Ivermectin inhibits extracellular vesicle secretion from parasitic nematodes

Hannah J. Loghry[1], Wang Yuan[1], Mostafa Zamanian[2], Nicolas J. Wheeler[2], Timothy A. Day[1], Michael J. Kimber[1*]

[1]Department of Biomedical Sciences, College of Veterinary Medicine, Iowa State University, Ames, Iowa, United States of America

[2] Department of Pathobiological Sciences, University of Wisconsin-Madison, Madison, Wisconsin, United States of America

*Corresponding author: michaelk@iastate.edu

This work was supported by the National Institutes of Health under Grant AI117204.

Word Count: 10,674

Abstract

Lymphatic filariasis (LF) is a disease caused by parasitic filarial nematodes that is endemic in 49 countries and affects or threatens over 890 million people. Strategies to control LF rely heavily on mass administration of anthelmintic drugs including ivermectin (IVM), a macrocyclic lactone drug considered an Essential Medicine by the WHO. However, despite its widespread use the therapeutic mode of action of IVM against filarial nematodes is not clear. We have previously reported that filarial nematodes secrete extracellular vesicles (EVs) and that their cargo has immunomodulatory properties. Here we investigate the effects of IVM and other anti-filarial drugs on parasitic nematode EV secretion, motility, and protein secretion. We show that inhibition of EV secretion was a specific property of IVM, which had consistent and significant inhibitory effects across nematode life stages and species (with the exception of male parasites). IVM inhibited EV secretion, but not parasite motility, at therapeutically relevant concentrations. Protein secretion was inhibited by IVM in the microfilariae stage, but not in any other stage tested. Our data provides evidence that inhibiting the secretion of immunomodulatory EVs by parasitic nematodes could explain, at least in part, IVM mode of action and provides a phenotype for novel drug discovery.

Keywords: ivermectin, macrocyclic lactone, extracellular vesicle, lymphatic filariasis, *Brugia malayi*, parasite

1. Introduction

Lymphatic filariasis (LF) is a Neglected Tropical Disease caused by thread-like parasitic filarial nematodes, including *Brugia malayi*, that establish in the lymphatic vasculature. LF is often asymptomatic but symptoms manifest in approximately 40% of cases with lymphedema, hydrocoele, dermatitis and long-term disability characterizing clinical disease. It is estimated that LF is endemic in 49 countries and that over 890 million people are infected or at risk of infection (World Health Organization, 2019). In 2000 the Global Programme to Eliminate Lymphatic Filariasis was created with the goal of eliminating this disease by 2020 and although there has been reduction in the prevalence of LF in some areas this disease is far from being eliminated. Strategies to control LF and other filarial parasitic nematode infections rely heavily on mass administration of the anthelmintic drugs ivermectin (IVM), albendazole (ABZ) and diethylcarbamazine (DEC) in endemic areas. IVM is classified as an essential medication by the World Health Organization (World Health Organization, 2019) and since 2000, over 7 billion treatments have been delivered to at risk individuals (World Health Organization, 2019), however, this disease still remains an issue. One challenge to eliminating LF centers on the inadequate drugs that are currently available; neither IVM, ABZ or DEC effectively kill adult parasites, thus established infections are incurable. Compounding this and despite their widespread use, the therapeutic modes of action of IVM, and to a lesser extent DEC, are not entirely clear.

A current working hypothesis for the therapeutic activity of IVM is that it inhibits the release of excretory-secretory (ES) products from parasites; this inhibition is postulated to “unmask” the parasite allowing for host recognition and parasite clearance (Moreno *et al*., 2010). In support is the acceptance that the host immune system is involved in filarial parasite elimination, especially in the clearance of microfilaria (mf) stage worms (Carithers, 2017; Wolstenholme *et al*., 2016) and data from experiments such as Berrafato *et al.* showing that IVM enhanced leukocyte binding to *Dirofilaria immitis* mf and Semnani *et al*. who showed that IVM could reverse the modified Th2 phenotypes caused in filaria infected patients (Berrafato *et al*., 2019; Semnani *et al*., 2006). There is

widespread support that ES products from filarial nematodes do modulate host immune responses. Early filarial nematode infection elicits a canonical Th2 immune response characterized by increased production of the cytokines interleukin (IL)-4, IL-5, IL-9, IL-10, and IL-13 and the antibody isotypes IgG1, IgG4 (in humans), and IgE and increased production of Th2 cells, eosinophils, alternatively activated macrophages, and innate lymphoid cells 2 (ILC2) (Allen & Maizels, 2011; Geary *et al*., 2010). With development of chronic filarial infection the Th2 response becomes "modified" to a more tolerant, regulatory environment with increased IL-4, IL-10, T_{reg} and alternatively activated macrophage proliferation and reduction in IL-5, IL-13 and T cell proliferation coupled with T cell anergy and decreased antigen presenting capabilities (Babu & Nutman, 2014). There is considerable evidence that filarial nematode parasites contribute to this "modified" phenotype but the exact parasite factors driving this manipulation remain uncertain.

Extracellular vesicles (EVs) are membrane-bound vesicles secreted into the extracellular environment by eukaryotic and prokaryotic cells. Although once thought to be carriers of waste products, it has been shown that EVs function in many physiological processes and are important mediators of cell-to-cell signaling (Bobrie *et al*., 2011; Lee *et al*., 2012; Raposo *et al*., 1996; Valadi *et al*., 2007). EVs are considered a heterogenous group of sub-cellular structures that can be subdivided based on size and biogenesis. Primary focus has been on two subsets of EVs, microvesicles that range from 150-1,500 nm and a smaller grouping (30-150 nm) originally termed exosomes (Johnstone *et al*., 1987). Exosomes are products of the endosomal pathway and are derived from multivesicular bodies (MVBs) that fuse with the cell membrane to secrete the vesicles into the extracellular space (Catalano & O'Driscoll, 2020; Riaz & Cheng, 2017; Vlassov *et al*., 2012). Consistent with a role in cell-to-cell communication, EVs contain diverse functional cargo that varies depending on the cellular origin of the EVs, but in general include bioactive proteins, RNA and lipids (Thery *et al*., 2002; Valadi *et al*., 2007). EV secretion from diverse parasitic nematodes has been described (Buck *et al*., 2014; Coakley *et al*., 2017; Eichenberger, Ryan, *et al*., 2018; Eichenberger, Talukder, *et al*., 2018; Gu *et al*., 2017; Hansen *et al*., 2015, 2019; Harischandra

et al., 2018; Shears *et al*., 2018; Tritten *et al*., 2017; Tzelos *et al*., 2016; Zamanian *et al*., 2015) and the cargo of these EVs have immunomodulatory functions (Buck *et al*., 2014; Quintana *et al*., 2017; Tritten *et al*., 2016). We have previously reported that *B. malayi* secretes EVs and that their cargo has putative immunomodulatory properties (Harischandra *et al*., 2018; Zamanian *et al*., 2015). Driven by these emerging data, EVs have been advanced as a potential mechanism by which parasites modulate host immune responses (Buck *et al*., 2014; Coakley *et al*., 2017; Eichenberger, Sotillo, *et al*., 2018; Harischandra *et al*., 2018).

We propose that nematode EVs are essential for filarial nematode parasitism and hypothesize that effective anti-filarial drugs inhibit their secretion. To investigate this hypothesis, a panel of anti-filarial drugs was screened for their ability to reduce EV secretion from parasitic nematodes. We found that IVM had the most consistent inhibitory effects on EV secretion by various species and life stages of parasite. Importantly, however, IVM had insignificant effects on motility and limited effects on protein secretion at therapeutically relevant concentrations and timepoints. These observations provide insight into the mechanism of action of IVM and may support prioritizing inhibition of EV secretion as a screenable phenotype for novel anti-filarial drug development.

2. Materials and Methods

2.1 Parasite culture and maintenance

Brugia malayi and *B. pahangi* parasites were obtained from the NIH/NIAID Filariasis Research Reagent Resource Center (FR3) at the University of Georgia, USA. Persistent *B. malayi* infections at FR3 are maintained in domestic short-haired cats. To obtain adult stage *B. malayi* jirds were infected intraperitoneally with approximately 400 L3 stage parasites. 120 days post-infection jirds were necropsied to collect adult stage parasites. L3 stage *B. malayi* were obtained from dissection of anesthetized *Aedes aegypti* 14 days post-infection. Microfilaria stage *B. malayi* were obtained

from a lavage of the peritoneal cavity of a euthanized gerbil. *B. pahangi* stages were obtained in the same manner as *B. malayi* with the exception that infective L3 stage parasites were collected 11 days and 16 days post-infection, respectively. The *B. malayi* parasite supply from FR3 was supplemented with parasites from TRS Labs LLC (Athens, Georgia, USA). These supplemental parasites were tested and responded to treatments in the same manner as parasites from FR3. Upon receipt at ISU, all *B. malayi* and *B. pahangi* parasites were washed several times in warmed worm culture media (RPMI with 1% HEPES, 1% L-glutamine, 0.2% Penicillin/Streptomycin, and 1% w/v glucose [all Thermo Fisher Scientific, Waltham, MA]) and then counted and cultured at 37°C with 5% CO_2. Adult female *Ascaris suum* were collected from an abattoir in Marshalltown, Iowa, USA. These parasites were washed multiple times in warmed *Ascaris* Ringer's Solution (13.14 mM NaCl, 9.67 mM $CaCl_2$, 7.83 mM $MgCl_2$, 12.09 mM $C_4H_{11}NO_3$, 99.96 mM $C_2H_3NaO_2$, 19.64 mM KCl with Gentamycin (100 µg/ml), Ciprofloxacin Hydrochloride (20 µg/ml), penicillin (10,000 units/ml), streptomycin (10,000 µg/ml), and Amphotericin B (25 µg/ml) at pH 7.87 [all Sigma-Aldrich, St Louis, MO]) and then incubated overnight at 34°C. After 24 hrs in culture the parasites were checked for visible signs of bacterial or fungal contamination; if present the parasites were discarded.

2.2 Drug treatments of parasites

Parasites were cultured in the presence or absence of drug to examine effects on extracellular vesicle (EV) secretion. For *B. malayi* and *B. pahangi*, 10 adult female and 10 adult males were cultured as previously described for 24 hrs in 10 ml and 3 ml culture media, respectively, in 15 ml polypropylene centrifuge tubes (Thermo Fisher Scientific). 100 L3 or $1x10^6$ microfilariae were cultured as previously described for 24 hrs in 1 ml culture media in 1.5 ml microcentrifuge tubes (Thermo Fisher Scientific). Single adult female *A. suum* were cultured in 100 ml culture media in 250 ml sterile Erlenmeyer flask for 24 hrs as previously described. Four drugs, ivermectin, albendazole, diethylcarbamazine, and levamisole (all Sigma-Aldrich) were investigated for their

effects on each life stage of the parasite species. The various drugs or DMSO (vehicle control) were added to the culture media at a final concentration of 1 µM and 0.01% respectively for screening purposes. Spent media was collected after a 24 hr incubation. Additionally, drug and control treated *A. suum* and *B. pahangi* media was collected at 2, 4, 6, and 12-hr intervals to investigate the time course of the effects of the drugs. A dose curve for the effects of ivermectin on *B. malayi* were conducted in the same manner as described above with concentrations ranging from 0.1 nM – 10 µM.

2.3 EV Isolation and Quantification

EVs were collected as previously described using differential ultracentrifugation (Harischandra *et al*., 2018; Zamanian *et al*., 2015). Media was filtered through 0.2 µm PVDF filtered syringes (GE Healthcare, Chicago, IL) or PVDF vacuum filters (Sigma-Aldrich) and centrifuged at 120,000 x *g* for 90 minutes at 4ºC. The supernatant was decanted leaving approximately 1.5 ml media to ensure that the EV pellet was not disrupted. The retained media and pellet were filtered through a PVDF 0.2 µm syringe filter and centrifuged at 186,000 x *g* for a further two hrs at 4ºC. Pelleted EV samples were resuspended to 500 µl in dPBS (Thermo Fisher Scientific). EV quantification and size determination were performed using nanoparticle tracking analysis (NTA; Nano-Sight LM10, Malvern Instruments, Malvern, UK).

2.4 Motility Analysis

The Worminator system developed and described by Marcellino *et al.* (2012) was used to quantitatively measure motility of adult filarial nematodes in microtiter plates. Microscopic parasite life stages were quantitatively analyzed by the same software, but with methods previously described by Storey *et al*. (2014). Briefly, a single adult male or female worm was cultured in one

well of a standard 24-well cell culture plate (Sigma-Aldrich). For infective L3 stage worms, 10 worms were cultured per well of a 96-well plate (Corning Inc, Corning, NY). Drug or DMSO (vehicle control) was added to each well to a final concentration of 1 µM or 0.01% respectively. Worms were incubated at 37°C and 5% CO_2 and measurements were briefly taken, at room temperature, prior to treatment, immediately after treatment (0 hrs) and at 2, 4, 6 and 24-hrs post treatment. Measurements of the effects of doses of ivermectin ranging from 0.1 nM – 10 µM on adult female *B. malayi* were conducted at 24 hrs post treatment.

2.5 SF21 Cell Culture

Sf21 cells (Thermo Fisher Scientific) were maintained in Ex-Cell 420 serum free media (Sigma Aldrich) with 1% Penicillin/Streptomycin and 0.25 ug/ml Amphotericin B. Cells were seeded at a density of $3x10^5$ cells/well in a 6-well plate. After an overnight incubation at 28°C, cells were either treated with a final concentration of 1.0 µM ivermectin, 0.1 µM ivermectin or 0.01% DMSO (vehicle control). Spent media was collected after 24 hrs and processed for EV isolation as described above with the addition of an initial centrifugation step of 12,000 x *g* for 30 minutes at 4ºC to eliminate cellular debris.

2.6 Protein Quantification Assay

A single *B. malayi* adult or 100,000 microfilariae were cultured per well of a 24-well plate with either drug or DMSO at a final concentration of 1.0 µM or 0.01%, respectively, for 24 hrs. A concentration-response curve for ivermectin was conducted on adult female worms with concentrations ranging from 0.1 nM – 10 µM. Spent media was collected and filtered through a 0.2 µM PVDF membrane filter (GE Healthcare) 500 µl of media were concentrated using a 0.5 mL, 3,000 Da Amicon Ultra centrifugal filter unit (Sigma-Aldrich) according to manufacturer's

instructions. Media samples were concentrated by centrifuging at 14,000 x *g* for 30 minutes. Samples were then washed with 500 µl dPBS for 30 minutes at 14,000 x *g*. The washing step was repeated four times. The volume of each sample was determined, and all samples were normalized to 150 µl with dPBS. Adult female samples were then further diluted 4-fold with dPBS while adult male, L3 stage and microfilariae were diluted 2-fold with dPBS to ensure that readings would fall within the standard curve. Total protein was quantified with Pierce micro BCA kit (Thermo Fisher Scientific) according to manufacturers' instructions. Protein assay plates were quantified using a SpectraMax M2e plate reader (Molecular Devices, San Jose, CA).

2.7 Statistical Analysis

Due to some variation among individual parasites and between batches of parasites, experiments were conducted across multiple batches of parasite shipments, with each N representing parasites from independent shipments. Individual control and treated worms within each batch were paired together to help account for batch variation. EV NTA data was analyzed via a ratio-paired T-test with p-values less than 0.05 being considered significant. Non-linear regressions with least squares fit were used to analyze the dose response curves for ivermectin on *B. malayi* adult female EV secretion, motility, and protein secretion. Motility data was analyzed via a RM 2-way ANOVA with Geisser-Greenhouse correction followed by a Dunnet's multiple comparison test. Paired T-test between each treatment for each life stage were used to analyze data from the protein assay as the data contained values unsuited for a ratio-paired T-test. Due to the variability among batches and individual parasites the ROUT outlier identification method was used to identify outliers in the data (Q = 0.5%). All statistical analyses were performed using Prism 8.4.1. (GraphPad Software, San Diego, CA).

3. Results

3.1 Ivermectin inhibits EV secretion from *Brugia malayi* in a sex- and stage- specific manner

In this study we investigated the effects of ivermectin (IVM) on *B. malayi* EV secretion *in vitro*. Parasites were cultured at 37°C in the presence or absence of IVM and the number of EVs secreted by the worms was quantified by nanoparticle tracking analysis (NTA). An initial screening concentration of 1.0 μM IVM significantly reduced EV secretion after 24 hrs incubation from *B. malayi* adult females by 59% ($p = 0.0204$, $N = 13$) and from L3 stage parasites by 31% ($p = 0.0067$, $N = 19$). There was no significant effect on EV secretion from adult male ($p = 0.4028$, $N = 10$) or microfilariae (mf) ($p = 0.2081$, $N = 13$) life stages (Fig 1A-D). The IVM concentration response in adult female *B. malayi* was further profiled and the IC_{50} determined to be 7.7 nM (Fig 1E). Studies conducted on the pharmacokinetics of a single dose of IVM in human subjects have determined serum levels to be between 20-70 nM (González Canga *et al*., 2008). IVM therefore inhibits EV secretion in adult female parasites at therapeutically relevant concentrations suggesting that this phenomenon may contribute to IVM therapeutic mode of action.

These observations on the inhibitory effect of IVM on EV secretion from adult female and L3 stage *B. malayi* generally align with preliminary data previously reported, (Harischandra *et al*., 2018) with the exception of the lack of inhibition in adult males and a reduced inhibition in mf stages. The previously reported inhibitory effect on EV secretion from adult males was marginal, but the lack of effect on mf is more surprising considering its prior robustness. Previously, mf were incubated with IVM at ambient temperature whereas here they were incubated at 37°C. To test the impact of temperature on the IVM phenotype in mf, we repeated the mf IVM incubation at ambient temperature. Unlike at 37°C, 1.0 μM IVM significantly inhibited EV secretion by 46% ($p = 0.0177$, $N = 8$) at ambient temperature (22°C) (Fig 2A). Control and treated parasites were still viable at the end of the experiment indicating that it was not loss of viability or death of the parasites that had caused inhibition of EV secretion. There are clear temperature-dependent effects on EV secretion from mf stage worms, not only did incubation of mf at 37°C abrogate the inhibitory effect of IVM on EV secretion, but it also increased EV secretion in untreated worms by approximately a factor of

7. Whilst logical to assume temperature changes impact worm physiology, there is a lack of data on the effects of temperature on specific processes and functions in mf stage nematodes. We do know that host temperature has no effect on the nocturnal periodicity of mf (Hawking, 1967) or on the ability of mf to bind to vascular endothelial cells (Schroeder *et al*., 2017). Environmental temperature may affect other physiological processes in mf leading to this increased production of EVs, but further investigation into this phenomenon is necessary.

This temperature-dependent effect in mf prompted further efforts to determine the mechanism by which IVM is inhibiting EV secretion. One hypothesis is IVM inhibits EV biogenesis in these parasites. Nematode cell lines that might provide an opportunity to interrogate EV biogenesis are lacking so Sf21cells from the army fallworm, *Spodoptera frugiperda*, were used as an ecdysozoan surrogate to examine the effect of IVM on EV biogenesis *in vitro*. Sf21 cells were treated with high (1.0 µM) and low (0.1 µM) concentrations of IVM and EV secretion was analyzed by NTA after 24 hrs. Neither concentration of IVM inhibited EV secretion from Sf21 cells (Fig 2B), perhaps suggesting the drug does not directly impact EV biogenesis. Another hypothesis is that IVM disrupts the location from where EVs are secreted. The physical characteristics of EVs produced from IVM-treated or DMSO-treated *B. malayi* adult females were analyzed by NTA. Although EVs are, as a whole, a heterogenous population in regards to size, our data suggested that there was no obvious shift in EV size profile following IVM treatment (Fig 2C). It might be expected that disrupting the location from where EVs are secreted, for example the parasite GI tract or reproductive structures, might alter their physical characteristics, but this was not observed. The physical characteristics of EVs secreted by other *B. malayi* life stages were also analyzed (Supplemental Figure 2A-C). Consistent with the adult female data, there were no differences in EV size following IVM treatment of any life stage. Collectively, our data suggests that IVM works not by altering EV biogenesis or changing the location from where EVs are secreted, but rather restricts the numbers of EVs being secreted by pre-existing pathways.

3.2 Other drugs with anti-filarial activity do not inhibit EV secretion from *B. malayi*

To examine if inhibition of EV secretion is a general feature of drugs with anti-filarial activity, we tested a panel of drugs with known anti-filarial activity including albendazole (ABZ), diethylcarbamazine (DEC) and levamisole (LEV). LEV is a nicotinic agonist and although more typically used to treat gastrointestinal nematode infection, was included in the panel because it is an anthelmintic drug with known neuromuscular effects on filarial nematodes (Martin, 1997; Robertson *et al*., 2013) and also because of reported microfilaricidal effects on canine heartworm, *D. immitis* (Carlisle *et al*., 1984; Mills & Amis, 1975). Parasites were cultured with or without an initial screening concentration of 1.0 µM drug and EVs were quantified using NTA. 1.0 µM DEC significantly increased EV secretion from *B. malayi* mf by 43% ($p = 0.0177$, $N = 7$) after 24 hrs (Fig 1D). DEC also seemed to increase EV secretion in L3 (Fig 1C) though not significantly ($p = 0.2236$, $N = 10$). Additionally, DEC had a moderate, but not significant, inhibition on EV secretion in adult females ($p = 0.1704$, $N = 10$) and had no effect on adult males ($p = 0.2323$, $N = 9$) (Fig 1A-B). There was a minor, but not significant, inhibition of EV secretion due to ABZ on *B. malayi* adult females ($p = 0.4042$, $N = 10$), L3 ($p = 0.1564$, $N = 11$), and mf ($p = 0.7815$, $N = 7$) (Fig 1A,C,D). In contrast, ABZ treatment seemed to increase EV secretion from adult males (Fig 1B) though not significantly ($p = 0.0821$, $N = 9$). LEV did not have an effect on EV secretion from either male ($p = 0.1091$, $N = 11$) or female adults ($p = 0.8659$, $N = 9$) (Fig 1A-B). L3 stage parasites showed a minor although not significant inhibition in EV secretion due to LEV treatment ($p = 0.0719$, $N = 10$) while mf had a moderate, but not significant increase in EV secretion due to LEV treatment ($p = 0.0770$, $N= 7$) (Fig 1C-D). In summary, none of the drugs in the panel significantly inhibited EV secretion from any of the *B. malayi* life stages when tested at 1.0 µM, except for DEC which significantly increased EV secretion from *B. malayi* microfilariae. This is significant because it suggests that inhibition of EV secretion by filarial nematodes may be a phenotype specific to IVM treatment and is not observed upon treatment with other anthelmintic drugs that are known to have anti-filarial activity, including a drug (LEV) that has clear neuromuscular effects on filarial worms.

This helps support the hypothesis that the mechanism of action of IVM, and perhaps other macrocyclic lactones, includes inhibition of EV secretion.

3.3 Ivermectin has broad inhibitory effects on EV secretion across other filarial and gastrointestinal nematode parasites

To test whether the inhibitory IVM phenotype in *B. malayi* was broadly consistent in nematodes, we repeated the same screening experiment with our same drug panel using first a related species of filarial nematode, *B. pahangi*. Our analysis was limited to *B. pahangi* adult females, adult males and mf based on worm availability. All drugs in the panel significantly inhibited EV secretion from *B. pahangi* adult female parasites. IVM treatment had the greatest reduction in EV secretion with an inhibition of 63% ($p = 0.0083$, $N = 10$), while ABZ had an inhibition of 61% ($p = 0.0066$, $N = 12$), DEC by 59% ($p = 0.0322$, $N = 12$) and LEV by 44% ($p = 0.0416$, $N = 11$) (Fig 3A). The IVM, ABZ and DEC results generally paralleled the trends seen in *B. malayi* adult females, but with LEV now also active. Neither IVM ($p = 0.9897$, $N = 11$), ABZ ($p = 0.9369$, $N = 13$), DEC ($p = 0.0925$, $N = 12$), nor LEV ($p = 0.7558$, $N = 11$) had any effect on adult male *B. pahangi* (Fig 3B). Again, this is consistent with the results seen in *B. malayi* adult male parasites. In mf stage *B. pahangi*, IVM significantly reduced EV secretion by 40% at 37°C ($p = 0.0358$, $N = 4$) (Fig 3C), which contrasts sharply with what was seen in *B. malayi* mf and perhaps better aligns with the expected bioactivity of IVM at this life stage (Moreno *et al*., 2010). While DEC significantly increased EV secretion in *B. malayi* mf it did not have any effect on *B. pahangi* mf ($p = 0.6428$, $N = 3$) (Fig 3C). Lastly, ABZ ($p = 0.6605$, $N= 3$) and LEV ($p = 0.4125$, $N = 3$) had no effect on EV secretion from *B. pahangi* mf (Fig 3C). To investigate whether the inhibitory effects of IVM on EV secretion were seen in more divergent nematode species we again repeated our screen on single adult female *Ascaris suum*, a soil-transmitted gastrointestinal nematode. IVM ($p = 0.0013$, $N = 14$) and LEV ($p = 0.0021$, $N = 16$) both significantly inhibited EV secretion from individual adult female *A. suum* after 24 hrs by

311 99.4% and 99.1% respectively (Fig 3E). However, neither ABZ (p = 0.3769, N = 12) nor DEC (p =
312 0.9680, N = 16) had any effect on EV secretion (Fig 3E).

313 The size characteristics of those EVs that continued to be secreted by both adult female *B. pahangi*
314 (Fig 3D) and *A. suum* (Fig 3F) following IVM treatment was examined using NTA. As was seen in
315 *B. malayi*, IVM did not affect the physical characteristics of EVs produced from these other species
316 of parasites, providing additional evidence that the inhibitory bioactivity of IVM may not be
317 involved in changing from where EVs are secreted. The effects of the other drugs in our screening
318 panel on the size of EVs secreted by *B. malayi* and *B. pahangi* life stages were also analyzed
319 (Supplemental Fig 2A-E). None of the drugs tested had any effect on the physical characteristics of
320 EVs secreted.

321 *B. pahangi* and *A. suum* adult female worms secrete EVs more robustly than *B. malayi.* In the case
322 of *A.suum*, they secrete approximately 250 times more EVs than *B. malayi* in 24 hrs. This
323 positioned us to use these two species to better understand how rapidly IVM inhibits EV secretion
324 from susceptible parasitic nematodes. Adult female *B. pahangi* and *A.suum* parasites were treated
325 with 1.0 µM IVM as before and spent media collected at 2, 4, 6, 12 and 24 hrs post IVM treatment.
326 IVM significantly inhibited both *B. pahangi* and *A. suum* EV secretion by 63% (p = 0.0076, N = 3)
327 and 99.4% (p = 0.0054, N = 3), respectively, at the 24 hrs post-treatment timepoint (Fig 3G-H). In
328 addition, EV secretion was inhibited as early as at 12 hours post-treatment by 72% (p = 0.1929, N =
329 3) for *A. suum* and by 57% (p = 0.0762, N = 3) for *B. pahangi*, though not statistically significant
330 (Fig 3G-H). *In vivo* studies have shown IVM reduces microfilaremia in mice experimentally
331 infected with *B. malayi* 24 hrs post-treatment (Halliday *et al*., 2014). Thus, the rapidity of onset for
332 this EV secretion phenotype is consistent with the therapeutic action of IVM. For context, other
333 IVM phenotypes have been identified at 24 hrs post-treatment timepoint, *in vitro* IVM reduces the
334 release of *B. malayi* mf from adult female worms (Tompkins *et al*., 2010) and increases the binding
335 of polymorphonuclear leukocytes to *B. malayi* mf (Berrafato *et al*., 2019) after 24 hrs. In canine

heartworms, IVM inhibits the motility of Missouri strain *D. immitis* 24 hrs post-treatment *in vitro* (Maclean *et al*., 2017).

3.4 Ivermectin inhibition of EV secretion is not driven by loss of gross motor function

Glutamate-gated chloride channels (GluCl) and nicotinic acetylcholine receptors (nAChRs), are known targets for IVM and LEV, respectively (Arena *et al*., 1991, 1992; Harrow & Gration, 1985). GluCl have been identified in motor neurons and interneurons in various parasitic nematode species (Adrian J. Wolstenholme & Rogers, 2005) and nAChRs have been identified at the neuromuscular junction in filarial nematodes (Martin, 1997). Their locations lead to the discovery that IVM can induce paralysis of pharyngeal pumping in *Haemonchus contortus* (Geary et al., 1993) and both IVM and LEV can cause paralysis of *B. malayi* parasites (Mostafa *et al*., 2015; Tompkins *et al*., 2010). Due to these documented effects it is plausible that the EV phenotype is driven by gross motor function defects. To test this we examined the effect of our screening panel on gross motor function by analyzing motility quantified using the Worminator software system (Marcellino *et al*., 2012). Our analysis was limited to *B. malayi* adults and L3 stage parasites due to parasite availability and difficulties in consistently recording the smaller mf life stage. A single *B. malayi* adult or 10 L3 stage parasites were cultured in a 24-well or 96-well plate respectively with or without 1.0 µM drug. Video recordings were taken prior to treatment, immediately after treatment (0 hrs), and at 2, 4, 6 and 24 hrs post-treatment. IVM significantly reduced adult female motility by 57% beginning at 4 hrs post-treatment ($p < 0.0001$, N=5) (Fig 4A). However, when the kinetics of IVM treatment on adult female parasites was investigated it was observed that more therapeutically relevant concentrations did not affect motility (Fig 4D). This is corroborated by other data that shows that motility of *B. malayi* parasites was not inhibited by concentrations of IVM less than 2 µM (Storey *et al*., 2014; Tompkins *et al*., 2010). This provides additional evidence that therapeutically relevant concentrations of IVM do not affect filarial nematode motility. The IC_{50} for IVM was determined to be 0.203 µM. The IC_{50} for EV secretion (7.7 nM) was below that of

362 motility indicating that IVM is not reducing EV secretion by paralyzing the parasites. DEC ($p <$
363 0.001, N=5) significantly inhibited adult female motility immediately upon treatment, but parasites
364 began to recover at one hr ($p < 0.01$, N=5) and completely recovered by 4 hrs post-treatment. LEV
365 ($p < 0.001$, N=5) significantly inhibited adult female motility by 88% immediately upon treatment,
366 but parasites began to recover during the remaining 24 hrs. At one hr post treatment LEV
367 significantly inhibited adult female motility by 71%, at four hrs by 40%, at six hrs by 35% and at 24
368 hrs by 29% (1-6 hrs post-treatment: $p < 0.0001$, N=5; 24 hrs: $P < 0.01$, $N = 5$). As was discussed
369 earlier, no drug in our panel had any effect on EV secretion in adult male *B. malayi*, but it was
370 discovered that LEV was a potent inhibitor of motility in adult male *B. malayi* (Fig 4B). Motility in
371 adult males was significantly inhibited by 70% upon treatment with LEV. Adult male parasites
372 treated with LEV did not recover with significant inhibition ranging from 70-76% over the 24 hrs
373 tested (1-24 hrs post treatment: $p < 0.0001$, N=5). The only drug that had any effect on *B. malayi* L3
374 parasites was also LEV with inhibition of motility by 90% immediately upon treatment ($p < 0.001$,
375 N=5) (Fig 4C). However, a very quick recovery of motility was seen in just one hour. In summary,
376 1.0 µM IVM had inhibitory effects on *B. malayi* adult female motility, but this concentration does
377 not compare to therapeutically relevant concentrations or to the concentrations that inhibited EV
378 secretion. LEV also had inhibitory effects on motility in all life stages tested, but adult female and
379 L3 life stages recovered over 24 hrs while adult males did not recover. Due to the differences in
380 IVM effects on motility compared to EV secretion we can conclude that inhibition of EV secretion
381 is not a factor of parasite gross motor function being compromised.

382

383 **3.5 Ivermectin does not have parallel effects on EV and protein secretion**

384 Data indicate that IVM and ABZ inhibit protein secretion from *B. malayi* mf (Moreno *et al*., 2010).
385 We have already shown that IVM can inhibit EV secretion from *B. malayi* so we hypothesized that
386 excretory-secretory (ES) proteins and EVs would similarly be affected by the screening drug panel.
387 Parasites were cultured with or without drug and ES protein secreted into the culture media was

quantified 24 hrs after treatment using BCA. We chose to examine the 24 hr time point as it is the most consistent with IVM therapeutic mechanism of action. Unlike EV secretion, neither 1.0 µM IVM ($p = 0.8152$, $N = 5$), ABZ ($p = 0.9962$, $N = 5$), DEC ($p = 0.8863$, $N=5$) or LEV ($p = 0.1571$, $N = 5$) had any effect on protein secretion from *B. malayi* adult females (Fig 5A,D). Similarly, no effect of any drug on protein secretion was observed in adult males (IVM $p = 0.9400$, $N = 5$, ABZ $p = 0.4906$, $N=5$, DEC $p = 0.9902$, $N = 5$, LEV $p = 0.8043$, $N = 5$) (Fig 5B). Inhibition of ES protein secretion from *B. malayi* mf was noted after treatment with 1.0 µM IVM (23%, $p = 0.0535$, $N = 5$) (Fig 5C), however, ABZ had no effect on protein secretion ($p = 0.5827$, $N = 5$). DEC (0.5789, $N=5$) and LEV ($p = 0.1648$, $N = 5$) also had no inhibitory effect on ES protein secretion from *B. malayi* mf. The data do not exactly correlate to previously published work describing clear inhibitory effects of IVM and ABZ protein release from *B. malayi* mf (Moreno *et al*., 2010). The assay we used to quantify ES protein secretion from *B. malayi* was slightly modified from that study but was fundamentally the same. Despite tight technical replication, there was challenging biological variability between worm batches and the low quantities of ES protein secreted necessitated a concentration step, potentially exacerbating variability. Despite this, we have high confidence in comparing this data with those of Moreno *et al* (2010). Both studies observed a rapid inhibition of ES protein secretion from *B. malayi* mf following 24 hr treatment with 1.0 µM IVM, although our observed inhibition was moderately higher at 23% inhibition than Moreno *et al* with 14%. Further, Moreno *et al* described an inhibitory effect of ABZ (more potent than that of IVM) that was not observed here.

4. Discussion

IVM is a broad spectrum, anti-parasitic drug that is commonly used to treat and prevent multiple diseases caused by parasitic nematodes. Even with its extensive usage, the therapeutic mechanism of action of this drug is not completely understood (Wolstenholme *et al*., 2016). The current accepted hypothesis is that it functions, at least in part, by inhibiting secretion of ES proteins from

parasites thereby "unmasking" the parasites and facilitating host clearance (Moreno *et al*., 2010). Recent work has led to the characterization of the *B. malayi* secretome which, combined with RNA sequencing approaches, has defined a complex milieu of proteins and miRNAs secreted from these and other filarial parasites (Bennuru *et al*., 2009; Hewitson *et al*., 2008; Hoy *et al*., 2014; Kaushal *et al*., 1982; Tritten *et al*., 2016). Within this heterogenous mix of ES products are documented host immunomodulatory effectors, including leucyl aminopeptidase (ES-62) and macrophage inhibiting factor 1 (MIF-1), among others (Harnett *et al*., 1998; Lal *et al*., 1990; Pastrana *et al*., 1998). Therefore, the observation that IVM can inhibit the secretion of immunomodulatory ES products is consistent with the rapid mf clearance observed after treatment in infected individuals. Although there is evidence tying the inhibition of ES product secretion to the mode of action of IVM, the critical ES products being inhibited are not immediately clear. In addition to freely secreted proteins, we have identified that prodigious numbers of EVs are also found in the ES products of filarial nematodes (Zamanian *et al*., 2015). In this study we show that IVM significantly and consistently inhibits EV secretion from *B. malayi* adult female, L3 and mf life stages, from *B. pahangi adult* females and mfs, and from female gastrointestinal *A. suum* nematodes. This inhibition occurs at therapeutically relevant concentrations (IC_{50} = 7.7 nM in adult female *B. malayi*) and time frame (within 24 hrs and perhaps even by 12 hrs). Given these properties, it is reasonable to hypothesize that the therapeutic mechanism of action of IVM against filarial nematodes may, in part, involve inhibition of EV secretion. Although premature, there is growing evidence to support this hypothesis. First, filarial nematode EVs are discrete structures that are enriched in immunomodulatory molecules. The cargo of *B. malayi* EVs includes proteins and miRNAs that have immunomodulatory functions and include modulatory proteins such as galectins and MIF-1 as well as miRNAs with identity to immunomodulatory host miRNAs (Harischandra *et al*., 2018; Zamanian *et al*., 2015). EVs from other nematode species are similarly imbued; protein and small RNA profiling of EV cargo from a range of gastrointestinal and filarial nematodes reveals a multitude of putative effector molecules with emerging functionality at the host-parasite interface

(Buck *et al*., 2014; Eichenberger, Ryan, *et al*., 2018; Eichenberger, Talukder, *et al*., 2018; Gu *et al*., 2017; Hansen *et al*., 2015, 2019; Shears *et al*., 2018; Tritten *et al*., 2017). It is reasonable to posit that specifically inhibiting EV secretion would obstruct the immunomodulatory capabilities of these parasites. Second, the pharmacological distruption of EV secretion does not perfectly correlate with the secretion of other ES products, hinting that the regulation of EV secretion may be distinct to that of other ES products and therefore differentially "druggable". IVM (1.0 μM) inhibited EV secretion from mf stage *B. malayi* and *B. pahangi* after 24 hrs by 46% and 40%, respectively. In comparison, we found the same treatment inhibited protein secretion by *B. malayi* mf more modestly at 23%. Further, whilst IVM (1.0 μM) inhibited EV secretion in adult female *B. malayi* by 59% after 24 hrs, the same treatment had no significant effect on protein secretion from those worms. Clearly, more work is needed to understand how parasite secretions are regulated but moving forward it may be advisable to disentangle the broad panoply of ES products and investigate them individually to help better understand host-parasite interactions and particularly how drugs affect the secretion of parasite effector molecules.

Parasite motility has long been used as an assay to identify and measure the anthelmintic activity of compounds and as marker of parasite health and viability. Impaired motility alone, however, does not adequately account for the therapeutic effects of IVM in filarial disease. The *in vitro* IVM concentrations that are required to produce detrimental effects on gross filarial nematode motility are significantly higher than the bioavailable concentrations found *in vivo* after therapeutic administration (González Canga *et al*., 2008; Marcellino *et al*., 2012; Storey *et al*., 2014; Tompkins *et al*., 2010). Our data support this – the IC_{50} of IVM in the *B. malayi* adult female EV secretion assay was 7.7 nM but was over 200 nM for the motility assay. IVM inhibits EV secretion but not motility in key stages at therapeutically relevant concentrations, supporting inhibition of EV secretion as a component of IVM mode of action. IVM also exerted inhibitory effects on *B. malayi* adult females but not adult males, and larval stages. This stage- and sex-specific activity does correlate to the expression patterns of genes encoding subunits of glutamate-gated chloride channels

(GluCls), a proposed target for IVM (Arena *et al*., 1991, 1992). Li *et al* (2014) found that *avr-14*, a gene encoding a putative GluCl subunit in *B. malayi*, was expressed in both female and male reproductive tissues but consistently more strongly in female tissues (ovaries and surrounding body wall muscle) than male. This differential expression profile may help explain why EV secretion was inhibited in female worms but not male worms and may also point to reproductive structures as a source of these EVs in adult female worms. Proteomic analyses of EV cargo has proved valuable in identifying markers of tissue origin in other nematodes (Buck *et al*., 2014) but our previous nano-scale proteome profiling of *B. malayi* female and male EVs did not identify any clear markers supporting a reproductive tissue origin for these vesicles (Harischandra *et al*., 2018). A more focused investigation of the fluid found in these structures may prove more illuminating, as would demonstration that putative IVM targets are similarly expressed in reproductive tissues of adult female *B. pahangi* and *A. suum* to account for the potent IVM activity we noted in those species. Li *et al*, (2014) also observed tissue-specific *avr-14* expression in embryonic stages within gravid females. This corroborates the findings of Moreno *et al*. (2014) who noted strong localization of *avr-14* around the ES pore of *B. malayi* mfs, earmarking this structure as another, perhaps more predictable, site of EV secretion in this stage that lacks reproductive tissues or a through gut.

Whether these EVs have their biogenesis in reproductive tissues, the excretory system or some other secretory route, the pathways by which IVM inhibits their secretion is obscure and will require a more thorough description of the microscopic anatomy of key tissues and a better understanding of IVM targets expressed therein. For example, despite the recognition that parasitic nematode ES systems secrete a complex suite of molecules believed to be essential for successful parasitism (Allen & Maizels, 2011; Hewitson *et al*., 2009; Hoerauf *et al*., 2005; van Riet *et al*., 2007), the ultrastructure and transcriptional topography of the ES pore region has largely been uninvestigated. The intersection between EVs as an important mechanism for host manipulation during infection, the inhibition of their secretion by IVM at therapeutically relevant concentrations

and time frames, and the localization of putative IVM targets in critical stage-specific tissues, provides strong rationale for addressing this knowledge gap.

A significant outcome from the work presented here is the demonstration that EV secretion from adult female and mf stage filarial nematodes (the stages that one could argue are most relevant to LF control programs) can be quantified and the effect of extraneously applied compounds on this secretion measured. Using this assay, we detected an IVM-sensitive EV secretion phenotype that perhaps correlates better with therapeutically relevant IVM concentrations than does assaying parasite motility, and in our experience is a more convenient and reproducible assay than that used to measure protein secretion from these worms. It may also be a better predictor of IVM mode of action. If the therapeutic mechanism of action of IVM is to inhibit immunomodulatory protein secretion from mf parasites then albendazole, which has been reported to inhibit protein secretion from *B. malayi* mf faster and more comprehensively than IVM (Moreno *et al*., 2010) (although we did not observe this), should also be an effective microfilaricide. Albendazole, however, is ineffective against mf stage filarial nematodes (Critchley *et al*., 2005). This suggests inhibition of EV secretion may be a preferred characteristic of anti-filarial drugs and therefore assaying this phenotype would be of significance to future drug discovery efforts aimed at developing new anti-filarial compounds, certainly those that function like IVM. In its favor, EV quantification would provide a consistent screening assay that would be comparable across different species of parasites, however, EV quantification is not high-throughput and does require additional EV isolation steps and specialized equipment for EV visualization. Recent technological advances may provide platforms that could be leveraged to streamline EV quantification assays and overcome these drawbacks. For example, we have contributed to an on-chip microfluidic device that utilizes a label-free photonic crystal biosensor to detect and discriminate host EVs from those secreted by parasitic nematodes based on differential expression of EV surface markers (Wang *et al*., 2018). This type of platform combines minimal sample processing with high throughput potential and does not require EV labelling, overcoming the disadvantages of traditional EV quantification and could be leveraged

in drug discovery efforts centered on EV secretion as an assay endpoint. Another potential use for this type of platform are the early detection of parasite infection. The use of EVs as a nematode diagnostic has been seeded by the focus on EVs as diagnostic markers for cancer detection. Current advanced technologies involve using surface enhanced Raman scattering (SERS) or localized surface plasmon resonance to detect tumor-derived EVs in body fluids (Mehmet *et al*., 2017; Thakur *et al*., 2017; Zong *et al*., 2016). In addition, the miRNA cargo of EVs has been of interest as biomarkers for various cancers (Kosaka *et al*., 2019). Similarly, a microfluidic on-chip device has potential to identify parasite EVs from host biofluids. Current efforts towards this goal are aimed at identifying secreted parasite markers that that could be incorporated in such a design, or used in more simple assay formats such as PCR (Quintana *et al*., 2017; Tritten *et al*., 2014). Finally, the assay we describe here may be an example of a relatively simple *in vitro* assay to test or validate the emergence of anthelmintic resistance. The Fecal Egg Count Reduction Test is the gold standard for detecting resistance to anthelmintics like IVM. Alternative *in vitro* assays complement FECRT and include hatching and development assays, molecular tests and, of course, motility assays (Kotze & Prichard, 2016). The EV secretion assay could be added to this list if it could reliably, and with sensitivity, detect resistance to drugs such as IVM in a standardized fashion. There is some evidence for this potential; we previously detected differences in IVM susceptibility based on EV secretion for two strains of canine heartworm, *D. immitis* (Harischandra *et al*., 2018).

Collectively, our data show that the secretion of EVs from different parasitic nematode species can be assayed and the effects of anthelmintic drugs or lead compounds on this physiological process can be measured. IVM consistently inhibited EV secretion against all species and most life stages investigated, with the exception of male worms; other anti-filarial drugs did not. These findings provide new insight into the stage-, sex- and species-specific pathways and pharmacological regulation of EV secretion in parasitic nematodes. The data is significant because, given the emerging immunomodulatory role of EVs at the host-parasite interface, it provides new evidence that the therapeutic mechanism of IVM, in part, involves inhibition of parasite EV secretion.

Disclosure of Interest

The authors report no conflict of interest.

References

Allen, J. E., & Maizels, R. M. (2011). Diversity and dialogue in immunity to helminths. *Nature Reviews Immunology*, *11*(6), 375–388. https://doi.org/10.1038/nri2992

Arena, Joseph P., Liu, K. K., Paress, P. S., & Cully, D. F. (1991). Avermectin-sensitive chloride currents induced by Caenorhabditis elegans RNA in Xenopus oocytes. *Molecular Pharmacology*, *40*(3), 368–374.

Arena, Joseph P., Liu, K. K., Paress, P. S., Schaeffer, J. M., & Cully, D. F. (1992). Expression of a glutamate-activated chloride current in Xenopus oocytes injected with Caenorhabditis elegans RNA: Evidence for modulation by avermectin. *Molecular Brain Research*, *15*(3), 339–348. https://doi.org/10.1016/0169-328X(92)90127-W

Babu, S., & Nutman, T. B. (2014). Immunology of lymphatic filariasis. *Parasite Immunology*, *36*(8), 338–346. https://doi.org/10.1111/pim.12081

Bennuru, S., Semnani, R., Meng, Z., Ribeiro, J. M. C., Veenstra, T. D., & Nutman, T. B. (2009). Brugia malayi Excreted/Secreted Proteins at the Host/Parasite Interface: Stage- and Gender-Specific Proteomic Profiling. *PLOS Neglected Tropical Diseases*, *3*(4), e410. https://doi.org/10.1371/journal.pntd.0000410

Berrafato, T., Coates, R., Reaves, B. J., Kulke, D., & Wolstenholme, A. J. (2019). Macrocyclic lactone anthelmintic-induced leukocyte binding to Dirofilaria immitis microfilariae: Influence of the drug resistance status of the parasite. *International Journal for Parasitology: Drugs and Drug Resistance*, *10*, 45–50. https://doi.org/10.1016/j.ijpddr.2019.04.004

Bobrie, A., Colombo, M., Raposo, G., & Théry, C. (2011). Exosome Secretion: Molecular Mechanisms and Roles in Immune Responses. *Traffic*, *12*(12), 1659–1668. https://doi.org/10.1111/j.1600-0854.2011.01225.x

Buck, A. H., Coakley, G., Simbari, F., McSorley, H. J., Quintana, J. F., Le Bihan, T., Kumar, S., Abreu-Goodger, C., Lear, M., Harcus, Y., Ceroni, A., Babayan, S. A., Blaxter, M., Ivens,

572 A., & Maizels, R. M. (2014). Exosomes secreted by nematode parasites transfer small RNAs
573 to mammalian cells and modulate innate immunity. *Nature Communications*, *5*(1), 5488.
574 https://doi.org/10.1038/ncomms6488
575 Carithers, D. S. (2017). Examining the role of macrolides and host immunity in combatting filarial
576 parasites. *Parasites & Vectors*, *10*(1), 182. https://doi.org/10.1186/s13071-017-2116-6
577 Carlisle, C. H., Atwell, R. B., & Robinson, S. (1984). The effectiveness of levamisole
578 hydrochloride against the microfilaria of Dirofilaria immitis. *Australian Veterinary Journal*,
579 *61*(9), 282–284. https://doi.org/10.1111/j.1751-0813.1984.tb06010.x
580 Catalano, M., & O'Driscoll, L. (2020). Inhibiting extracellular vesicles formation and release: A
581 review of EV inhibitors. *Journal of Extracellular Vesicles*, *9*(1), 1703244.
582 https://doi.org/10.1080/20013078.2019.1703244
583 Coakley, G., McCaskill, J. L., Borger, J. G., Simbari, F., Robertson, E., Millar, M., Harcus, Y.,
584 McSorley, H. J., Maizels, R. M., & Buck, A. H. (2017). Extracellular Vesicles from a
585 Helminth Parasite Suppress Macrophage Activation and Constitute an Effective Vaccine for
586 Protective Immunity. *Cell Reports*, *19*(8), 1545–1557.
587 https://doi.org/10.1016/j.celrep.2017.05.001
588 Critchley, J., Addiss, D., Ejere, H., Gamble, C., Garner, P., & Gelband, H. (2005). Albendazole for
589 the control and elimination of lymphatic filariasis: Systematic review. *Tropical Medicine &*
590 *International Health*, *10*(9), 818–825. https://doi.org/10.1111/j.1365-3156.2005.01458.x
591 Eichenberger, R. M., Ryan, S., Jones, L., Buitrago, G., Polster, R., Montes de Oca, M., Zuvelek, J.,
592 Giacomin, P. R., Dent, L. A., Engwerda, C. R., Field, M. A., Sotillo, J., & Loukas, A.
593 (2018). Hookworm Secreted Extracellular Vesicles Interact With Host Cells and Prevent
594 Inducible Colitis in Mice. *Frontiers in Immunology*, *9*.
595 https://doi.org/10.3389/fimmu.2018.00850

596 Eichenberger, R. M., Sotillo, J., & Loukas, A. (2018). Immunobiology of parasitic worm
597 extracellular vesicles. *Immunology & Cell Biology*, *96*(7), 704–713.
598 https://doi.org/10.1111/imcb.12171
599 Eichenberger, R. M., Talukder, M. H., Field, M. A., Wangchuk, P., Giacomin, P., Loukas, A., &
600 Sotillo, J. (2018). Characterization of Trichuris muris secreted proteins and extracellular
601 vesicles provides new insights into host–parasite communication. *Journal of Extracellular*
602 *Vesicles*, *7*(1), 1428004. https://doi.org/10.1080/20013078.2018.1428004
603 Geary, Timothy G., Sims, S. M., Thomas, E. M., Vanover, L., Davis, J. P., Winterrowd, C. A.,
604 Klein, R. D., Ho, N. F. H., & Thompson, D. P. (1993). Haemonchus contortus: Ivermectin-
605 Induced Paralysis of the Pharynx. *Experimental Parasitology*, *77*(1), 88–96.
606 https://doi.org/10.1006/expr.1993.1064
607 Geary, Timothy G., Woo, K., McCarthy, J. S., Mackenzie, C. D., Horton, J., Prichard, R. K., de
608 Silva, N. R., Olliaro, P. L., Lazdins-Helds, J. K., Engels, D. A., & Bundy, D. A. (2010).
609 Unresolved issues in anthelmintic pharmacology for helminthiases of humans. *International*
610 *Journal for Parasitology*, *40*(1), 1–13. https://doi.org/10.1016/j.ijpara.2009.11.001
611 González Canga, A., Sahagún Prieto, A. M., Diez Liébana, M. J., Fernández Martínez, N., Sierra
612 Vega, M., & García Vieitez, J. J. (2008). The Pharmacokinetics and Interactions of
613 Ivermectin in Humans—A Mini-review. *The AAPS Journal*, *10*(1), 42–46.
614 https://doi.org/10.1208/s12248-007-9000-9
615 Gu, H. Y., Marks, N. D., Winter, A. D., Weir, W., Tzelos, T., McNeilly, T. N., Britton, C., &
616 Devaney, E. (2017). Conservation of a microRNA cluster in parasitic nematodes and
617 profiling of miRNAs in excretory-secretory products and microvesicles of Haemonchus
618 contortus. *PLOS Neglected Tropical Diseases*, *11*(11), e0006056.
619 https://doi.org/10.1371/journal.pntd.0006056
620 Halliday, A., Guimaraes, A. F., Tyrer, H. E., Metuge, H. M., Patrick, C. N. W., Arnaud, K.-O. J.,
621 Kwenti, T. D. B., Forsbrook, G., Steven, A., Cook, D., Enyong, P., Wanji, S., Taylor, M. J.,

& Turner, J. D. (2014). A murine macrofilaricide pre-clinical screening model for onchocerciasis and lymphatic filariasis. *Parasites & Vectors*, *7*(1), 472. https://doi.org/10.1186/s13071-014-0472-z

Hansen, Eline P., Fromm, B., Andersen, S. D., Marcilla, A., Andersen, K. L., Borup, A., Williams, A. R., Jex, A. R., Gasser, R. B., Young, N. D., Hall, R. S., Stensballe, A., Ovchinnikov, V., Yan, Y., Fredholm, M., Thamsborg, S. M., & Nejsum, P. (2019). Exploration of extracellular vesicles from Ascaris suum provides evidence of parasite–host cross talk. *Journal of Extracellular Vesicles*, *8*(1), 1578116. https://doi.org/10.1080/20013078.2019.1578116

Hansen, Eline P., Kringel, H., Williams, A. R., & Nejsum, P. (2015). SECRETION OF RNA-CONTAINING EXTRACELLULAR VESICLES BY THE PORCINE WHIPWORM, TRICHURIS SUIS. *The Journal of Parasitology*, *101*(3), 336–340. JSTOR.

Harischandra, H., Yuan, W., Loghry, H. J., Zamanian, M., & Kimber, M. J. (2018). Profiling extracellular vesicle release by the filarial nematode Brugia malayi reveals sex-specific differences in cargo and a sensitivity to ivermectin. *PLOS Neglected Tropical Diseases*, *12*(4), e0006438. https://doi.org/10.1371/journal.pntd.0006438

Harnett, M. M., Deehan, M. R., Williams, D. M., & Harnett, W. (1998). Induction of signalling anergy via the T-cell receptor in cultured Jurkat T cells by pre-exposure to a filarial nematode secreted product. *Parasite Immunology*, *20*(11), 551–563. https://doi.org/10.1046/j.1365-3024.1998.00181.x

Harrow, I. D., & Gration, K. A. F. (1985). Mode of action of the anthelmintics morantel, pyrantel and levamisole on muscle cell membrane of the nematode Ascaris suum. *Pesticide Science*, *16*(6), 662–672. https://doi.org/10.1002/ps.2780160612

Hewitson, J. P., Grainger, J. R., & Maizels, R. M. (2009). Helminth immunoregulation: The role of parasite secreted proteins in modulating host immunity. *Molecular and Biochemical Parasitology*, *167*(1), 1–11. https://doi.org/10.1016/j.molbiopara.2009.04.008

Hewitson, J. P., Harcus, Y. M., Curwen, R. S., Dowle, A. A., Atmadja, A. K., Ashton, P. D., Wilson, A., & Maizels, R. M. (2008). The secretome of the filarial parasite, Brugia malayi: Proteomic profile of adult excretory–secretory products. *Molecular and Biochemical Parasitology*, *160*(1), 8–21. https://doi.org/10.1016/j.molbiopara.2008.02.007

Hoerauf, A., Satoguina, J., Saeftel, M., & Specht, S. (2005). Immunomodulation by filarial nematodes. *Parasite Immunology*, *27*(10–11), 417–429. https://doi.org/10.1111/j.1365-3024.2005.00792.x

Hoy, A. M., Lundie, R. J., Ivens, A., Quintana, J. F., Nausch, N., Forster, T., Jones, F., Kabatereine, N. B., Dunne, D. W., Mutapi, F., MacDonald, A. S., & Buck, A. H. (2014). Parasite-derived microRnas in host serum as novel biomarkers of helminth infection. *PLoS Neglected Tropical Diseases*, *8*(2). Gale Academic OneFile. https://link.gale.com/apps/doc/A364854680/AONE?u=iastu_main&sid=zotero&xid=72d7c0d1

Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., & Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *Journal of Biological Chemistry*, *262*(19), 9412–9420.

Kaushal, N. A., Hussain, R., Nash, T. E., & Ottesen, E. A. (1982). Identification and characterization of excretory-secretory products of Brugia malayi, adult filarial parasites. *The Journal of Immunology*, *129*(1), 338–343.

Kosaka, N., Kogure, A., Yamamoto, T., Urabe, F., Usuba, W., Prieto-Vila, M., & Ochiya, T. (2019). Exploiting the message from cancer: The diagnostic value of extracellular vesicles for clinical applications. *Experimental & Molecular Medicine*, *51*(3), 1–9. https://doi.org/10.1038/s12276-019-0219-1

Kotze, A. C., & Prichard, R. K. (2016). Chapter Nine - Anthelmintic Resistance in Haemonchus contortus: History, Mechanisms and Diagnosis. In R. B. Gasser & G. V. Samson-

673 Himmelstjerna (Eds.), *Advances in Parasitology* (Vol. 93, pp. 397–428). Academic Press.
674 https://doi.org/10.1016/bs.apar.2016.02.012
675 Lal, R. B., Kumaraswami, V., Steel, C., & Nutman, T. B. (1990). Phosphocholine-Containing
676 Antigens of Brugia Malayi Nonspecifically Suppress Lymphocyte Function. *The American*
677 *Journal of Tropical Medicine and Hygiene*, *42*(1), 56–64.
678 https://doi.org/10.4269/ajtmh.1990.42.56
679 Lee, Y., EL Andaloussi, S., & Wood, M. J. A. (2012). Exosomes and microvesicles: Extracellular
680 vesicles for genetic information transfer and gene therapy. *Human Molecular Genetics*,
681 *21*(R1), R125–R134. https://doi.org/10.1093/hmg/dds317
682 Maclean, M. J., Savadelis, M. D., Coates, R., Dzimianski, M. T., Jones, C., Benbow, C., Storey, B.
683 E., Kaplan, R. M., Moorhead, A. R., & Wolstenholme, A. J. (2017). Does evaluation of in
684 vitro microfilarial motility reflect the resistance status of Dirofilaria immitis isolates to
685 macrocyclic lactones? *Parasites & Vectors*, *10*(2), 480. https://doi.org/10.1186/s13071-017-
686 2436-6
687 Marcellino, C., Gut, J., Lim, K. C., Singh, R., McKerrow, J., & Sakanari, J. (2012). WormAssay: A
688 Novel Computer Application for Whole-Plate Motion-based Screening of Macroscopic
689 Parasites. *PLOS Neglected Tropical Diseases*, *6*(1), e1494.
690 https://doi.org/10.1371/journal.pntd.0001494
691 Martin, R. J. (1997). Modes of action of anthelmintic drugs. *The Veterinary Journal*, *154*(1), 11–34.
692 https://doi.org/10.1016/S1090-0233(05)80005-X
693 Mehmet, K., R, M. E., Aysun, K., & Sebastian, W.-H. (2017). Fundamentals and applications of
694 SERS-based bioanalytical sensing. *Nanophotonics*, *6*(5), 831–852.
695 https://doi.org/10.1515/nanoph-2016-0174
696 Mills, J. N., & Amis, T. C. (1975). Levamisole as a Microfilaricidal Agent in the Control of Canine
697 Dirofilariasis. *Australian Veterinary Journal*, *51*(6), 310–314.
698 https://doi.org/10.1111/j.1751-0813.1975.tb06962.x

699 Moreno, Y., Nabhan, J. F., Solomon, J., Mackenzie, C. D., Geary, T. G., & Campbell, W. C.
700 (2010). Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae
701 of Brugia malayi. *Proceedings of the National Academy of Sciences of the United States of*
702 *America*, *107*(46), 20120–20125. JSTOR.
703 Mostafa, E., Storey, B., Farghaly, A. M., Afify, H. A. E.-H., Taha, A. A. E.-R., & Wolstenholme,
704 A. J. (2015). Transient effects of levamisole on Brugia malayi microfilariae. *Invertebrate*
705 *Neuroscience*, *15*(3), 5. https://doi.org/10.1007/s10158-015-0181-0
706 Pastrana, D. V., Raghavan, N., FitzGerald, P., Eisinger, S. W., Metz, C., Bucala, R., Schleimer, R.
707 P., Bickel, C., & Scott, A. L. (1998). Filarial Nematode Parasites Secrete a Homologue of
708 the Human Cytokine Macrophage Migration Inhibitory Factor. *Infection and Immunity*,
709 *66*(12), 5955–5963. https://doi.org/10.1128/IAI.66.12.5955-5963.1998
710 Quintana, J. F., Babayan, S. A., & Buck, A. H. (2017). Small RNAs and extracellular vesicles in
711 filarial nematodes: From nematode development to diagnostics. *Parasite Immunology*,
712 *39*(2), e12395. https://doi.org/10.1111/pim.12395
713 Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., &
714 Geuze, H. J. (1996). B lymphocytes secrete antigen-presenting vesicles. *Journal of*
715 *Experimental Medicine*, *183*(3), 1161–1172. https://doi.org/10.1084/jem.183.3.1161
716 Riaz, F., & Cheng, G. (2017). Exosome-like vesicles of helminths: Implication of pathogenesis and
717 vaccine development. *Annals of Translational Medicine*, *5*(7), 22.
718 https://doi.org/10.21037/atm.2017.03.45
719 Robertson, A. P., Buxton, S. K., & Martin, R. J. (2013). Whole-cell patch-clamp recording of
720 nicotinic acetylcholine receptors in adult Brugia malayi muscle. *Parasitology International*,
721 *62*(6), 616–618. https://doi.org/10.1016/j.parint.2013.03.008
722 Semnani, R. T., Keiser, P. B., Coulibaly, Y. I., Keita, F., Diallo, A. A., Traore, D., Diallo, D. A.,
723 Doumbo, O. K., Traore, S. F., Kubofcik, J., Klion, A. D., & Nutman, T. B. (2006). Filaria-

724 Induced Monocyte Dysfunction and Its Reversal following Treatment. *Infection and*
725 *Immunity*, *74*(8), 4409–4417. https://doi.org/10.1128/IAI.01106-05
726 Shears, R. K., Bancroft, A. J., Hughes, G. W., Grencis, R. K., & Thornton, D. J. (2018).
727 Extracellular vesicles induce protective immunity against Trichuris muris. *Parasite*
728 *Immunology*, *40*(7), e12536. https://doi.org/10.1111/pim.12536
729 Storey, B., Marcellino, C., Miller, M., Maclean, M., Mostafa, E., Howell, S., Sakanari, J.,
730 Wolstenholme, A., & Kaplan, R. (2014). Utilization of computer processed high definition
731 video imaging for measuring motility of microscopic nematode stages on a quantitative
732 scale: "The Worminator." *International Journal for Parasitology: Drugs and Drug*
733 *Resistance*, *4*(3), 233–243. https://doi.org/10.1016/j.ijpddr.2014.08.003
734 Thakur, A., Qiu, G., Ng, S.-P., Guan, J., Yue, J., Lee, Y., & Wu, C.-M. L. (2017). Direct detection
735 of two different tumor-derived extracellular vesicles by SAM-AuNIs LSPR biosensor.
736 *Biosensors and Bioelectronics*, *94*, 400–407. https://doi.org/10.1016/j.bios.2017.03.036
737 Thery, C., Zitvogel, L., & Amigorena, S. (2002). Exosomes: Composition, biogenesis and function.
738 *Nature Reviews Immunology*, *2*(8), 569-. Gale Academic OneFile.
739 Tompkins, J. B., Stitt, L. E., & Ardelli, B. F. (2010). Brugia malayi: In vitro effects of ivermectin
740 and moxidectin on adults and microfilariae. *Experimental Parasitology*, *124*(4), 394–402.
741 https://doi.org/10.1016/j.exppara.2009.12.003
742 Tritten, L., Burkman, E., Moorhead, A., Satti, M., Geary, J., Mackenzie, C., & Geary, T. (2014).
743 Detection of circulating parasite-derived microRNAs in filarial infections. *PLoS Neglected*
744 *Tropical Diseases*, *8*(7). Gale Academic OneFile.
745 https://link.gale.com/apps/doc/A383328488/AONE?u=iastu_main&sid=zotero&xid=960749
746 0a
747 Tritten, L., Clarke, D., Timmins, S., McTier, T., & Geary, T. G. (2016). Dirofilaria immitis exhibits
748 sex- and stage-specific differences in excretory/secretory miRNA and protein profiles.
749 *Veterinary Parasitology*, *232*, 1–7. https://doi.org/10.1016/j.vetpar.2016.11.005

750 Tritten, L., Tam, M., Vargas, M., Jardim, A., Stevenson, M. M., Keiser, J., & Geary, T. G. (2017).
751 Excretory/secretory products from the gastrointestinal nematode Trichuris muris.
752 *Experimental Parasitology*, *178*, 30–36. https://doi.org/10.1016/j.exppara.2017.05.003
753 Tzelos, T., Matthews, J. B., Buck, A. H., Simbari, F., Frew, D., Inglis, N. F., McLean, K., Nisbet,
754 A. J., Whitelaw, C. B. A., Knox, D. P., & McNeilly, T. N. (2016). A preliminary proteomic
755 characterisation of extracellular vesicles released by the ovine parasitic nematode,
756 Teladorsagia circumcincta. *Veterinary Parasitology*, *221*, 84–92.
757 https://doi.org/10.1016/j.vetpar.2016.03.008
758 Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J. J., & Lotvall, J. O. (2007). Exosome-
759 mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange
760 between cells. *Nature Cell Biology*, *9*(6), 654-. Gale Academic OneFile.
761 van Riet, E., Hartgers, F. C., & Yazdanbakhsh, M. (2007). Chronic helminth infections induce
762 immunomodulation: Consequences and mechanisms. *Immunobiology*, *212*(6), 475–490.
763 https://doi.org/10.1016/j.imbio.2007.03.009
764 Vlassov, A. V., Magdaleno, S., Setterquist, R., & Conrad, R. (2012). Exosomes: Current knowledge
765 of their composition, biological functions, and diagnostic and therapeutic potentials.
766 *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1820*(7), 940–948.
767 https://doi.org/10.1016/j.bbagen.2012.03.017
768 Wang, Y., Yuan, W., Kimber, M., Lu, M., & Dong, L. (2018). Rapid Differentiation of Host and
769 Parasitic Exosome Vesicles Using Microfluidic Photonic Crystal Biosensor. *ACS Sensors*,
770 *3*(9), 1616–1621. https://doi.org/10.1021/acssensors.8b00360
771 Wolstenholme, Adrian J., Maclean, M. J., Coates, R., McCoy, C. J., & Reaves, B. J. (2016). How
772 do the macrocyclic lactones kill filarial nematode larvae? *Invertebrate Neuroscience*, *16*(3),
773 7. https://doi.org/10.1007/s10158-016-0190-7

774 Wolstenholme, Adrian J., & Rogers, A. T. (2005). Glutamate-gated chloride channels and the mode
775 of action of the avermectin/milbemycin anthelmintics. *Parasitology*, *131*(S1), S85–S95.
776 https://doi.org/10.1017/S0031182005008218
777 World Health Organization. (2019). *World Health Organization model list of essential medicines:*
778 *21st list 2019* (WHO/MVP/EMP/IAU/2019.06). Article WHO/MVP/EMP/IAU/2019.06.
779 https://apps.who.int/iris/handle/10665/325771
780 World Health Organization. (2019). Global programme to eliminate lymphatic filariasis: Progress
781 report, 2018. *Weekly Epidemiological Record*, *94*(41), 457–472.
782 Zamanian, M., Fraser, L. M., Agbedanu, P. N., Harischandra, H., Moorhead, A. R., Day, T. A.,
783 Bartholomay, L. C., & Kimber, M. J. (2015). Release of Small RNA-containing Exosome-
784 like Vesicles from the Human Filarial Parasite Brugia malayi. *PLoS Neglected Tropical*
785 *Diseases*, *9*(9). https://doi.org/10.1371/journal.pntd.0004069
786 Zong, S., Wang, L., Chen, C., Lu, J., Zhu, D., Zhang, Y., Wang, Z., & Cui, Y. (2016). Facile
787 detection of tumor-derived exosomes using magnetic nanobeads and SERS nanoprobes.
788 *Analytical Methods*, *8*(25), 5001–5008. https://doi.org/10.1039/C6AY00406G
789

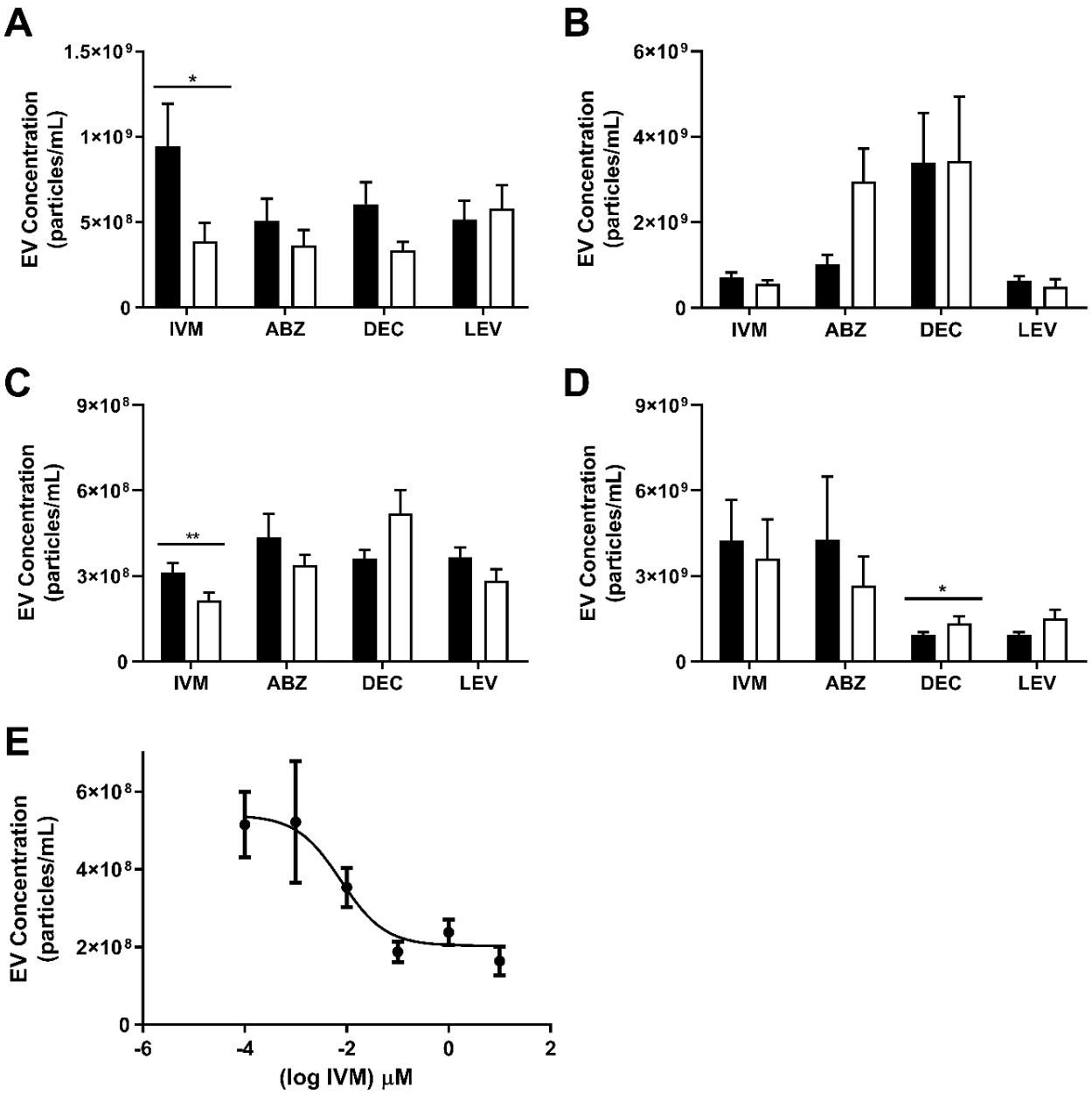


Figure 1. Ivermectin inhibits *Brugia malayi* EV secretion in a stage- and sex- specific manner

B. malayi life stages were cultured at 37°C in RPMI with either drug (1.0 μM) or DMSO (vehicle control). Media was collected after 24 hrs and EVs were isolated and quantified. 1.0 μM IVM significantly reduced EV secretion from adult female worms (A) and L3 stage parasites (C) but not from adult males (B) or microfilaria (D). Albendazole (ABZ), diethylcarbamazine (DEC) or levamisole (LEV) had no effect on EV secretion from any life stage except in microfilaria, where DEC increased EV secretion. (E) The IC_{50} for IVM on adult female worms was determined to be 7.7 nM. N = 7 (minimum), Mean ± SEM, *P<0.05, **P<0.01. ■ = DMSO control, □ = Treatment

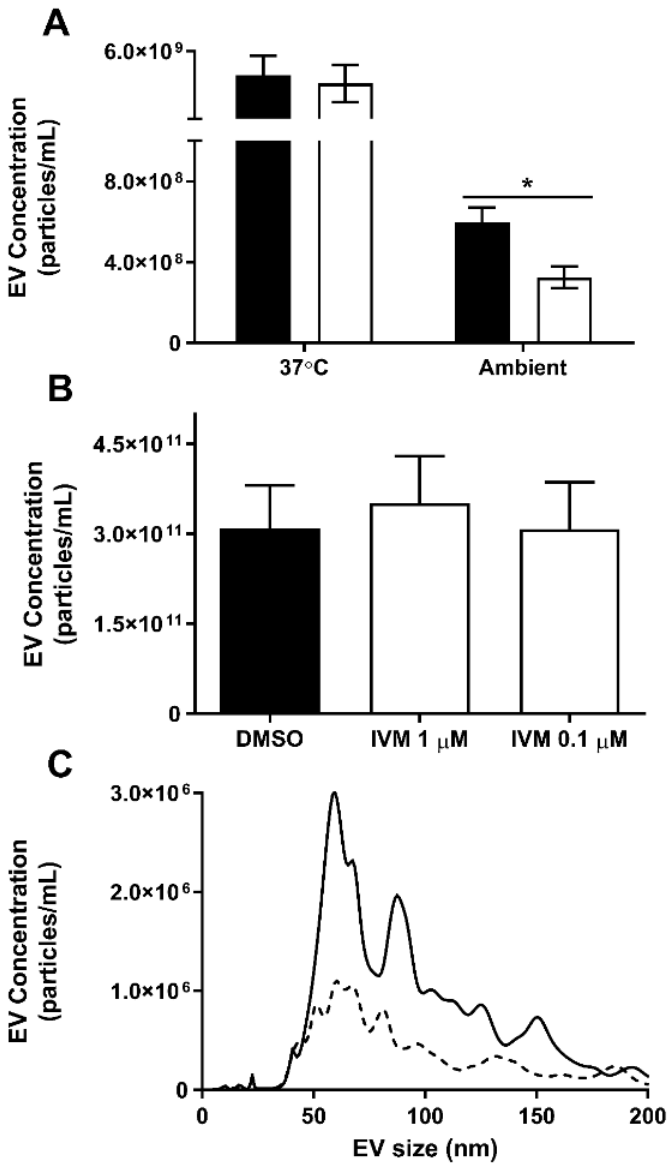


Figure 2. Ivermectin activity against mf stage worms is temperature dependent and does not alter EV biogenesis

B. malayi life stages were cultured at 37°C in RPMI with either drug (1.0 µM) or DMSO (vehicle control). Media was collected after 24 hrs and EVs were isolated and quantified. The activity of IVM on *B. malayi* microfilariae was temperature dependent (A). Inhibition of EV secretion occurred at ambient temperature, but not at 37°C. To investigate IVM mechanism of inhibition Sf21 cells were treated with either DMSO, high (1.0 µM) or low (0.1µM) concentrations of IVM. Spent media was collected after 24 hrs and EVs were collected and quantified. (B) there were no differences in the ability of DMSO control, high, or low concentrations of IVM treated cells to secrete EVs. To further investigate the potential effect on biogenesis the size of EVs secreted from adult female parasites were evaluated (C). The mean size of EVs secreted from adult females was highly similar between DMSO and IVM treated parasites. N = 3 (minimum). *P<0.05. ■ = DMSO, □ = Treatment. Solid line = DMSO, Dashed line = IVM.

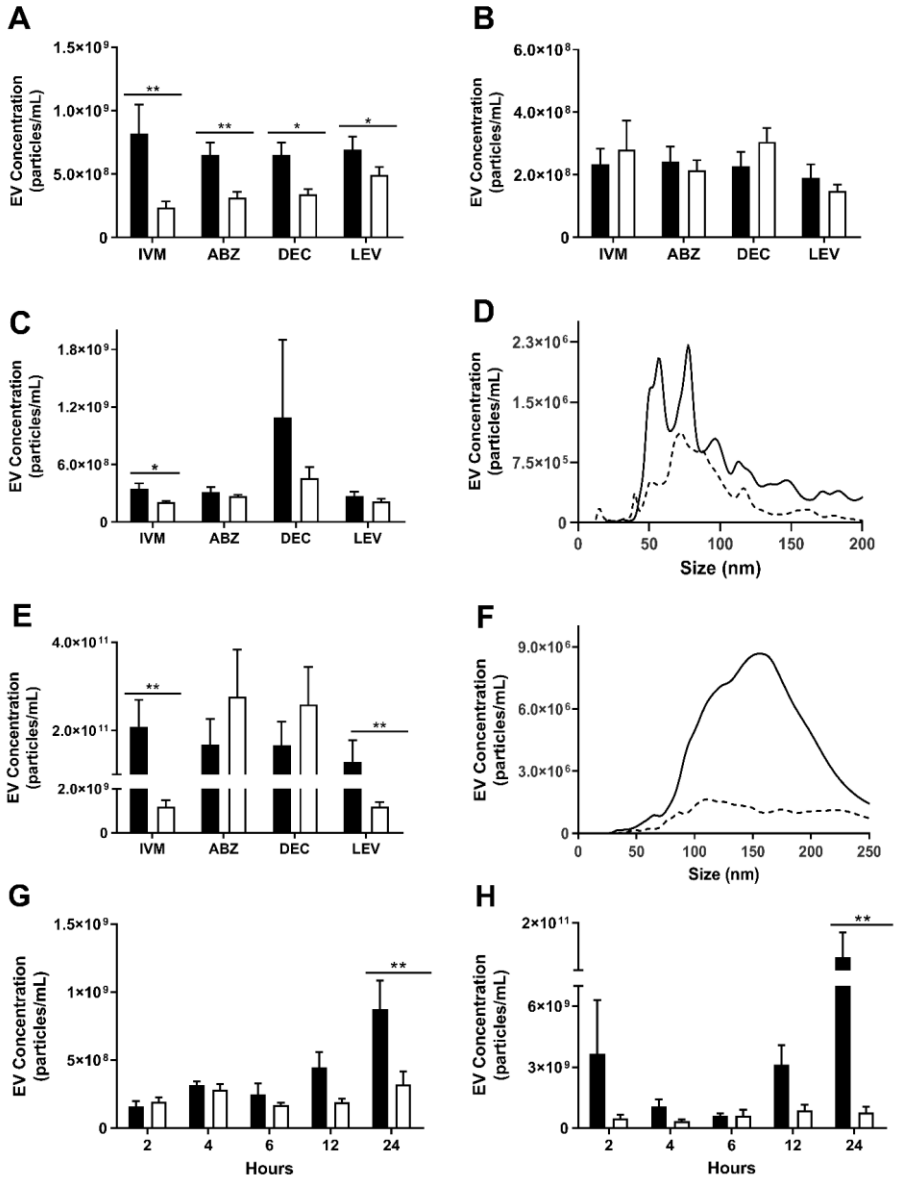
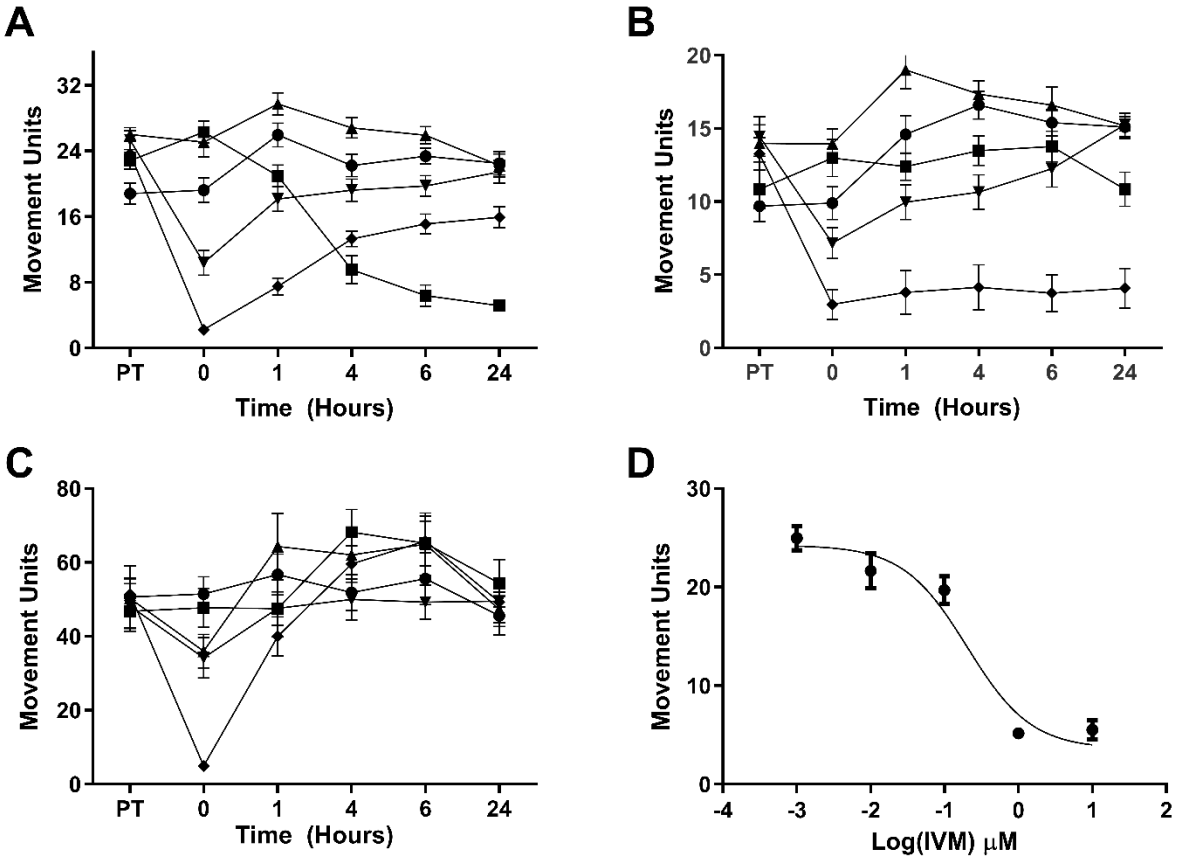


Figure 3. Ivermectin has broad inhibitory effects across filarial and gastrointestinal parasites *B. pahangi* life stages in RPMI and adult female *A. suum* in *Ascaris* Ringers solution were cultured at 37°C with either drug (1.0 uM) or DMSO (vehicle control). Media was collected after 24 hrs and EVs were isolated and quantified. 1.0 µM IVM, ABZ, DEC, and LEV all significantly inhibited EV secretion from *B. pahangi* adult female parasites (A) while only IVM significantly reduced EV secretion in the microfilariae life stage (C). No treatment had any effect on EV secretion from *B. pahangi* adult male parasites (B). For *A. suum* adult females, both IVM and LEV significantly inhibited EV secretion (E). The size distribution of EVs secreted from DMSO and IVM treated *B. pahangi* adult females (D) and *A. suum* adult females (F) were highly similar indicating that IVM does not affect the physical characteristics of EVs produced (F). IVM rapidly inhibits EV secretion from adult female *B. pahangi* and *A. suum* 24 hrs post-treatment (G-H). N = 3 (minimum). Mean ± SEM, *P<0.05, **P<0.01. ■ = DMSO, □ = Treatment. Solid line = DMSO, Dashed line = IVM.

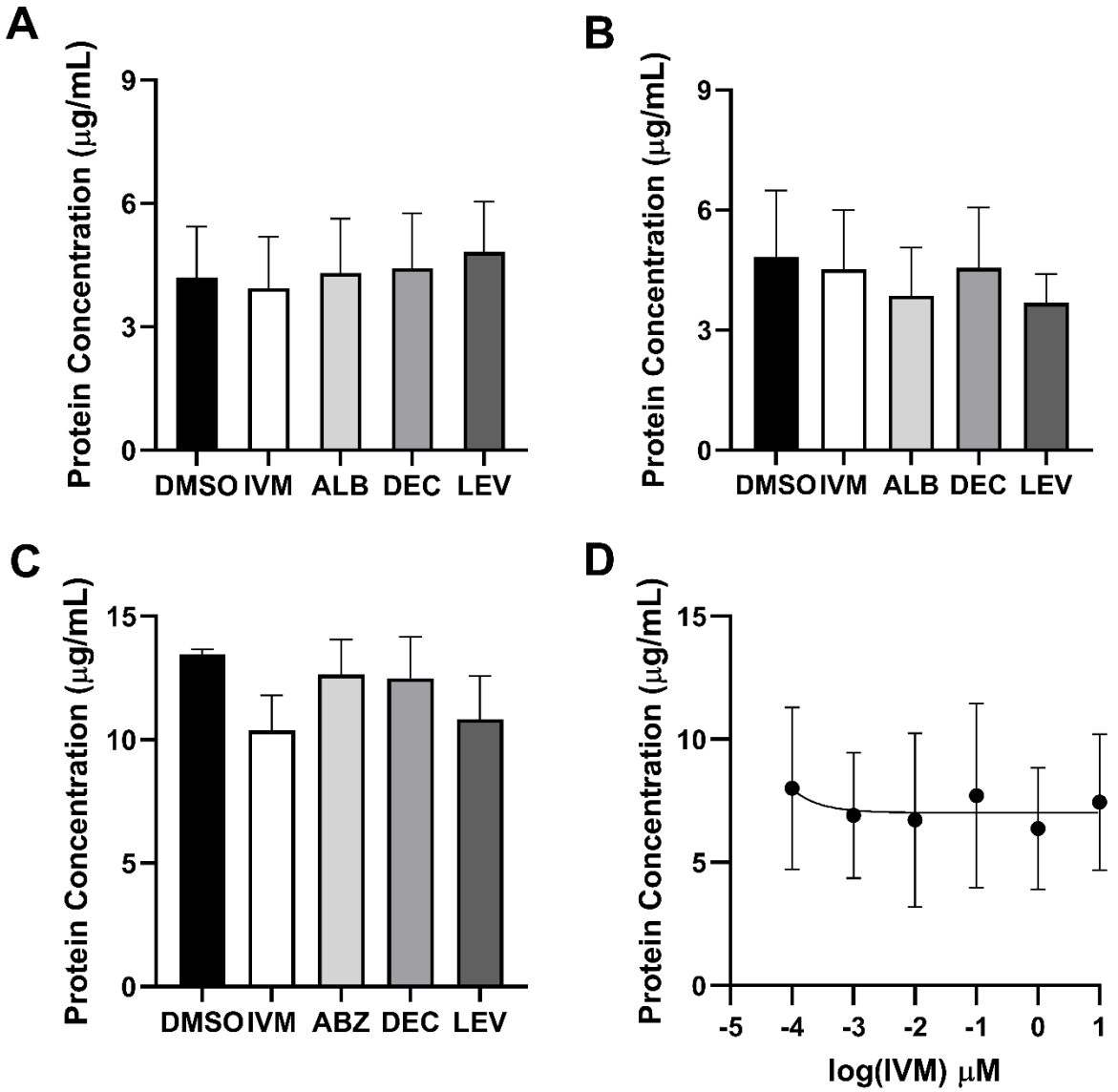


826

827 **Figure 4. Ivermectin inhibition of EV secretion is not driven by loss of gross motor function**

828 Single adult or 10 L3 stage *Brugia malayi* parasites were cultured in a 24-well plate with either 1.0
829 μM drug or DMSO (vehicle control). Video recordings of worms were taken at timepoints ranging
830 from pre-treatment to 24 hrs using the Worminator system. (A) IVM significantly reduced adult
831 female *B. malayi* motility as compared to control from 4-24 hrs post treatment ($p<0.0001$).
832 However, further investigation revealed that more therapeutically relevant concentrations of IVM
833 did not affect adult female motility. The IC_{50} for IVM on adult female parasites was determined to
834 be 203 nM. (D). DEC significantly reduced adult female motility immediately upon treatment and
835 one hour post treatment ($p<0.001$). LEV significantly reduced adult female motility from 0-24 hrs
836 ($p<0.0001$-$p<0.01$) though the parasites began to recover after initial treatment. (B) DEC
837 significantly reduced adult male motility from one to four hours post treatment ($p<0.05$, $p<0.01$)
838 and IVM began to reduce motility at 24 hrs post treatment ($p<0.05$). LEV significantly reduced
839 motility with no recovery from 0-24 hrs post treatment ($p<0.0001$). (C) LEV significantly reduced
840 L3 stage motility immediately upon treatment ($p<0.0001$), but the parasites quickly recovered
841 within one hr. N = 11 (minimum), Mean ± SEM. ● = DMSO, ■ = IVM, ▲ = ABZ, ▼ = DEC, ♦ =
842 LEV

843



844

845 **Figure 5. EV and protein secretion are differentially affected by ivermectin**

846 Single adult or 100,000 microfilariae *Brugia malayi* parasites were cultured per well of a 24-well
847 plate with either drug (1.0 µM) or DMSO (0.01%) for 24 hrs. Spent media was collected and
848 proteins were concentrated and washed. Protein concentration was determined by absorbance at 562
849 nm. No drug had any effect on protein secretion from *B. malayi* adult females (A). *B. malayi* adult
850 males (B) had a decrease in protein secretion due to LEV though not statistically significant.
851 Microfilariae protein secretion (C) was inhibited by ivermectin ($p = 0.0535$). (D) A dose response
852 curve for IVM on adult female parasites showed no effect from any concentration of IVM (10 µM –
853 0.1 nM). N = 5 (minimum), Mean ± SEM.

854