIOMARKERS

The data presented in this chapter is contributing to two manuscripts in preparation. The data describing the effect of calcium channel inhibitors on ELV release will form part of a currently untitled manuscript in collaboration with John Siekierka's group at Montclair State University, NJ, validating a family of calcium channel blockers as novel antifilarial drugs. The data evaluating the use of secreted miRNAs as biomarkers of filarial disease will be published part of a broader study examining small RNA release from *B. malayi*. A running title for this manuscript is: "Comparison of secreted and endogenously expressed microRNAs (miRNAs) in the filarial nematode parasite, *Brugia malayi."* Zamanian M., Harischandra H., Day T.A. and Kimber M.J.

Introduction

Lymphatic filariasis (LF) is a widespread Neglected Tropical Disease that is caused by the mosquito-borne parasitic filarial nematode worms *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. It is a major public health problem with nearly 947 million individuals at risk of infection in 54 tropical and subtropical countries worldwide [1] resulting over US $100.5 billion economic burden for treatment, cost of healthcare and potential income loss [2]. The key component of LF control and elimination programs is mass drug administration (MDA) to reduce the infection levels in at-risk populations and interrupt disease transmission. The preventative chemotherapy drugs currently used in these MDA programs are either albendazole and diethylcarbamazine (DEC) or albendazole and ivermectin (in areas co-endemic with onchocerciasis). Although these drugs are generally regarded as safe and have been widely used

as anthelmintics to target filarial nematode parasites, their physiological modes of action are still not well understood [3]. Illustrating this, Ivermectin is a highly effective microfilaricide in that its effect can be seen as early as one day post treatment, leading to complete clearance of Bancroftian microfilariae and about 90% of Brugian microfilariae by one week after treatment [4]. Ivermectin exerts its effects by acting as a glutamate gated chloride channel ($GluCl^-$) potentiator [5, 6] but how this results in rapid microfilaricide activity is not well understood. Studies have shown that high doses of ivermectin paralyze nematode muscle but therapeutic concentrations have no apparent effect [6, 7]. This has led to the hypothesis that a host component is responsible for the rapid clearance observed *in vivo* [8, 9]. Hinting at another mechanism of action, Moreno *et al*. (2010) showed ivermectin inhibits protein release from the excretory-secretory pore of *B. malayi* [10]. This is interesting because analysis of the *Brugia* secretome identified proteins that could potentially have a host immunomodulatory role [11-13] and the Moreno *et al*. (2010) study adds support to the hypothesis that the anthelmintic effects of ivermectin are partly due to synergistic effects between the host and parasite. A thorough understanding of how ivermectin and other antifilarial drugs exert their effects is not only important in optimizing their use but also to inform the constant search for more efficacious compounds that can be used to combat this socially stigmatizing disease.

In addition to chemotherapy, surveillance and diagnostics form a critical component of LF control programs to both evaluate the effectiveness of MDA and to provide an informed basis for continued implementation. Current methods for monitoring and evaluation of LF infection have largely relied on the detection of microfilariae (mf) stage parasites in a blood smear [14], however, such diagnostic methods are sub-optimal and carry inherent problems that may contribute to continued disease prevalence. Given that mf nocturnal periodicity, blood for these tests must be

collected at night and is therefore inconvenient and presents reliability issues. Although detection of antigenemia and *W. bancrofti* antibodies in human populations have been developed as sensitive and accurate approaches to identify adult and mf infection [15], they are ineffective at recognizing larval stage parasites during patent infection. This is a significant obstacle because these stages are vulnerable to chemotherapeutics whereas adults are not, such that early diagnosis would allow a curative therapeutic intervention rather than one that simply manages the infection. Hence, to bridge this gap, there is an urgent need to develop affordable, reliable and quantitative diagnostic tests for the early detection of LF.

Exosomes are nano-sized vesicles (50-150 nm) of endosomal origin that are released into the extracellular space by many cell types [16, 17] and are widely distributed in various body fluids [18-22]. Exosomes have been shown to contain a diverse range of bioactive molecules including proteins, mRNA and miRNA [23-26]. Importantly, these exosomally packaged miRNAs are emerging as novel regulators of cellular function and interest has emerged over the years in exosome research because of the potential role for these extracellular vesicles in disease pathogenesis and biomarker discovery [19, 27-29]. Although proteins secreted by *Brugia* with potential immunomodulatory properties have been reported [11-13], the mechanism by which these proteins may be delivered to target cells and exert their effect has not been elucidated. Given the role of exosomes in cell to cell communication in various biological contexts [30-33], we hypothesize that exosome-like vesicles are a mechanism by which *Brugia* can package and release parasite-specific effector molecules (both protein and miRNA) to modulate the human host and successfully establish and maintain infection. Extending this hypothesis, if vesicle release is a critical mechanism for parasite pathogenicity and disrupting the process may have therapeutic or prophylactic benefit. Additionally, blood-based assays for circulating vesicle-bound extracellular

RNAs could prove particularly fruitful as biomarkers in diagnosing LF. A recent study has identified stage specific, differentially expressed miRNAs in *B. malayi* [34] and we have previously shown that *B. malayi* exosome-like vesicles released into the host contain a diverse small RNA (microRNA, miRNA) cargo [23] which suggest a potential role in host manipulation through ELVs.

In this current study, we apply our central hypothesis that *Brugia* release exosome-like vesicles containing protein and small RNA effectors that modulate host biology. We demonstrate that *Brugia* vesicle release is inhibited by current anthelmintics used to treat LF, confirming this is a druggable biology and suggesting a novel mechanism of anthelmintic activity. Further, we explore the use of specific miRNA molecules secreted into host biofluids as novel, stage-specific biomarkers of filarial nematode infection.

Results and Discussion

Current anthelmintics inhibit ELV release.

Ivermectin (IVM), diethylcarbamazine (DEC) and albendazole (ALB) are the chemotherapeutic drugs currently used in mass drug administration (MDA) programs to treat Lymphatic filariasis (LF). Linking the parasite and host derived mechanisms, Moreno *et al.* (2010), showed that ivermectin sensitive glutamate-gated chloride channels were expressed around the ES pore of *Brugia* mf and that ivermectin treatment reduced protein secretion by this stage. It has been speculated that this reduced protein secretion may facilitate host mechanisms to clear the parasites. Our previous work led to the identification of the ES pore as a site of ELV release in microfilariae (Chapter 1). In light if this and our previous findings that *Brugia malayi* ELVs have immunomodulatory properties [23], we hypothesized that ivermectin may function in part by

inhibiting ELV release of these parasites, thus making them vulnerable to host defense mechanisms. To test this hypothesis, we incubated *Brugia malayi* mf, L3, AF and AM parasites with ivermectin, as well as the other anthelmintics DEC and albendazole and quantified ELV release. Interestingly, ELV release across the *Brugia* life cycle was potently inhibited by IVM (Figure 1) and might reveal a new mode of action for IVM induced parasite clearance. We hpothesize that *Brugia* parasites modulate the host via molecules secreted in ELVs such that the host is unable to mount an immune reaction effective enough to clear the parasites. The fact that IVM inhibits ELV release supports this hypothesis in that by inhibiting ELV release, IVM prevents the parasite from modulating the host immune system, making the parasite vulnerable to host defense mechanisms. Interestingly, DEC and ALB also inhibited ELVS release although host mediated parasite clearance has not been associated with these drugs, especially abendazole. The conclusions that can be derived from this observation are two-fold: 1) that there may be a host driven component that has not been discovered driven by inhibition of vesicle release or 2) the inhibition of vesicle release is a non-specific consequence of drugs that disrupt neuromuscular function, which is still an interesting observation because it demonstrates that vesicle release is a regulated process, controlled by the worm. An interesting observation was that all drugs effectively blocked adult ELV release eventhough none are effective adulticides. The reason for this could be that it is too challenging to expel the adult worm from the host due to the sheer mass of it. Niche could also factor into this. For example, the adults are in the lymphatics whereas larval stages are circulating and relatively accessible.

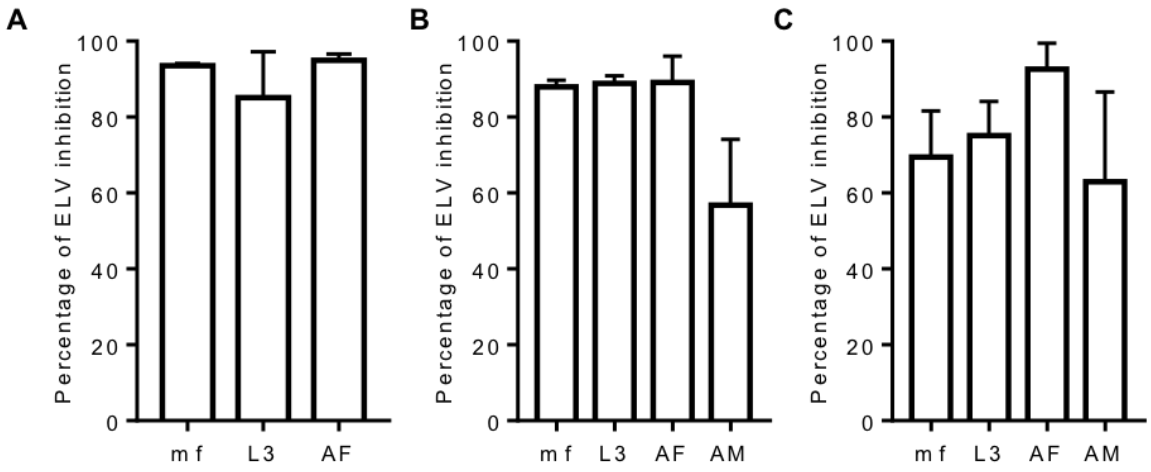

Figure 1. Current anthelmintics inhibit ELV release across the *B. malayi* life cycle. Worms were incubated with anthelmintics used to treat lymphatic filariasis (A) albendazole, (B) diethylcarbamazine and (C) ivermectin, media was collected and exosomes were purified. Quantification of exosomes via nanosight particle analysis showed inhiition of ELV release across the life cycle by all three drugs.

Calcium channel blockers inhibit ELV release

Drugs that alter Ca homeostasis in flatworms, for example praziquantel are currently being used as anthelmintics to treat Schistosomiasis suggesting that Ca homeostasis is a good, druggable target for helminths. Here we wanted to extend this principle to filarial nematodes and investigate the potential of targeting ca metabolism in parasite to inhibit vesicle release. A class of voltage-dependent L-type calcium channel blockers has recently been identified as a source of potential novel antifilarial drug candidates in a diversity library compound screen (John Siekierka, personal communication). Family members rapidly paralyze adult female and mf stage parasites and also inhibit mf release from adult females. Given the potent neuromuscular effects on *B. malayi* and inhibition of mf release, we wanted to test the hypothesis that L-type calcium channel blockers also inhibit other similar release mechanisms such as ELV release by *Brugia*. To test this hypothesis, we incubated *B. malayi* mf and L3 parasites with the most potent L-type calcium

channel inhibitors identified in the above study, namely amlodipine, benedipine, isradipine, nisoldipine and lacidipine. We also incubated worms with DMSO control and ivermectin for reference. All compounds potently inhibit ELV release in mf (Figure 2A), with the most potent compounds being amlodipine, benedipine, isradipine and nisoldipine. All four compounds inhibited ELV release more than 95% which was significantly higher than the inhibition by ivermectin which was approximately 60% (Figure 2C). Interestingly, the ELV inhibition pattern of L3 was markedly different from that of mf. Lacidipine, which was the least potent inhibitor of mf ELV release (Figure 2A), was the most potent ELV inhibitor of L3 stage parasites over control (Figure 2B), followed by amplodipine, ivermectin and nisoldipine. There was no significant difference in the inhibition over ivermectin in any of the compounds (Figure 2D). These data provide indicate that L-type calcium channel blockers are potent inhibitors of ELV release in larval stage *B. malayi* parasites. Moreover, they demonstrate differences of patterns of pharmacological inhibition between the two larval stages that could underpin different physiological mechanisms of ELV release between different life stages. Although we demonstrated that ELV release in microfilarial stage parasites occurs via the excretory-secretory pore, we were unable to visualize a site of release for the other life stages and postulated one reason may be that other life stages do not release ELV via this structure. It is possible that the different pharmacological profiles revealed in this study reflect the different sensitivities to L-type calcium channel blockers in different parasite structures; ES pore in the case of mf, intestinal tract (for example) in the case of L3. Knowledge on the L3 and adult ELV protein content will allow us to perform a more targeted immunochemistry (IHC) approach to show localize ELV release in distinct parasite life stages. Stage- or sex-specific ELV proteomic analysis, as performed in Chapter 2 of this thesis, will position us to identify stage- or sex-specific markers of ELV release that may allow selective

visualization of ELV release sites through IHC. Alternative approaches that may also be valuable to this end include in situ hybridization or variants such as RNAscope technology. These techniques may permit the visualization of the small RNA cargo contained within filarial nematode ELVs [103].

Our observation that ELV release is a ‘druggable’ process, and that we can assay for compounds that affect this process, is also significant. If inhibition of vesicle release proves to be a valuable paradigm upon which to base novel anthelmintic discovery, we have established that known anthelmintic drugs can elicit a potent phenotype, defined that phenotype, and established a baseline assay for lead compound screening. This initial assay will require refinement; it is impractical and decidedly low throughput to screen hundreds of individual parasite with a single drug/concentration, collect vesicles by ultracentrifugation and quantify by nanoparticle tracking analysis. A major improvement would be to incorporate more sensitive methods to detect and quantify vesicle release, and the automation of such methods. This would allow fewer parasites to be used per treatment and may enable transition towards a plate based assay. Exciting developments to this end are emerging from the interface of biology and engineering with microfluidics and lab-on-a-chip type devices showing promise for sensitive vesicle detection approaches [35].

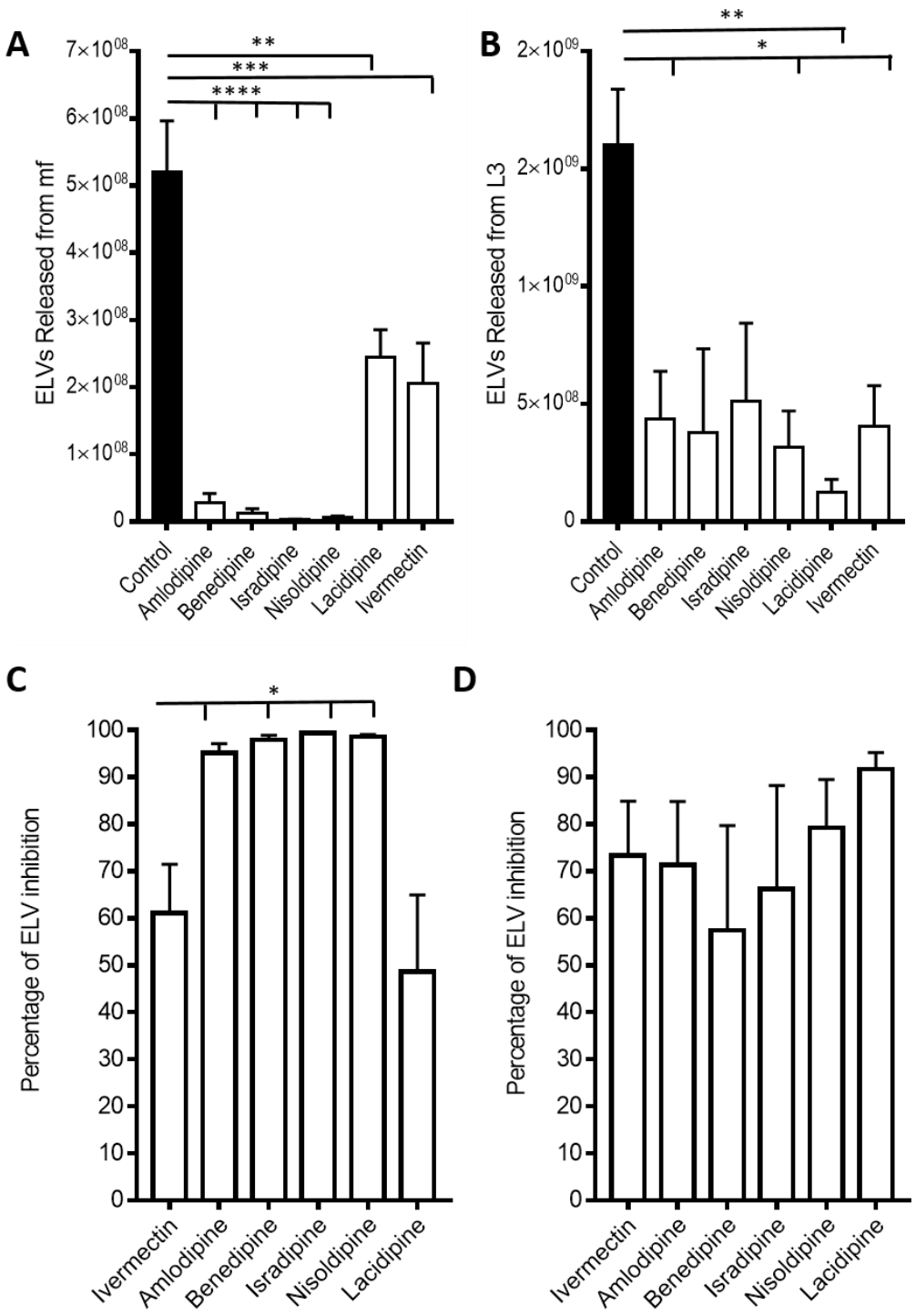

Figure 2: L-type calcium channel blockers potently inhibit ELV release by mf and L3 stage parasites. (A) Mf and (B) L3 parasites were incubated with chosen L-type calcium channel inhibitors. They were also incubated with ivermectin for reference. ELVs were purified from media and quantified via nanosight particle tracking analysis. Significance of ELV release inhibition against ivermectin was analyzed for both (C) mf and (D) L3 stages.

Investigating the potential of secreted miRNA as biomarkers

Among the different methods of LF diagnosis, confirming the presence of mf in finger-prick blood is the current gold standard. However, due to nocturnal periodicity of mf, blood for this method needs to be drawn between 10 p.m. and 2 a.m. making the approach both inconvenient and unreliable. A more reliable test is the immunochromatographic card test or ELISA based assay on circulating filarial antigens. These tests are only available for *W. bancrofti* and not yet available for *B. malayi* [36]. An emerging body of studies investigate the potential of specific molecules to serve as biomarkers for different disease conditions such as various types of cancers and neurodegenerative diseases [29, 37-39]. In fact, serological markers CEA, CA 125 and CA 19.9 are currently being used in follow up visits for patients with prostate cancer [40, 41]. An attractive aspect of using liquid biopsies i.e. molecules circulating biological fluid [42], is the non-invasiveness in obtaining samples. In Chapter 2 of this thesis we demonstrated stage specific cargo contained within *B. malayi* ELVs. Identification of secreted miRNAs unique to each stage of *B. malayi* might enable the development of a non-invasive, accurate detection platforms, a parsite-specific liquid biopsy if you will, which not only detects the infection but also it's maturity. The goal of the present study was to explore this idea and develop novel diagnostic assays based on identification of parasite-derived extracellular small RNA packaged in exosome-like vesicles. Four parasite-derived miRNAs were identified as specific to the parasite and not found in the circulating host miRNA pool using a robust bioinformatics pipeline established in our lab. Our hypothesis was that these parasite-derived miRNA will have differential expression between *B. malayi* infective and adult life stages, demonstrating proof-of principle that they may have biomarker potential.

We were able to reproducibly amplify all four miRNAs (lin4, unk2, mir100d and bantam) from worm culture media. (Figure 3). This suggests that all four miRNAs are being actively secreted by these worms and therefore, have the potential of being used as a biomarker to identify patent LF infections. Of these, there was a significant increase in the level of mir100d miRNA secreted by adult females when compared to that of L3 and adult male secretions (Figure 4C). The fact that the amount of secreted mir100d varies between life stages is interesting because it implies mir100d has the potential of serving as a candidate to predict the maturity of the infection. Although the other three candidates lin4, unk2 and bantam are all secreted by the parasite, there is no apparent difference in secretion levels between the life stages and therefore, have the potential of being used to identify LF infection but not the stage of infection. Identification of more targets that are differentially secreted by the larvae and adults will enable the development of a robust qPCR based infection identification platform that can be used reliably to predict infection.

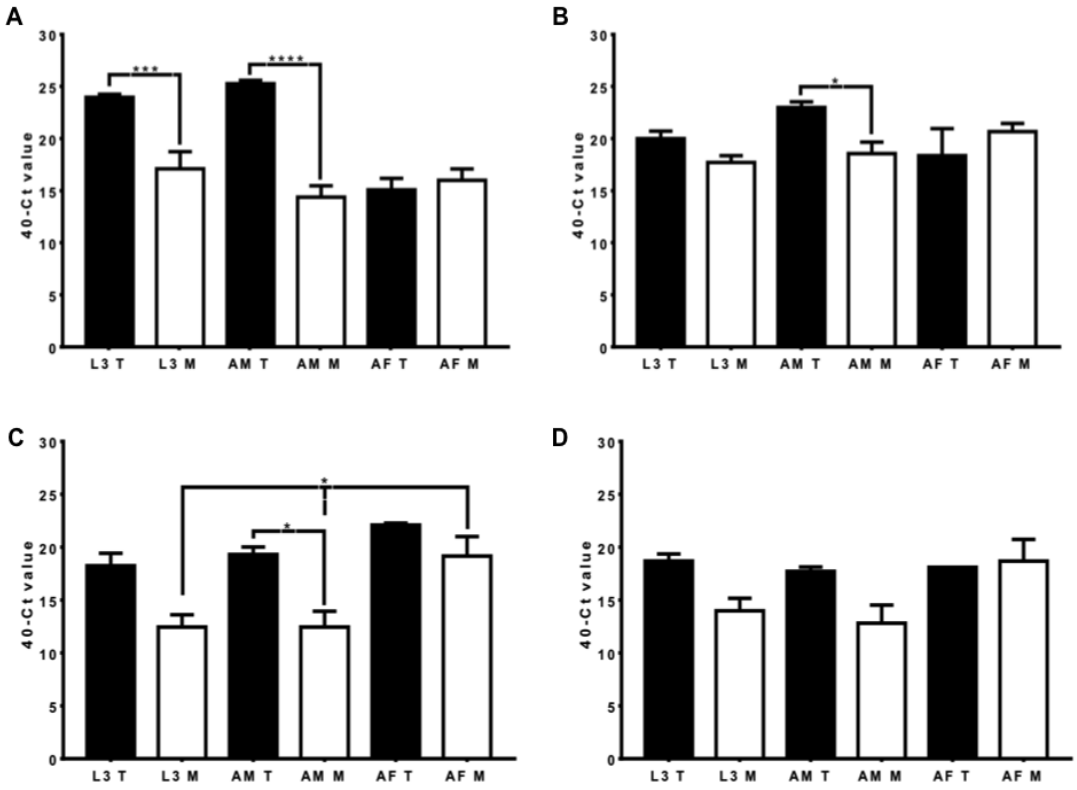

Figure 3: miRNA targets are differentially expressed between tissue (solid) and secretory samples (clear). The expression of four miRNA targets (A) lin4, (B) unk2, (C) mir100d and (D) bantam were analyzed. miRNA targets unk2, lin4, bantam and mir100d secreted by *B. malayi* parasitic nematodes were detected in culture media of (A) L3, (B) AF and (C) AM life stages.

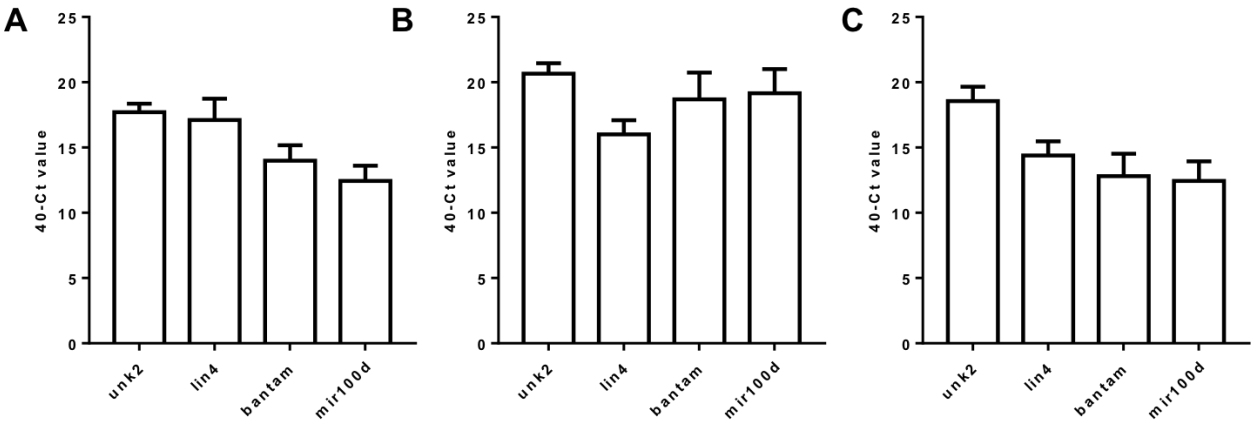

Figure 4. miRNA targets unk2, lin4, bantam and mir100d secreted by *B. malayi* parasitic nematodes were detected in culture media of (A) L3, (B) AF and (C) AM life stages.

Materials and Methods

ELV release assay

Brugia malayi microfilariae (mf) and infective L3 stage larvae were obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, Athens GA. Mf were filter purified using standard protocols. Briefly, they were centrifuged at 2000 rpm for 10 minutes and resuspended in 5 mls of 1X dPBS (Life Technologies). The mf suspension was overlaid on 5 mls of Histopaque (Sigma Aldrich?) in a 15 ml tube and centrifuged at 2000 rpm for 15 minutes to separate worms from host contaminants. The worms were then washed twice in 10 mls of dPBS and centrifuged at 2000 rpm for 5 minutes. Red blood cells were lysed by adding 3 mls of water for 15 seconds. 12 mls of 1X dPBS was added and centrifuged for

5 minutes at 200 rpm. Purified worms were cultured in RPMI 1640 (Life Technologies) containing 10 g/L glucose and pen-strep (penicillin 0.4 units/mL, streptomycin 0.4 mcg/mL) at room temperature. L3 worms were cultured in identical medium at 37°C in the presence of 5% CO_2.

For the ELV release assay, one million mfs, 100 L3s, 10 adult male and 10 adult female parasites per treatment were cultured in 1.3, 1.3, 3 and 10 mls of media containing the test compound at 1μM final concentration or DMSO (vehicle) control respectively. Culture media was collected after 24 hrs and ELVs purified via differential centrifugation as described previously [23]. Briefly, media was centrifuged at 12,000 x *g* for 45 min at 4°C and filtered through 0.22 μm filters to remove debris followed by ultracentrifugation at 55,000 rpm for 2 hrs at 4°C in 1.5 ml ultracentrifuge tubes to pellet secreted ELVs. Supernatant was aspirated leaving a residual media volume of 25 μL and the purified ELV preparations were re-suspended to 500 μl using cold 1X dPBS (Life Technologies). Nanoparticle Tracking Analysis (NanoSight LM10, Malvern Instruments, UK) was used to quantify the purified ELV preparations.

Biomarker qPCR assay

L3, adult female and adult male worms were cultured for 24 hours in RPMI 1640 (Life Technologies) containing 10 g/L glucose and pen-strep (penicillin 0.4 units/mL, streptomycin 0.4 mcg/mL) at 37°C in the presence of 5% CO_2. Microfilariae (mf) were culture in identical media at room temperature. RNA was extracted from culture media using the Norgen total urine kit as per manufacturer's instructions. RNA was also extracted from IP fluid collected from infected and uninfected jirds (*Meriones* spp). RNA preparations were DNAse treated using the Turbo DNASe kit (Ambion) and converted to cDNA using the miRNA cDNA synthesis kit (Quanta Bioscineces). qPCR analysis was done to validate *in silico* findings of four parasite-derived sequences identified

by Dr. Mostafa Zamanian using a robust bioinformatics pipeline. The qPCR was performed in a GeneAmp 5700 machine at 95°C for 5 mins followed by 40 cycles of 30 seconds at 95°C and 45 seconds at 60°C using the universal primer (Quanta Biosciences) and the following gene specific primers.

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CONCLUSIONS AND FUTURE DIRECTIONS

Lymphatic filariasis (LF) is a debilitating, socially stigmatizing neglected tropical disease which puts 947 million people at risk worldwide [1]. It is caused by filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* of which the infective larval stage is transferred to humans via a mosquito bite. The L3 larval stage migrates to the lymphatics where it molts twice and matures into adults. These adults mate and the female nematode produces millions of microfilariae. There are no vaccines for this disease. Chemotherapy through mass drug administration (MDA) programs and prevention measures are the only solution. However, even after 15 years of MDA, Lymphatic filariasis still remains a global health concern. Successful parasitism depends on specific interactions between the host and the parasite and our efforts of delineating these interactions resulted in the identification of a novel mechanism by which these parasites may manipulate the host to its benefit (Figure 1).

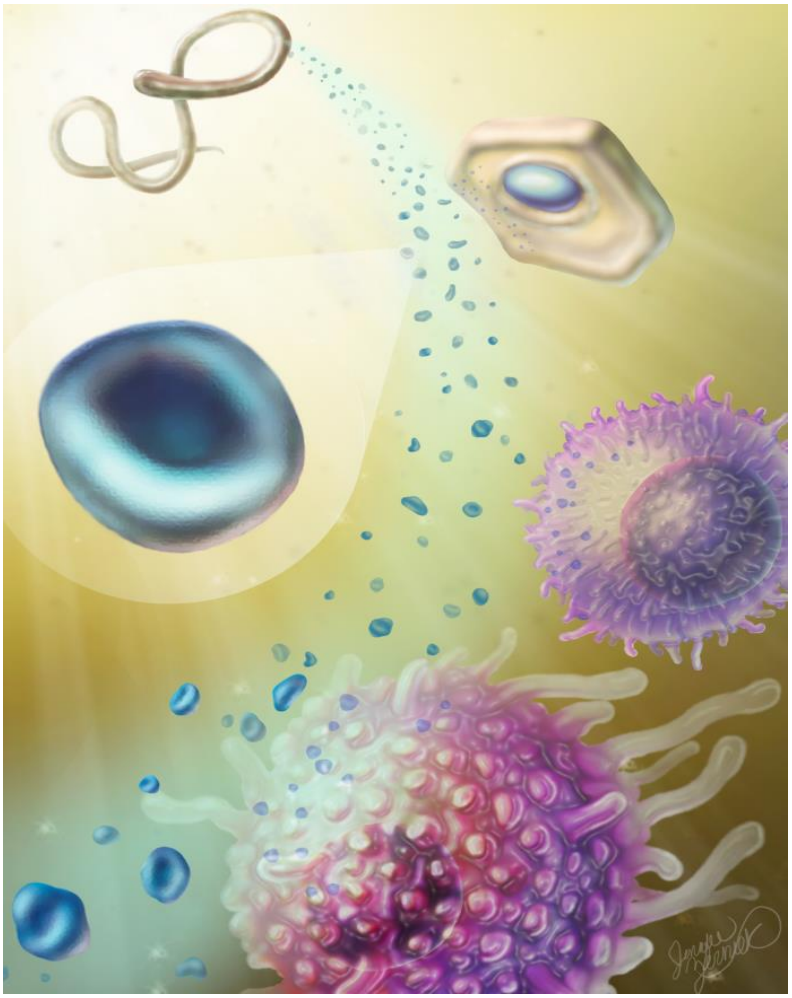

Figure 1. A proposed model by which exosome-like vesicles (ELVs) secreted by the filarial nematode, *Brugia malayi*, are released into the host environment and interact with host cells to facilitate infection.

The immune profile of a Lymphatic filariasis infection is mainly immunosuppressive. Although secretome studies have identified proteins with immunomodulatory capacity [2-4], neither how these proteins enter the respective cells nor how immunosuppression is achieved has been known to date. Exosomes are extracellular vesicles containing effector molecules. Previously thought to be a mechanism of waste disposal, with the discovery that these vesicles harbor bioactive molecules exosomes have gained renewed interest during the past few years. Previously we found that infective L3 stage *B. malayi* release exosome-like vesicles (ELVs) that contain putative host effector molecules including microRNAs (miRNAs) with the potential to manipulate host gene expression [5]. Further, these ELVs were internalized by host macrophages, early mediators of the host immune response for filarial infection [6-8]. Our overarching hypothesis is that filarial nematodes, including *B. malayi*, use ELVs to release effector molecules that facilitate the establishment or maintenance of parasitemia, specifically by actively directing the host response to infection. In Chapter 3 of this thesis we describe data that support this hypothesis.

We have demonstrated that vesicles bearing characteristics of exosomes are secreted by all life stages of the parasitic filarial nematode *B. malayi.* No other study thus far has demonstrated this phenomenon throughout the life cycle; all studies on parasitic helminth ELV release have focused a single life stage, mostly adults [9-15] and one on the fourth larval stage [16]. Pan life cycle secretion suggests that ELV release is a conserved process. The proteomic profile of adult and infective L3 larval stage ELVs contained canonical exosomal markers and proteins of endocytic pathway origin, providing evidence that the observed vesicles are exosomes and not another form of extracellular vesicles. Previous studies on parasitic nematode ELVs have discussed the possibility of the release of heterologous vesicle population and therefore question whether the effects seen are due to exosomes or a different subset of extracellular vesicle; our

observations suggest a homologous exosome population as discussed in chapter 2 in this thesis and functional assays attribute the effects observed to ELVs.

Previous studies on the *Brugia* secretome [2-4] have revealed proteins with immunomodulatory capacity such as galectins and yet how these proteins enter cells nor how they exert their effect is unknown. We hypothesized that *Brugia* employ ELVs as a mode of transferring these effector molecules to host cells, thereby aiding in host manipulation. Indeed, analysis of the proteomic content of the ELVs revealed immunomodulatory cargo supporting this hypothesis; Adult female ELVs were abundant in proteins with immunomodulatory capacity such as macrophage migration inhibitory factor, galectins, annexin, DAF-21. The proteomic profiles of the different *B. malayi* life stages compare well with the existing profiles of ELVs from other helminths [11-13, 16, 17]. They all contain structural proteins such as actin, tubulin, nuclear proteins such as histones, peroxiredoxins such as thioredoxin peroxidase, and exosomal proteins such as HSP70. Interestingly, thioredoxin peroxidase which has been shown to be contained in these ELVs and annexin which was shown in *D. dendriticum, S. Japonicum and S. mansoni* [12, 13, 17] ELVs are the only immunomodulatory proteins identified in other ELV proteomic studies, while our current study on adult ELVs identified many other immunomodulatory proteins contained within them. Also of interest was the stage and gender specificity of the cargo within these ELVs; the proteomic profiles of L3 and adult male ELVs were mainly of structural proteins, unlike the female profile. Not only does this demonstrate stage specific secretion, hence possibly stage specific host modulation but also possibly sexual dimorphism. Our studies uncovered new information that adds to the wealth of knowledge in the field of nematode biology and parasitology. Here, we have identified a mechanism by which effector molecules are released from the parasite and delivered to the host. The fact that the release of these effector molecules is via a regulated

defined process provides further support of specific functions and targeted delivery. It has also uncovered a host of novel molecules that can be targeted in drug discovery efforts. Moreover, this is the 1st report of ELV secretion of a medically relevant human parasite and the second of a parasitic nematode. A recent report on the secretion of exosomes harboring effector molecules by the intestinal parasitic nematode *Heligsomoides polygyrus* [9] suggest exosome release is a process shared among nematodes and warrants further investigation with other nematode species.

We also demonstrated that *B. malayi* ELVs are internalized by host macrophages and lymphatic endothelial cells. Previous studies have shown that exposure to *B. malayi* parasites can induce specific immune responses. A Th2 response with elevated levels of anti-inflammatory cytokines like IL-4, IL-5, IL-10 and IL-13 is prototypical of a Lymphatic filariasis infection [18]. Studies have shown that L3 larval stage parasites [19] and adult worms induce IL-4 production [20]. Moreover, mfs initially induce a more Th1-like response later followed by increased IL-4 and IL-5 production [20, 21]. In addition to changes in the immune system, studies have also shown that these parasites induce endothelial rearrangement [22]. Although it is apparent that these parasites influence multiple host processes how this is achieved has not been elucidated to date. Interaction of parasitic ELVs with host cells suggest a mechanism for effector delivery to these tissues and explains how parasites might be mediating their host. Exosome-like vesicles (ELVs) released by the infective larval stage (L3) *B. malayi* trigger the classical activation pathway in murine macrophages upon internalization. However, no other stage elicits such a response. This stage specific activation of host cells in response to internalization suggests that ELVs are released by the parasite to cater to each stage and suggests its importance in establishing and maintaining infection. A recent study on *Heligmosomoides polygyrus* corroborates our findings [23] and suggest exosome mediated host manipulation might be a mechanism employed by parasitic

nematodes. This is an interesting paradigm and studies on other parasitic nematode species are essential to investigate the universality of this process. This would help explore the possibility of developing disease control methods targeting multiple nematode diseases, which would be useful for areas co-endemic with many.

Further, In Chapter 4, we demonstrated that ELV secretion by *B. malayi* parasites can be significantly decreased by anthelmintics. Previous efforts to delineate the mechanisms of action of anthelmintics have suggested a host component to the effective larval clearance observed *in vivo* [24-27] although neither what it is nor how it achieves this clearance has been explained to date. Our finding that anthelmintics decrease *Brugia* ELV secretion might provide a mechanistic basis for how this is achieved, partially if not completely, adding another piece to the puzzle of how these parasites manipulate host mechanisms. Our hypothesis is that the parasite releases ELVs packaged with effector molecules that can manipulate and combat defense mechanism mounted by the host in response to the parasites, enabling them to establish and maintain infection. The immunomodulatory proteins contained within these ELVs, as revealed by the proteomic analysis, provides support in favor of this hypothesis. Furthermore, we identified calcium channel blockers that inhibit ELV secretion more potently than ivermectin. If ELVs are a method of host manipulation, then the discovery of compounds targeting this process more potently than available anthelmintics is very exciting. It also suggests that ELV release could represent a novel screening platform for the next generation of anthelmintics.

Early detection of the disease is important to help keep it at bay. Here we demonstrated that secreted miRNA can be isolated from host mileu and detected via a qPCR platform. This discovery is important because it demonstrates proof-of-principle of using secreted miRNA as biomarkers to detect infection. ELV antigens are protected against degradation over freely

circulating macromolecules due to being enclosed within a vesicle and can therefore circulate throughout the body unharmed to reach distal organs. This characteristic enables the use of ELVs as liquid biopsies for identification of the disease. Different cargo within ELVs allow us to exploit this further to establish stage-specific biomarkers to identify disease progression, providing medical practitioners the option to optimize treatment on a case by case basis. Accurate identification is important to ensure it's targeted and treated so that infected individuals cannot serve as reservoirs for parasites, prolonging transmission. Targets that can accurately identify the presence of L3s in patients would be of paramount importance since most LF infections are asymptomatic and this allows for detection prior to a patent infection being established. Furthermore, adult parasites could be residing in patients even though microfilaria cannot be detected in blood samples. This scenario cannot be detected by the traditional finger-prick method since it probes for microfilariae but can be identified by miRNA biomarkers specific to the adult stage. In a hypothetical world, treating these patients will help minimize the damage inflicted by the parasites. Having said that, the 3 antifilarial drugs currently being used are only larvicidal and therefore, will have no effect against the adult parasites. For this reason, there is no use in treating non-patent infections at the present but once an adulticide is discovered, it will be important that these infections are also treated. For the time being, it is important however, that these patients are routinely checked for repeated infections so that they can be treated early if microfilariae re-surface.

Since MDA is carried out to populations at risk, diagnosis of LF is mainly to evaluate the effectiveness of the MDA treatments. Blood can be drawn at random from populations to check for the presence of miRNA biomarkers that indicate not only the presence or absence of infection but also potentially the stage of it. There are many advantages to this method. It is a more

convenient method than the conventional finger-prick method because blood drawings are not constrained to evenings. In the conventional method, absence of microfilariae in the blood sample can leave a degree of uncertainty; microfilariae have nighttime periodicity and it could be that they had not yet surfaced at the time blood was drawn. The biomarker method is more reliable and accurate because nighttime periodicity has no effect on circulating miRNA. It is also less time consuming and therefore, can serve as a high throughput screening method; even up to 384 samples can be run on a single plate. Moreover, no special skills are needed to conduct this screening process. Another advantage is that it can be developed further to identify markers of various diseases at the same time using a single sample. This is a cost-effective and more efficient method when diagnosis of multiple diseases is concerned. A major disadvantage is that this screening method requires the access to a qPCR machine and one will most probably not be available at every diagnostic center in these areas. Another disadvantage might be that it is difficult to parse out the parasitic miRNA or the biomarker from host material at low levels and therefore, could lead to inaccurate conclusions. A recent study questioned the applicability of parasitic miRNA as biomarkers due to the difficulty of parsing out parasitic miRNA from host biofluid [28]. Similar studies in *Brugia* infected hosts will help inform the transferability of these conclusions to the *Brugia* model.

Future experiments

It is important to analyze the proteomic profile of the microfilariae and the fourth larval stage parasite ELVs as well in order to identify cargo specific to those stages and fully understand the role of ELVs in pathogenesis. Our previous study showed that the infective L3 larval stage parasite ELVs contained miRNA with potential immunomodulatory capacity [5]. These may be critical regulators of host gene expression in this model and therefore it is also important to identify the small RNA cargo of all other stages. This information will be important in furthering the understanding of the role of these vesicles and the identification of stage specific cargo that can be exploited in disease control and drug discovery efforts as discussed below.

The fact that these ELVs are internalized by J774A.1 and RAW 264.7 murine macrophages and Human Lymphatic Endothelial Cells (HLEC), which all life stages of *B. malayi* interact with within the human host strengthens the hypothesis that the ELVs are secreted by the parasite for host immunomodulation. Demonstration that ELVs elicit stage specific differential activation profiles in the murine macrophage model further suppots this hypothesis. Although the usage of murine models is accepted for the study of early LF infection by larval stage *B. malayi,* it is not the best model for studying adults due to their inability to survive to adulthood in these animals. Therefore, an important next step will be to investigate the response to ELVs in a more translatable model such as human macrophage cell lines such as THP-1 and U937, or macrophages from athymic nude mice (C3H/HeN) in which a related species, *Brugia pahangi* has been reported to survive to adulthood [29]. Moreover, although studying the interaction between parasite ELVs and explicit macrophage lines is necessary to define the specificity and outcome of this particular host-parasite interaction, taken in isolation, these phenotypes may not reveal the global effect of ELV secretion on the host immune response. Therefore it is also important to examine the global

response to ELV secretion using a whole animal murine model for a comprehensive understanding of this interface. C57/BL6 mice can be footpad injected with ELVs purified from different life stages of *B. malayi*. Draining lymph nodes can be collected and changes in the global cytokine profile can be explored using MCYTOMAG-70K, MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel Immunology Multiplex Assay on the Luminex platform. This high throughput assay will allow the simultaneous identification and quantification of chemokines/cytokines, allowing us to explicitly describe the global response to parasite ELVs. Since we looked at only classical activation profiles in cells, a more inclusive cytokine array should be used in this experiment that will also check for cytokines associated with the alternative activation pathway. The control mice will be injected with PBS. Lymphotoxin α or retinoic acid-related orphan receptor (Ror)-γt knock-out mice can be used as an additional control. These mice will also be injected with ELVs but since they lack or have reduced lymph nodes, the cytokine profile in these mice should be more closely related to control mice than the ELV injected mice, hence attributing any changes in the cytokine profile to ELVs. Further, immunohistochemistry can be performed on cross sections of lymph nodes. The staining patterns of different cytokines can provide additional information about the immune response generated. Moreover, dendritic cells, T cells, B cells, monocytes granulocytes can be isolated from the lymph nodes and assessed for morphological changes, which could further inform which cells are involved in generating the immune response observed. One of the biggest advantages of looking at the effect of ELVs in the whole mouse model is that it provides direct evidence of immunomodulation by ELVs as opposed to a cell based method. A disadvantage of this method is that it is terminal.

Another option would be to inject ELVs and collect blood from the ocular vein or submandibular and look for cytokines in the serum. An advantage of this method is that a time

course study can be done using the same mice, which has many advantages; it provides more information such as when a robust immune response is generated, how long it lasts. It is also cost-effective and reduces the mouse to mouse variation if the same set are used. I believe this method would indeed identify immunomodulation as long as it is optimized for the amount of ELV injected and when the reaction is monitored. Whether the amount of ELVs that need to be injected to observe a change in the cytokine profile is physiologically relevant is a different question that needs to be addressed.

While ELVs might be employed by *B. malayi* to manipulate the host immune system, their immunomodulatory characteristics suggest these very ELVs may be exploited therapeutically to provide protection against filarial nematode infection. Controlling filarial infection relies exclusively on chemotherapy. Issues with drug efficacy, regimen, safety and resistance make a broadly protective approach to disease control highly appealing. This is a novel concept and has not been demonstrated in a medically relevant human filarial parasite to date. Historically, efforts to develop vaccines against *Brugia* have used both attenuated parasite and sub-unit approaches. Irradiated L3 infective stage larvae [30], ALT-1 and ALT-2 major gene product of the L3 stage [31] and recombinant Trehalose-6-Phosphate Phosphatase [32] antigens have all yielded varying degrees of protection against the L3 stage, with the Bm-TPP experiments also leading to reduced microfilarial load [32]. A study on the protective effects of excretory-secretory products of the L3 larval stage of *B. pahangi*, a related species to *B. malayi*, observed similar results with no effect against the adult parasites [33]. Whilst these efforts have successfully elicited protective effects against the infective stage, their poor efficacy against adult parasites mean that although transmission can be reduced, it cannot be halted. Moreover, suboptimal vaccines pose a threat. Previous studies with *L. sigmodontis*, a filarial parasite closely related to *B. malayi*, reported that

phenotypic plasticity can occur in response to host immune responses and that they can adjust their development based on environmental cues as soon as they enter the host [34]. Therefore, it is important to target a mechanism that is universal across the life cycle so that parasites that escape the initial defense will still be targeted. The Th-1/ Th-2 response in endemic normal individuals makes the discovery of the proinflammatory nature of ELVs that much more interesting. The antigenic nature of *Brugia* ELVs suggest the potential of mounting a rapid response in the face of repeated infection and therefore, validate them as potential novel vaccine candidates. Specialized adhesion and recognition molecules on ELVs may associate with antigen-presenting cells more efficiently and will be more effective in cross-priming since ELVs are a known mechanism for transporting antigens between cells [35, 36]. ELV antigens are protected against degradation over freely circulating macromolecules due to being enclosed within a vesicle and can therefore circulate throughout the body unharmed to reach distal organs. This further opens up the possibility of pre-exposure to potential antigens identified in the future encapsulated within these ELVs to ensure minimal degradation and optimal delivery to cells. Moreover, ELVs are secreted by diverse parasitic nematodes [5, 9] and therefore, vaccines developed with this approach have the potential of being used for trans-species control. We believe parasite ELVs are attractive novel protective antigens and warrant further studies to investigate the possibility of exploiting these structures for therapeutic benefit.

A recent study conducted using exosomes secreted by an intestinal parasitic nematode *Heligmosomoides polygyrus*, showed that mice injected with exosomes secreted by these parasites demonstrated protective effects against larval challenge [23]. An earlier study with *Leishmania* spp. (a single celled parasite that is also known to secrete ELVs) has also similar protective effects upon reinfection when pre-exposured to Leismania ELVs has a protective effect upon reinfection

[37]. *B. malayi* L3 larvae or adults can be surgically implanted into the peritoneal cavity of vaccinated or control animals as previously described [38]. Jirds (*Meriones* spp) can be used in these studies because of the permissiveness of them to *Brugia* infection and the ability of these parasites to develop into adulthood within this host as have been shown by studies where L3 larvae inoculated into the peritoneal cavity of jirds developed within this location [39, 40]. Short-term (2 weeks post immunization) and long term (5 months post immunization) effects can be observed to confirm that any initial clearance observed with the short –term study is clearance due to immunological memory and not just due to the persistent inflammation from the vaccine. The viability of the inoculated and implanted parasites and other measures of parasitemia in the ELV-challenged jirds can be compared to control animals at several time points; 2 weeks, 4 weeks and 4 months post immunization. The expected result would be reduced parasitemia and worm burden in the animals pre-exposed to ELVs. The importance of this study lies in the demonstration of proof-of-concept that vaccines may be generated using *B. malayi* ELVs to prime the immune system, which can then be administered to human or animal populations at high risk for filarial nematode disease. Priming the immune system in this way will result in reduced susceptibility to the parasite, helping disrupt transmission of the disease.

In conclusion, we identified a novel mechanism by which *B. malayi*, one of the etiological agents of Lymphatic filariasis might be manipulating the host to its benefit via exosome-like vesicles (Figure 1). We have also demonstrated differential cargo and host activation patterns of these vesicles, lending support for stage and gender specific roles of these vesicles. Furthermore, we showed the antigenic nature of these vesicles. Our discoveries not only further the understanding of nematode biology and parasitology but also have demonstrated a novel

mechanism of host manipulation, suggest a mechanism of anthelmintic mode of action and uncovered a host of molecules that can be targeted in disease control and drug discovery efforts.

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APPENDIX I – IDENTIFICATION AND CHARACTERIZATION OF NEMATODE G PROTEIN-COUPLED RECEPTORS

Introduction

Lymphatic filariasis (LF) is a debilitating and socially stigmatizing neglected tropical disease (NTD) caused by parasitic nematodes including *Brugia malayi*. In 2000, the world health organization launched a Global Programme to Eliminate Lymphatic Filariasis (GPEFL) by 2020 through mass drug administration (MDA). Although the MDA program has celebrated some success [1], the chemotherapeutics being used are suboptimal. All three drugs used are only effective against larval stages and therefore can merely reduce prevalence and not completely disrupt transmission. Ivermectin and diethylcarbamazine (DEC) cannot be used in certain areas where LF is co-endemic with certain other NTDs due to severe adverse reactions if administered to those with loiasis and onchocerciasis respectively. Due to these reasons, there is an urgent need for novel anthelmintics for *B. malayi*.

Acetylcholine (ACh) functions as a major neuro-transmitter in both vertebrates and invertebrates [2]. The two types of cholinergic receptor in nematodes are the ligand gated chloride channels, which are comprised of the nicotinic ACh receptors (nAChRs) and the acetylcholine gated chloride channels (ACC), and the slower acting muscarinic ACh receptors (mAChRs – in nematodes these are referred to as G protein-linked acetylcholine receptors, or GARs) [3]. A number of current anthelmintics such as pyrantel, morantel and levamisole target nAChRs [4], acting as selective agonists at the receptors to increase cation conductance, eliciting membrane depolarization and muscle contraction thereby facilitating parasite expulsion. The widespread use of such anthelmintics to control primarily gastrointestinal parasites in both human and veterinary

medicine has ensured that the pharmacology and physiological importance of nematode nAChRs are well characterized. However, there are significant knowledge gaps in our understanding of the pharmacology and physiological importance of the other types of nematode cholinergic receptor, in particular, the GARs.

Despite our limited understanding of nematode GARs, there is evidence supporting the argument that parasitic nematode GARs are strong candidates as novel anti-nematodal drug targets. GARs are reported to be expressed in the body wall and pharyngeal muscle of the model free-living nematode *Caenorhabditis elegans* [5] and may be involved in regulating or modulating locomotory and feeding behaviors in nematodes. These processes have historically been prioritized as targets in the development of novel anthelmintics. Further, *C. elegans* GARs also have a role in mating behavior and egg laying [6]. Whilst no known anthelmintics disrupt parasite reproductive biology as their primary mechanism of action, compounds targeting this process would have potential in controlling parasite infection through breaking the parasite life cycle – an overall strategy that has proven effective in LF control. Lastly, as members of the wider G protein-coupled receptor (GPCR) superfamily, GARs have specific advantages from a drug development perspective. GPCRs are highly druggable proteins and are targeted by a diverse array of therapeutics for many diseases [7]. A broad range of high throughput platforms and technologies allow screening and pharmacological analysis of these receptors, facilitating the quest for compounds that act against specific GPCRs. Lastly, although GARs appear pharmacologically distinct from vertebrate mAChRs, they do share responsiveness to acetylcholine – a fact that may allow the exploitation of established chemical libraries that include small molecules used to target cholinergic GPCRs.

The goal of this study was to identify and characterize parasitic nematode GARs using the filarial nematode *B. malayi*, and provide new information on their role in parasitic nematode physiology. *B. malayi* genomic data was queried using *C. elegans* GAR sequences as bait to identify fragments of GAR-encoding genes which were extended using RACE PCR to elucidate the full length sequence of a GAR-3 homolog, termed *Bmgar-3*. *Bmgar-3* homologs were also identified in the genomes of other parasitic nematode species. The expression pattern of BmGAR-3 and the *Ascaris* homolog were examined using a combination of RT-PCR and western blot to reveal specific temporal and spatial expression; GAR-3 homologs showed increased expression in male worms of both species. An auxotrophic yeast expression system was used to pharmacologically characterize BmGAR-3 but positive functional expression could not be consistently replicated despite exhaustive optimization efforts. Similarly, functional expression in HEK cells interfaced with a novel phosphorylation assay to reveal receptor activation yielded inconsistent results. Our data point to issues expressing nematode GPCRs in heterologous cell lines that must be addressed before parasitic nematode GPCRs can be exploited as novel anthelmintic drug targets.

Results and discussion

Identification of BmGAR-3, the *Brugia malayi* GAR-3 homolog

Three G protein-coupled acetylcholine receptors (GARs) have been identified and characterized in the model worm, *Caenorhabditis elegans* [2, 8, 9]. Our hypothesis is that *B. malayi* possesses a similar GAR complement. To identify *B. malayi* GAR-encoding genes, we used a tBLASTn search of the *Brugia* draft genome using the three *C. elegans* GAR sequences as bait. We identified three fragments with homology to the *C. elegans* receptors [WGS reads BRBCS25TJ

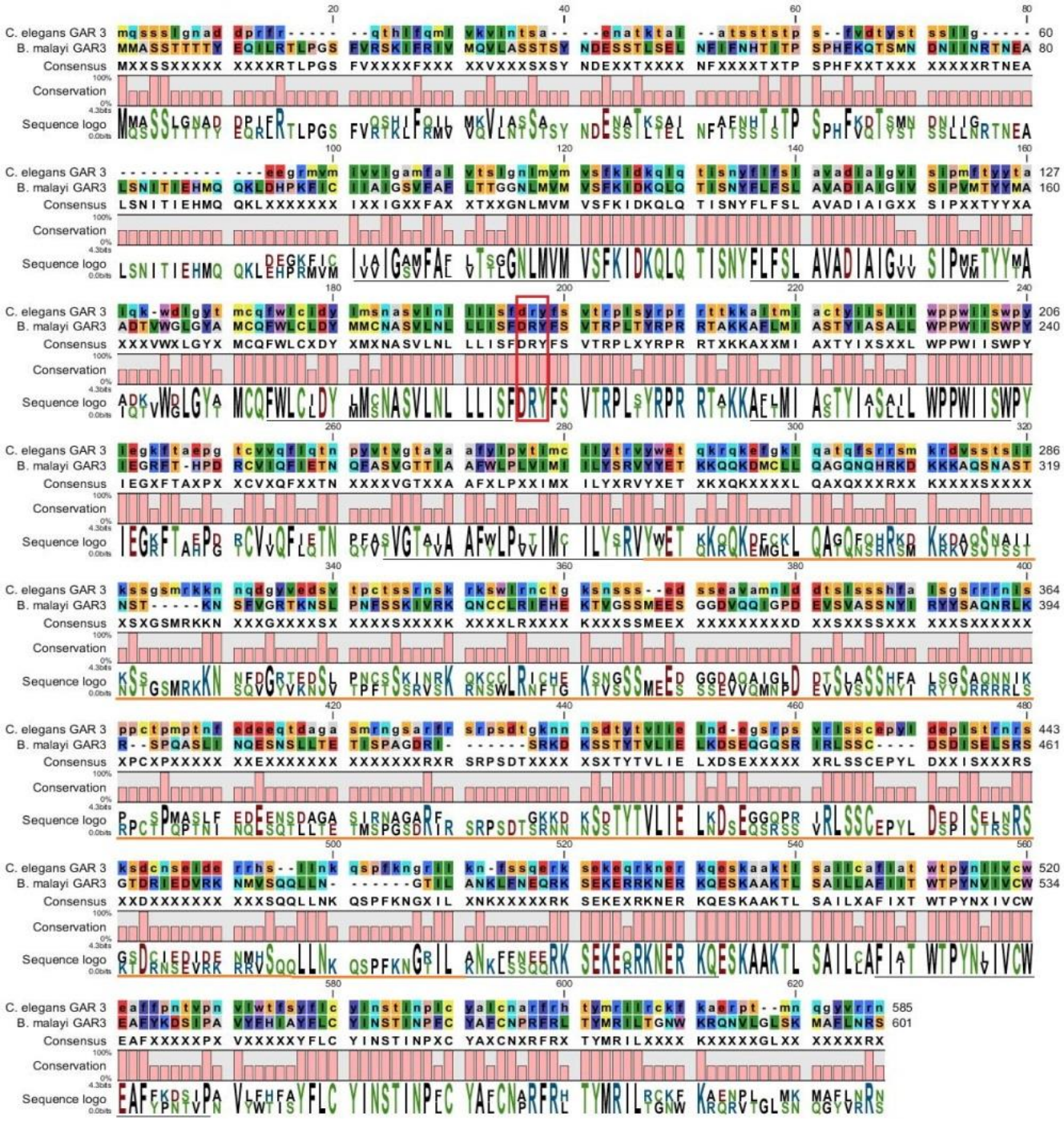
(putative BmGAR-1) and BRKPQ43TF (putative BmGAR-3), and XM_001893774 (putative BmGAR-2)]. To extend these fragments into full-length sequence we used RACE (Rapid Amplification of cDNA Ends) PCR using primers designed from the genomic data. We extended the known *Bmgar-1* and *Bmgar-2* sequences but were not able to identify complete transcripts for these two genes. However, we were able to generate a full-length transcript for *Bmgar-3* (Figure 1A). This transcript included a 1803 nt open reading frame encoding a 601 aa protein. A reciprocal BLAST returned GAR-3 homologs as the most similar proteins, strongly indicating that this molecule is the *B. malayi* GAR-3 homolog.

BmGAR-3 sequence analysis

G protein coupled receptors (GPCR) have a characteristic seven transmembrane (TM) domain arrangement. Crossing the membrane seven times generates an amino terminus and extracellular region with three extracellular loops, an intracellular region with three intracellular loops and a carboxyl terminus [7, 10]. The greatest homology between GPCRs and GPCR families lies in the TM domains, while they differ the most at the amino and carboxyl termini and third intracellular loop [7]. Throughout the GPCR sequence there are key conserved motifs that are related to receptor functions, such as ligand binding and G-protein interaction sites. For example, the DRY motif at the end of the third intracellular loop is a highly conserved sequence [11] that functions in receptor conformation, ligand binding and G-protein interaction [12] . Our hypothesis is *BmGAR-3* encodes a *B. malayi* acetylcholine responsive GPCR. To test this hypothesis we examined the encoded *BmGAR-3* protein for features consistent with this designation. To describe transmembrane topology, the BmGAR-3 sequence was analyzed using TMHMM 2.0 (cbs.dtu.dk), an online tool for transmembrane helix prediction. This model takes into account hydrophobicity,

the charge of each residue (positively charged residues are more abundant in the intracellular or extracellular regions [13]), helix length and the GPCR "grammar" where the cytoplasmic and non-cytoplasmic loops must alternate [14]. TMHMM 2.0 output identified seven TM domains in *BmGAR-3* (Figure 1B). The predicted extracellular orientation of the N terminus (low N-in value = 0.048), intracellular orientation of the C terminus, and presence of these seven TM domains corresponds well with the standard structure of GPCRs and supports the hypothesis that *BmGAR-3* is a GPCR (Figure 1C). The sequence of *Bmgar-3* was then aligned to *C. elegans gar-3* using CLC DNA Workbench 6 software to assess the homology between the two receptors (Figure 1A). The highest conservation of sequence is found across the seven TM domains (underlined in black) with the lowest homology across the long third intracellular loop (underlined in orange) and the N- and C-termini. The highly conserved DRY motif was also observed (196-198 aa). Collectively, these sequence features strongly support the hypothesis that BmGAR-3 is indeed a GPCR.

A


C. elegans GAR 3
B. malayi GAR3
Consensus
Conservation
Sequence logo

B

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# Sequence Exp number, first 60 AAs:  0.50882
# Sequence Total prob of N-in:        0.04756
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Sequence        TMHMM2.0        TMhelix    101   123
Sequence        TMHMM2.0        inside     124   135
Sequence        TMHMM2.0        TMhelix    136   158
Sequence        TMHMM2.0        outside    159   172
Sequence        TMHMM2.0        TMhelix    173   195
Sequence        TMHMM2.0        inside     196   215
Sequence        TMHMM2.0        TMhelix    216   238
Sequence        TMHMM2.0        outside    239   263
Sequence        TMHMM2.0        TMhelix    264   286
Sequence        TMHMM2.0        inside     287   509
Sequence        TMHMM2.0        TMhelix    510   532
Sequence        TMHMM2.0        outside    533   546
Sequence        TMHMM2.0        TMhelix    547   569
Sequence        TMHMM2.0        inside     570   601
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C

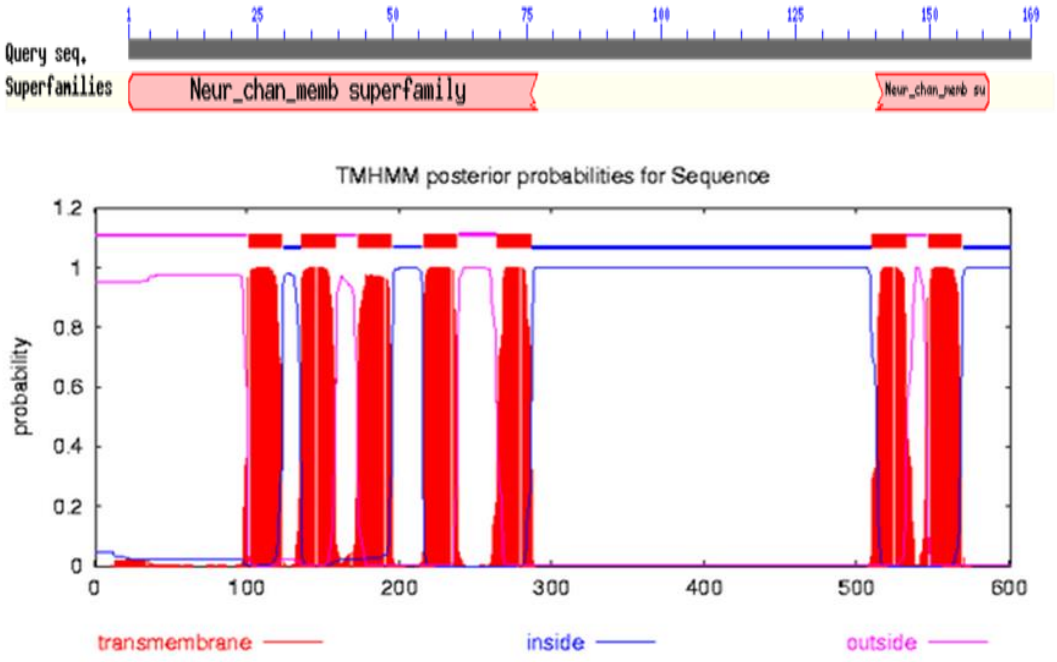


Figure 1. Analysis of BmGAR3 sequence. (A) alignment of BmGAR3 with C. elegans GAR3. (B) Probability values of the sequence matching to GPCR structure. (C) Topolgy of BmGAR3 as predicted by TMHMM2.0

Muscarinic acetylcholine receptors (mAChRs) are the family of acetylcholine GPCRs in vertebrates. We have shown that GARs are structurally and functionally distinct from mAChR [15], an important consideration if GARs are to be exploited as novel anthelmintic drug candidates. However, both GARs and mAChRs are GPCRs that are activated by acetylcholine and other cholinergic ligands. Sequence features of mAChRs that are important for cholinergic ligand binding are known. Our hypothesis is that *BmGAR-3* is a GPCR that responds to acetylcholine. To support this hypothesis we examined the *BmGAR-3* sequence for features that are important for ligand binding and which are shared with mAChRs. There are 11 amino acid positions important for acetylcholine binding in mAChRs (Table 1, Figure 2). Ten of these amino acids are conserved in *BmGAR-3* (denoted by red asterisks), with the only difference being substitution of the valine at position 266 to an alanine in *BmGAR-3* (highlighted by the black asterisk). The cysteine residues at position 175 and 255, engaged in a disulfide bond, are another key characteristic of muscarinic receptors and are also present in *BmGAR-3*, shown by the yellow circles. This high conservation of critical ligand interaction residues with mAChRs supports the prediction that *BmGAR-3* is an acetylcholine GPCR.

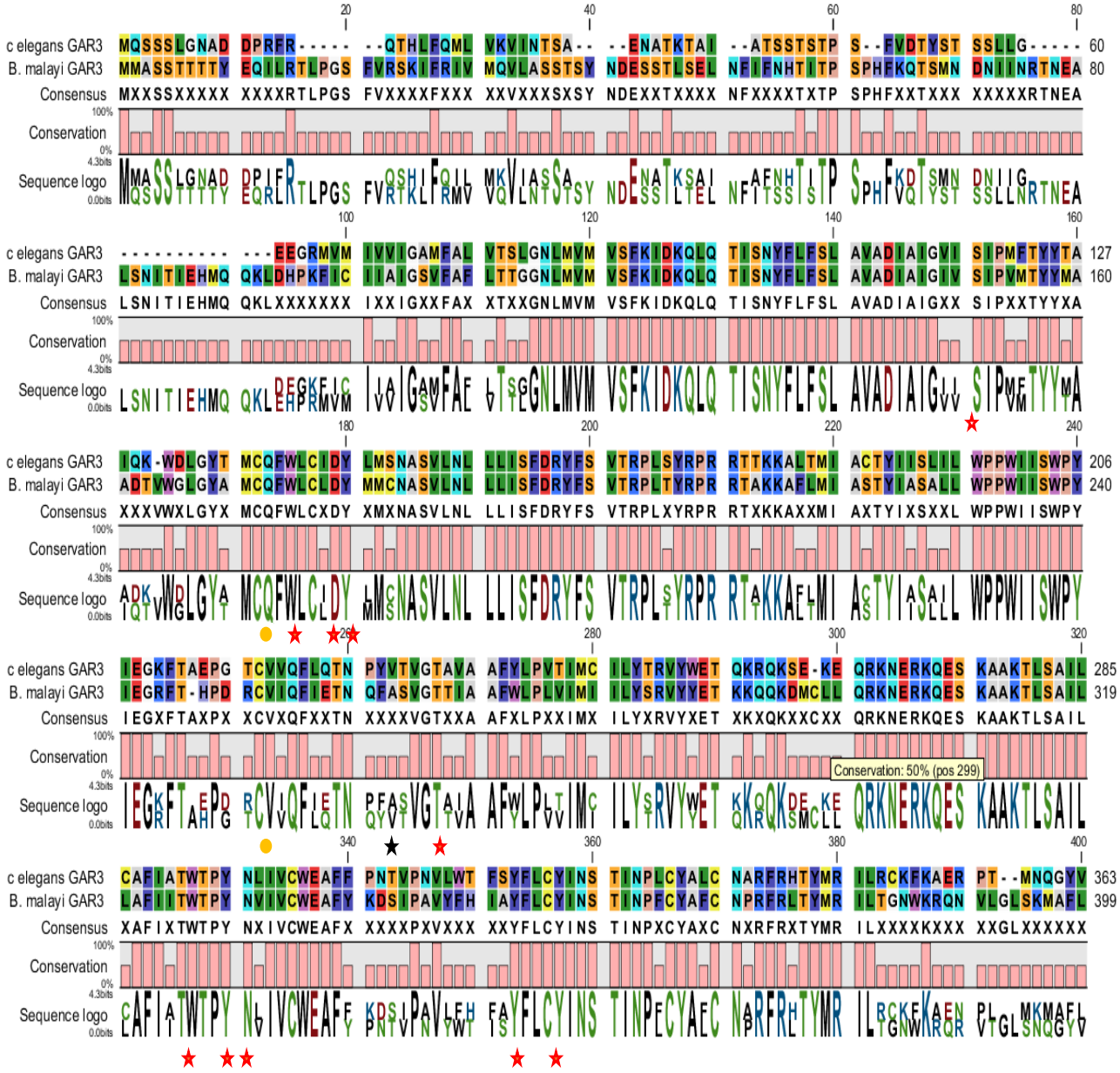


Figure 2. *BmGAR-3* chare characteristics with muscarinic acetylcholine receptors.

Table 1. Amino acids important for muscarinic ligand binding.

Amino acid	Position
(S)	154
(W)	179
(D)	182
(Y)	183
Valine (V)*	266
(T)	270
(W)	559
(Y)	562
(N)	563
(Y)	585
(Y)	589

Expression analysis of *BmGAR-3* and a related receptor

Current chemotherapeutics used to treat LF are only effective against larval stages. An anthelmintic with macrofilaricidal properties would be of great value and help expedite the eradication process of LF. Acetylcholine is the primary excitatory neurotransmitter in nematodes and GARs mediate potent contractile activities, affecting worm body wall muscle [16], egg laying [17], and pharyngeal muscle [5]. Given the expression of GARs throughout the *C. elegans* life cycle, their putative physiological significance to adult nematode biology, and the conservation between *C. elegans* and *B. malayi*, we hypothesized that *BmGAR-3* would be expressed in all *B. malayi* life stages. To test this hypothesis, we explored the spatial and temporal expression patterns

of *Bmgar-3* via semi-quantitative RT-PCR where target gene expression at each stage was normalized to *Bm-tph-1*, a stably expressed internal reference gene. *Bmgar-3* was expressed in infective L3 larvae, adult female (AF) and adult male (AM) life stages but could not be amplified from microfilaria (mf) or the L4 larval stage (Figure 3A). Notably, relative expression levels of *Bm-gar*-3 were higher in the adult stages compared to the larval L3, and markedly higher in adult male preparations than adult female. Enrichment in adult worms and specifically in the male suggests *Bmgar-3* may function in worm reproductive behaviors. This is consistent with *C. elegans* where GARs are known to have a functional role in worm reproduction [17, 18]. To support this transcriptional data, we commercially raised polyclonal antisera against *BmGAR*-3 using extracellular epitopes in the third and sixth TM domains. Western blot analysis revealed reactivity in adult male and adult female protein preparations at the expected size. As with the RT-PCR findings, *BmGAR*-3 protein expression was higher in male worms than female (Figure 3B). We also used the *BmGAR*-3 antisera in whole worm immunocytochemical studies to determine tissue localization of *BmGAR*-3. Unfortunately, despite significant optimization studies, no specific staining was observed in *Brugia* preparations and we were unable to elucidate spatial expression patterns. We know the *BmGAR*-3 antisera works in Western blot so failure of the immunocytochemical approach is likely due to technical challenges of performing immunocytochemistry on *B. malayi* worms and subsequent anecdotal evidence from other investigators in the field supports this.

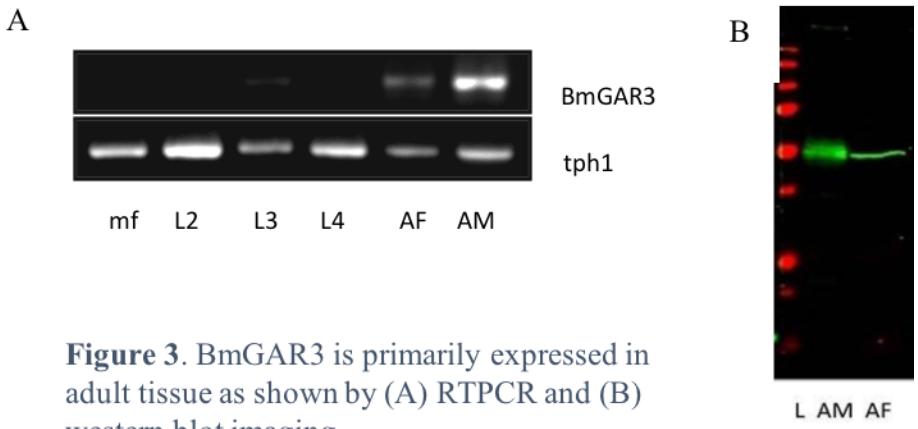


Figure 3. BmGAR3 is primarily expressed in adult tissue as shown by (A) RTPCR and (B) western blot imaging.

In an effort to determine tissue-specific spatial expression patterns of GAR-3 homologs, we transitioned to the pig gastrointestinal nematode, *Ascaris suum*. During our reciprocal BLAST analysis of *Bmgar-3* we identified a putative GAR-3 homolog in *Ascaris*, which we called *AsGAR-3*. Significant *AsGAR-3* identity in the *BmGAR-3* epitope regions (Figure 4A) led us to speculate if we could use the *BmGAR-3* antisera to specifically localize AsGAR-3. Using *Ascaris* as a model also allows us to exploit the much larger size of this parasite; it is possible to dissect multiple tissue types from these worms and individually assay them for protein or transcript expression [15]. Western blot analysis of *Ascaris* tissue revealed specific immunoreactivity of the expected size in female body wall muscle preparations (Figure 4B). Refinement of these tissue preparations and a shift to *AsGAR-3* transcript analysis using RT-PCR showed that *As-gar-3* is expressed in the pharynx of female worms, and in the body wall muscle and pharynx of male worms (Figure 4C). Similar to *Bm-gar-3*, transcript expression is higher in male preparations and expression is restricted to distinct subsets of parasite tissue. Although the Western blot and RT-PCR results are

somewhat incongruous, they suggest roles for *AsGAR-3* (and by extension BmGAR-3) in specific worm behaviors that may have sex-specific relevance.

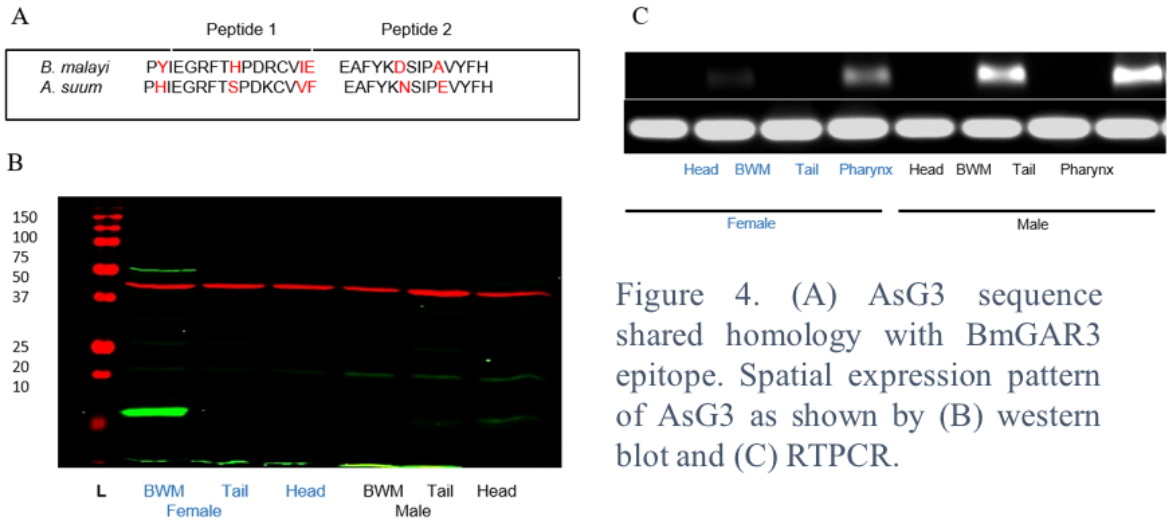
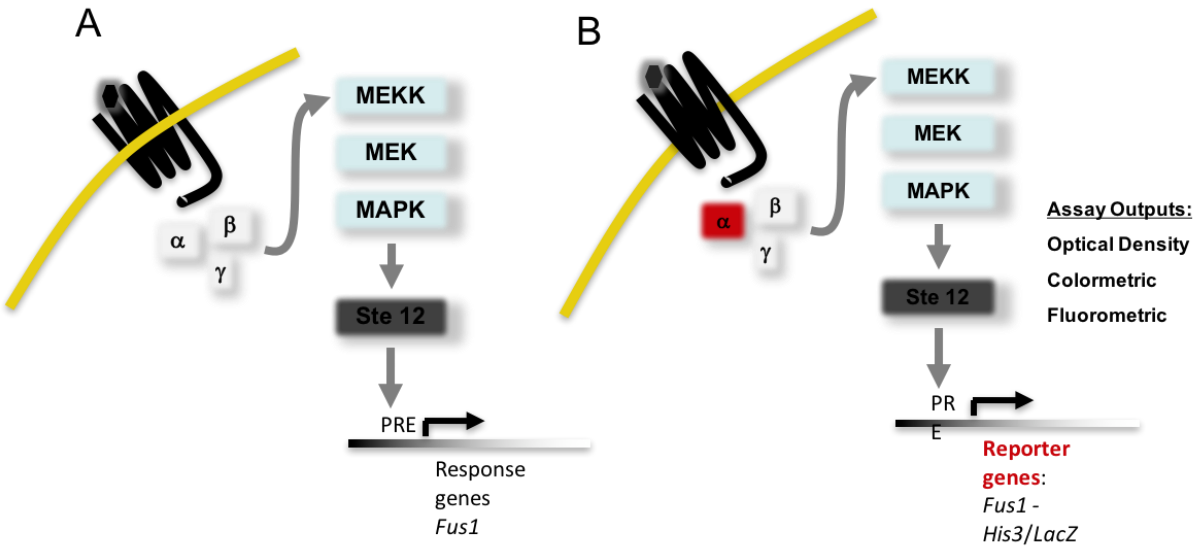


Figure 4. (A) AsG3 sequence shared homology with BmGAR3 epitope. Spatial expression pattern of AsG3 as shown by (B) western blot and (C) RTPCR.

Pharmacological characterization of *BmGAR-3*

Although nematode GARs are GPCRs that respond to cholinergic ligands, they have limited structural homology to mAChRs, are phylogenetically distinct to mAChRs, and our hypothesis is that they have a different pharmacological profile to mAChRs. Supporting this hypothesis, Kimber *et al*. (2009) functionally characterized a GAR from *Ascaris* (*AsGAR-1*) and found different pharmacology to mAChRs. Specifically, some mAChR agonists had no effect and all mAChR anatagonists were ineffective. To test the hypothesis that *BmGAR-3* is pharmacologically different from its most closely related mACh (M5), we attempted to functionally express *BmGAR-3* and human M5, and directly compare agonist and antagonist profiles. We used an auxotrophic yeast functional expression system (Figure 5 A and B) where auxotrophic *S. cerevisiae* strains are transformed with GPCRs of interest and receptor activation is reported by yeast growth in histidine-deficient media. Initial optimization efforts on M5 using

this system were encouraging. The M5 receptor showed strong agonist-driven growth in response to carbachol (10^{-4}M; Figure 5C). *BmGAR-3* was more challenging; over 100 individual transfection positive clones from multiple yeast strains were assayed for activity and whilst agonist-driven growth could be generated in some clones (Figure 5D), it could not be maintained over repeated experiments despite exhaustive efforts (Figure 5E). Included in our optimization experiments was the transfection with *BmGAR-3* codon optimized for yeast expression (Figure 5F). This also failed to generate agonist-driven yeast growth, however, we were able to isolate a number of constitutively active clones. These cells expressed a constitutively active *BmGAR-3*, that is, the receptor was always active and driving yeast growth even in the absence of ligand. Whilst unsuitable for agonist studies, these receptors could be useful for identifying inverse agonists, compounds that elicit the opposite response to agonists rather than simply acting as antagonists. We screened the constitutively active *BmGAR-3* with a panel of compounds known to act as inverse agonists at mAChRs. None of these compounds showed dose-dependent activity at *BmGAR-3* (Figure 5G). This observation is not unexpected since we previously found mAChR antagonists were mostly ineffective against the *Ascaris* GAR, *AsGAR-1* in the same yeast expression system.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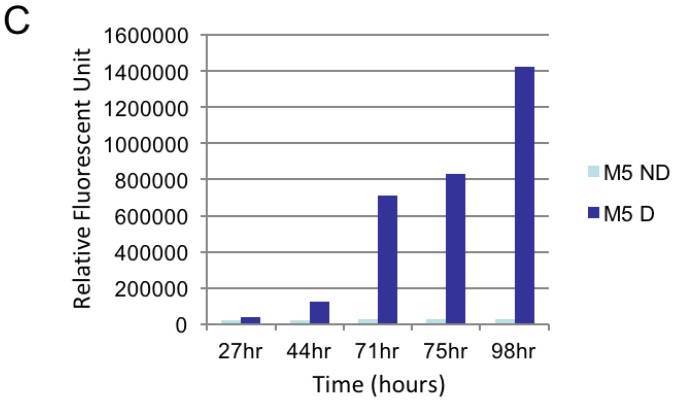
Reporter genes:
Fus1 - His3/LacZ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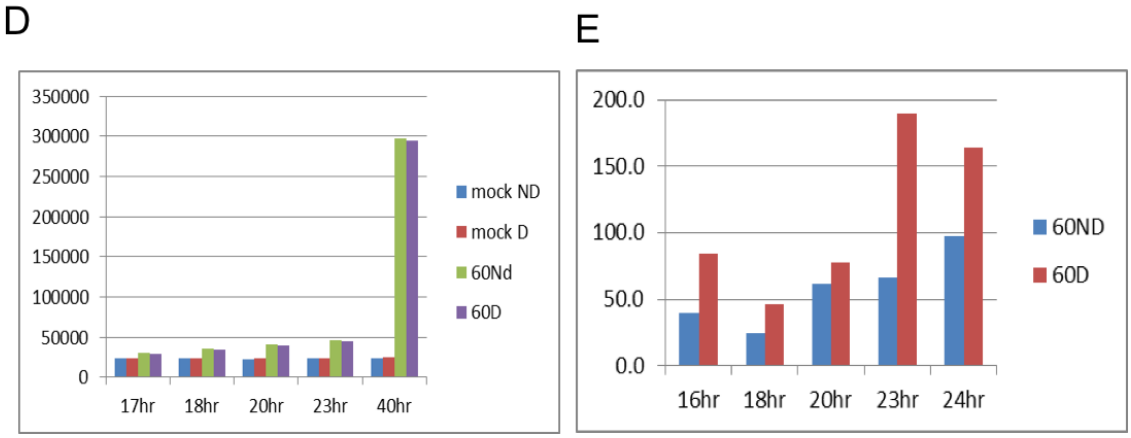
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                                    A  S  S            T  T
Optimized 1 ATGATGGCCTCTTCCACAACAACTACTTATGAACAAATCTTGAGAACATTACCTGGTTCC
Original 1  ATGATGGCATCATCAACAACAACAACATATGAACAAATCTTGCGTACATTACCTGGATCT
                                    A  S  S            T  T

Optimized 61 TTCGTCAGATCCAAAATCTTCAGAATCGTTATGCAAGTATTAGCATCTTCAACCTCATAT
Original 61  TTCGTTCGTTCCAAAATATTTCGAATTGTTATGCAGGTGCTTGCATCGTCAACTTCTTAT

Optimized 121 AACGATGAATCCAGTACTTTGTCCGAATTGAACTTCATATTCAACCATACAATCACCCCA
Original 121  AATGATGAAAGTTCAACGCTATCTGAATTAAATTTTATTTTTAATCACACAATAACACCT

Optimized 181 TCACCTCACTTCAAGCAAACTTCCATGAACGACAACATTATTAACAGAACAAACGAAGCA
Original 181  TCTCCTCATTTCAAGCAAACATCAATGAACGATAATATAATTAATCGTACTAATGAAGCG

Optimized 241 TTGTCTAATATAACCATCGAACACATGCAACAAAAGTTGGATCACCCAAAGTTTATTTGT
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Optimized 301 ATCATCGCAATCGGTTCAGTTTTTGCCTTCTTAACTACAGGTGGTAACTTGATGGTTATG
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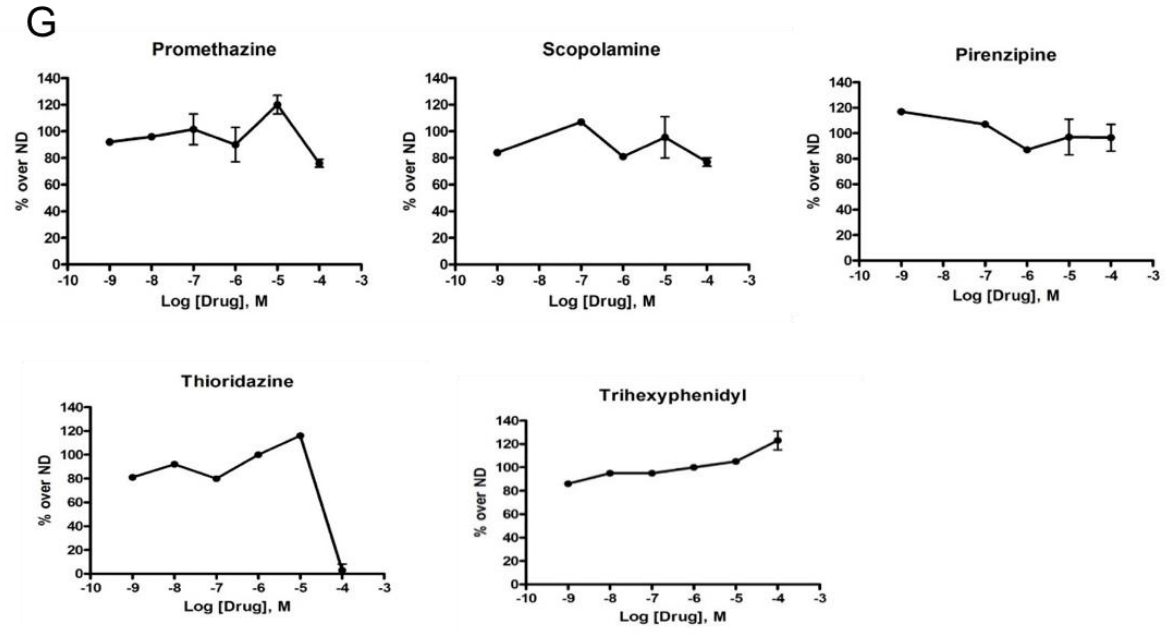

Figure 5: Auxotrophic yeast pheremone pathway (A) without and (B) with modifications to be employed as a screening platform for pharmacological compounds. (C) Muscarinic receptor M5 is potently expressed in the yeast system. (D and E) BmGAR3 showed poor reproducibility. (F)BmGAR3 was codon optimized for yeast. (G) Dose response curves for uscarinic agonist/inverse agonists.

Pharmacological characterization in a mammalian expression platform.

Mitogen-activated protein kinase (MAPKs) pathways are one of the major pathways activated by G protein coupled receptors [19, 20]. The most prominent MAP kinases are extracellular signal-related kinases (ERK1 and 2 also known as p44mapk or MAPK3 and p42mapk or MAPK1 respectively) [19, 21], stress activated kinases or Jun N-terminal kinase (SAPK/JNK), p38MAPK and big MAPK (BMK) [19, 22]. Unphosphorylated ERK is tethered to the cytoplasm in a 2-kinase complex together with raf kinases and MEK1/2. Upon activation, ERK is released from this complex and phosphorylates downstream reactions [21]. Signaling of the ERK/MAPK pathway has previously been studied in HEK293T cells [20, 23] and led us to hypothesize that it can be lobbied to heterologously express GARs of our interest to pharmacologically characterize them. *C. elegans gar-3* was cloned into pcDNA3.1, a mammalian cloning vector and transfected into HEK293 cells. Carbachol was added and cells were collected after 5 and 10 minutes of exposure, lysed and processed. Both total and phosphorylated ERK were measured. Total ERK was constant between treatment groups (Figure 6A). However, nearly a two-fold difference was observed in phosphorylated ERK in the *CeGAR3* transformed cells over no drug and untransfected cells. This suggested that the ERK/MAPK system in HEK293 cells is a viable assay for the pharmacological characterization of GARs.

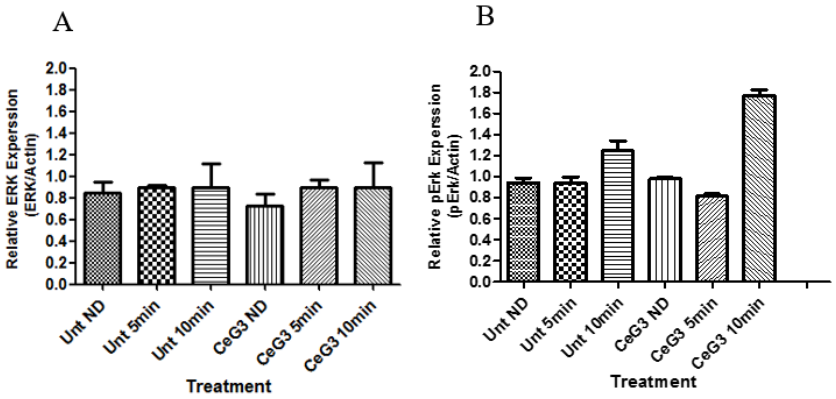

Figure 6. *C. elegans* GAR3 is expressed in HEK293 cells as shown by (A) total ERK and (B) phosphorylated ERK assays.

Next, we cloned *Bmgar-3* into pmaxgfp, a mammalian expression vector expressing gfp and transfected into HEK293 cells (Figure 7). Empty pmaxgfp vector and M5, which was the most closely related muscarinic receptor, were also transfected as controls. Transfection and expression efficiency of the vector and receptors were observed at different time points. Some expression of the empty vector was observed after incubating for 16hrs at 37℃, but no expression was observed in either the M5 or *Bmgar-3* transfected cells (Figure 7A). pmaxgfp empty vector transfected cells were highly expressed after 32hrs of incubation at37℃ although the receptor transfected cells were barely expressed (Figure 7B). Following anecdotal evidence that temperature shifts can activate nematode GPCRs, a parallel experiment was carried out where the cells were transferred to 30℃ after the first 16hrs and were incubated for 16hrs at 30℃ (Figure 7C). Indeed, this drastically improved the expression of M5. However, it had no effect on *BmGAR-3* expression. Uncertainty whether this poor expression efficacy would translate into the pharmacological assays halted further experimentation with this system. If time was not of the essence, the next logical step would have been to select the cells that expressed the fluorescently tagged *Bm-gar3* gene by Fluorescence-activated cell sorting (FACS) and use the same number of control cells to do experiments with. Furthermore, a stably transfected cell line containing *Bm-gar3* could also have been established by selecting cells containing the *Bm-gar3* gene by FACS and growing them on media with neomycin. Since the plasmid has a G418 neomycin resistant gene, only plasmids that have been successfully be transfected will grow on the selective media. This cell line could have then been used for future exeriments.

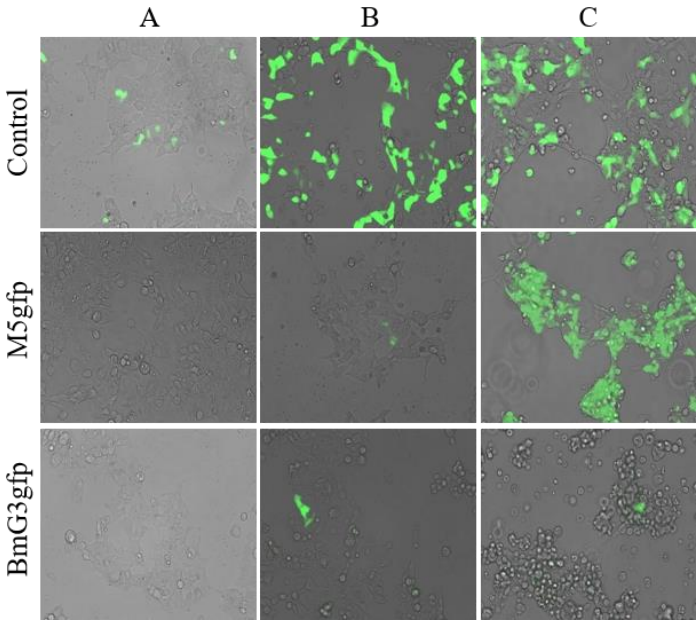

Figure 7. Expression patterns of HEK293 cells transfected with empty vector control, muscarinic receptor M5 and BmGAR3 at (A) 16hrs at 37℃, (B) 32hrs at 37℃ and (C) 16hrs at 30℃.

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APPENDIX II – RELEASE OF SMALL RNA-CONTAINING EXOSOME-LIKE VESICLES FROM THE HUMAN FILARIAL PARASITE BRUGIA MALAYI

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This manuscript was published in PLoS Neglected Tropical Diseases in September 2015. I performed exosome collections and experiments contributing to figure 9 of this manuscript.

Abstract

Lymphatic filariasis (LF) is a socio-economically devastating mosquito-borne Neglected Tropical Disease caused by parasitic filarial nematodes. The interaction between the parasite and host, both mosquito and human, during infection, development and persistence is dynamic and delicately balanced. Manipulation of this interface to the detriment of the parasite is a promising potential avenue to develop disease therapies but is prevented by our very limited understanding of the host-parasite relationship. Exosomes are bioactive small vesicles (30–120 nm) secreted by a wide range of cell types and involved in a wide range of physiological processes. Here, we report the identification and partial characterization of exosome-like vesicles (ELVs) released from the infective L3 stage of the human filarial parasite Brugia malayi. Exosome-like vesicles were isolated from parasites in culture media and electron microscopy and nanoparticle tracking analysis were used to confirm that vesicles produced by juvenile B. malayi are exosome-like based on size and morphology. We show that loss of parasite viability correlates with a time-dependent decay in vesicle size specificity and rate of release. The protein cargo of these vesicles is shown to include common exosomal protein markers and putative effector proteins. These Brugia-derived vesicles contain small RNA species that include microRNAs with host homology, suggesting a potential role in host manipulation. Confocal microscopy shows J774A.1, a murine macrophage cell line, internalize purified ELVs, and we demonstrate that these ELVs effectively stimulate a classically activated macrophage phenotype in J774A.1. To our knowledge, this is the first report of exosome-like vesicle release by a human parasitic nematode and our data suggest a novel mechanism by which human parasitic nematodes may actively direct the host responses to infection. Further interrogation of the makeup and function of these bioactive vesicles could seed new therapeutic strategies and unearth stage-specific diagnostic biomarkers.

OPEN ACCESS

Citation: Zamanian M, Fraser LM, Agbedanu PN, Harischandra H, Moorhead AR, Day TA, et al. (2015) Release of Small RNA-containing Exosome-like Vesicles from the Human Filarial Parasite Brugia malayi. PLoS Negl Trop Dis 9(9): e0004069. doi:10.1371/journal.pntd.0004069

Editor: Achim Hoerauf, Institute of Medical Microbiology, Immunology and Parasitology, GERMANY

Received: March 10, 2015

Accepted: August 18, 2015

Published: September 24, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by National Institute of Food and Agriculture Award 1001501 to MJK. URL: www.csrees.usda.gov. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Author Summary

Lymphatic filariasis is caused by parasitic nematodes that invade and occupy the host lymphatic system. The extent of lymphatic filariasis is staggering, with over 120 million people infected in 73 endemic countries and an estimated 40 million people suffering from a range of disfiguring and debilitating clinical manifestations of this disease. The mechanisms by which these medically important parasites navigate the host immune response to establish infection are not yet fully understood. In this study, we identify exosome-like vesicles (ELVs) that are abundantly released from infective stage L3 Brugia malayi, an etiological agent of human lymphatic filariasis. We show that these vesicles have a narrow size distribution and morphology consistent with classical exosomes, and that they contain common exosomal protein markers, putative effector proteins, as well as small regulatory RNAs. We show that ELVs are enriched with microRNAs that are perfectly conserved between parasite and host, suggesting a potentially novel mechanism by which filarial worms can actively manipulate host gene expression. We demonstrate that parasite ELVs are internalized by macrophages and elicit a classically activated phenotype in these host cells. The discovery of exosome-like vesicle release by human nematode parasites newly enlightens the roadmap to understanding the pathology of LF and related helminthiases. These vesicles also present promising new targets for intervention and diagnostics.

Introduction

The parasitic filarial nematodes Wuchereria bancrofti, Brugia malayi and B. timori are etiological agents of Lymphatic filariasis (LF), a chronic and debilitating disease infecting over 120 million people in 73 endemic countries [1]. Adult parasites reside in the lymphatic vasculature of infected individuals and release larvae called microfilariae, which are taken up by vector mosquitoes during the blood meal. Parasites rapidly develop within the mosquito, molting twice to the infective L3 stage [2, 3] before transmission to the definitive host during a subsequent blood meal. Following penetration of the vertebrate host via the puncture wound left by the mosquito, L3 stage parasites migrate to the lymphatics and undergo further growth and development, molting to the L4 stage and again to adulthood. The longevity of patent infection is remarkable; adults live for at least 8–10 years by general consensus. The ability of larval stages to successfully invade the host, and for adult worms to maintain infection for such an extended period of time, suggest filarial worms have developed strategies to both facilitate the establishment of infection and evade or manipulate the host immune response. Although the immunomodulatory capabilities of infecting larval and adult stage filarial worms have been well documented and reviewed [4–8], the parasite effector molecules responsible for manipulating host biology and their mechanisms of release have been difficult to define. Actively secreted proteins have historically been considered the principal candidates and several secreted proteins have been identified with demonstrable bioactivity at the host-parasite interface [9–12]. Adding to these, the characterization of parasitic nematode secretomes has revealed a complex array of potential proteinaceous effectors [13–16]. Other types of effector, including molecules expressed on the parasite surface may have a role [17] and the emergence of small noncoding RNAs as cell-to-cell agents of genetic regulation [18–22] hint at exciting alternative mechanisms.

Exosomes are a subtype of extracellular vesicle categorized by size (30–120 nm diameter) and defined by a particular biogenic pathway [23]; exosomes are formed by inward budding of vesicles in the late endosomal pathway to create multivesicular endosomes that fuse with the

plasma membrane to effect release [24, 25]. Originally thought to be a means of cellular waste disposal, exosomes are now considered highly bioactive extracellular vesicles that facilitate cell-to-cell communication and are the focus of renewed investigation. The cargo of exosomes is complex and variable, containing bioactive proteins, functional mRNA, miRNA and other small non-coding RNA species [18, 26], likely reflecting both source and target environments. Fusion of the exosome to a target cell delivers this heterogeneous bioactive cargo and selectively alters the biology of the target tissue [19, 21, 26, 27]; the isolation of exosomes from circulatory systems and an array of biofluids suggests effector sites can be far from the point of release. Parasites are known to release exosome-like vesicles [27–30] and it is compelling to hypothesize that bioactive molecules secreted by parasitic nematodes, packaged in exosomes, function as cell-to-cell effectors in the host-parasite interaction. Indeed recently, extracellular vesicles secreted by the gastrointestinal nematode Heligmosomoides polygyrus, containing proteins and small RNA species, have been shown to alter gene expression in host cells and suppress innate immune responses in mice [26].

Here we show that larval and adult stage B. malayi secrete prodigious quantities of extracellular vesicles in vitro whose size and morphology are consistent with exosomes. These exosome-like extracellular vesicles (ELVs) contain small RNA species, including specific miRNA and are enriched in miRNA that are identical to host miRNAs with known immunomodulatory roles [31–34]. The protein cargo of the vesicles is relatively scant but includes bioactive proteins, proteins with putative RNA binding properties and proteins commonly associated with exosomes [35]. The parasite ELVs are internalized by host macrophages and elicit a classically activated phenotype in these cells. The demonstration that filarial nematodes secrete exosomal RNA and proteins that potentially function at the host-parasite interface is significant. Defining this parasite effector toolkit exposes an array of new molecules that may be exploited in novel LF control strategies.

Results and Discussion

Infective-stage B. malayi release exosome-like vesicles

In order to ascertain whether exosomes are released by B. malayi, extracellular vesicles were isolated from parasites incubated in culture media using a filtration and ultracentrifugation protocol. We focused our initial discovery efforts on larval and adult stage parasites. L3, adult male, and adult female B. malayi were incubated in vitro for 24 hour periods under standard culture conditions, and purified vesicle preparations were evaluated with electron microscopy (EM). Infectious stage L3 parasites in culture release abundant 50–120 nm microvesicles consistent with the classical "deflated ball" morphology of mammalian and non-mammalian exosomes reported in the literature [36] (Fig 1A & 1B). We refer to these as exosome-like vesicles (ELVs) throughout this manuscript, in recognition that they cannot be unequivocally designated as exosomes, rather than another class of extracellular vesicles, because their biogenesis has not been determined. Preparations from adult stage B. malayi were more heterogenous and dilute, not allowing for the definitive categorization of putative exosome-like vesicles (Fig 1C). This, despite the fact a much higher mass of total parasite tissue was used for adult preparations as compared to larval preparations. These data suggest ELV release to be a predominantly larval phenomenon in B. malayi, a working hypothesis supported by analysis of RNA associated with the vesicles. We therefore chose to focus our subsequent experiments on L3 stage parasites. A compelling overall hypothesis for the function of B. malayi ELVs is that they mediate the secretion and trafficking to host cells of effector molecules that facilitate parasitism and the observation that ELV secretion occurs primarily in those parasite stages that infect the host and establish parasitemia is consistent with this narrative.

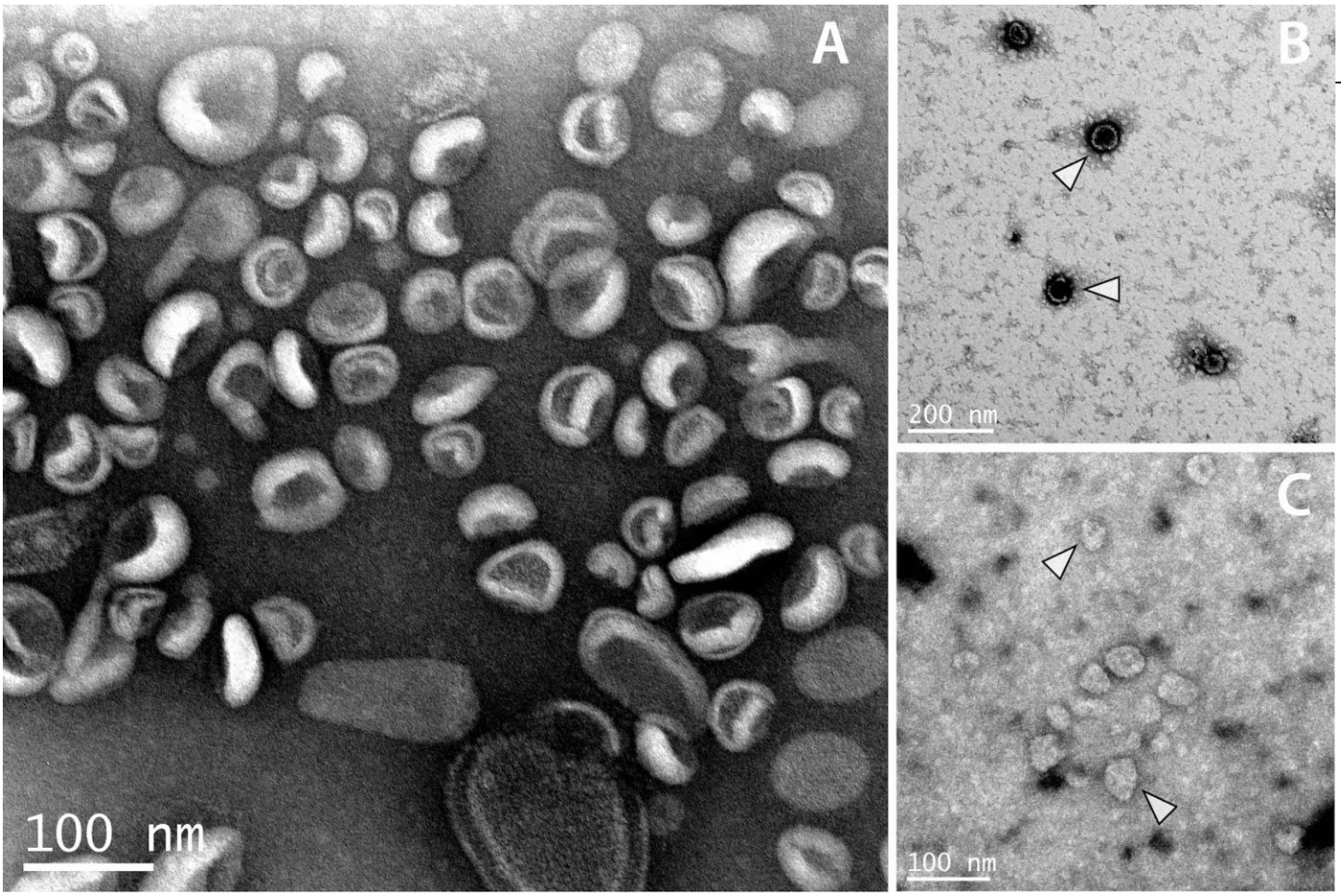
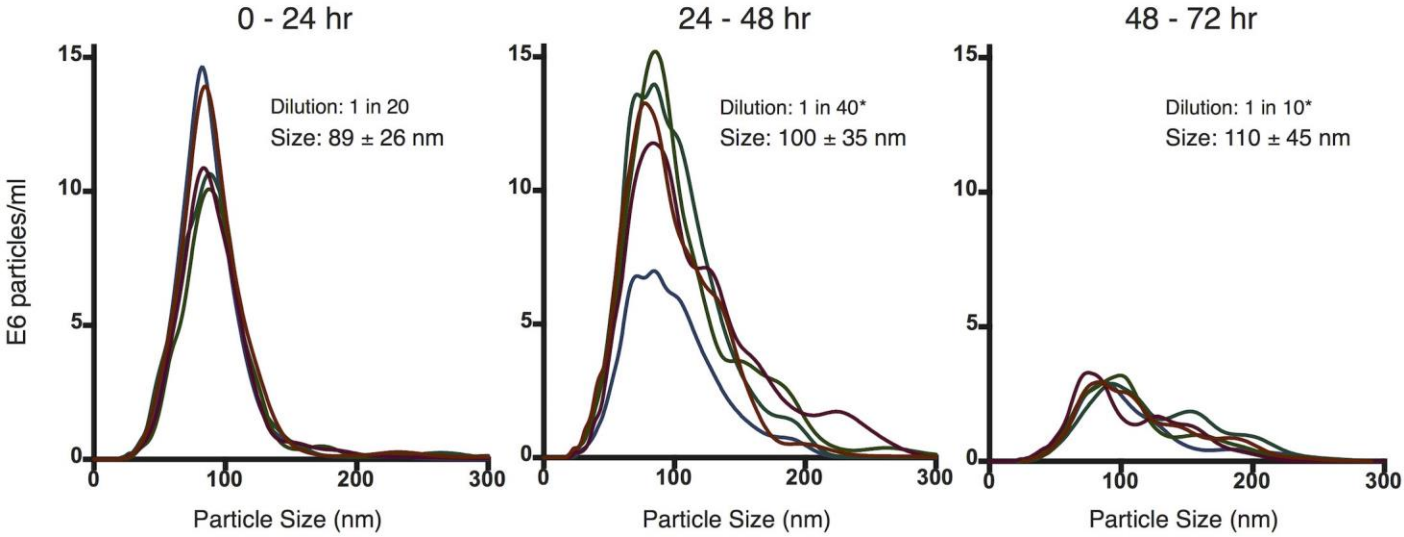


Fig 1. Electron microscopy confirms secretion of exosome-like vesicles in intra-host stages of B. malayi. TEM images of L3 (A and B) and adult female (C) ELV preparations are shown. L3 vesicles take on a distinct morphology often reported in the literature. Adult isolations are more heterogenous and may require further optimization to achieve uniform vesicle preparation. White arrows show canonical L3 ELVs (B) and putative adult ELVs (C). This provides evidence for the release of exosome-like vesicles in the human-infective L3 stage of the parasite and much of the rest of the work we report is focused on vesicles derived from this larval stage.

doi:10.1371/journal.pntd.0004069.g001

Time course profile of exosome-like vesicle release from infectious stage B. malayi

To more accurately resolve the dynamics of ELV release in L3 B. malayi, we used a nanoparticle tracking analysis (NTA) system to measure vesicle output in a 72 hr in vitro time course. Media was collected from 300 worms after three successive 24 hr incubation periods, vesicles were purified by ultracentrifugation as before and individual vesicle preparations were analyzed via NanoSight LM10 as shown in Fig 2 (sample recording: S1 Video). Day 1 (0–24 hr in culture) preparations reveal a prolific ELV release rate (> 9,000 ELVs/parasite/min) with a very narrow size distribution centered at ~90 nm. Day 2 (24–48 hr in culture) preparations show an essentially equivalent rate of release, but a stark broadening of the size distribution. Day 3 (48–72 hr in culture) preparations are associated with significantly lower levels of release (<4,000 ELVs/parasite/min) and an even wider multimodal size distribution. These data



	ELVs	/parasite/min
0 - 24 hr	4.0×10^9	9,300
24 - 48 hr	4.5×10^9	10,400
48 - 72 hr	1.7×10^9	3,900

Fig 2. Particle tracking analysis reveals prolific larval Brugia exosome-like vesicle release rate. Profile of ELVs isolated from culture media incubated with 300 L3 parasites for successive 24 hr incubations. The size distribution of L3-derived ELVs from Day 1 (left), Day 2 (center) and Day 3 (right) incubations are shown (mean ± SD). Calculated vesicle release rates are provided in tabular format. ELV rate of release and size specificity decay in a time-dependent manner in vitro. * re-scaled based on dilution for comparison to 0–24 hour (1:20) dilution.

doi:10.1371/journal.pntd.0004069.g002

suggest an overall time-dependent decay in vesicle rate of release and size specificity, which correlates to decreased L3 viability in vitro. The release of considerable quantities of precisely-sized ELVs in viable worms (Days 1–2) is followed by the release of smaller quantities of a broader size range of particles that potentially include larger membrane vesicles and apoptotic blebs (Days 2–3). This suggests an active and regulated mechanism of ELV release in healthy and viable L3 stage parasites, as opposed to a passive mode of noisy cellular deterioration.

The protein cargo of Brugia exosome-like vesicles

The protein content of B. malayi ELVs was determined using nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC/MS/MS). A total of 32 proteins each containing at least two unique peptides were identified using MASCOT (Table 1). Specific proteins identified within the pellet included characteristic markers of exosomes including Hsp70, elongation factor-1α, elongation factor-2, actin, and Rab-1. In addition, over 80% of the proteins identified are orthologous to proteins identified in mammalian exosome proteomes, strongly suggesting that these vesicles are exosome-like in nature and supporting our ELV designation here. Interestingly, this set of vesicle-specific proteins is entirely distinct from the proteins previously identified in pre- and post-molt L3 secretions [37].

Table 1. Annotation of Brugia ELV proteome.

Brugia Protein ID	UniProt ID	Annotation
BM-ACT-5	A8P5A0_BRUMA	Actin
Bm-HSP-1	A8P6Q6_BRUMA	HSP70
Bm5195	A8PJ17_BRUMA	Elongation factor 1-alpha
BM-EEF-2	A8PJV1_BRUMA	Elongation factor 2
Bm4733	A8PHP7_BRUMA	Beta-tubulin
BM-MEC-12	A8PN52_BRUMA	Alpha-tubulin like
BM-DPY-23	A8PZJ6_BRUMA	Adaptin
Bm13837	A8QAR6_BRUMA	ATP synthase subunit alpha
Bm-ATP-2	A8Q895_BRUMA	ATP synthase subunit beta
Bm5931	A8P4C6_BRUMA	Alpha-1,4 glucan phosphorylase
BM-CPL-1	A8NCV6_BRUMA	Cathepsin L-like cysteine protease
BM-EAT-6	A8Q4C9_BRUMA	Na^+K^+ATPase
Bm-SCA-1	A8QET1_BRUMA	Calcium-transporting ATPase
BM-RAB-1	A8Q8U0_BRUMA	Ras-related protein
Bm4628	A8NSV0_BRUMA	Ubiquitin
Bm5528	A8NHQ1_BRUMA	1,4-alpha-glucan branching enzyme
BM-DLST-1	A8PU77_BRUMA	2-oxoglutarate dehydrogenase
Bm3206	A8NHD8_BRUMA	Histone H2B
Bm3425	A8NHD2_BRUMA	Histone H3
Bm4113	A8Q1K1_BRUMA	Histone H4
Ribosomal Proteins		
BM-RPS-16	A8P1D4_BRUMA	40S ribosomal protein S16
Bm2853	A8Q0J1_BRUMA	40S ribosomal protein S2
Bm2320	A8NXR7_BRUMA	40S ribosomal protein S3
BM-SECS-1	A8PJH5_BRUMA	40S ribosomal protein S5
BM-RPS-9	A8P2X1_BRUMA	40S ribosomal protein S9
Bm13774	A8PTY7_BRUMA	60S ribosomal protein L10
Bm13718	A8NKQ0_BRUMA	60S ribosomal protein L11
BM-RPL-3	A8P136_BRUMA	60S ribosomal protein L3
BM-RPL-9	A8QHP9_BRUMA	60S ribosomal protein L9
Bm3930	A8ND23_BRUMA	Ribosomal protein
BM-RPL-1	A8NG31_BRUMA	Ribosomal protein

Homology-based annotation of B. malayi ELV proteins reveals hallmarks of mammalian exosomes, including HSP70 and translation elongation factors. Ribosomal proteins, histones, ras-related proteins, cathepsins, ATP synthase subunits, and other homologs of identified Brugia ELV proteins have also been reported in exosomes derived from various cell types.

doi:10.1371/journal.pntd.0004069.t001

UniProt-GOA and quickGO were used to sort proteins into functional groups based on assigned gene ontology (GO) terms [38, 39], as shown in Fig 3. Based on GO annotations, 20% of the proteins identified are involved in binding of bioactive molecules including nucleic acids and other proteins, 16% function in the transport of various ions and proteins and 14% are ribosomal proteins. In addition, a large fraction of proteins identified (21%) appear to be involved in various metabolic processes including hydrolase and transferase activities while the remaining 29% comprises proteins with translational, cytoskeletal and other functions.

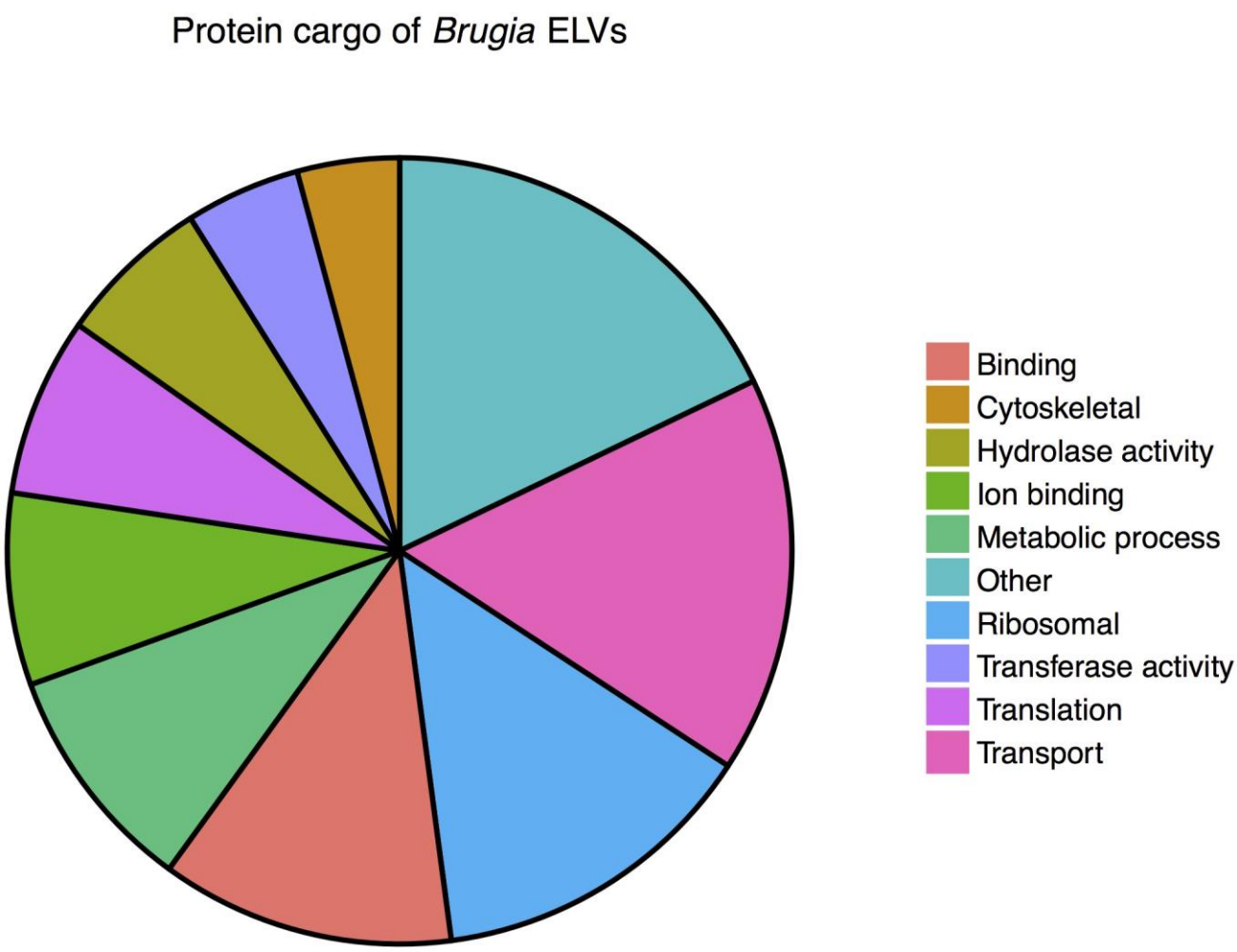


Fig 3. Protein content of B. malayi exosome-like vesicles. GO functional annotation of 32 proteins identified in ELVs isolated from B. malayi L3 stage parasites.

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Included in the list of Brugia ELV proteins are potential effector molecules. Bm-CPL-1 is a cathepsin L-like cysteine protease robustly expressed across the B. malayi life cycle [40]. Upregulation of Bm-cpl-1 expression coincides with transition between life cycle stages and an important role in the modulation of parasite molting has been confirmed [41–43]. This is the first demonstration that B. malayi secretes CPL-1 although other cathepsin-like cysteine proteases have been identified in the B. malayi secretome [14, 37] and a cathepsin L-like molecule is secreted by intra-mammalian stage Haemonchus contortus [44]. The exogenous function of exosomal Bm-CPL-1 is not clear but evidence points to some manipulation of the host-parasite interface. In a previous study, we suppressed Bm-cpl-1 expression using in vivo RNAi during the mosquito life stages [42]. Loss-of-function reduced prevalence of infection in mosquitoes by nearly 40%, suggesting Bm-CPL-1 is important for establishing or maintaining parasitemia. In flatworms, an immunomodulatory role for secreted cathepsin L-like proteases is better established [45]; in Fasciola infection cathepsin L contributes to the permissive polarized Th2 > Th1 host response.

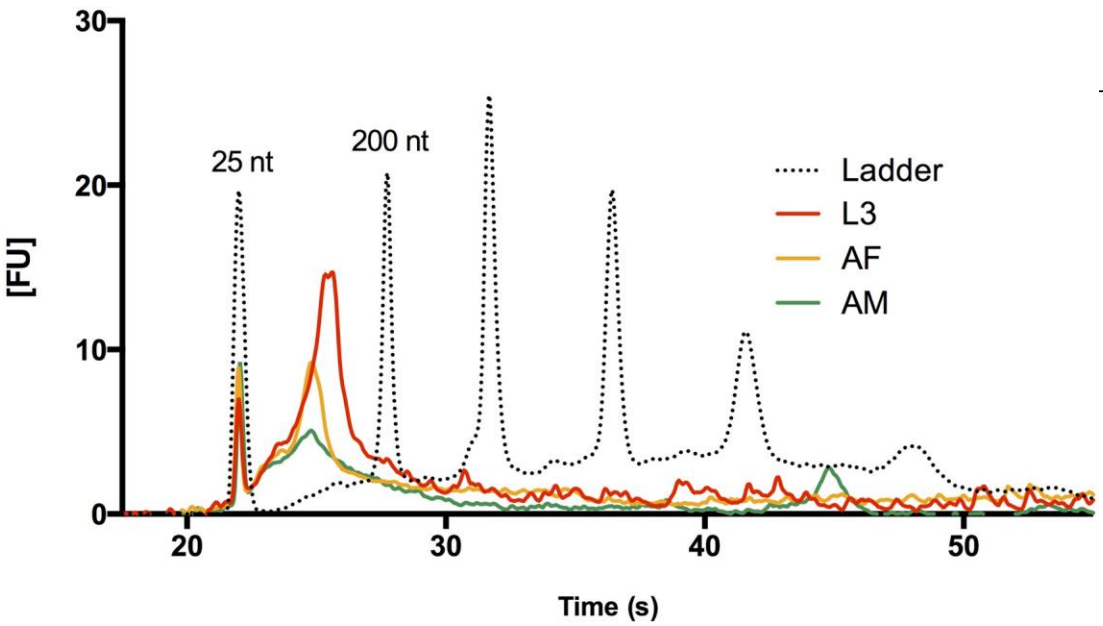


Fig 4. Isolation of Small RNAs from larval and adult B. malayi ELV fractions. Bioanalyzer data are shown for RNAs isolated from L3, adult male, and adult female Brugia preparations. L3 ELVs contain significant amounts of small RNAs in the 25–200 nt range (25 and 200 nt reference peaks labeled), while adult male and female vesicle preparations yield fewer RNAs. Vesicle fractions were prepared from 300 L3 and 30 adults in 24 hr culture incubations. Despite the much higher total tissue amounts used in adult culture, we detect much higher levels of small RNAs in L3-derived ELVs.

doi:10.1371/journal.pntd.0004069.g004

The proteomic profiles of parasitic helminth exosomes are broad in range; for example, over 350 proteins were identified in the putative exosomes secreted by Heligmosomoides polygyrus [26] whilst 45 and 79 proteins were identified in exosome-like vesicles from Echinostoma caproni and Fasciola hepatica, respectively [46]. The B. malayi L3 stage profile identified here is relatively scant but consistent with this broad distribution. It may be that this is a stage-specific observation and ELV secreted by other B. malayi life stages display a more complex and abundant protein cargo tailored to distinct functional demands. Reflecting the small RNA component of these ELVs (see later sections), it may also be that larval stage Brugia ELVs are primarily vehicles for protected RNA secretion. Replication of the experiments conducted here might add depth to the MS data set and identify further ELV-associated proteins.

B. malayi ELVs contain small RNA including miRNAs with potential host targets

We probed larval and adult microvesicle preparations for the presence of small RNA species. Exosomes have been found to contain both non-coding RNAs (ncRNAs) and messenger RNAs (mRNAs) in a diverse range of species and cell types. Of particular interest to us was the potential presence of small non-coding RNAs, including microRNAs (miRNAs), that could potentially mediate parasite-parasite communication or modulate host gene expression. Small RNA species were preferentially isolated from putative ELV-containing pellets and examined with an Agilent Bioanalyzer. The microvesicle fractions of L3 B. malayi (24 hr incubations of 300 worms) revealed an abundance of small RNA species in the 25–200 nt range (Fig 4). Much less RNA was detected from incubations of adult male and female B. malayi (24 hr incubations of 30 adult worms), despite the much higher mass of tissue in adult stage culture media. This lack

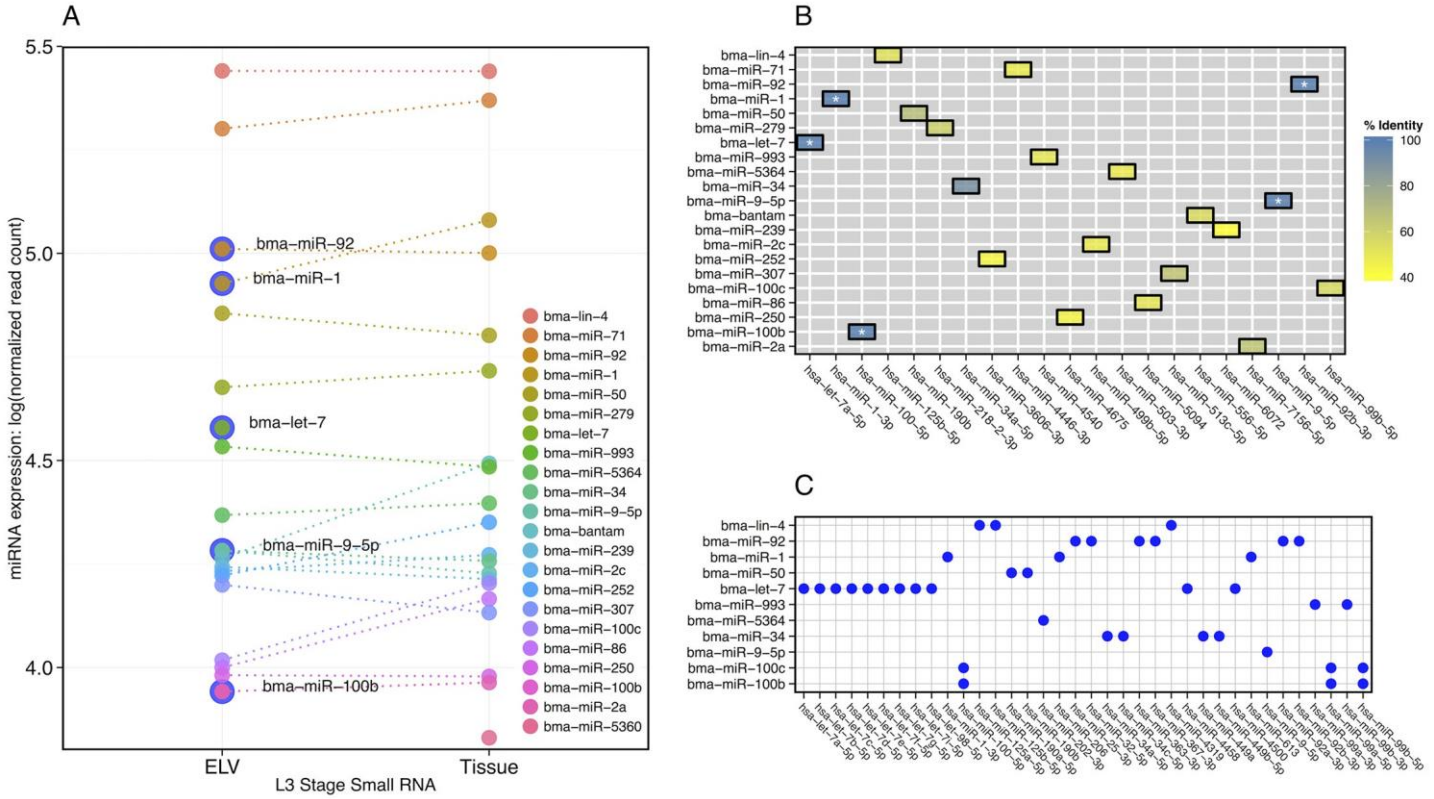


Fig 5. Discovery and profiling of miRNAs in B. malayi exosome-like vesicles. (A) Comparative abundance of miRNAs in L3 ELV and tissue-derived samples. miRNA discovery and abundance estimation was carried out using the mirDeep2 pipeline. The 20 miRNAs with highest expression in each sample were retained for comparison and abundance was normalized with respect to total miRNA-mapping reads within each sample. Normalized read count is plotted on a log scale for ELV and tissue miRNAs to provide a relative ordering of fractional abundance. Bma-let-7 only appears in the highly expressed subset, and a number of miRNAs with perfect mature sequence identity to host homologs are highlighted (outer blue circle). (B) Sequence conservation between B. malayi ELV-origin miRNAs and the host (H. sapiens) miRNA complement. Reduced heat map showing one-to-one homology between a given B. malayi miRNA and its nearest matching human counterpart in terms of percent identity. Bma-let-7, bma-miR-1, bma-miR-9, bma-miR-92, and bma-miR-100b (white asterisks) share 100% identity with a host miRNA, while bma-miR-34 shows high identity with a host miRNA (21/23 nucleotides). This B. malayi miRNA subset (shown in blue) contains potential modulators of host gene expression. (C) Sequence conservation between B. malayi ELV-origin miRNA seed sites and host (H. sapiens) miRNA seed sites. miRNAs sharing perfectly conserved seed sites, defined here as nucleotides 2–8 of the mature miRNA, are marked (blue circles).

doi:10.1371/journal.pntd.0004069.g005

of correlation between total parasite tissue material and RNA yield, coupled to the differential quality of larval and adult microvesicle preparations as evaluated by EM, further indicates that ELV release is primarily a characteristic of larval-stage parasites and perhaps more biologically relevant to early parasite infection.

To more fully investigate the nucleic acid contents of these newly discovered vesicles, we carried out RNA-Seq with both L3 ELV and tissue-derived small RNAs. Reads generated by Illumina sequencing were processed and used to seed an miRNA discovery and abundance estimation pipeline using miRDeep2 [47] (read statistics and raw miRNA abundances can be found in S1 Table). To compare ELV and cellular RNA abundance, miRNA expression was normalized to the total miRNA read count within each sample. miRNA discovery and profiling was augmented with data from previously discovered miRNAs in closely related nematode species to help overcome gaps in the B. malayi draft genome assembly (see Methods). Fig 5A compares normalized miRNA expression between ELV and tissue for the 20 most abundant

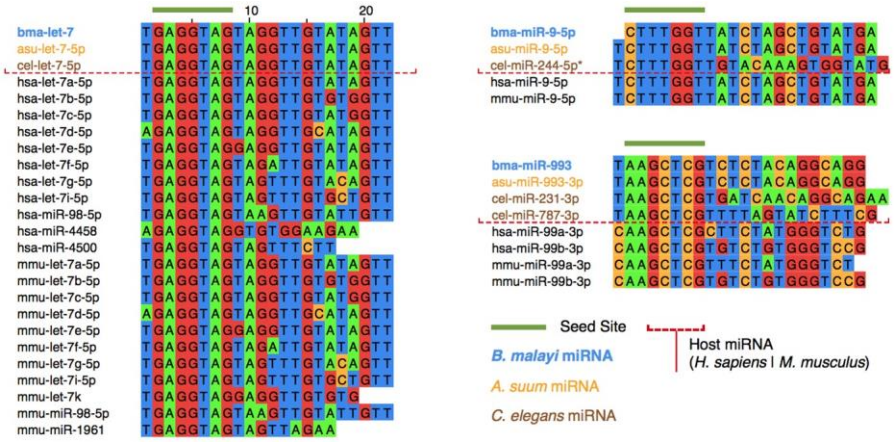


Fig 6. Brugia malayi ELV miRNA sequence homology to nematode and mammalian host miRNAs. miRNAs from B. malayi, A. suum, C. elegans, H. sapiens, and M. musculus were grouped by seed site sequence identity (nucleotides 2–8) for multiple sequence alignments. Alignments are shown for bma-let-7, bma-miR-9 and bma-miR-993. bma-let-7 is shown as an example of a Brugia ELV miRNA that exhibits both seed site and full length sequence conservation extending to other parasitic and free-living nematodes, as well as to mammalian host species. bma-miR-9 and bma-miR-993 are presented as examples where conserved parasite miRNAs have clear host homologs, yet lack one-to-one C. elegans orthologs. The complete set of alignments can be found in S1 Fig.

doi:10.1371/journal.pntd.0004069.g006

miRNAs in each sample. Although there is considerable conservation in relative miRNAs abundances, there are some notable observations and exceptions.

Bma-let-7 is significantly enriched in L3 ELVs as compared to L3 tissue, where it does not appear among the 20 most abundant miRNAs. Bma-let-7, along with four other B. malayi mature miRNAs found in ELVs (bma-miR-1, bma-miR-9, bma-miR-92, and bma-miR-100b), share perfect sequence identity with host (Homo sapiens) mature miRNAs, as shown in Fig 5B. Additionally, bma-miR-34 shares near perfect sequence identity with its H. sapiens homolog. 11 B. malayi miRNAs also share common seed sites with H. sapiens miRNAs (Fig 5C). Brugia ELV miRNA sequences were more broadly clustered by putative seed site and aligned to miRNAs from the soil-transmitted parasitic nematode Ascaris suum, the free living model nematode Caenorhabditis elegans, and mammalian host species H. sapiens and Mus musculus (Fig 6 and S1 Fig). In all cases, Brugia ELV miRNAs that share common seed sites with host miRNAs have one-to-one A. suum orthologs. In some cases, parasite miRNAs are better conserved in mammalian hosts than in C. elegans (e.g., bma-miR-9, bma-miR-993, and bma-miR-100b/c).

We examined the complement of the most abundant Brugia ELV-associated miRNAs with respect to very recent investigations of miRNAs released by other parasitic nematode species and found circulating in host biofluids [26, 48–50]. Common markers include let-7, lin-4, miR-34, miR-71, miR-92, and miR-100c (Fig 7A and 7B). While all members of this subset share seed site sequence identity with mammalian host miRNAs, lin-4, miR-34, miR-71, and miR-100c are sufficiently diverged from host miRNAs over their full length mature miRNA sequence and can potentially serve as biomarkers of filarial infection. Additionally, we compared the complements of the 20 most abundant Brugia ELV and H. polygyrus exosomal [26] miRNAs, identifying six miRNAs shared between these vesicles and a large number of miRNAs unique to each species (Fig 7C).

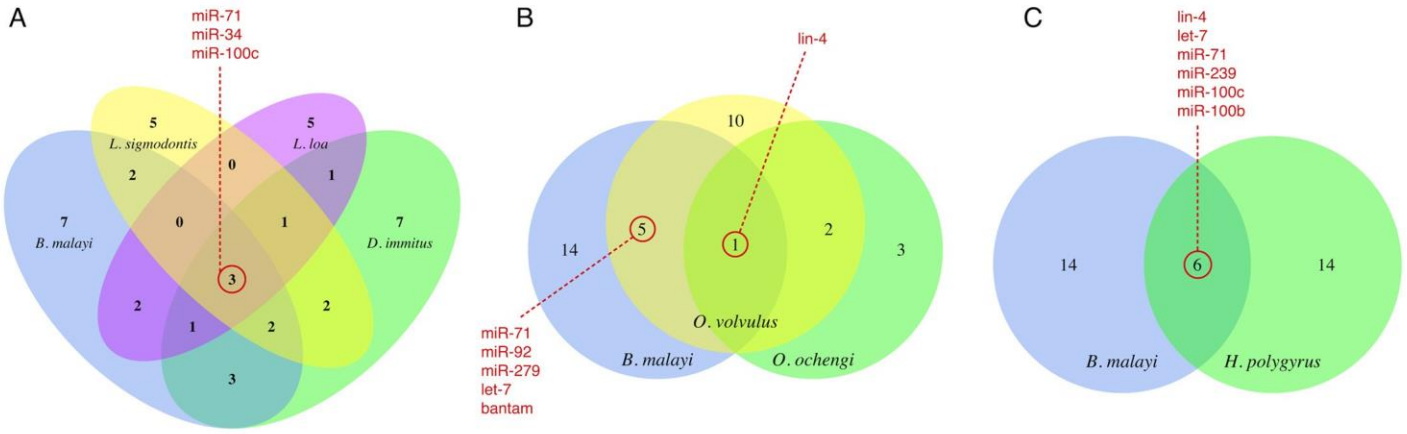


Fig 7. Comparison of the B. malayi ELV miRNA complement to miRNAs secreted by other parasitic nematodes species. (A & B) Comparison of the 20 most abundant B. malayi ELV miRNAs with the complements of miRNAs found circulating in the serum and plasma of definitive and model mammal hosts burdened with filarial infection (Litomosoides sigmodontis [26], Dirofilaria immitis [48], Loa loa [49], Onchocerca volvulus [48, 50], and Onchocerca ochengi [49]). The D. immitis miRNAs in (A) are restricted to the 20 most abundant miRNAs, and the O. volvulus miRNAs in (B) represent the combination of two non-overlapping sets arising from separate reports. (C) Comparison of the 20 most abundant miRNAs identified in B. malayi ELVs and H. polygyrus exosomes. These analyses reveal sets of common markers and a number of miRNAs unique to each species.

doi:10.1371/journal.pntd.0004069.g007

Enrichment of bma-let-7 and the high fractional presence of other parasite miRNAs sharing perfect or high homology to host miRNAs, leads us to speculate about a potential ELV-mediated mechanism by which parasite RNAs can be used to efficiently direct aspects of gene expression in host cells. Targets of endogenous let-7 family miRNAs in vertebrates include oncogenes, as well as genes involved in proliferation, apoptosis, and innate immunity [51–53]. Let-7 is intricately involved in macrophage polarization and responses to pathogen challenge [31, 33, 54], and the altering of host let-7 expression therefore represents a potentially advantageous point of intervention for an invading parasite. Live pathogens down-regulate the expression of let-7 family miRNAs, and let-7 miRNAs act on toll-like receptors (e.g. TLR4) that directly mediate macrophage responses [54–56]. Clearly, there is an important association between macrophage response to pathogens and let-7 expression. Our observation that B. malayi secrete let-7 and other potential modulators of host gene expression identifies a mechanism by which this host immune response might be manipulated. Supporting this hypothesis, let-7 and other miRNAs with host conservation have been identified in immunomodulatory H. polygyrus adult stage exosomes [26]. To fully dissect this hypothesis, a broad investigation of the interaction of ELV miRNAs and host immune cells in vivo is needed.

Brugia ELVs are internalized by host macrophages

Macrophages are critical mediators of the early immune response to invasive Brugia parasites [8]. To test the hypothesis that secreted Brugia ELVs interact with host macrophages, we used fluorescent lipophilic dyes to visualize the interaction between J774A.1 murine macrophages and ELVs. This cell line was chosen because it is commercially available, can be cultured readily and because it recapitulates the biology of primary macrophages and dendritic cells [57]. ELVs were labeled with PKH67, a green fluorescent dye, and incubated with J774A.1 labeled with PKH26, a red fluorescent dye. Confocal microscopy revealed efficient internalization of the

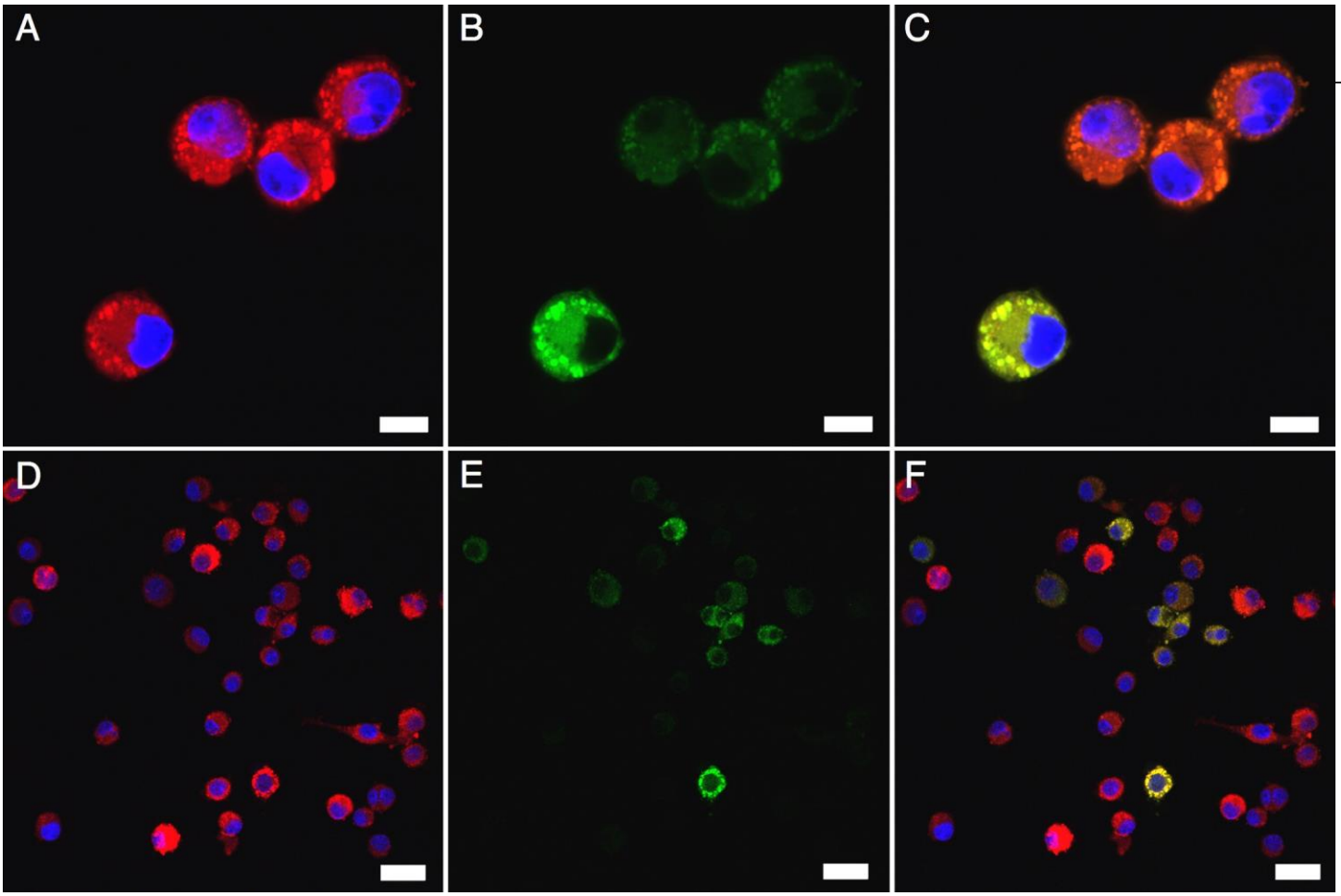


Fig 8. Brugia exosome-like vesicles (ELVs) are internalized by J774A.1 macrophages. (A and D) J774A.1 macrophages were labeled with PKH26 (red) and counterstained with DAPI (blue) to visualize nuclei. (B and E) B. malayi L3 stage ELVs were purified from a 24 hr parasite culture and labeled with PKH67 (green). 3×10^5 J774A.1 were co-incubated with approximately 3×10^7 labeled ELVs for 6 hrs at 37°C and washed repeatedly to remove unbound ELVs. Vesicles internalized by macrophages appear diffusely throughout cytoplasm and focused in discrete puncta associated with the cell membrane. (C and F) Merged images showing internalization of parasite ELVs. All images were acquired using a using a Leica TCS SP5 X Confocal/multiphoton microscope system with 20X (A-C) or 60X (D-F) objectives. Scale bars: 10 μm (A-C) and 25 μm (D-F).

doi:10.1371/journal.pntd.0004069.g008

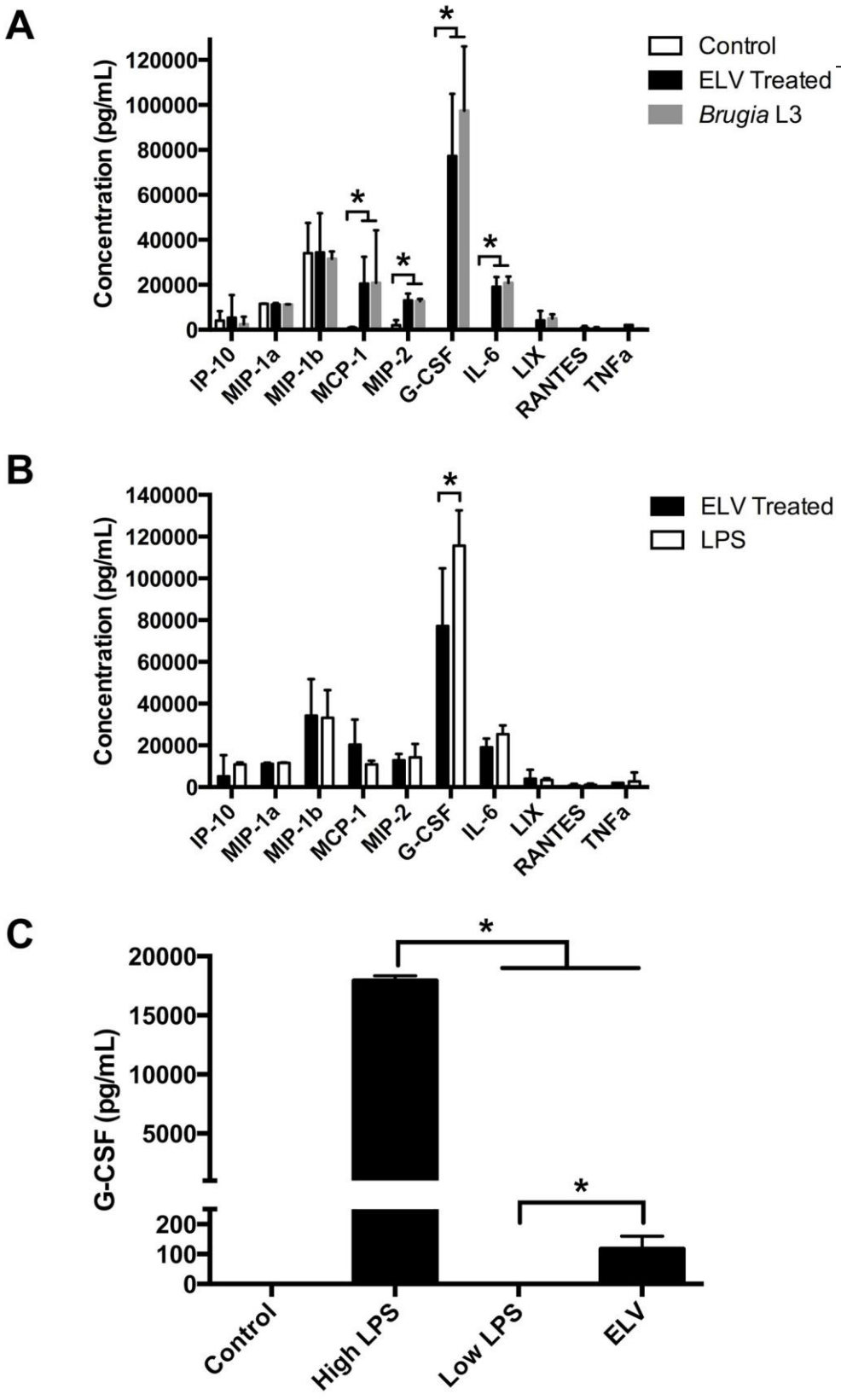
ELVs by this macrophage cell line (Fig 8). Internalization was observed diffusely throughout the cell cytoplasm with focus around membrane-rich puncta associated with the surface of the macrophages (Fig 8B). This pattern of internalization is consistent with other studies describing a phagocytic route of vesicle internalization [58, 59]. Macrophages were counterstained with DAPI to determine the efficiency of cell labeling and ELV uptake. PKH26-labeling of J774A.1 was very efficient and all cells were visualized although intensity of labeling was variable (Fig 8D). Approximately 40–50% of macrophages internalized labeled ELVs to some degree (Fig 8E) with approximately 10% of macrophages internalizing ELVs at markedly higher levels (Fig 8E). There was no correlation between strong PKH 26-labelling of macrophages and vesicle uptake indicating internalization is not a factor of receptiveness to labeling.

Brugia ELVs elicit a classically activated phenotype in host macrophages

Macrophage activation is dichotomous; classically activated macrophages (CAMF) are elicited by LPS or IFN-γ and have a generally pro-inflammatory phenotype whereas alternatively activated macrophages (AAMF), driven by IL-4 and IL-13, appear immunosuppressive or anti-inflammatory. Helminth infection is typically associated with the AAMF pathway although both CAMF and AAMF are involved in the immune response to, and immunopathology caused by, Brugia infection. Experiments demonstrate different Brugia preparations can generate both CAMF and AAMF activation phenotypes; dead and moribund worms and worm lysates produce CAMF [60] but live worms and complete excretory/secretory (ES) preparations drive AAMF [61–63]. To test the hypothesis that ELVs activate host macrophages, J774A.1 were treated with purified ELV preparations and their cytokine/chemokine responses monitored. J774A.1 were treated for 48 hrs with approximately 4×10^8 L3 stage vesicles, purified from in vitro culture medium by ultracentrifugation. The macrophage response was assayed using the Milliplex MAP Mouse Cytokine/Chemokine kit (EDM Millipore) interfaced with a Bio-Plex System (Bio-Rad) utilizing Luminex xMAP technology, a platform capable of simultaneously identifying and quantifying 32 cytokines/chemokines. Vesicle treatment effectively activated J774A.1 macrophages with significant increases in G-CSF, MCP-1, IL-6 and MIP-2 levels compared to control macrophages treated with naïve RPMI 1640 culture media, (p 0.001)(Fig 9A). Smaller increases in LIX, RANTES and TNF-α were also noted. Healthy, viable L3 stage parasites produced an almost identical response (Fig 9A), the only difference being a modest but significant enhancement of G-CSF stimulation by the viable parasites ($p < 0.001$), suggesting that the dominant parasite immunogen(s) are found in the vesicle pellet. Finally, parasite culture media from which the ELVs had been removed by centrifugation did not produce this response, nor did live schistosomes (S. mansoni cercaria) or their secreted vesicles (S2 Fig) suggesting the Brugia-associated activation is specific to this parasite and not a general response to helminths or their secreted vesicles.

The activation profile observed would be considered more indicative of a CAMF response than AAMF; to confirm the response was CAMF-like, we compared it to the response elicited by LPS (200 ng/mL). The only significant differences were that ELV treatment stimulated G-CSF and IL-6 less effectively ($p < 0.001$) and stimulated MCP-1 more effectively ($p < 0.001$) than LPS (Fig 9B). The overall conservation of response, however, indicates these ELVs generate a CAMF phenotype. Since Wolbachia, the endosymbiont present in filarial nematodes, lack LPS biosynthetic capacity it seemed unlikely our CAMF-like response was driven by LPS-like contamination but to rule this out, endotoxin levels in our vesicle preparation were determined commercially (Lonza, Walkersville, MD). LPS-like activity was present (0.003 ng/mL) but at a concentration several orders of magnitude lower than the minimum dose required to stimulate J774A.1 macrophages [64]. As expected, treatment of macrophages with this low LPS dose was insufficient for activation (Fig 9C) indicating that the CAMF response we observe is not due to an LPS-like component in our preparation.

Since the stimulation of an AAMF phenotype by live Brugia and ES preparations thereof in vivo and in vitro has been well established [61–63] it might be expected that Brugia ELV preparations also stimulate a AAMF phenotype, especially since complete Brugia ES preparations are likely to include ELVs similar to those examined here, albeit at reduced concentrations. We observed a response consistent with a CAMF phenotype, however, although without the acute elevation in IL-β and TNF-α production others have seen in response to LPS [60]. One interpretation is that the CAMF > AAMF phenotype may be a somewhat artificial function of the homogenous J774A.1 monoculture used here as other studies describing a AAMF phenotype


A
Concentration (pg/mL)
120000
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80000
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40000
20000
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IP-10
MIP-1a
MIP-1b
MCP-1
MIP-2
G-CSF
IL-6
LIX
RANTES
TNFa
Control
ELV Treated
Brugia L3
*
B
Concentration (pg/mL)
140000
120000
100000
80000
60000
40000
20000
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IP-10
MIP-1a
MIP-1b
MCP-1
MIP-2
G-CSF
IL-6
LIX
RANTES
TNFa
ELV Treated
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High LPS
Low LPS
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Fig 9. Brugia exosome-like vesicles (ELVs) elicit a classically activated phenotype in J774A.1 macrophages. (A) J774A.1 (5×10^5) were treated with approximately 4×10^8 purified L3 stage ELVs, live L3 stage parasites (10 worms) or naïve culture media (control) and supernatents collected after 48 hr. The presence of 32 cytokines/chemokines was simultaneously assayed using the Milliplex MAP Mouse Cytokine/Chemokine kit (EDM Millipore) interfaced with a Bio-Plex System (Bio-Rad) utilizing Luminex xMAP technology (Luminex). The quantification of identified cytokines is presented. The cytokine profile generated by ELV treatment is consistent with a classically activated phenotype. (B) Cytokine response to ELV treatment is compared to LPS (200 ng/mL). The close correlation of responses indicates ELV treatment generates a classically activated phenotype. (C) J774A.1 (5×10^5) were treated with high dose LPS (200 ng/mL), low dose LPS (0.003 ng/mL), ELV or naïve culture media (control) for 24 hr, supernatant collected and assayed for G-CSF using a Mouse G-CSF Quantikine ELISA kit (R&D Systems). The absence of response to low dose LPS suggests the classically activated response is not due to LPS-like contamination.

doi:10.1371/journal.pntd.0004069.g009

often use PBMC or other heterogeneous primary cell types. It would be instructive to monitor the responses of such mixed cell populations to the ELV preparation. Additionally, although the murine model is regarded as valuable for illuminating both how parasites establish themselves and the early host immune response, J774A.1 may not be optimal for studying this particular Brugia-host interaction and optimization with other murine or human cells may be required. Another interpretation, however, is that the purified ELVs examined here should be considered a distinct and specific fraction of the highly complex immunogenic facade presented by filarial parasites and may elicit a genuine CAMF phenotype when examined in isolation. Supporting this interpretation, exosomes isolated from other biological systems effectively generate a CAMF phenotype [59, 65, 66]. A key mediator of this pro-inflammatory response is Hsp70 [65], which was identified in our ELV proteomic profile. In summary, irrespective of the polarity of macrophage activation phenotype, our results unequivocally identify secreted ELVs as distinct parasite-derived structures capable of activating the host immune system.

A picture is emerging that parasitic helminths secrete functional exosome-like vesicles. The protein and small RNA cargo of these vesicles have putative effector functions at the host-parasite interface and potentially serve to create conditions favorable to the establishment or maintenance of infection. The identification of these cell-to-cell effector structures is exciting and prompts further investigation of their functional relevance. In particular, it will be important to describe the roles of individual miRNAs and proteins contained within the ELVs, to identify the host molecular targets being manipulated in vivo, and reveal any conserved or stage-specific effectors secreted across the parasite life cycle. Another intriguing question is whether or not there is any specificity or selectivity in host cells or tissues targeted and if so, what molecular mechanisms underscore this specificity. Addressing such questions will illuminate the fundamental interactions that occur between parasite and host, and may open previously unexploited opportunities for parasite control and diagnostics.

Materials and Methods

Mosquito maintenance

Aedes aegypti (Black eyed Liverpool strain, LVP), previously selected for susceptibility to infection with Brugia malayi [67], were maintained in controlled conditions (27°C ± 1°C and 75% ± 5% relative humidity) with a 16:8 photoperiod. Adult mosquitoes were fed a diet of 10% sucrose. Approximately 4,000 and 2,600 mosquitos were used for proteomics and RNA sequencing, respectively.

Establishing Brugia malayi infection

For proteomics and transcriptomics, B. malayi microfilaria (mf) infected cat blood was obtained from the University of Georgia NIH/NIAID Filariasis Research Reagent Resource Center (FR3). Blood containing the parasites was diluted with defibrinated sheep's blood (Hemostat Laboratories, CA, USA) to achieve a concentration of 80–100 mf per 20μL. To establish infection, 3- to 5-day-old Ae. Aegypti (LVP) were allowed to feed for one hour on a glass membrane feeder. Mosquitoes were sucrose-starved for 24 hrs prior to blood feeding and those that did not take a blood meal were removed. Infected mosquitoes were maintained in the above described conditions for 13–15 days post infection (dpi) to allow development of parasites.

Brugia malayi maintenance and collection of vesicle-containing media

In exploratory studies, larval (300 L3) and adult (30 male or 30 female) B. malayi were procured from the FR3. On arrival, parasites were cultured in 50 mL RPMI 1640 (Sigma-Aldrich, St. Louis, MO) at 37°C (5% CO_2). Cell culture media was collected and replaced at 24 hr intervals for up to 72 hrs to collect secreted ELVs. For downstream sequencing and proteomics, B. malayi (13–15 dpi) were locally collected using methods described by FR3. Briefly, infected mosquitoes were immobilized by cooling to 4°C for 15 minutes. Immobilized mosquitoes were crushed in a mortar containing 5 ml of chilled Hanks' balanced salt solution (HBSS, pH 7.0) containing pen-strep (0.4 units penicillin/ml, 0.4 mcg streptomycin/ml). Mosquitoes were then rinsed onto a 150 mesh sieve contained in a deep well plastic petri dish and washed 3–4 times using fresh chilled HBSS + pen-strep. Sieves were then placed into petri dishes containing warm (40°C) HBSS + pen-strep to allow infective larvae to migrate out. Sieves were transferred to new deep well petri dishes containing fresh warm HBSS every 30 minutes. Collected parasites were washed twice with warm HBSS + pen-strep, placed into 25 mL RPMI 1640 containing pen-strep (0.4 units penicillin/ml, 0.4 mcg streptomycin/ml) and held at 37°C, 5% CO_2 for 24 hrs to collect secreted ELVs.

Exosome-like vesicle purification

Differential centrifugation was used to isolate ELVs from 25 or 50 mL aliquots of Brugia culture media. Aliquots were collected from 24 hr incubations of larval or adult worms in culture media. Lower speed centrifugation and filtration steps were used to remove contaminating cells (300 × g, 10 mins) and cellular debris (10,000 × g, 15 mins). The resulting supernatants underwent filtration through 0.22 μm filters and ultracentrifugation at 105,000 × g for 90 mins to pellet ELVs. Pellets were then washed with cold phosphate-buffered saline (PBS) and a final spin was carried out at 105,000 × g for 90 mins. Supernatants were discarded and pellets were resuspended in small volumes (30–250 uL) of PBS for imaging, sequencing, and proteomics, and RPMI for immunological assays. Samples were kept on ice and centrifugation steps were carried out at 4°C. Resuspended ELVs were stored at −80°C.

Electron microscopy and nanoparticle tracking analysis

Small aliquots of ELV suspension (3 μl) were applied to carbon coated 200 mesh copper grids and negatively stained with 2% uranyl acetate. Images were taken using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optics Laboratories, Akishima, Japan) at the Microscopy and NanoImaging Facility (Iowa State University). Nanoparticle tracking analysis was carried out with the NanoSight LM10 (NanoSight Ltd., Amesbury, UK) to ascertain the size and frequency distribution of individual vesicle preparations, assayed in triplicate. The

Brownian motion of particles in solution is related back to particle sizes and numbers, allowing better statistical resolution of vesicle size and concentration [68].

LC-MS/MS and proteomic analysis

Protein was isolated from purified exosome-like vesicles for proteomic analysis (System Biosciences). Briefly, samples were modified with 10% SDS to a final concentration of 2% SDS, heated at 100°C for 15 minutes and clarified by centrifugation. Protein concentration was determined using a Qubit fluorometry assay (Invitrogen). 15 μg of material was processed by SDS-PAGE using a 10% Bis-Tris homogeneous gel and the MES buffer system. In-gel digestion with trypsin was done at 37°C for 4 hrs using a ProGest robot (DigiLab, Marlborough, MA). The digested sample was analyzed by nano LC-MS/MS analysis using a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Data were searched against a copy of the B. malayi UniProt database (taxon ID: 6278) using a locally running copy of MASCOT (Matrix Science Ltd., London, UK). The search was restricted using the following parameters; maximum missed cleavages = 2, fixed modifications = carbamidomethyl (C), variable modifications = Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q) and Deamidation (N, Q), a peptide mass tolerance of 10 ppm, and a fragment mass tolerance of 0.02 Da. Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a nonredundant list per sample. Data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Prophet scores) and requiring at least two unique peptides per protein.

RNA isolation and sequencing

For detection of RNA species in ELV preparations, small RNAs were preferentially isolated from vesicle-containing pellets using the miRCURY RNA Isolation Kit (Exiqon, Vedbaek, Denmark) and RNA samples were examined with an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit. For small RNA sequencing (RNA-Seq), total RNA was isolated from ELVs released by ~5,000 L3s over a 24 hr incubation period using the Total RNA and Protein Isolation Kit (Invitrogen, Carlsbad, CA). In parallel, total RNA was isolated from whole worm tissue using a TRIzol (Invitrogen) protocol, where a 6 hr precipitation step was carried out at -80°C to improve small RNA recovery. RNA NGS libraries were constructed using modified Illumina adapter methods using SBI's XRNA Sample Preparation Kit (System Biosciences, Mountain View, CA) and indexed with separate bar codes for multiplex sequencing on an Illumina MiSeq v3 instrument using a 2 × 75 bp paired end run setting.

miRNA discovery and abundance estimation

Raw reads were trimmed to remove adapter sequences, filtered by quality score, and de-multiplexed using the FASTX-Toolkit [69] (sequencing data are deposited with the NCBI SRA under project number PRJNA285132). The miRDeep2 pipeline was used to map short RNA reads (>15 nt) to the B. malayi genome for miRNA discovery, and to estimate and normalize miRNA abundances with respect to total miRNA read count. Nematode precursor and mature miRNA sequences deposited into miRBase [70] were used in the pipeline, including known B. pahangi, Caenorhabditis elegans, Ascaris suum, Haemonchus contortus, and Strongyloides ratti miRNAs. Non-mapped reads were ranked by abundance, filtered for homology against known miRNAs in the phylum Nematoda using BLASTn [71], and incorporated for final quantification of abundance with the miRDeep quantifier script, allowing for capture of miRNAs that did not map to the B. malayi assembly due to sequencing gaps. The ggplot2 package [72] of the statistical programming language R was used to organize and visualize comparisons between vesicular and tissue RNA samples.

Cell culture

J774A.1 murine macrophages (ATCC, Manassas, VA) were maintained in complete tissue culture medium (Dulbecco's modified Eagle's medium, 25 mM HEPES, pH 7.4 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.05 μM 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum) at 37°C and 5% CO_2. 24 hrs prior to assays, 400 μL cells were plated in standard 24-well plates at a density of 5×10^5 cells/well.

Vesicle labeling and uptake

Exosome-like vesicles were purified from a 24 hr culture of 300 Brugia malayi L3 parasites as described above and labeled with the green fluorescent dye, PKH67 (Sigma-Aldrich, St Louis, MO,USA), according to the manufacturer's instructions. ELVs were incubated with PKH67 for 5 min at room temperature and the reaction terminated by addition of 1% BSA in PBS. RPMI 1640 media was added, mixed and centrifuged at 105,000 × g for 1 hr to separate ELV-bound PKH67 from excess PKH67. Labeled ELV were washed again then resuspended in an appropriate volume of complete tissue culture medium (Dulbecco's modified Eagles medium, 25 mM HEPES, pH 7.4 supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.05 μM 2-mercaptoethanol and 10% heat-inactivated fetal bovine serum).

J774A.1 were labeled with red fluorescent lipophilic dye, PKH26 (Sigma-Aldrich, St Louis, MO), according to the manufacturer's instructions. Macrophages were incubated with PKH26 for 5 min at room temperature and the reaction terminated by addition of 1% BSA. To remove excess unbound dye, samples were centrifuged at 400 × g for 10 minutes at room temperature and the supernatant discarded. Centrifugation was repeated three more times using 10 ml of complete media to ensure full removal of unbound dye and the cells were re-suspended in 1 mL of complete medium. Approximately 3×10^5 labeled cells were plated onto sterile coverslips and incubated overnight at 37°C/5% CO_2. Labeled ELV suspension (approximately 3×10^7 per coverslip) was added to labeled J774A.1 and incubated for 6 hrs. Cells were washed 5 times with ice-cold PBS to remove excess labeled ELVs, the cells fixed in 4% paraformaldehyde (Sigma-Aldrich), washed and counterstained with DAPI before mounting and storage at 4°C. Preparations were visualized using a Leica TCS SP5 X Confocal/multiphoton microscope system (Leica Microsystems Inc., Buffalo Grove, IL).

Detection of macrophage modulation by Luminex assay

Triplicate wells of adhered J774A.1 were treated with approximately 4×10^8 purified L3 stage ELVs. The ELVs were purified by ultracentrifugation as previously described, resuspended in RPMI 1640 medium (Gibco/Life Technologies, Carlsbad, CA) and quantified by nanoparticle tracking analysis. Other treatments were similar volumes of vesicle depleted L3 culture medium (supernatant created following pelleting of ELV fraction from spent parasite culture medium), live B. malayi L3 parasites (10 worms/well), lipopolysaccharide (LPS; final concentration 200 ng/mL)(Sigma-Aldrich, St. Louis, MO), naïve RPMI 1640 culture medium and various combinations of these conditions. Supernatants from these cell cultures (400 μL/well) were collected 24 or 48 hrs post-treatment and centrifuged briefly (2,000 × g for 10 min) to remove non-adhered cells and cell debris before being analyzed for the presence of cytokines/chemokines. The Milliplex MAP Mouse Cytokine/Chemokine kit (EDM Millipore, Billerica, MA) interfaced with a Bio-Plex System (Bio-Rad, Hercules, CA) utilizing Luminex xMAP technology (Luminex, Austin, TX) allowed the simultaneous identification and quantification of the following analytes in the cell culture supernatant: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, M-CSF, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p40), IL-13, IL-15, IL-17, IP-10, MIP-2, KC, LIF, LIX, MCP-1, MIP-1α, MIP-1β, MIG, RANTES, TNFα, IL-12(p70), VEGF,

IL-9. Briefly, experimental samples, background, standards and controls were added to a 96-well plate and combined with equal volumes of pre-mixed, antibody coated magnetic beads; the plate was sealed and incubated overnight at 4°C. Following washing, 25 μL of detection antibody was added and the plate incubated for one hour at room temperature with shaking. Streptavidin-Phycoerythrin (25 μL) was added to each well and the plate incubated for a further hour at room temperature before washing. Finally, 150 μL assay buffer was added to all wells and fluorescence immediately recorded. Median fluorescent intensity data were analyzed as recommended using a five-parameter logistic curve-fitting method for calculating cytokine/chemokine concentration.

G-CSF ELISA

Triplicate wells of adhered J774A.1 cells, prepared as described above, were treated with LPS (final concentration 200 ng/mL or 0.003 ng/mL), approximately 4×10^8 purified L3 stage ELVs as described above, or RPMI 1640 as negative control. Cell culture supernatants were collected 24 hrs after treatment, cleared via centrifugation as described previously and assayed for G-CSF using a Mouse G-CSF Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Standard curves were generated using Prism 6 software (GraphPad Software, San Diego, CA) and sample G-CSF concentrations determined by regression analysis.

Statistical analysis

For analysis of Luminex data, Tukey's test was used to compare overall treatments while multiple t-tests, incorporating the Holm-Sidak method to correct for multiple comparisons, were used to compare individual chemokines/cytokines following treatments. t-tests were used to compare treatment groups following ELISA analysis. All statistical analyses were performed using Prism 6 for Mac (Graphpad).

Supporting Information

S1 Video. Brugia malayi L3 ELVs recorded via NanoSight.
(MP4)

S1 Table. Small RNA-Seq miRNA abundances and read statistics.
(XLSX)

S1 Fig. Brugia malayi ELV miRNA sequence homology to nematode and mammalian host miRNAs. miRNAs are grouped by putative seed site and aligned.
(TIFF)

S2 Fig. Macrophage activation is a specific function of Brugia ELVs. (A) J774A.1 macrophages (5×10^5) were treated with approximately 4×10^8 purified L3 stage ELVs, an equivalent volume of culture media supernatant from which ELVs had been depleted by centrifugation (SN Treated) or naïve culture media (control). The presence of 32 cytokines/chemokines was simultaneously assayed using the Milliplex MAP Mouse Cytokine/Chemokine kit (EDM Millipore) interfaced with a Bio-Plex System (Bio-Rad) utilizing Luminex xMAP technology (Luminex). The quantification of identified cytokines is presented. ELV treatment, but not the ELV depleted culture media, generates a classically activated phenotype. (B) J774A.1 macrophages were treated with approximately 4×10^8 ELVs collected from a culture of Schistosoma mansoni invasive stage schistosomules as described for Brugia (Sm ELV), live S. mansoni schistosomules (300 per well; Sm) and naïve RPMI 1640 culture media (control). Macrophages were not

activated by either schistosome preparation.
(TIFF)

Acknowledgments

The authors would like to thank Marie Bockenstedt, Mary J. Long, and Dr. Douglas E. Jones for helpful discussions and technical guidance relating to the luminex experiments. The authors would also like to acknowledge parasite materials provided by Erica Burkman and the NIH-NIAID Filariasis Research Reagent Resource Center (FR3). The authors thank Michael Nazarchyk and Brendan Dunphy for technical assistance with mosquito infections and mosquito maintenance, and Morgan Pearson for assistance with vesicle isolation.

Author Contributions

Conceived and designed the experiments: MZ LMF LCB MJK ARM. Performed the experiments: MZ LMF HH PNA. Analyzed the data: MZ LMF TAD LCB MJK. Contributed reagents/materials/analysis tools: ARM. Wrote the paper: MZ LMF LCB MJK.

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