Characterization of Secretory Processes and the Secretome of Parasitic Nematodes

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July, 2011

A thesis submitted to Graduate and Postdoctoral Studies office in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Abstract

Relatively little is known about the molecular mechanisms displayed by parasitic nematodes to infect a host; however, it has been generally recognized that successful parasitic nematode infections rely on their ability to release a variety of products commonly named Excretory-Secretory Products (ESP). To gain a deeper understanding of the mechanisms that lead to the establishment of filarial nematode infections, we collected and analyzed through 1D-SDS PAGE and LC-MS/MS the ESP of *Brugia malayi* adult females, adult males and microfilariae, one of the etiological agents of human lymphatic filariasis. 228 proteins were identified through this approach, including several proteins with potential immunoregulatory properties. Subsequent work using an immunohistochemical approach allowed for the definition of 3 anatomical expression patterns in *B. malayi* microfilariae for a representative group of 5 ESP. All of these patterns involved localization in the microfilarial Excretory-Secretory apparatus, a specialized anatomical feature involved in protein release that in this life stage was found to be associated to a muscle structure. Glutamate-gated chloride channels (GluCls), the main target of the antiparasitic drug ivermectin (IVM), were also located at this structure, suggesting that protein release from the Excretory-Secretory apparatus is enhanced by neuromuscular activity regulated by GluCls. Consistent with these observations, a marked reduction in protein release by microfilariae upon *in vitro* IVM exposure was shown. It is proposed that under *in vivo* conditions, the rapid microfilarial clearance induced by IVM treatment is the result of the suppression of the parasite's ability to secrete proteins that enable evasion of the host immune system. Finally, it is anticipated that elucidation of specific features associated with each of the different parasitic nematode lifestyles would require the comparison of ESP composition from several nematode species. To overcome the limitations associated with the lack of sequence information in most nematode species required for the database searching strategy in MS-based proteomic analysis, the use of transcriptomic next-generation sequencing (RNA-seq) *de novo* assemblies is explored to identify the ESP composition of the mouse gastrointestinal (GI) parasitic nematode *Heligmosomoides polygyrus*. 209 proteins were identified using this strategy. The list also includes proteins with potential involvement in immunoregulation, modulation of

signalling pathways and nutrient transport and/or uptake. The results presented here are useful to understand the roles of proteins released by filarial and GI nematodes in immune evasion events and other aspects of the host-parasite relationship.

Abrégé

Les mécanismes moléculaires déployés par les parasites pour s'établir dans leur hôtes sont relativement méconnus; néanmoins, il est généralement accepté que le succès des infections par les nématodes parasitaires dépend de leur capacité à libérer une variété de produits dénommés produits d'excrétion-sécrétion (PES). Afin d'acquérir une meilleure connaissance des mécanismes qui conduisent à l'établissement d'infections de nématodes filaires, nous avons recueilli et analysé par électrophorèse sur gel de polyacrylamide (1D-SDS-PAGE) et par chromatographie liquide couplée à la spectrométrie de mases (LC-MS/MS) les PES des adultes mâles et femelles et des microfilaires de *Brugia malayi*, l'un des agents étiologique de la filariose lymphatique humaine. Grâce à cette approche, 228 protéines ont été identifiées, incluant plusieurs protéines possédant potentiellement des propriétés immunorégulatrices. Des travaux ultérieurs utilisant une approche immunohistochimique ont permis de définir trois profils d'expression anatomique pour un groupe représentatif de 5 PES chez les microfilaires de *B. malayi*. Pour tous ces profils, les protéines ont été localisées au niveau de l'appareil d'excrétion-sécrétion des microfilaires. Cette structure anatomique spécialisée est impliquée dans la libération de protéines et, chez ce stade de développement du parasite, elle est associée à une structure musculaire. Nous avons aussi montré que les canaux chloriques glutamate-dépendants (GluCls), la cible principale du médicament antiparasitaire ivermectine (IVM), étaient également localisés dans cette structure, ce qui suggère que la libération de protéines par l'appareil d'excrétion-sécrétion est renforcée par l'activité neuromusculaire régulée par des GluCls. Ces résultats concordent avec l'observation *in vitro* d'une réduction marquée de la libération de protéines par les microfilaires exposés à l'IVM. Il est proposé que, dans des conditions *in vivo*, l'élimination rapide des microfilaires induite par traitement avec l'IVM est causée par la suppression de la capacité du parasite à sécréter des protéines lui permettant d'évader le système immunitaire de l'hôte. Finalement, il est prévu que l'élucidation des caractéristiques spécifiques associées à chacun des différents modes de vie des nématodes parasitaires requerrait la comparaison de la composition des PES de plusieurs espèces de nématodes. Ces comparaisons, qui requièrent l'emploi d'une stratégie de recherche des résultats des MS générés lors des analyses protéomiques sur

des banques de données des protéines, sont fortement limitées par l'absence d'information sur les séquences pour la plupart des espèces de nématodes. Pour contourner ces limitations, nous avons essayé l'utilisation des assemblages *de novo* du séquençage transcriptomique de nouvelle génération (RNA-seq) afin d'identifier la composition des PES du nématode gastrointestinal (GI) chez la souris *Heligmosomoides polygyrus*. 209 protéines ont été identifiées en utilisant cette stratégie. La liste comprend aussi des protéines ayant une implication potentielle dans l'immunorégulation, la modulation des voies de signalisation et le transport et/ou l'absorption de nutriments. Les résultats présentés dans cette thèse permettent de mieux comprendre le rôle des protéines libérées par des nématodes filaires et GI dans les événements d'évasion immunitaire et dans d'autres aspects de la relation hôte-parasite.

Acknowledgments

I feel a great sense of personal accomplishment and proud for the final output of my PhD studies. There is no doubt that I have certainly fulfilled all the expectations I had when deciding pursuing these goals. I am conscious that it would not be possible without the great environment that has surrounded me during these years of study. I want to acknowledge all the persons that provided it and who finally helped me and contributed for the realization of this thesis.

I am immensely grateful with Prof. Timothy G. Geary for his mentorship, friendship and support. In simple words, Prof. Geary IS great. It has been an amazing privilege to learn from him and a truly honour to be his very first PhD student at McGill. Prof. Geary engagement with both the personal and intellectual growth of each of his students along with his astonishing ability to approach and handle all kind of situations in a constructive manner, are facts that I deeply admire from him. I will always appreciate his kindness, sense of humour, generosity and always positive attitude towards life and research.

Also, I would like to thank both the former and current members of the Geary-Beech Lab for providing me with such a great environment to work, learn and have fun: John Lee, Rushini Perera, Che Hua, Dr. Walter Tita, Vicki Muise, Karine Sonzogni, Dr. Lucien Rufener, Coby Stals, George Hr Woo. I want to specially thank Sergei Dernovici, Dr. Anne Schwab-Gaucher, Jim Geary, Karen Geukers, Vanessa Dufour, Dr. Joseph Nabhan, Jonathan Solomon, and Elizabeth Ruiz-Lancheros from which I have shared the lab for long hours for their friendship, help and continuous support. I am also grateful with Serge for assisting me in the acquisition of the confocal microscopy images that I present in this work and with Vanessa for helping me with the translation of the abstract to French.

I am also grateful with all the faculty, staff and students of the Institute of Parasitology. Special mention goes to Dr. Robin Beech, Barkha Bhatnagar, Gordon Bingham, Dr. Catherine Bourguinat, Dr. Celia Caballero-Franco, Norm Cyr, Dr. Florence Dzierszinski, Jenny Eng, Dr. Fouad El-Shehabi, Kathy Keller, Dr. Armando Jardim, Shirley Mongeau,

Dr. Maria de Lourdes Mottier, Nick Patocka, Dr. Roger Prichard, Dr. Paula Ribeiro, Dr. Reza Salavati, Dr. Rita Schildknecht, Dr. James Smith, Mary Solana, Rona Strasser, Christiane Trudeau, Dr. Vijay Rao, Marie-Claire Rioux for their friendship, help, assistance, continuous input and advice.

I want also to acknowledge Prof. Mary M. Stevenson and her research group for involving me in the project aiming for the identification of the excretory-secretory products from *Heligmosomoides polygyrus*. This project was a continuous source of new and exciting challenges and the opportunity to learn and interact with a group of scientist with different interests and backgrounds from which I have been normally exposed.

The advice and technical support of from the proteomics and sequencing platforms at the McGill University Genome Quebec Innovation Centre was greatly appreciated: Daniel Boismenu, Lyne Roy, Dr. Silvie LaBoissière, Nathalie Hamel and Louis Letourneau. Likewise, the advice and training of Dr. Elke Küster-Schöck who helped me during the running of the 2D-difference gel electrophoresis experiments at the Cell Imaging and Analysis Network (Dept Biology).

I also want to thank my family and friends in Colombia and Canada for their constant support. Finalmente, quiero dedicar este trabajo a mi mamá, Graciela, a mi hermana, Sandra y a mi esposa, Zaava. Mi mamá y mi hermana me han enseñado a través de su constante ejemplo el valor detrás de la consecución de cualquier objetivo a partir de la honestidad y el trabajo fuerte. Mi madre ha hecho grandes esfuerzos para proveernos a mi hermana y a mí de una buena educación y para impulsarnos hacia la realización de nuestros planes de vida. Zaava ha compartido y me ha apoyado en esta y muchas otras aventuras de manera incondicional. Su inmenso amor y paciencia me han acompañado en cada momento y son una fuente inagotable de alegría, bienestar, enseñanzas y motivación. Ellas son las grandes bendiciones de mi vida.

Thanks to everybody!
YM

List of Abbreviations

2D-DiGE: Two-dimensional difference gel electrophoresis
ABZ: Albendazole
AAMΦ: Alternatively activated macrophages
APC: Antigen presenting cells
ASP: *Ancylostoma* secreted protein
CAP: Cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1 protein
cDNA: Complimentary DNA
CID: Collision induced dissociation
CLSM: Confocal scanning laser microscopy
COP: cargo-containing coat protein complex
CP: Chronic pathology
CPI: Cysteine protease inhibitor
CRISP: Cysteine-rich sperm protein
CTL: C-type lectin
DALY: Disability-adjusted life years
DAPI: 4',6'-diamidino-2-phenylindole
DC: Dendritic cells
DEC: Diethylcarbazamine
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
ER: Endoplasmic reticulum
IFN: Interferon
EN: Endemic normals
ENO: Enolase
ES: Excretory – Secretory
ESP: ES products
EST: Expressed sequence tag
ESV: ES vesicle
F: Female
FBPA: Fructose bisphosphate aldolase A
FAB: Fatty acid binding protein
FAR: Fatty acid and retinol binding protein
FDR: False discovery rate
FGF: Fibroblast growth receptor
FGP: Filarial genome project
GABA: γ-Aminobutyric acid
GAL: Galectin
GI: Gastrointestinal
GluCl: Glutamate-gated chloride channel
GMCSf: Granulocyte-macrophage colony-stimulating factor
GO: Gene ontology
GPELF: Global program for elimination of LF
GPX: Glutathione peroxidase
GT: Glutamyl transpeptidase
GST: Glutathione S-transferase
HHVI: Human hookworm vaccine initiative

IgG: Immunoglobulin G
IL: Interleukin
IVM: Ivermectin
L1: First stage larvae
L2: Second stage larvae
L3: Third stage larvae
L4: Fourth stage larvae
LC: Liquid chromatography
LF: Limphatic filariasis
LGIC: Ligand-gated ion channels
LLTP: Large lipid transfer protein
M: Male
Mb: Mebagase
MDA: Mass drug administration
MeOH: Methanol
Mf: Microfilariae
Mf+: Microfilaremic
Mf-: Amicrofilaremic, microfilariae free
MHC II: Major histocompatibility complex class II
MIF: Macrophage migration inhibitory factor
ML: Macrocyclic lactone
MS: Mass spectrometry
MS/MS: Tandem MS
MSP: Major sperm protein
MW: Molecular weight
MWCO: MW cut-off
m/z: Mass-to-charge
NAR: Nematode polyprotein allergens/antigens
NJ: Neighbour-joining
NDPK: Nucleoside diphosphate kinases
NQPCT: Prorated Query Count percentage
ORF: Open reading frame
PBS: Phosphate-buffered saline
PC: Phosphorylcholine
PCR: Polymerase chain reaction
PRX: Peroxiredoxin
PtdIns(4,5)P2: Phosphatidylinositol-4,5-bisphosphate
ROS: Reactive oxygen species
RNR: Ribonucleotide reductases
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE: soluble N-ethylmaleimide-sensitive fusion protein accessory protein receptors
SOD: Superoxide dismutase
SP: Signal peptide
SPN: Serine protease inhibitor
RACE: Rapid amplification of cDNA ends
RNA-seq: Transcriptome next generation sequencing
TBS: Tris-buffered saline
TCA: Trichloroacetic acid
TCTP: Translationally controlled tumor protein-like protein
TGF: Tumor growth factor
TGH: TGF homologue

Th: T helper cell

TLRs: Toll-like receptors

TM: Transmembrane

TNF: Tumor necrosis factor

TPI: Triose phosphate isomerase

Treg: Regulatory T cells

TRX: Thioredoxin

VAL: Venom allergen like

VEGF: Vascular endothelial growth factors

Vtg: Vitellogenin

Statement of Originality

Manuscript I. Moreno Y & Geary TG (2008). Stage- and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products. *PLoS Neglected Tropical Diseases* 2(10):e326

In this manuscript, the identification of the excretory-secretory products (ESP) from *Brugia malayi* was assessed using a proteomic approach. In 2008, Hewitson *et al* (*Mol Biochem Parasitol. (2008). 160(1):8-21*) also reported the identification of 80 proteins from co-cultured adult worms. Our work expanded these findings by presenting 228 identified proteins from independent incubations of the microfilarial stage as well as of female and male adult forms. In addition, functional annotations were compared between the different forms of the parasite, suggesting that the different forms of the parasite display different strategies for survival and immunoregulation.

Manuscript II. Moreno Y, Nabhan JF, Solomon J, Mackenzie CD and Geary TG (2010). Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*. *Proc Natl Acad Sci USA*. 107 (46): 20120 - 20125.

Ivermectin (IVM) is widely used in programs for the elimination of filariasis. The results presented in this manuscript suggest a mechanism to reconcile the rapid microfilaricidal activity observed *in vivo* with the lack of apparent *in vitro* effect of IVM on filarial parasites. The localization of glutamate-gate chloride channels in the excretory-secretory apparatus has not been reported in any other nematode. The association of this neurotransmitter with a muscle structure in the excretory-secretory apparatus suggests the requirement of neuromuscular activity to regulate parasite protein release. We associated the excretory-secretory apparatus with 3 different localization patterns obtained from the immunolocalization of 5 different *B.malayi* microfilarial ESP confirming this apparatus as the main anatomical pathway for protein release. This is also the first time that confocal laser microscopy on whole mounted microfilariae is presented on literature which provides a very convenient tool for visualization and subsequent inference of biological processes in these parasites. We determined the effect *in vitro* relevant IVM concentrations on total protein release by *B. malayi* microfilariae.

Manuscript III. Moreno Y, Gros PP, Tam M, Segura M, Valanparambil R, Geary TG and Stevenson MM (2011). Proteomic analysis of Excretory-Secretory Products of *Heligmosomoides polygyrus* assessed with next-generation sequence transcriptomic information. Accepted PLoS Neglected Tropical Diseases.

In this manuscript, a transcriptomic analysis as well as the identification of the ESP using a proteomic approach was assessed for the first time in the mouse gastrointestinal nematode *Heligmosomoides polygyrus*. We show the utility of transcriptomic next-generation sequencing *de novo* assemblies for database searches to asses protein identity from mass spectra data acquired during a proteomics experiment. The report of 209 proteins identified from this parasite along with their functional annotation expands the current knowledge on the mechanisms leading to survival and immunomodulation of GI nematodes in their host. The release of this assembly can be useful for the scientific community to initiate further molecular biology work related to this parasite.

Author's Contributions

The design and execution of experiments presented in this thesis were carried out by the author with the supervision of Prof. Timothy G. Geary. In the second manuscript, Dr. Joseph Nabhan and Jonathan Solomon contributed by raising and purification of antibodies against recombinant Bma-MIF-1, Bma-CPI-2, Bma-TCTP and, with the execution of some preliminary assays, for the immunolocalization of proteins in *Brugia malayi*. Additionally, Prof. Charles Mackenzie (Michigan State University) contributed with the design and analysis of data on this manuscript. In the third manuscript, Pierre-Paul Gros and Dr. Mifong Tam contributed with the purification and incubation of *Heligmosomoides polygyrus* as well as with the sample preparation for RNA extraction and proteomic analysis. Rajesh Valanparambil collaborated with the functional annotation of *H. polygirus* excreted-secreted proteins. Dr. Mariela Segura and Prof. Mary M. Stevenson also contributed with the design and analysis of the data of this manuscript.

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Chapter I

Introduction

Shaped by evolution, parasites are designed to invade a host and survive in this organism at least until they are ready to be transmited [1]. To succeed in infection, parasites must develop ways to reach the host, penetrate its tissues and avoid its defense system by deploying mechanisms to avoid or suppress host defense responses. The influence of the parasite on host immune function can be exerted over long periods of time, since many parasitic infections are not fatal and are able to remain within their host for years and even for life. This characteristic provides them particular advantages by giving enough time to guarantee both offspring production and host reproduction, maintaining the host population for subsequent generations of the parasite to infect [2].

While we lack a complete understanding of the molecular mechanisms by which parasites achieve protection from host immune responses, it is generally accepted that parasitic nematodes release a variety of products, primarily proteins, to infect a host by penetrating tissue barriers, to migrate through host tissues and to evade immune responses. The characteristics and functions of these products are diverse and must depend on, among other factors, the lifestyle of each parasite. Even though their importance for establishing and maintaining the host-parasite interaction is well accepted, in the case of parasitic nematodes, there is still much to know about the mechanisms by which these products regulate the immune system. However, recent advances in biochemistry, molecular biology and genomic and proteomic data acquisition have helped to give the first indications of some of the molecular bases of immune evasion by parasitic nematodes.

Several *in vitro* released proteins from these parasites (conventionally named Excretory/Secretory products - ESP), have been identified and characterized, particularly from *Brugia malayi*, an organism that causes human lymphatic filariasis and can also be maintained in the laboratory as a model. ESP from *Brugia* and other parasitic nematodes has been a field of study for many decades. Nevertheless, some information on the role of

these products in filarial parasitism has been recently obtained thanks to the functional identification of proteins through advances in culture techniques, analytical capabilities and assays of immune functional status.

The original central initiative of this work was the exploitation of the remarkable sensitivity of new proteomic tools with the availability of a completely sequenced genome [3] to identify proteins secreted *in vitro* by *B. malayi*. The results of this initial phase of the project constitute the first manuscript of this thesis. The identification of these proteins has allowed us and others to expand our knowledge on the biology of secretory processes in this organism and to establish a path for developing an understanding of how these parasite proteins function in immune evasion events.

In the second part of this thesis, we focused on the identification of the anatomical pathways involved in protein release from the first larval stage (the microfilariae) of this parasite. Simultaneously, as we developed methodologies for protein immunolocalization in this parasitic stage, we also became interested in the localization of glutamate-gated chloride channels (GluCls) in this particular stage. These channels are the putative target for ivermectin (IVM), a drug that is essential for filariasis control and elimination programs [4]. IVM acts by clearing the microfilariae (mf) from the host and suppressing the production and release of new mf from adult worms [5]; however, the apparent lack of *in vitro* effects on mf motility and/or viability of this drug as well as the dynamic of mf clearance suggested the possibility that IVM disrupts filarial processes that result in the modulation of the host immune system [6]. The results presented in this part provide experimental support for this hypothesis by linking two different biological processes: protein release and neuromuscular activity controlled by GluCls. This allowed us to gain new insights into the mode of action of IVM.

The final manuscript of this document shows how the experiences gained and methodologies developed during the characterization of *B. malayi* ESP can be applied for other parasitic nematode systems. In addition, the identification and comparison of ESP components from different parasitic nematode species might provide new clues for the

identification of both particular and more generalized features of nematode parasitism. In this case, we aimed for the identification of ESP from the parasitic nematode *Heligmosomoides polygyrus*. This parasite is able to establish chronic gastrointestinal infections in mice, making it an attractive model for the study of gastrointestinal nematode biology and the pathology associated with their chronic infections [7]. Contrary to *B. malayi*, there is no genome sequence information available for *H. polygyrus*; limiting to a considerable degree the protein identification process in which mass spectra data acquired during a proteomics experiment are used to interrogate *in silico* a database that contains the sequences of the proteins or homologues of this proteins present in the analyzed sample. We overcame this limitation by performing a transcriptome next generation sequencing (RNA-seq) analysis and employing the resulting assembly for the protein identification process. Methods and analyses employed in this part can be useful and affordable to unlock the study of biochemical and molecular aspects in species for which sequence information is not available.

Both filarial and gastrointestinal nematode parasitic infections are considerable sources of morbidity that continue to pose significant threats to human and animal health. The identification of ESP from species causing these diseases and the mechanisms of protein release to the host is a step forward toward understanding the molecular mechanisms that allow parasites to survive in their host. This is expected to contribute to the development of new and better therapeutic and diagnostic strategies.

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Chapter II
Literature Review

Lymphatic Filariasis

Lymphatic filariasis (LF) is a disabling and disfiguring parasitic disease caused by the developing and adult forms of filarial parasites residing in the lymphatic system. In humans, it is caused by the infection of any of three parasites belonging to two genera: *Wuchereria bancrofti*, *Brugia malayi* and *B. timori* [1]. The infection is most common in subtropical and tropical regions of the world with a total population at risk currently estimated to be 1334 million people in 81 LF-endemic countries [2]. Approximately 120 million people are infected, with 91% of the cases associated with *W. bancrofti* and the remaining with *B. malayi* [3,4]. *B. timori* replaces *B. malayi* in the eastern islands of the lesser Sunda archipelago (Nusa Tenggara Timur) [5].

The infection is transmitted by blood-feeding mosquitoes. *W. bancrofti* is primarily transmitted by *Culex quiquefasciatus* and by *Anopheles* spp. in urban areas and by *Aedes* spp. in rural ones. *Mansonia, Anopheles* and *Aedes* spp. are the main vectors of *Brugia* spp. infections [1].

Although LF is rarely life-threatening, it causes chronic suffering, social stigma and disability, primarily due to the development of elephantiasis in some infected individuals. With a morbidity estimated for 2004 at 5.9 million Disability Adjusted Life-Years (DALYs) (http://apps.who.int/ghodata/), it is considered the second leading cause of permanent and long-term disability world-wide, after mental illness. Disability resulting from infection greatly hinders the ability to work and the economic impact of this disease is considerable.

Clinical manifestations of the disease range from acute to chronic depending on the parasite specie, host immune status and geographical area. An acute stage of filariasis is

characterized by episodic adenolymphangitis, followed by obstructive lesions after one or more decades [6]. It is now recognized that recurrent bacterial infections are an essential factor for the development and progression of the chronic symptoms [7]. In Brugian filariasis, adenolymphangitis is most commonly observed at the inguinal region, and elephantiasis predominantly involves the leg below the knee. In bancroftian filariasis, the lymphatics of the male genitalia are frequently affected, leading to epididymo-orchitis and hydrocele [6].

For the treatment of LF, three drugs are currently used due to their effectiveness when administered on their own or in combination: Diethylcarbamazine (DEC), ivermectin (IVM) and albendazole (ABZ) [8]. The use of these drugs is a major part of strategies for interruption of the disease transmission and reduction of morbidity of the global program to eliminate LF (GPELF) [3]. General recommendations of GPELF include community directed mass treatment programs with combinations of ABZ (400 mg) and IVM (200 mg/kg) in areas endemic for onchocerciasis or elsewhere with combinations of ABZ (400 mg) and DEC (6 mg/kg) for 4-6 years. Similar to evidence for selection and spread of resistance to the anthelmintic treatment [9,10,11], the emergence of parasite resistance to these drugs is a concern since there is a poor understanding of their complete mechanism of actions [12].

An important aspect in the biology of several filarial nematodes, including *W. bancrofti* and *Brugia* spp., is the presence of *Wolbachia*; an intracellular alpha-proteobacteria. This endosymbiont is predominantly found in the adult lateral chords as well as in ovaries, oocytes and developing embryos in female worms [13]. Although the mechanisms that underlie this symbiotic relationship remain elusive, *Wolbachia* is essential for the growth, development, embryogenesis, and survival of its nematode host [14]. This characteristic makes anti-*Wolbachia* therapy an alternative for filariasis control in situations where current strategies fail or are unable to be delivered [13,15]. For this purpose, the combined use of antibiotic therapies, particularly doxycycline with other anthelmintics, is currently under evaluation [16,17]

Lymphatic Filariae – Biology and Life Cycle [18].

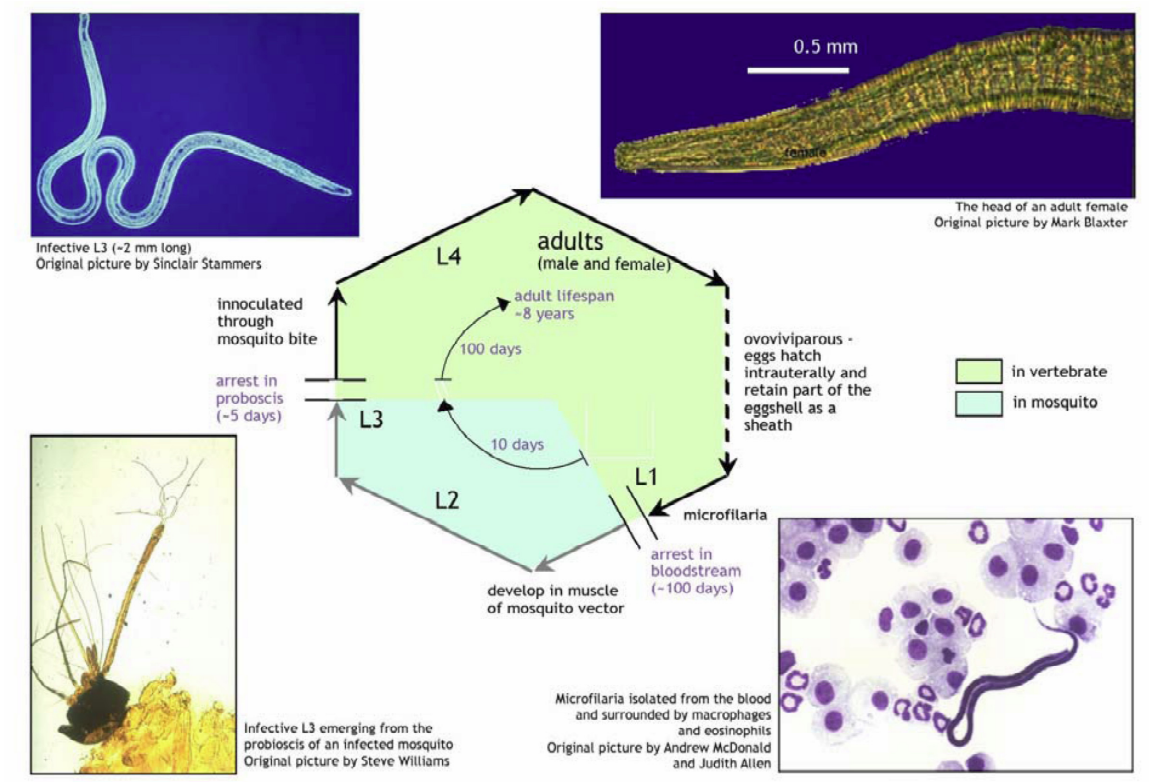

Figure 1 Life cycle of *B. malayi* [19].

Lymphatic filariae are nematodes belonging to the order Spirurida and the family Onchocercidae. The lymphatic filariae parasites have a biphasic life cycle in which larval development takes place in the mosquito or intermediate host, and larval and adult development occurs in the human or definitive host (Figure 1). There are no free-living forms of these parasites.

Infection in the human host is initiated with the release by the mosquito during feeding of third stage larvae (L3) on the skin of the human host. The larvae enter the host at the puncture site, penetrate the host's dermis and enter the lymphatic system. In the definitive host, L3 parasites initiate a developmental program that culminates in a molt to fourth stage larvae (L4) between days 9 and 14 post-infection. L4 undergo dramatic growth during the next 6 to 12 months as they develop into mature adults. The adult worms tend

to localize in the varices of the lymphatic vessels of the lower extremities. After insemination, zygotes develop *in utero* in microfilariae over a three week period. Adult female parasites can remain reproductively active for more than 5 years. The upper limit for reproductive lifespan is not known.

Females release thousands of fully-formed, sheathed microfilariae (Mf) per day into the lymph circulation of the host. From the lymph, they find their way into the peripheral circulation. In most endemic areas, the lymphatic filariae are nocturnally periodic. During the day, the Mf are concentrated in the microvasculature of the deep tissues, predominantly in the lungs.

In the mosquito, infection is initiated by ingestion of Mf from an infected host as part of the blood meal. Within a few hours, Mf penetrate the midgut wall and begin their migration via the haemolymph to the flight muscles, where they initiate their molt to the second larval stage (L2). The L1 to L2 molt takes place between 6 and 10 days post-infection. Between days 11 to 13 post-infection, there is an L2 to L3 molt. Within 1 or 2 days, the L3 larvae migrate from the flight muscles to the head of the mosquito where they associate with the feeding structures that will mediate their transmission to the definitive host.

Immunological aspects of filarial nematode infections

Host responses to lymphatic filarial nematode infection are complex and can exhibit a significant degree of variation from individual to individual. Studies from endemic areas have provided information to delineate 3 main groups based on the spectrum of symptoms and immunological responses exhibited. The first group is composed of those individuals who are clinically asymptomatic but microfilaraemic (Mf+) and represents the majority of the infected population. These individuals may exhibit some degree of subclinical disease [20]. Mf+ individuals usually harbour fertile adults in their lymphatics and are considered to be immunologically tolerant of the parasite. The second group is referred to as the chronic pathology (CP) group and is defined by those individuals are exhibit pathology (lymphedema/hydrocele) associated with the filarial infection but are

generally amicrofilaraemic. Studies from animal models suggest that patients in this group are resistant to re-infection and can kill adult nematodes [21]. The third group constitutes a small proportion of the population in endemic areas that may be immune to invading L3 as they remain both symptom and Mf-free. They are referred to as 'endemic normals' (EN) [22].

The Mf+ asymptomatic state is characterized by a suppression of both Th1 and Th2 responses, which permits high parasite loads and reduced immune-related damage to the host. Down-regulation of these immune responses is characterized by unresponsiveness and impaired proliferation of T cells, increased production of the anti-inflammatory cytokine IL-10 and higher levels of IgG4 [23]. Associated with the T-cell unresponsiveness, there is a decreased production of both IFN-γ and IL-5 in Mf+ asymptomatic patients [24]. The immune hyporeactivity in filarial infections is associated with the induction of TGF-β [25], which along with IL-10 is involved in the induction and modulation of the activity of regulatory T (Treg) cells. Tregs together with the expansion of alternatively activated macrophages (AAMΦ) may act as determinants in the maintenance of immunologic self-tolerance and/or suppression of inflammatory responses [26,27,28].

IL-10 is associated with the induction of IgG4 while suppressing IgE production by B cells [29]. This seems to be central to the development of LF pathology as IgG4/IgE or IgG4/IgG ratios are considered to be a main criterion to determine whether a filarial infection turns into the asymptomatic or the CP state [29]. Levels of IgG4 in Mf+ asymptomatic individuals can rise to 90% of the total immunoglobulin content, and although CP patients also exhibit high levels of IgG4; the IgG4/IgE ratio appears to be still higher in Mf+ patients [30,31]. Based on these findings, it has been proposed that IgG4, a non-cytolytic antibody, can protect Mf by competing with antigens of other cytolytic antibodies (IgG1, IgG2, IgG3 and IgE) that may induce various effector mechanisms such as complement activation and antibody-dependent cell-mediated cytotoxicity [29].

In contrast to the Mf+ asymptomatic patients, CP individuals exhibit increased CD4+ responses to filarial antigens [32,33]. In addition, CP patients show higher levels of IFN-γ and equivalent levels of filarial-specific IL-4 secreting lymphocytes when compared to Mf+ asymptomatics, indicative of bias towards a Th1-type of response [34]. Further analyses have shown that upon stimulation with filarial antigens, there is also higher production of TNF-α and up-regulation of Th17 cytokines (IL-17A, IL-17F, IL-21, and IL-23) in CP patients. Additionally, decreased expression of cell markers associated to Tregs (Foxp3, GITR, TGFβ, and CTLA-4) has been found in CP patients together with significantly higher expression of TLR 2, 4, 7, and 9 as well Nod1 and 2; indicative of increased Th1/Th17 responses, decreased Tregs and regulation of Toll and Nod-like mediated receptors signalling in the pathogenesis of filarial infections [35]. Associated with the increase in the levels of pro-inflamatory cytokines in CP patients, there is up-regulation of the expression of particular isoforms of vascular endothelial growth factors (VEGF) which are involved in controlling angiogenesis and lymphangiogenesis in humans and might promote in the case of infected patients, lymph vessel hyperplasia as a first step to lymphoedema and other chronic pathologies [36,37].

Lymphatic damage and pathology is therefore, the result of a series of factors, including the effect of alterations in tissues induced by both living and nonliving adult parasites, the host inflammatory response to the parasites, and particularly the *Wolbachia* endosymbiont and/or secondary bacterial or fungal infections, which are thought to be major sources of these pro-inflamatory stimuli [14,35].

Protective immunity to LF in human is still source of debate beyond the observation of EN individuals in endemic areas. This is due to the notion that differences in size between filarial nematodes and host immune effector cells impose an operational constraint for parasite clearance. In addition, the particular anatomical localization of the worms in the lymphatics as well as their ability to persist in the host for long periods of time contribute to refute the possibility of immunity [38,39]. However, the use of mathematical and comparative data analysis framework modeling provided evidence that group immunity

can be the result of exposure-driven acquisition, as reflected by variations in the observed age-prevalence patterns of infection in human filariasis [40].

When evaluated, immune responses of EN individuals markedly differ from those infected people [39]. For example, Mf- subjects from an endemic area of the Cook Islands have significantly higher adult worm antigen-driven cellular proliferation responses accompanied by generation of IL-2, IL-5, IL-10, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GMCSf) compared to infected subjects [41]. In this group, it is envisaged that a balance of both Th1 and 2 types of responses, probably controlled by the activity of Treg cells, are of sufficient magnitude to promote parasite killing [42].

Brugia malayi: a Model for the Study of Filarial Parasites

The paucity of laboratory animal models that accurately imitate the immunobiology and pathology of filarial infection in humans is one of the main obstacles that limit the understanding of the biology and immunobiology of lymphatic filarial parasites [18]. Although several animal models have been used to study filarial infections, aspects such as availability, maintenance costs, and societal concerns about animal experimentation have dictated rodent models to be the most desirable for future studies [43].

The *Brugia*-gerbil (*Meriones unguiculatus*) model is the most widely used. The gerbil is permissive for the development of *B. malayi* and *B. pahangi* (a cat parasite) from L3s to reproductive adults [18], a characteristic that has been exploited for immunological studies [43,44,45,46]. The availability of material from mosquito-phase stages and the ability to be maintained *in vitro* account for the choice of *B. malayi* as the representative parasitic nematode to be analyzed for the Filarial Genome Project (FGP) [47,48,49].

The FGP was initiated in 1994 with funds from the World Health Organization (WHO/TDR/UNDP/World Bank), with the aim of promoting drug target and vaccine candidate identification for filariasis by gene discovery using high-throughput methods [50]. Final results of this sequencing project were published in 2007 [19] and represented at that time, the first genome of a parasitic nematode to be sequenced [19,48]. Other

finished sequencing projects include the plant parasitic nematode *Meloidogyne incognita* [51] and more recently, the food-borne zoonotic parasite *Trichinella spiralis* [52]. Moreover, many other projects are underway and are expected to provide new elements for comparative and experimental analysis of nematode parasitism. Among these, genomic sequence information for the filarial nematodes *W. bancrofti, Onchocerca volvulus* and *Loa loa* have recently become available through the Filarial Worm Database at the Broad Institute of Harvard and Massachusetts Institute of Technology (Boston, MA) (http://www.broadinstitute.org/annotation/genome/filarial_worms/Info.html).

The *B. malayi* genome project employed a whole-genome shotgun approach to provide a more than eight-fold coverage for close to 88 megabases (Mb) assembled into 8180 scaffolds. Annotation was performed using gene finding algorithms trained on *B. malayi* genes and ESTs available in the public databases. Close to 11,500 protein coding genes were proposed in 71 Mb of robustly assembled sequence [19].

Availability of sequence information for *B. malayi* opened the possibility to employ a variety of genomic and post-genomic tools to understand basic aspects of the biology of filarial nematode parasites. These include the use of bioinformatic comparative tools, to identify putative drug targets [53] as well as microarray tools, to identify stage- and gender-associated gene expression [54,55], genes associated with mosquito infectivity [56] and the effect on gene expression of anti-*Wolbachia* treatment [57]. Also, it provided the possibility to use more reliable proteomic approaches for protein identification, initially from their ESP [58,59, this work] and later in the identification of adult worm antigens [60], determination of protein changes in response to anti-*Wolbachia* treatment [61] and, more recently, for the characterization of the full proteome and validation of protein models [62].

Gastrointestinal Nematode Infections.

Gastrointestinal (GI) nematode infections are major causes of disease in both humans and animals [63,64]. Human GI nematodes are mostly prevalent in developing countries, affecting ~1 billion people and posing a burden estimated at ~2 M DALYs (http://apps.who.int/ghodata). Infections with Ascaris lumbricoides, hookworms (Necator americanus and Ancylostoma duodenalis) and Trichuris trichiura are most commonly observed. Infection rates are highest in children living in sub-Saharan Africa, followed by Asia and then Latin America and the Caribbean [65].

Although most human GI nematode infections are asymptomatic, heavy infections in children are usually associated with anemia, wasting, stunting, cognitive impairment and lowered educational achievement, which in the long term may interfere with productivity and the wage earning capacity in adults [66].

Control programs targeting high risk groups have been implemented to reduce the diseases burden in many endemic countries. These programs are based on the administration of anthelmintics, mostly on single doses of the benzimidazoles ABZ (400 mg, 200 mg for children between 12 - 24 months) and mebendazole (500 mg). To a lesser degree, levamisole and pyrantel are also administered for control of GI nematode infections [67]. ABZ is preferred over the other drugs as it is relatively more effective against hookworm and whipworm. However, ABZ is not completely effective for removal of these parasites, as cure rates after single dose treatments are in the range of 88% for roundworms and of 78% for hookworms [67].

Emergence of drug resistance is also a concern for the control of GI nematode infections. ABZ acts by binding to β-tubulin, leading to the disruption of microtubule-based processes [68]. It has been shown that resistance to benzimidazoles can be conferred by single amino acid substitutions from phenylalanine to tyrosine in the β-tubulin gene [69]. This imposes an urgency to develop novel or more efficient approaches for the control of these diseases.

Alternatives to chemotherapeutical control of GI nematode infections are currently under investigation. These include the development of the vaccine against hookworms by the Human Hookworm Vaccine Initiative (HHVI) [65]. Targeting approach for this vaccine consists in the selection of two different components that would eventually result in the control of both the larval and adult stages of the hookworm life cycle. Current candidates include the homologue of an *Ancylostoma* Secreted Protein (ASP) from *N. americanus* (*Na*-ASP-2) as a potential larval component of the vaccine and 2 hemoglobinases and a glutathione-S-transferase for targeting of the adult forms [70].

Human GI nematodes life cycles [71]

GI nematodes establish chronic infections, surviving in the host for considerable periods of time. Anatomical localization varies among species. Adult hookworms reside in the upper part of the human small intestine, whereas *Ascaris* roundworms occur in the entire small intestine and adult *Trichuris* whipworms are associated with the large intestine, particularly the caecum. After insemination, female worms produce thousands of eggs per day that leave the body in the faeces.

Human infection with *T. trichiura* and *A. lumbricoides* occurs by ingestion of fully developed eggs. In the case of *N. americanus* and *A. duodenale* hookworms, eggs hatch in the soil and molt twice to become motile infective larvae (L3). Infection in this case occurs by attachment and penetration of human skin by the L3.

The ingestion of *T. trichiura* eggs is followed by the release of the contained larvae that molt and migrate to the colon where they burrow into the epithelia and develop into adult whipworms within ~12 weeks. Larvae of *A. lumbricoides* require several migration steps for development: they initially penetrate the intestinal mucosa and migrate to the liver then the lungs. Re-entering into the gastrointestinal tract occurs via the epiglottis. *Ascaris* egg-laying adult worms develop ~9–11 weeks after egg ingestion.

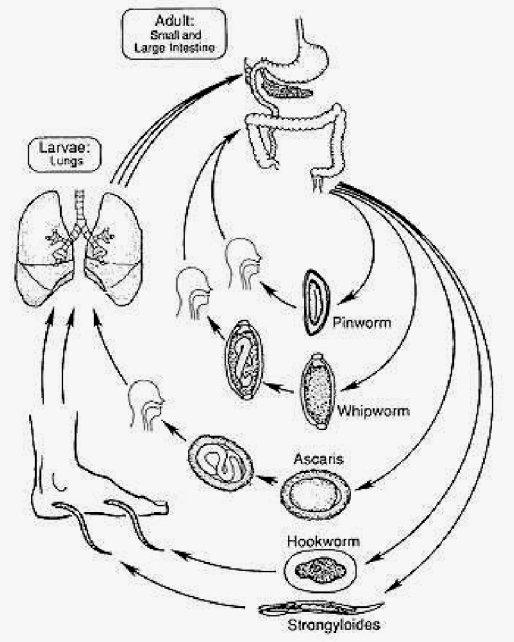

Figure 2. Generalized life cycle of intestinal nematodes [72]

~5–9 weeks are usually required from hookworm larvae skin penetration until development of egg-laying adults. After skin penetration, hookworms larvae reach the host circulation by entering the subcutaneous venules and lymphatic vessels. Migration to the GI tract starts after trapping in the pulmonary capillaries, to entering the air space of the lungs and passing over the epiglottis.

Immunological aspects of GI nematode infections as revealed through the use of animal models

A feature characteristic of most GI nematode infections is the inability to multiply inside the host. Each worm in an infected patient is the result of an individual infectious event [71]. The peak of infection occurs in early childhood and is then reduced during adulthood, suggesting the possibility of acquired immunity and linking the presence of heavy and chronic infections in children from endemic areas to repeated parasite exposure and inadequacy of parasite expulsion [73].

Human studies on the immune response to GI nematode have been largely conducted using peripheral blood sampling due to the extreme invasiveness of the methods required to obtain material in local infected areas [73]. These studies have demonstrated that an active, acquired response is generated as an outcome of the infection. In general, the overall GI nematode immune response is of the Th2- type, characterized by the production of the cytokines interleukin-4 (IL-4), IL-5, IL-10, and IL-13; parasite-specific immunoglobulins, non-specific IgE, and expansion and mobilisation of mast cells, eosinophils, and basophils [71].

More information about the immune events following GI nematode infections has been obtained from different animal models. Laboratory mouse models include infections with *Trichuris muris*, *Trichinella spiralis* and the hookworms *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* [74]. The rates and longevity of these infections vary from model to model. Only *T. muris* and *H. polygyrus* are able to generate chronic heavy infections. *N. brasiliensis* only develops at low-dose infections and stimulates a strong protective Th2 response at normal-high standard doses. The *T. spiralis* transmission strategy relies on rapid development to maturity and reproduction previous prior to a Th2 mediated expulsion [74,75].

In general, mouse GI nematode models lead to a strong polarization towards a Th2 -type immune response. It has been shown that the induction of this type of response is protective, so chronic establishment of the parasite is thought to be associated with parasite ability to modify this response. For instance, strong immunomodulatory effects have been identified in these models, characterized by the display of down-regulatory mechanisms such as the induction of AAMΦ and Tregs [74].

Although the molecular mechanisms involved in this induction are not completely understood, studies in the two chronic models have revealed that the strategies that lead to long term GI nematode infections may be different depending on the parasite. On one hand, *H. polygyrus* infections are mainly associated with immunosupresion of both Th1 and Th2 [75]. In contrast, early downregulation of host Th2 responses occurs during *T.*

muris infections, favoring a Th1-type of response that may led to an uncoordinated host effector clearance response [74].

Dendritic cells (DC) play a central role in the fate of host adaptative responses, particularly in determining T cell differentiation towards Th1- or Th2 -types. Chronic establishment of GI nematode infections may exploit DC from the early stage of infection to facilitate survival within the host. These responses seem to be stimulated from products release from the parasite. While the development of a Th-2 driving DC population has been observed upon stimulation with *N. brasiliensis* ESP, DC treatment with *H. polygyrus* ESP lead to impairment of DC function characterized by the suppression of both Th1 and Th2 -type responses [76,77].

The role of Tregs and AAMΦs in immunoregulation is a current area of study in GI nematode infections. Tregs differentiate upon antigen stimulation of conventional T cells in the presence of mediators such as TGF-β, IL-2 and retinoic acid [78,79]. Induction of Tregs results in the dampening down of Th1 or Th2-mediated responses. In the case of *H. polygyrus*, ESP from this parasite induce *de novo* Foxp3-expressing T cells (also Tregs) via ligation of the TGF-β receptor [79]. AAMΦs immunoregulatory activity is based on their suppressive effects on the proliferation of APC in the context of Th2-dominated infections. Stimulation of AAMΦs has been observed in *H. polygyrus* infections as well as the induction of markers associated with AAMΦs (Ym1 and Fizz1 and other mammalian chitinases) in *N. brasiliensis* infections [80,81].

Secreted Products from Parasitic Nematodes

General Overview

Secretory processes are thought to be essential for the establishment of the parasitic lifestyle. Several functions have been associated with these processes, ranging from penetration of host tissue barriers and feeding to evasion or modulation of the host immune response [82].

Parasitic nematodes exhibit a variety of life stages and strategies for the establishment of infection. Consequently, the profile and function of their secretory products must vary according to the developmental stage and probably in response to external signals. Additionally, it is expected that these products can induce forms of immunological tolerance by affecting functions involved in activation and/or inhibition of host immune responses to permit long survival [83]. This idea is supported by the general observation that in nematode parasitism, the development of disease tends to be slow, with cumulative body burden generating pathology, often exacerbated by inappropriate immune responses from the human host [84].

Secreted products from helminths have been identified using both functional assays (i.e. enzymatic activity) and proteomic approaches. Proteins identified include several proteinases, inhibitors of proteases (cystatins, aspins and serpins), acetylcholinesterase, antioxidant enzymes (superoxide dismutase, peroxidases), lectins, nucleotide metabolizing enzymes and immunomodulatory components (complement binding factors, eosinophil attractants and immunosuppressants) [85,86].

A wide variety of direct immunomodulatory effects of ESP from parasitic nematodes has been extensively reported and may at least partially explain how their mechanisms of immune evasion are orchestrated [85]. These include impairment of antigen-driven lymphocyte proliferation [87] and induction of suppressive peritoneal exudate cells (AAMΦ) by *Brugia* spp [88]. Also, inhibition of T cell proliferation and macrophage nitric oxide production along with impairment of DC function and induction and regulation of the activity of Treg has been reported for ESP from gastrointestinal parasitic nematodes such as *H. polygyrus* [76,79,89].

Another role of nematode ESP is related to processes such as feeding and invasion which may involve the degradation of host tissues by parasite proteases. Other proteins, such as protease inhibitors and enzymes with anti-oxidant activities, may play important roles in defence against host immune responses, either by limiting direct molecular damage by inhibiting host proteolytic enzymes or by detoxification of reactive oxygen species

produced by host phagocytic cells [86]. Proteinase inhibitors also participate in subversion of the host immune system by altering intracellular processing for antigen presentation by the Major Histocompatibility Complex class II (MHC II) [90].

Many ESP from nematodes are modified at the post-transcriptional level forming antigenic glycoconjugates which can dissipate more extensively into the nematode's environment [91]. Special attention has been paid to ESP modified with N-Type glycans containing phosphorylcholine (PC-glycans). These are unusual structures found in filarial nematodes that are important human pathogens and in the ES-62 product from *Acanthocheilonema viteae* [92]. In ES-62, PC is attached to a glycan with a trimannosyl core, with or without core fucosylation, carrying between one and four additional N-acetylglucosamine residues [93].

ES-62 exerts an immunomodulatory effect on a variety of cells of the murine immune system, including B and T lymphocytes as well as antigen presenting cells (APCs) such as DC and macrophages. Rather than acting in an immunosuppressive manner, the molecule induces a Th2 anti-inflammatory phenotype, characterized by the production of IL-10, reduced levels of IL-12, IFN-γ and pro-inflammatory cytokines, and IgG1 rather than IgG2a antibodies [94]. The immunomodulatory properties of this ESP and particularly its ability to inhibit TLR4 signalling by blocking TLR2- and TLR4-driven inflammatory responses has began to be explored for its potential in the therapeutic treatment of inflammatory and allergic diseases [95,96].

Eukaryote Cellular Pathways of Protein Secretion

Protein secretion is a central process for most if not all living cells and organisms. Studies on the mechanisms involved in this process started soon after the discovery of ribosomes and the association of some of them to the endoplasmic reticulum (ER) in 1955 [97]. The recognition of the synthesis of secreted proteins on ER-bound ribosomes and the subsequent identification of a signal sequence in several secreted proteins that was involved in ER targeting and membrane translocation led to the description of what is currently known as the classical/conventional secretory pathway [97].

The classical secretory protein transport pathway is involved in the export to the cell surface or the extracellular space of most signal peptide (SP) containing proteins [98]. The SP is a 15–30 aminoacid N-terminal peptide which is cleaved off during translocation of the protein across the ER membrane. Although there is no simple consensus sequence, SP are typically composed of three regions: the N-terminal region (n-region) which often contains positively charged residues; the hydrophobic region (h-region) of at least six residues and the C-terminal region (c-region) which contains polar uncharged residues with some degree of conservation at positions -3 and -1 relative to the cleavage site [99].

Nascent proteins enter the ER through the signal-peptide recognition particle. Exit from the ER occurs through the formation of vesicles coated with the cargo-containing coat protein complex II (COPII) at specialized membrane domains called ER exit sites (tER sites). The COPII coat is involved in cargo selection and consists in yeast of the small GTPase Sar1 and two protein heterodimers (Sec23–Sec24 and Sec13–Sec31). Vesicles containing the synthesized proteins reach the Golgi apparatus, where they are modified, processed, sorted and dispatched towards their final destination. Retrograde movement of components in the Golgi and back to the ER requires COPI-coated vesicles, which comprise the small GTPase ADP ribosylation factor 1 (ARF1) and a heptameric protein complex known as the coatomer [98].

Transport within the secretory pathway requires a series of fusion events between vesicular intermediates and organelles, catalysed by SNAREs (soluble N-ethylmaleimide-sensitive fusion protein (NSF) accessory protein (SNAP) receptors). Membrane fusion is undertaken by bringing opposing membranes to close proximity through the formation of tight trans-SNARE complexes from vesicle and target (v- and t-) SNARES. The trans-SNARE complexes are then dissociated through SNAP binding by the hexameric ATPase NSF which allows the recycling of v-SNAREs for another round of vesicular transport [98].

In addition to the classical secretory pathway, two types of non-conventional (also known as non-clasical) protein transport pathways to the eukaryotic cell surface have been discovered. One involves proteins containing SP that after insertion into the ER during their synthesis, reach the cell surface in a in a coat protein complex II (COPII) machinery- and/or Golgi-independent manner. The other route is exhibited by certain cytoplasmic and nuclear proteins lacking ER-SP. These proteins have shown to exit cells through ER- and Golgi-independent pathways [98].

Several mechanisms have been proposed to explain protein transport to the extracellular space for both types of non-conventional protein secretion. These include, in the case of SP-containing proteins, direct fusion of COPII-coated vesicles with the plasma membrane after ER translocation. Alternatively, COPII-coated vesicles can fuse with endosomal or lysosomal compartments that fuse with the plasma membrane. Proteins can also be packaged into non-COPII-coated vesicles that can fuse directly with the plasma membrane or can be targeted to the Golgi apparatus [98].

In the case of secretion of non SP-containing proteins, mechanisms of transport include the direct translocation of the protein from the cytoplasm across the plasma membrane, as in the case of fibroblast growth factor 2 (FGF2) which occurs via the recruitment at the inner leaflet of the plasma membrane by phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2). Additional mechanisms include lysosome fusion with the plasma membrane as well as the release of exosomes which can be formed from microvesicle shedding from the cell surface or through the formation of internal vesicles leading to the formation of multivesicular bodies [98,100,101].

Sources of Nematode Excreted – Secreted Products

The sources in nematodes for ESP include specialized excretory and secretory systems which exhibit certain anatomical diversity, the gut, uterine fluids and other surface components [102]. Among these specialized organs, glands such as the excretory and oesophageal glands in species of the genera *Nippostrongylus*, *Stephanurus* and *Trichostrongylus* are involved in the secretion *in vitro* of acetylcholinesterase [103,104,105]. Additionally, the stichocyte cells surrounding the oesophagus in members of the superfamily Trichuroidea and the anal and excretory pores of *Toxocara canis* infective larvae are sources of antigenic ESP [106,107,108].

In the model nematode *Caenorhabditis elegans*, the excretory-secretory system is composed of 4 cells: the pore cell, duct cell, excretory canal cell (H cell) and the excretory gland cell (A cell) (Figure 3). The excretory canal is filled with fluids, has the appearance of a letter "H and is the largest cell in *C. elegans* as it extends almost the entire length of the worm. The excretory gland cell presumably collects fluids and then empties them outside via the excretory duct and pore cells to which is connected by gap junctions. The duct cell and the pore cell surround the excretory duct. These two cells are connected by a gap junction [109,110].

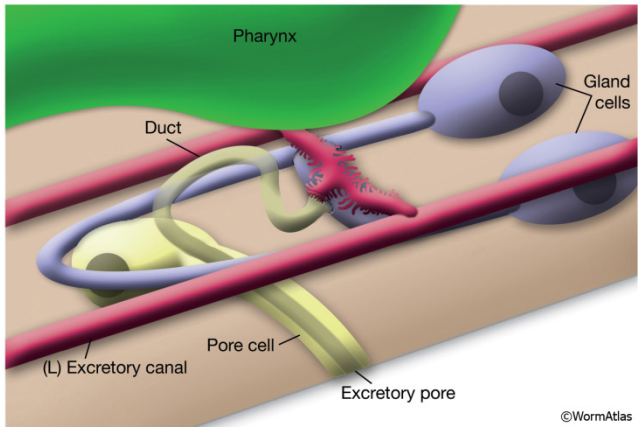

Figure 3. Schematic of the adult excretory-secretory system in *C. elegans*. Lateral oblique view. The excretory cell (red), the gland cell (blue) and the duct cell (brown) are all joined at a specialized intercellular junction called the excretory-secretory junction, where glandular secretions and excreted material are passed into the duct to be transported outside through the pore cell pore cell (yellow). In this view, excretory and duct cell bodies are removed to reveal the junction. (http://www.wormatlas.org)

The gut in nematodes is a continuous organ between an anterior mouth and a posterior anus. The anterior region of the nematode gut typically has specialized secretory organs which produce, in some parasite species, antigenic molecules in infected host. It can be predicted that molecules involved in enzymatic digestion of food, or protection of the parasite's gut epithelium from attack by its intestinal digestive enzymes, and material resulting from turnover would be shed from the gut of actively feeding adult nematodes [102].

Although they have not received detailed attention, another source of ESP is constituted by the fluids that are released from the shedding of eggs or larvae from female worms. In fact, comparison of the uterine fluids with other *A. suum* components revealed the high complexity of this fraction, with some of its components being recognized by sera from naturally infected pigs [111].

Molecules derived from the cuticle of nematodes make a substantial contribution to the contents of ESP of the parasites maintained *in vitro* and are probably also shed *in vivo* [102]. Surface antigens play a critical role in the expression of effective immune responses [112]. The active shedding of such surface molecules may have profound implications for the development of host-protective immune responses to parasitic nematodes since they may provide antigen suitable for stimulation of anti-surface immune responses [102].

In *C. elegans*, the cuticle is formed of collagens, insoluble proteins termed cuticlins, glycoproteins, lipids as well as Hedgehog-related peptides [113,114]. Cellular pathways involved in the formation of the *C. elegans* cuticle are thought to be diverse, as apical secretion of collagens seems to be unrelated to the V0-ATPase exosomal secretion of Hedgehog-related proteins [115].

Early studies revealed the dynamism of nematode cuticle by demonstrating active protein shedding and changes in surface composition over the course of the life cycle of *T. spiralis* [116,117]. The presence of surface components in ESP was also reported for

other parasitic nematodes, including adult and infective larvae of *T. canis* [118,119], adult *N. brasiliensis* [120], adults and microfilariae of *B. malayi* and adult *B. pahangi* [121].

Functional Roles of Proteins Secreted by *Brugia malayi*

As mentioned above, the number of identified and characterized proteins from filarial nematodes increased as a consequence of efforts toward the acquisition of genomic data, namely ESTs and genomic projects [122,123]. Nevertheless, the specific role of most released proteins remains unknown. Products from only 5 secreted gene families from *B. malayi* have been characterized at a functional level. Identification of such products has led to the acquisition of valuable knowledge that has enhanced understanding of the molecular basis of immune evasion by filarial and other nematodes [123].

Glutathione Peroxidase

Studies during the 1980's used biochemical and immunological approaches to characterize both secreted and surface antigens. Advances in filarial genomics led to the identification of a 29 kDa glutathione peroxidase (Bma-GPX-1) as a major surface glycoprotein of adult *B. malayi* [124,125]. This protein is secreted into *in vitro* culture media, and is believed to act as a lipid hydroperoxidase, protecting parasite membranes from peroxidation caused by oxygen free radicals [126].

Cystatins

Cystatins are a widely-distributed family of cysteine protease inhibitors which play essential roles in a spectrum of physiological processes [127]. A number of filarial cystatins have been characterized from *B. malayi*, *A. viteae*, *O. volvulus* [86]. There are two homologues in *B. malayi*, Bma-CPI-1 and Bma-CPI-2; both are located on the surface of *B. malayi* L3 larvae, while Bma-CPI-2 is also a significant adult surface antigen. Only Bma-CPI-2 is secreted by both L3 and adult stages of the parasite [123]. Bma-CPI-1 is selectively expressed by late L2 and L3 stages in the mosquito vector, but

expression ceases within 2 days of infection of the mammalian host. Bma-CPI-2 is constitutively expressed throughout the parasite life cycle. Sequence analysis reveals both common and unexpected features of the filarial cystatins [123]. These features include the presence of a consensus motif associated with binding to papain-like enzymes. Bma-CPI-2 can inhibit both papain-like and legumain-like enzymes, due to the presence of a SND motif in amino acids 76-78 previously found only in certain mammalian cystatins and involved in intracellular processing of antigens for presentation by MHC class II [90,123].

Bma-CPI-2 was tested for its ability to block class II mediated antigen processing in a model system in which human B cells process and present peptides from tetanus toxin to defined T cell clones. Results of these experiments showed complete inhibition of presentation of some epitopes, while others remained unaffected by the presence of the filarial cystatin, demonstrating that Bma-CPI-2 targets one of a set of alternative processing enzymes in the human lymphocyte [90].

Serine Protease Inhibitors

In *B. malayi*, two serine protease inhibitor (serpins) sharing only 27.8% identity (110/395) at the amino acid level have been identified. Even with the low percentage of identity, both *B. malayi* serpins show conserved `motif' and `signature' sequences [128]. Bma-SPN-2 was identified after screening a Mf cDNA library with antibodies against strongly stimulatory fraction to murine T cells [129,130]. Bma-SPN-2 seems to be Mf-specific in its expression [123]. Bma-SPN-1, in contrast to the Mf-specific expression of Bma-SPN-2, is preferentially produced by the L3 stage [131] and indeed is one of the most abundant mRNA species present in this stage.

Serpins have been previously identified in *Ascaris suum*, *O. volvulus*, *O. ochengi*, *Trichostrongylus vitrinus*, *T. spiralis*, *C. elegans*, *Schistosoma mansoni* and *S. haematobium* [86]. None of the *C. elegans* serpins is predicted to possess a signal sequence, indicating that the parasitic species have adapted these genes to fulfill an extracellular function [123].

Bma-SPN-2 was tested against a panel of serine proteases, with no observed reactivity against trypsin, chymotrypsin or pancreatic elastase. However, the parasite protein is a potent inhibitor of two neutrophil enzymes, cathepsin G and neutrophil elastase. There is an intriguing correlation between exclusive expression of Bm-SPN-2 by only the blood-dwelling filarial stage, and the ability of Bm-SPN-2 to block products of the most abundant nucleated cell type in the blood, the neutrophil [123,128,130].

Cytokine Homologues

Two sets of cytokine-homologous gene families have been identified in *B. malayi*: TGF-β (Bma-TGH-1 and -2) and MIF (Bma-MIF-1 and -2) [123].

The TGF-β (Transforming Growth Factor β) gene superfamily encompasses many proteins that regulate both developmental and immunological processes [132]. Bm-TGH-2 is secreted *in vitro* and is expressed by Mf as well as adult *Brugia* [123,133,134,135]. Additionally, it binds to host TGF-β receptors, providing an explanation for how this protein could participate in the down-regulation of host inflammatory responses, such as ablation of nitric oxide synthesis [123].

The MIF gene family is likewise derived from an ancient metazoan ancestor, but in mammalian organisms, these proteins are associated with largely pro-inflammatory cytokine activities The two *Brugia* MIF molecules are only 27% identical to each other, but both show very similar cytokine-like activity (e.g., macrophage kinesis, monocyte activation and induction of endogenous cytokine secretion) as well as an enzyme activity (dopachrome tautomerase) previously described for human MIF [123,131,136]. The crystal structure of Bma-MIF-2 has been determined and, despite being only 28% identical to human MIF, its 3-dimensional structure is highly similar [136]. It is speculated that continuous secretion of MIF molecules by filarial parasites may induce a counter-inflammatory phenotype, either by desensitization or by stimulating macrophages beyond the short-term acute time period during which it has generally been examined [122].

Experimental Approaches for the Identification of ESP from Parasitic Nematodes.

For years, attempts to identify components of parasitic nematode ESP have been motivated by the notion that ESP were especially important to understand functional immunity to the parasite and to establish specific diagnostic techniques in chronic infection [137]. ESP analysis is often restricted by limitations in the amount of recovered material, requiring increased sensitivity in analytical techniques. Early studies using immunochemical and radiolabelling techniques were able to determine of the immunogenic character of certain ESP as well as the contribution of the parasite surface as a source for the release of these products [112]. However, improvements in cloning and sequencing technologies have enable the robust these proteins. The availability of Expressed Sequenced Tags (EST) and cDNA expression libraries allowed the identification of antigens and potential secreted candidates based on immunological screening, homology searches and/or analysis of relative expression [122,123]. Later, the application of mass spectrometry (MS)-based proteomic technologies which have been greatly improved by the availability of large collections of genes in curated databases, became feasible [58,59,138]. Both gel- and gel free-based proteomic approaches have now been widely used for the analysis of ESP from parasitic nematodes [58,59,139,140,141].

The gel-based proteomic approach allows the separation of complex mixtures of proteins by one or two-dimensional (1D or 2D) gel electrophoresis. After fixation and staining, particular spots or bands can be excised from the gel and be analyzed by tandem MS after in-gel tryptic digestion [140]. Several limitations have been associated with this approach, most of them with the time and labour consuming requirements for sample manipulations. These include poor reproducibility between gels (particularly 2D-gels), low sensitivity for detection of proteins and limited sample capacity [140]. However, continuous advances in the state of this art have overcome most of these limitations.

In a proteomic experiment, the central initiative is to characterize proteins in a sample based on their molecular weights which is a function of the protein composition [142].

This can be possible through the analysis of the unmodified proteins. However, due to the fact that it is more difficult to measure differences in larger proteins, most proteomic experiments rely on the measurement of the mass of peptides derived from them by enzymatic cleavage. When analyzing a peptide mixture, a MS-spectrum is generated by plotting the mass-to-charge (*m/z*) ratios versus their ion current or mass spectrometric signal. To determine identity of the protein, the peptides are additionally fragmented through collision with an inner gas such as helium or nitrogen at low pressure (CID, collision induced dissociation). The resulting spectrum, called MS/MS (tandem or MS^2) spectrum, is a list of *m/z* ratios for different fragments with some of the differences corresponding to the specific mass of one amino acid. Peptide sequences can be inferred by connecting these fragments with increasing size and calculating the mass differences from the N terminus (b-ion series) or C terminus (y-ion series) (*de novo* sequencing) [142]. However, it is more common and easier to identify peptide sequences by correlating acquired fragment ion spectra with theoretical spectra predicted for each peptide contained in a protein sequence database (database searching), or with spectra libraries acquired from previous experiments (spectral library searching) [143]. For this purpose, a number of search engines have been designed, the most popular being MASCOT [144], SEQUEST [145] and X! Tandem [146].

The database search approach has been preferred for the identification of ESP from nematodes and other species. However, protein identification by database searching becomes problematical when working with organisms lacking sequenced genomes, as is the case for most parasitic nematodes. Approaches to cross species protein identification have been employed [147,148,149], but are limited if the organism being studied is distantly related to any organism with a sequenced genome [150]. Inclusion of EST sequences for database searches has been employed with great success in the identification of helminth ESP [151,152] and it is expected that recent developments in massive sequencing could help to overcome these limitations for the analysis of non-model species [153].

In the next chapter, we used a proteomic approach consisting of 1D gel electrophoresis along with tandem MS for the analysis of ESP from *B. malayi* Mf, adults female and male worms. The availability of predicted gene models from the *B. malayi* genome project allowed us to reach great sensitivity in the identification of proteins using a database search as the strategy for MS to peptide assignation. This along with the use of several bioinformatic tools provided substantial information regarding the strategies deployed by each form of the parasite to survive in the host.

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Chapter III

(Manuscript I)

Stage- and gender-specific proteomic analysis of *Brugia malayi* excretory-secretory products

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Running title: *B. malayi* secretome

Published in: PLoS Neglected Tropical Diseases (2008). 2(10):e326[1]

Abstract

Introduction: While we lack a complete understanding of the molecular mechanisms by which parasites establish and achieve protection from host immune responses, it is accepted that many of these processes are mediated by products, primarily proteins, released from the parasite. Parasitic nematodes occur in different life stages and anatomical compartments within the host. Little is known about the composition and variability of products released at different developmental stages and their contribution to parasite survival and progression of the infection.

Methodology /Principal Findings: To gain a deeper understanding on these aspects, we collected and analyzed through 1D-SDS PAGE and LC-MS/MS the Excretory-Secretory Products (ESP) of adult female, adult male and microfilariae of the filarial nematode *Brugia malayi*, one of the etiological agents of human lymphatic filariasis. This proteomic analysis led to the identification of 228 proteins. The list includes 76 proteins with unknown function as well as also proteins with potential immunoregulatory properties, such as protease inhibitors, cytokine homologues and carbohydrate-binding proteins. Larval and adult ESP differed in composition. Only 32 proteins were shared between all three stages/genders. Consistent with this observation, different gene ontology profiles were associated with the different ESP.

Conclusions/Significance: A comparative analysis of the proteins released *in vitro* by different forms of a parasitic nematode dwelling in the same host is presented. The catalog of secreted proteins reflects different stage- and gender-specific related processes and different strategies of immune evasion, providing valuable insights on the contribution of each form of the parasite for establishing the host-parasite interaction.

Author Summary

To succeed in infection, parasites must have ways to reach the host, penetrate its tissues and escape its defense systems. As they are not necessarily fatal, most helminth parasites remain viable within their host for many years, exerting a strong influence over the host immune function. Many of these functions are performed by products that are released from the parasite. We exploited the remarkable sensitivity of modern proteomics tools together with the availability of a sequenced genome to identify and compare the proteins released *in vitro* by adult males, adult females and the microfilariae of the filarial nematode *Brugia malayi*. This parasite is one of the etiological agents of lymphatic filariasis, a disease that poses continuing and significant threats to human health. The different forms of the parasite inhabit different compartments in the mammalian host. We found that the set of proteins released by each form is unique; they must reflect particular developmental processes and different strategies for evasion of host responses. The identification of these proteins will allow us to illuminate the biology of secretory processes in this organism and to establish a path for developing an understanding of how these parasite proteins function in immune evasion events.

Background

Lymphatic filariasis (LF) is a disabling and disfiguring parasitic disease caused by the adult and developing forms of filarial nematode parasites residing in the lymphatic system of a mammalian host. The infection in humans is caused by *Wuchereria bancrofti*, *Brugia malayi* or *B. timori* [1] and puts at risk an estimated 1307 million people in 83 endemic countries in subtropical and tropical regions of the world [2].

Lymphatic filarial parasites have a two-host life cycle. Infection is initiated with the release by the mosquito of third stage larvae (L3) during feeding on the host. The L3 enter the host at the puncture site, penetrate the dermis and enter the lymphatic system. L3 parasites initiate a developmental program that culminates in a molt to fourth stage larvae (L4) 9 - 14 days post-infection. L4 undergo dramatic growth during the next 6 to 12 months as they develop into mature adults. Adult worms tend to localize in the varices

of lymphatic vessels of the lower extremities. After insemination, zygotes develop *in utero* to microfilariae (Mf) over a three-week period. Adult female (F) parasites can remain reproductively active for > 5 years. Females release hundreds to thousands of fully-formed, sheathed microfilariae per day into the lymphatic circulation of the host. From the lymph, they transit into the peripheral circulation.

Dramatic clinical manifestations, including hydrocoele, recurrent adenolymphangitis, lymphedema and elephantiasis are associated with chronic infection. Nevertheless, the majority of infected individuals have no clinically apparent sequelae, despite the presence of circulating Mf (and parasite antigens) in the peripheral blood [3]. Associated with the asymptomatic state is a suppression of both Th1 and Th2 responses, which may lead to high parasite loads and reduced immune-related damage to the host. This down-regulation of the host immune response is characterized by impaired proliferation of T cells, increased production of the regulatory cytokine IL-10, and higher levels of IgG4 [4].

The complexity of immune responses in LF is due, among other factors, to the presence of different life cycle stages of the parasite and the different levels of anatomical compartmentalization in which they reside [5,6]. In addition, the presence in filarial nematodes of a *Wolbachia* endosymbiont, a matrilineally inherited obligate intracellular bacteria, contributes to this complexity, as *Wolbachia* antigens have been related to the development of inflammatory-mediated filarial disease [7,8,9].

While we lack a complete understanding of the molecular mechanisms by which pathogens achieve protection from host immune responses, it is generally accepted that parasitic nematodes release a variety of products, primarily proteins (many having posttranscriptional modifications), that enable infection by facilitating penetration of tissue barriers, migration through host tissues and evasion of immune responses. The characteristics and functions of these products are diverse and must reflect, among other factors, the lifestyle of each parasite. Even though their importance for establishing and maintaining the host-parasite interaction is accepted, relatively little is known about the mechanism(s) by which proteins secreted from nematodes regulate the immune system.

Proteins released from these parasites during culture i*n vitro* are conventionally named excretory/secretory products (ESP). Several have been identified and characterized, particularly from *Brugia malayi*, an organism that can be maintained in the laboratory as a model of filarial nematodes and was chosen as a representative species to be analyzed for the Filarial Genome Project [10,11,12]. A draft of this genome was recently released [13], allowing the identification through proteomic analysis of the ESP from adult males (M) and females (F) of this parasite maintained together *in vitro* [14]. To gain a deeper understanding of how these parasites survive in their particular host milieu and the contribution of each form to the progression of the infection, we present a comparative analysis of the ESP independently released by Mf, F and M *B. malayi*.

Materials and methods

Parasites

Mf and adult *B. malayi* were recovered >120 days post-infection from the peritoneal cavities of jirds (*Meriones unguiculatus*) infected subcutaneously with 200 - 300 L3. Infected jirds were obtained from the Filariasis Research Reagent Repository Center (Athens, Georgia USA). Adult worms were washed several times in RPMI 1640 medium [with L-glutamine, 20 mM HEPES, 100 μg/ml penicillin, 100 units/ml streptomycin (Gibco), pH 7.2] (henceforth, RPMI 1640) and separated by gender. Mf were obtained through several washes of the peritoneal cavity with 37˚C RPMI 1640. The combined washes were centrifuged 5 min at 1000 x g to pellet the Mf, which were subsequently purified from host cells by passage through PD-10 columns equilibrated with pre-warmed RPMI 1640 as described [15]. Animal procedures were reviewed and approved by the Facility Animal Care Committee of McGill University – Macdonald campus and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Parasite culture and preparation of ESP

Parasites were cultured in RPMI 1640 at 37˚C for 4 days with changes of media each 24 hr [16]. F, M and Mf were maintained at densities of 6, 15 and 2.5 x 10^5 parasites/ml, respectively. A cocktail of protease inhibitors [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; bestatin hydrochloride; N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; pepstatin A; phosphoramidon disodium salt] (Sigma No P8849, St. Louis, MO) was added to media containing ESP following sterilization by passage through a 0.22 μm filter. Media were stored at -30˚C until analysis.

The combined volume (30 – 165 ml) of 3 different incubations was concentrated to 1-1.5 ml in an Amicon Ultra 3000 MWCO (Millipore). Proteins were then precipitated with trichloroacetic acid (TCA, 20% final conc.). The pellet was washed 3 times with cold acetone (-30°C) and air-dried. Proteins were resuspended in Tris-HCl [20 mM, pH 8.0] and quantified (Quant-iT™ Protein Assay on a Qubit fluorimeter; Invitrogen).

1D electrophoresis and band excision

Concentrated ESP from Mf, F and M were centrifuged at 20000 x g for 3 min and resuspended in loading buffer containing 2-mercaptoethanol. Final amounts of 37 μg, 65 μg and 15 μg of protein, respectively, were separated by SDS-PAGE on a 2.4 cm gradient gel (7-15% acrylamide). The gel was stained with Coomassie Brilliant blue G. The entire lanes were subjected to automated band excision, to generate 15 bands per lane (see Figure 1). The Protein Picking Workstation ProXCISION (Perkin Elmer) was set to excise 5 to 7 pieces per band, depending on the width of the lane.

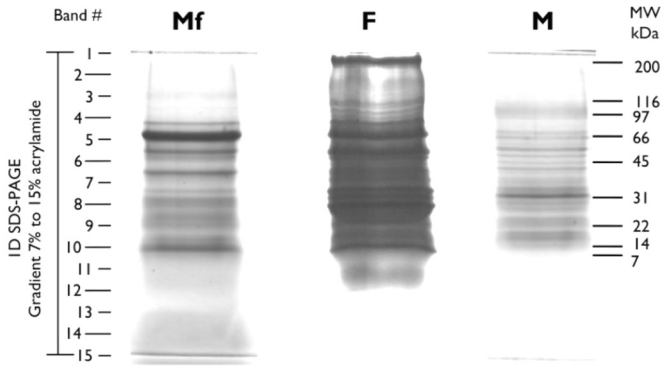

Figure 1. SDS-PAGE of ESP from microfilariae (Mf), females (F) and males (M) *B. malayi*. 37.3 µg, 65.3 µg and 15.4 µg of protein from Mf, F and M, respectively, were separated by electrophoresis through a 2.4 cm gradient SDS gel (7.5-14%). Protein loaded is the amount recovered from 3 pooled sets of independent incubations. Following staining with Coomassie Brilliant blue G, the entire lanes were subjected to automated band excision to obtain 15 pieces per lane. Proteins from gel bands were digested with trypsin and analyzed by LC-MS/MS. Intensity of lane F was adjusted in order to allow better visualization of the staining.

Tryptic digestion and Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) analysis

Proteins from gel bands (5 to 7 gel pieces per band/well) were subjected to reduction, cysteine-alkylation and in-gel tryptic digestion in a MassPrep Workstation (Micromass, Manchester, UK) as previously described [17]. Briefly, gel pieces were pre-washed twice in 100 µl HPLC grade water for 10 min. Gel pieces were destained in 2 - 10 min incubations in 50 µl 100 mM ammonium bicarbonate followed by 5 min in 50 µl 100% acetonitrile.

Destained and dehydrated gel pieces were reduced and alkylated by incubation in 50 µl 10 mM dithiothreitol for 30 min, followed by addition of 50 µl 55 mM iodoacetamide for 20 min and finally 100 µl 100% acetonitrile for 5 min. Gel pieces were washed by incubation for 10 min in 50 µl 100 mM ammonium bicarbonate, followed by a 5 min incubation in 50 µl 100% acetonitrile and were then dried for 30 min at 37°.

Proteins were digested in-gel by incubation in 25 µl trypsin solution (6 ng/µl in 50 mM ammonium bicarbonate, Promega) for 30 min at room temperature, followed by 4.5 hr at 37°C. Peptides were initially extracted with 30 µl of a mix containing 1% formic acid and 2% acetonitrile at room temperature and then by two successive extractions with 12 µl of a mix of 1% formic acid and 2% acetonitrile and 12 µl of 100% acetonitrile.

Using the Micro Well-plate sampler and the IsoPump modules of an an Agilent 1100 Series Nanoflow HPLC, 20 µl of the tryptic digest solution was injected on a Zorbax 300SB-C18 pre-column (5 x 0.3 mm, 5 µm) linked to an Agilent 1100 Series HPLC-system previously conditioned with water containing acetonitrile (3%) and formic acid (0.1%). The sample was washed for 5 min at 15 µl/min and subsequently the valve holding the pre-column was actuated to connect it between the NanoPump module and the flushed through a 75 µm ID PicoFrit column (New Objective, Woburn, MA) filled with 10 cm of BioBasic C18 (5 µm, 300 Å) in order to allow elution of the peptides towards the mass spectrometer at a flowrate of 200 nL/min. The acetonitrile concentration was first raised linearly from 10% to 40% in 40 min. It was increased linearly to 70% in 8 min, then to 95% in 5 min. The acetonitrile was held at 95% for 2 min then brought back to 10% in 2 min. The system was left at 10% acetronitrile for 3 min before starting the next injection. The total cycle time was 65 min. Eluted peptides were analyzed in a QTof micro (Waters Micromass, Manchester, UK)) equipped with a ZSpray Nanoflow stage modified with a ADPT-MZS nanospray adapter (New Objective, Woburn, MA). MS survey scan was set to 1 s (0.1 s interscan) and recorded from 350 to 1600 *m/z*. MS/MS scans were acquired from 50 to 1990 *m/z*, scan time was 1.35 s and the interscan interval was 0.15 s. The doubly and triply charged selected ions were selected for fragmentation with collision energies calculated using a linear curve from reference collision energies.

MS/MS raw data were transferred from the QTof micro computer to a 50 terabytes server and automatically manipulated for generation of peaklists by employing Distiller version 2.1.0 (http://www.matrixscience.com/distiller.htmls) software with peak picking parameters set at 30 as for Signal Noise Ratio (SNR) and at 0.6 for Correlation Threshold

(CT). The peaklisted data were searched against a copy of the Universal Protein Resource (UniProt) data base (March 03, 2008) by employing Mascot (http://www.matrixscience.com) version 2.1.04, and restricting the search to up to 1 missed (trypsin) cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, 0.5 mass unit tolerance on parent and fragment ions, and monoisotopic. The search was limited to the *Brugia* and *Wolbachia* taxonomies (Taxonomy ID: 6278 and 953, respectively) (17537 sequences; 5684760 residues).

Mascot results from bands 1 to 15 (based on spectra assigned to tryptic peptide sequences at the 95% confidence level) generated peptide identifications that were then linked to the proteins and sorted by protein to produce an initial list of protein identifications. The list was quite redundant since about 5% of the spectra matched more than one peptide and 40% of the peptides identified occur in more than one protein. Consequently, the sequences were processed by a grouping algorithm to generate a list of proteins defined by distinct sets of proteins [18]. That is, the minimum number of protein sequences needed to explain the peptides observed. This minimal list of proteins was summarized on a SubGroup Count Report.

Bioinformatics

Sequences from the minimal list of proteins were retrieved from the UniProt database and scanned for prediction of signal peptides and subcellular localization with SignalP 3.0 [19], TargetP 1.1 [20] and SecretomeP 2.0 [21].

Gene Ontology (GO)

GO annotations were performed using Blast2GO [22]. The initial Blastp search was performed against the NCBI nonredundant database with a minimum expectation value of 1×10^{-3} and a high scoring segment pair cut-off of 33. Annotations were made with default parameters; the pre-eValue-Hit-Filter was 1×10^{-6}, Annotation cut-off was 55, and GO Weight was 5. Annotation was augmented by using the Annotation Expander

(ANNEX) [23] and by addition of the GO terms associated with functional domains derived from scanning the InterPro database [24,25].

The statistical framework GOSSIP [26] was used to identify statistically enriched GO terms associated with gender- or stage-specific secreted proteins compared to the GO terms associated with the complete set of identified proteins. Contingency tables for each GO term in the test group were generated and P values calculated in a Fisher's exact test. The P values were adjusted for multiple testing by calculation of the false discovery rate and the family wise error rate.

Results

Protein identification

ESP were obtained from F, M and Mf under previously described conditions [16,27,28]. Average protein recoveries from several incubations were 71 and 41 ng protein per worm per day for F and M, respectively, and 165 ng protein per 1x 10^6 Mf per day, values that are in the same order of magnitude as reported by others [16,27]. No remarkable changes in motility or physical integrity of the worms were observed during the 4 day incubation, suggesting that the collected media contained the products of normal physiological activity of the parasites and not the products of death or leaking caused by the procedures.

The analysis of ESP from parasitic nematodes is limited by the very low amount of protein usually recovered from *in vitro* incubations. We addressed this issue by comparing several techniques for concentration and desalting at small scale. Although trichloroacetic acid (TCA) precipitation gave higher yields of protein than Amicon (MWCO 3000) ultrafiltration, scaling the TCA precipitation up to 20 ml caused the retention of a significant amount of salt in the preparation. This generated wide lane profiles on SDS-PAGE, resulting in dilution of the proteins in the gel and diminishing the chances for identification. A preliminary analysis under these conditions led to the identification of 15 proteins in F, M, and Mf preparations, which ranged in amount from 6.5 to 13 μg protein (not shown).

As an alternative, we concentrated and desalted ESP by using Amicon devices to attain a final volume of 1 – 1.5 ml, and then precipitated the proteins with TCA. Preparations obtained in this way provided narrower running profiles with no apparent signs of proteolytic degradation (Figure 1). For this analysis, we pooled and concentrated the total media recovered from 3 sets of independent incubations. One set of incubations corresponds to the total number of worms and larvae recovered from one infected animal. This procedure led to the recovery of 37.3 μg, 65.3 μg and 15.4 μg of protein for Mf, F and M, respectively, subsequently subjected to SDS-PAGE (Figure 1).

For protein identification, each lane of a gradient 2.4 cm SDS-PAGE gel was excised, digested with trypsin and analyzed by Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS). Initial peptide matches led to the preliminary identification of 286 proteins in the total ESP set. Refinement of the assignment of peptides to authentic proteins as described in the Methods section. This approach allowed us to identify 228 distinct proteins as part of the ESP of Mf, F and M (Table 1 and S1). All except one, annotated as Mmc-1(UniProt ID: Q9NDV4), were matched to proteins annotated in the *B. malayi* genome database. Peptides identified in the MS/MS analysis were assigned with high confidence to *B. pahangi* Mmc-1, which was also previously identified in Mf-ESP from this parasite [29]. A homolog nucleic acid sequence for the *B. pahangi* Mmc-1gene is also present in the *B. malayi* EST data set (Gene Index: TC11321). Its absence at the moment from the *B. malayi* protein models likely reflects the incomplete assembly available in the current version of the database and not a false-positive assignment.

		Uniprot ID	Pub_locus	TIGR locus	DESCRIPTION	SS	SecP	NQPCT		
								Mf	Fw	Mw
Microfilariae	1	A8NW22	Bm1_11105	13673.m00035	Recombinant antigen R1, identical	Y	0.70	14.35	0.27	0.15
	2	P29030	Bm1_17035	14274.m00229	Endochitinase precursor	Y	0.53	13.04	0.00	0.00
	3	A8PJW0	Bm1_28525	14931.m00318	Serpin	Y	0.49	9.42	0.00	0.00
	4	A8Q2C4	Bm1_41005	14973.m02599	OV-16 antigen, putative	N	0.60	4.64	0.75	1.83
	5	A8QEB1	Bm1_51000	14992.m10974	Putative uncharacterized protein	N	0.51	4.20	5.19	5.55
	6	A8NQM6	Bm1_07780	13333.m00082	Immunogenic protein 3, putative	Y	0.88	3.91	0.98	1.32
	7	A8PKM4	Bm1_29130	14940.m00172	Triosephosphate isomerase, putative	N	0.36	3.48	16.87	3.73
	8	O97392	Bm1_09950	13531.m00015	Gamma-glutamyl transpeptidase precursor	Y	0.69	2.75	3.35	0.81
	9	A8P664	Bm1_17400	14293.m00075	Trypsin inhibitor, putative	N	0.69	2.61	0.00	0.00
	10	A8PGM6	Bm1_24940	14731.m01012	Galectin, putative	N	0.35	2.61	5.22	0.56
	11	A8PVV9	Bm1_35870	14971.m02814	Copper type II ascorbate-dependent monooxygenase, C-terminal domain containing protein	N	0.65	2.03	0.00	0.00
	12	Q9BJC9	-	-	Major allergen	Y	0.76	2.03	1.69	1.02
	13	A8QFZ3	Bm1_54345	15148.m00017	Zinc finger, C2H2 type family protein	N	0.55	1.80	0.00	0.00
	14	O16159	Bm1_56600	15418.m00009	Cystatin-type cysteine proteinase inhibitor	Y	0.96	1.74	1.83	1.39
	15	Q6T8C4	-	-	Superoxide dismutase [Cu-Zn]	N	0.39	1.74	0.58	1.17
Female worm	1	A8PKM4	Bm1_29130	14940.m00172	Triosephosphate isomerase, putative	N	0.36	3.48	16.87	3.73
	2	A8PJU3	Bm1_28435	14930.m00337	Bm-MIF-1, identical	N	0.51	0.72	8.91	0.15
	3	A8QH34	Bm1_56305	15373.m00009	Leucyl aminopeptidase, putative	Y	0.86	0.58	7.72	1.83
	4	A8QFI4	Bm1_53510	15059.m00091	Myotactin form B, putative	N	0.35	0.00	5.24	7.17
	5	A8PGM6	Bm1_24940	14731.m01012	Galectin, putative	N	0.35	2.61	5.22	0.56
	6	A8QEB1	Bm1_51000	14992.m10974	Putative uncharacterized protein	N	0.51	4.20	5.19	5.55
	7	O97392	Bm1_09950	13531.m00015	Gamma-glutamyl transpeptidase precursor	Y	0.69	2.75	3.35	0.81
	8	P67877	Bm1_40465	14972.m07803	Cuticular glutathione peroxidase precursor	Y	0.59	0.29	2.74	7.69
	9	A8PFE3	Bm1_24115	14703.m00079	Enolase, putative	N	0.53	1.16	2.54	4.69
	10	O16159	Bm1_56600	15418.m00009	Cystatin-type cysteine proteinase inhibitor	Y	0.96	1.74	1.83	1.39
	11	Q9BJC9	-	-	Major allergen	Y	0.76	2.03	1.69	1.02
	12	A8Q119	Bm1_40580	14972.m07829	Glycosyl hydrolases family 31 protein	N	0.59	0.00	1.49	1.39
	13	A8Q0F4	Bm1_40185	14972.m07743	Calsequestrin family protein	Y	0.76	0.00	1.32	0.22
	14	A8P3E5	Bm1_15350	14176.m00093	Fructose-bisphosphate aldolase 1, putative	N	0.40	0.00	1.29	6.00
	15	A8QEM1	Bm1_51495	14992.m11078	Heat shock protein 90, putative	N	0.18	0.00	1.08	0.44
Male worm	1	P67877	Bm1_40465	14972.m07803	Cuticular glutathione peroxidase precursor	Y	0.59	0.29	2.74	7.69
	2	A8QFI4	Bm1_53510	15059.m00091	Myotactin form B, putative	N	0.35	0.00	5.24	7.17
	3	A8P3E5	Bm1_15350	14176.m00093	Fructose-bisphosphate aldolase 1, putative	N	0.40	0.00	1.29	6.00
	4	A8NPW6	Bm1_07275	13311.m00333	Core-2/I-Branching enzyme family protein	N	0.67	0.00	0.10	5.68
	5	A8QEB1	Bm1_51000	14992.m10974	Putative uncharacterized protein	N	0.51	4.20	5.19	5.55
	6	A8PFE3	Bm1_24115	14703.m00079	Enolase, putative	N	0.53	1.16	2.54	4.69
	7	A8P0Q6	Bm1_13605	14015.m00091	Major Sperm Protein	N	0.62	0.00	0.38	4.63
	8	A8PKM4	Bm1_29130	14940.m00172	Triosephosphate isomerase, putative	N	0.36	3.48	16.87	3.73
	9	A8NLB0	Bm1_04870	13066.m00251	Putative uncharacterized protein	N	0.78	0.00	0.10	3.18
	10	A8NZQ9	Bm1_12945	13929.m00009	Lethal protein 805, isoform d, putative	Y	0.60	0.00	0.37	2.53
	11	Q4VWF8	Bm1_04665	13047.m00009	Independent phosphoglycerate mutase isoform 1	N	0.49	0.29	0.58	2.42
	12	A9XG48	Bm1_50995	14992.m10973	L3R15 repetitive antigen	N	0.63	0.00	1.04	1.85
	13	A8Q2C4	Bm1_41005	14973.m02599	OV-16 antigen, putative	N	0.60	4.64	0.75	1.83
	14	A8QH34	Bm1_56305	15373.m00009	Leucyl aminopeptidase, putative	Y	0.86	0.58	7.72	1.83
	15	A8P0Q4	Bm1_13600	14015.m00090	Major sperm protein 2 , putative cytoskeletal MSP	N	0.75	0.00	0.13	1.74

Table 1. Most abundant proteins identified in the ESP of *B. malayi*. SS: Signal Peptide Prediction from SignalP. SecP: SecretomeP score; values > 0.5 not having predicted Signal Sequence predict the possibility of non-classical secretion in mammalian cells. NQPCT: Prorated Query Count Percentage values.

Comparison with previous ESP analyses

Many proteins have been identified as being secreted by *B. malayi* and related filarial nematodes by more traditional biochemical techniques (Table S2). All but two of the proteins reported to be secreted by *B. malayi* were found in the current survey. Neither of these two (acetylcholinesterase and Transforming growth factor - 2) were reported in the initial proteomic analysis [14]. Proteins reported to be secreted by other filariae all had homologues in the *B. malayi* ESP set.

This set included 59 of the 80 proteins (73.8%) reported in [14] from incubations of M and F worms in joint culture. Proteins not identified in the current study included 8 assigned in [14] through screening of the *B. malayi* EST database (Gene Index: TC7940, AI783143, TC9625, TC7985, TC8258, TC8116, TC7986 and AA592049). None of these is found in the current assembly of the *B. malayi* genome database, suggesting that further analysis may alter the assignment of the peptides to a genomic locus. In addition, nuclear function associated proteins (Pub locus: Bm1_03115, Bm1_46120, Bm1_25620), several with undetermined function (Bm1_19065, Bm1_57465, Bm1_46475, Bm1_11505, Bm1_01245, Bm1_09845 and Bm1_33310), 6-phosphofructokinase (Bm1_01930), a tropomyosin family protein (Bm1_02060) and phosphatidylethanolamine-binding protein 2 (Bm1_31500) were identified as relatively low abundance ESP proteins in [14] but were not detected in the current study. As reported in [14], none of the proteins detected in *B. malayi* ESP in the current study could be assigned to *Wolbachia* proteins.

According to the Prorated Query Count percentage values (NQPCT), which provide a measure of relative abundance of a protein in a sample [18], we found triosephosphate isomerase (TPI) to be one of the most abundant proteins in the ESP of all gender/stages, indeed the most abundant in F-ESP, consistent with other observations [14]. Although results from our separate incubations of M ad F worms generally agreed with those from the combined culture [14], we found some differences. A fasciclin domain containing protein (UniProt ID: A8P605) and an endochitinase (P29030) were exclusively associated with Mf-ESP and were not present in ESP from adults. These two proteins have relatively

high NQPCT values in the Mf-ESP sample and, therefore, their identification as low abundance ESP proteins in [14] may be related to release of Mf in adult worm cultures.

62 (27.2%) of the identified proteins were predicted by SignalP [19] to have an amino-terminal secretion signal peptide and therefore may be secreted through the classical pathway. This value represents an enrichment of 11.8% in comparison to the proportion of total gene models in the *B. malayi* database having a predicted signal peptide. In addition, 81 (35.5%) of these proteins may be secreted through non-classical secretory pathways, as they were identified by SecretomeP [21] to share features with mammalian proteins secreted in this manner. The proportion of proteins bearing a secretion motif is similar in the current study compared to the proteins identified in [14], suggesting that the presence in ESP of proteins lacking known secretion motifs is not an artifact.

Mf, M and F worms secrete different sets of proteins.

76 proteins were identified in Mf-ESP. 160 and 119 were identified in F and M-ESP sets, respectively. Only 32 proteins (14.0%) were shared by all three stages/genders (Figure 2). Approximately half of these proteins had no annotated function or were poorly annotated (assigned a named match but with no associated functional domain or Gene Ontology term). F and M shared 54 proteins (23.7%), whereas Mf showed a much lower degree of similarity with the adult stages, with only 7 (3.1%) and 2 (0.9%) proteins shared with F and M-ESP, respectively.

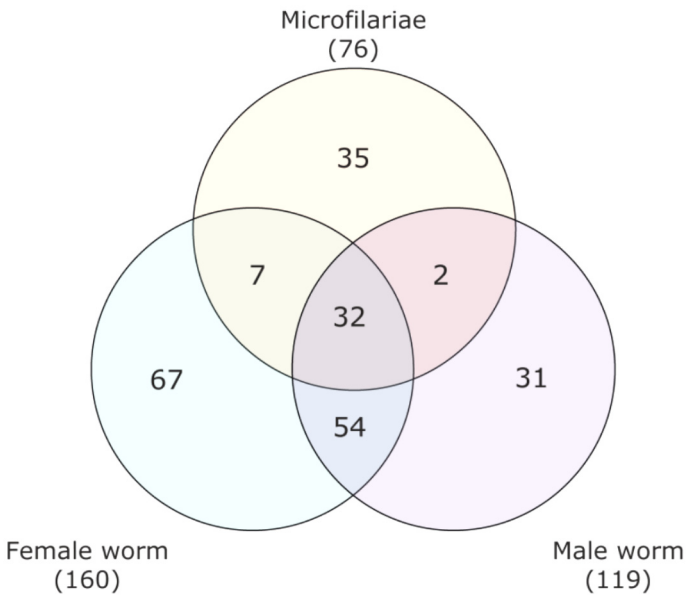

Figure 2 Venn diagram showing the distribution of proteins identified in ESP from microfilariae, female and male *B. malayi.*

Differences expressed as presence/absence of a protein can be extended to protein abundance. Table 1 presents a list of the 15 most abundant proteins as determined by NQPCT in each ESP. Most of the Mf-ESP proteins presented in this table were only identified at this stage or were not found as highly abundant proteins in ESP from adults. Differences in ESP composition between M and F were also observed; notable among these are the presence of Major Sperm Protein (MSP) family proteins as highly abundant in M-ESP, the appearance of a homolog of the human macrophage migration inhibitory factor (MIF-1, A8PJU3) as an abundant protein in F-ESP but not in M-ESP and numerous differences in the relative abundances of other proteins between the sexes.

GO Analysis

We used the Blast2Go analysis tool to mine the GO based data to illuminate the different functions and processes in which the proteins identified in the ESP are putatively involved, The initial annotation was augmented by using the Annotation Expander (ANNEX) [23] and by addition of the GO terms associated with functional domains resulting from scanning the InterPro database [24]. This analysis provided ≥1 GO terms

for 171 sequences (75%) from the total set. Of these, 157 (68.8% of the total) sequences could be assigned to terms associated with molecular functions and 136 (59.6% of the total) with biological functions.

In the total set of ESP and the individual subsets (Mf, F and M), catalytic activity (GO:0003824) and binding (GO:0005488) were the two major molecular function categories (Figure 3A). Other molecular function categories include enzyme regulator activity (GO:0030234) and antioxidant activity (GO:0016209). Structural molecule activity (GO:0005198) was not found in Mf-ESP but was common in M-ESP (Figure S1). At a higher level of ontology (Figure 4A), most of the assigned binding activity could be assigned to metal ion binding and cation binding (GO:0046872 and GO:0043169), purine nucleotide binding (GO:0017076), ribonucleotide binding (GO:0032553) and, to a lesser extent, sugar binding (GO:0005529) and several terms related to protein binding (GO:0051082, GO:0008092 and GO:0042802). The catalytic activity function is populated by diverse types of reactions, with a major contribution from peptidase activity (GO:0008233). Interestingly, the protease inhibitor activity term (GO:0030414) was the most common in the enzyme regulator activity category.

The most common biological function categories (Figure 3B) were cellular process (GO: 0009987), metabolic process, multicellular organism process (GO:0032501), developmental process (GO:0065007) and, less commonly, biological regulation (GO:0065007), growth (GO:0040007) and reproduction (GO:0000003). As with the molecular function classes, proteins in these categories were found in the total set and individual subsets of ESP (Figure S2). In addition and as expected, proteins associated with reproduction (GO:0000003) were predominantly found in adult ESP.

A higher level of ontology (Figure 4B) shows that most of the cellular and metabolic processes are related to synthesis and degradation of macromolecules, particularly proteins and carbohydrates (GO:0044260, GO:0019538, GO:0043283, GO:0005975, GO:0009057), whereas the largest contribution to the multicellular organism and developmental processes came from terms such as embryonic development ending in

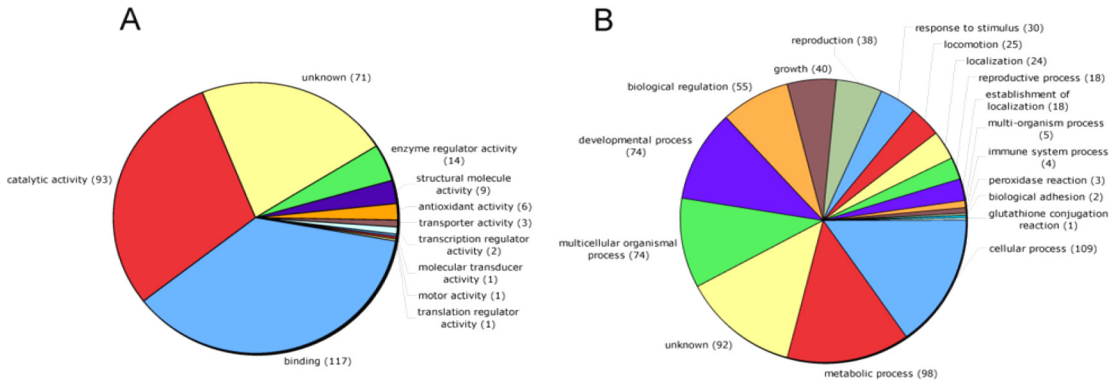

Figure 3. Distribution of Gene Ontology terms (level 2) for proteins identified in ESP from microfilariae, female and male *B. malayi* . A. Molecular Function. B. Biological Process

birth or egg hatching (GO:0009792) and larval development (GO:0002164) that can be associated with the release and development of Mf.

The GOSSIP statistical framework [26] was used to determine the enrichment of particular functions or processes in the ESP from Mf, M and F. We compared the terms associated with the proteins identified in each of the 3 ESP sets against those from the total GO term annotated proteins. Several processes and functions had significant *P* values ($P < 0.05$) in the single test (Figure 5). Nevertheless, to correct for multiple testing, a more stringent comparison using both a false discovery rate and a family-wise error rate was performed, and only the terms in the Mf-ESP were found to be enriched. These terms are all children of the parent ion binding term (GO:0043167), with zinc ion binding (GO:0008270) the term with the highest level of ontology.

Discussion

Filarial infections pose continuing and significant threats to human and animal health. Although drugs such as ivermectin, diethylcarbamazine (DEC) and albendazole are currently used to interrupt disease transmission and reduce morbidity [30], there are concerns about the emergence of resistance for these drugs [31,32]. Secreted products are

thought to be essential for the establishment of the parasitic lifestyle and therefore their identification in filarial nematodes may lead to the discovery of novel drug and vaccine targets [33,34,35,36]. Moreover, their recognition will help to illuminate the biology of secretory processes in these organisms and to establish a path for developing a deeper understanding of how parasite proteins function in immune evasion.

We exploited the remarkable sensitivity of mass spectrometry and the availability of a genome with > 11,500 predicted gene models [13] to identify proteins secreted *in vitro* by *B. malayi.* It is reasonable to assume that the profile and function of secreted proteins will vary with developmental stage and sex, and may also vary in response to signals from the host.

To begin an investigation into this area, we analyzed ESP from independently incubated Mf, M and F worms; this analysis thus extends in a new way the previous work done on ESP obtained from adult male and female *B. malayi* in co-culture [14]. In addition to the identification of 148 novel secreted proteins from filarial nematodes, the approach employed here allowed us to compare the ESP composition for each group. As we recognize in global terms these differences in the ESP from the different forms of a parasitic nematode, it should enable us to start to infer the different roles and processes in which these proteins are involved, in particular to gain a better understanding of how filarial parasites orchestrate immune evasion. This will open a new dimension in the understanding of the biological significance of protein secretion by parasitic nematodes.

Until recently, all previous work on the identification of ESP from filarial nematodes was performed by the study of a few, primarily immunoreactive, proteins. Comprehensive analyses of ESP are still limited by the low amounts of proteins that can be recovered from *in vitro* incubations of living worms. In this context, the use of 1D-SDS PAGE and LC-MS/MS may be a more efficient way to identify protein sequences in filarial ESP compared to the shotgun LC–MS/MS approach or the excision of particular spots from 2D-gels followed by MALDI-ToF/ToF [14]. This technical approach, in conjunction with homology-based analyses for mining of functional information on each of the hits, allows

for a more complete understanding of the probable events and functions in which these components may be involved.

Effective prediction of ESP through the identification of signal sequences or other domain features is not feasible because, as we show here, many of the proteins released in culture would have been missed by secretome prediction tools, indicating that the processes involved in the release of these components are not fully understood. This issue, along with the need to identify non-protein components and post-transcriptional modifications of proteins in ESP that may have functional and essential roles, remain goals for the development of new tools to understand the basic biology of filarial and other parasitic infections.

Proteins common to all stages/genders

About 14% of secreted proteins were found in all stages/genders. One can speculate that this group is likely to include proteins that are essential for survival in the host, for instance, by deflecting the immune response. However, a significant portion of the common ESP proteins have no annotated function or GO term that can help in inferring their roles; therefore, the challenge is to elucidate their possible functions as determinants of parasitism. This includes proteins putatively assigned to the transthyrethin-like family, recently reported in [14], a set of hypothetical proteins and previously identified but functionally uncharacterized antigens.

An intriguing aspect of the common ESP set is the prominent presence of the glycolytic enzymes enolase (A8PFE3) and TPI (A8PKM4). Although this finding could be due to death or compromised integrity of the parasites in culture, we believe this is not the case. First, other glycolytic enzymes, at least as abundant in cytoplasm as these two, do not appear in ESP. Secondly, these proteins have been previously identified in ESP from *B. malayi* and other parasitic nematodes [14,35,37,38]. Finally, evidence has appeared about their multiple roles and interaction with surface components in eukaryotic cells; an extracellular role thus cannot be excluded [39,40,41].

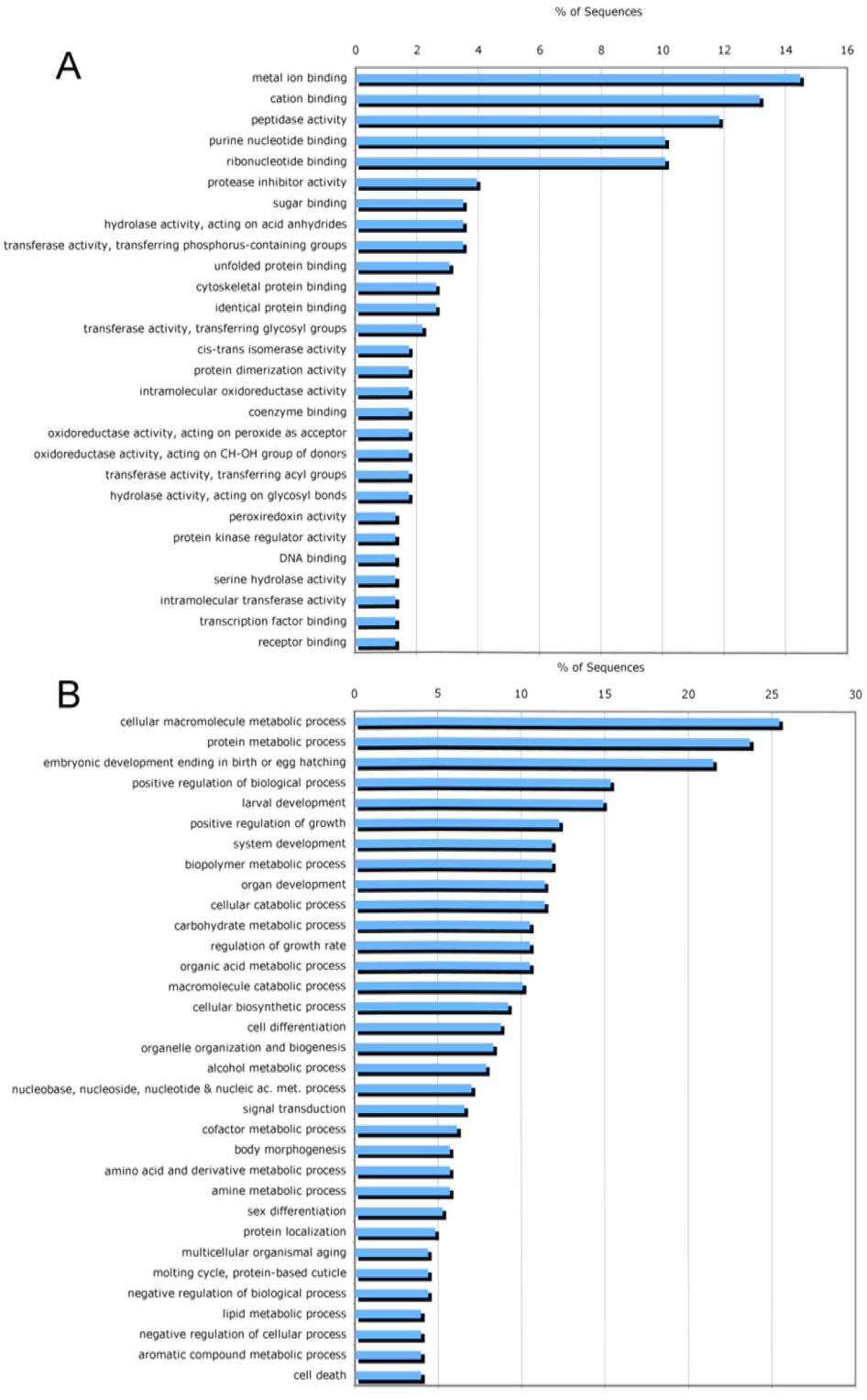

Figure 4. Distribution of the most abundant Gene Ontology terms (level 4) assigned for proteins identified in ESP from microfilariae, female and male worms of *B. malayi.* A. Molecular Function. B. Biological Process.

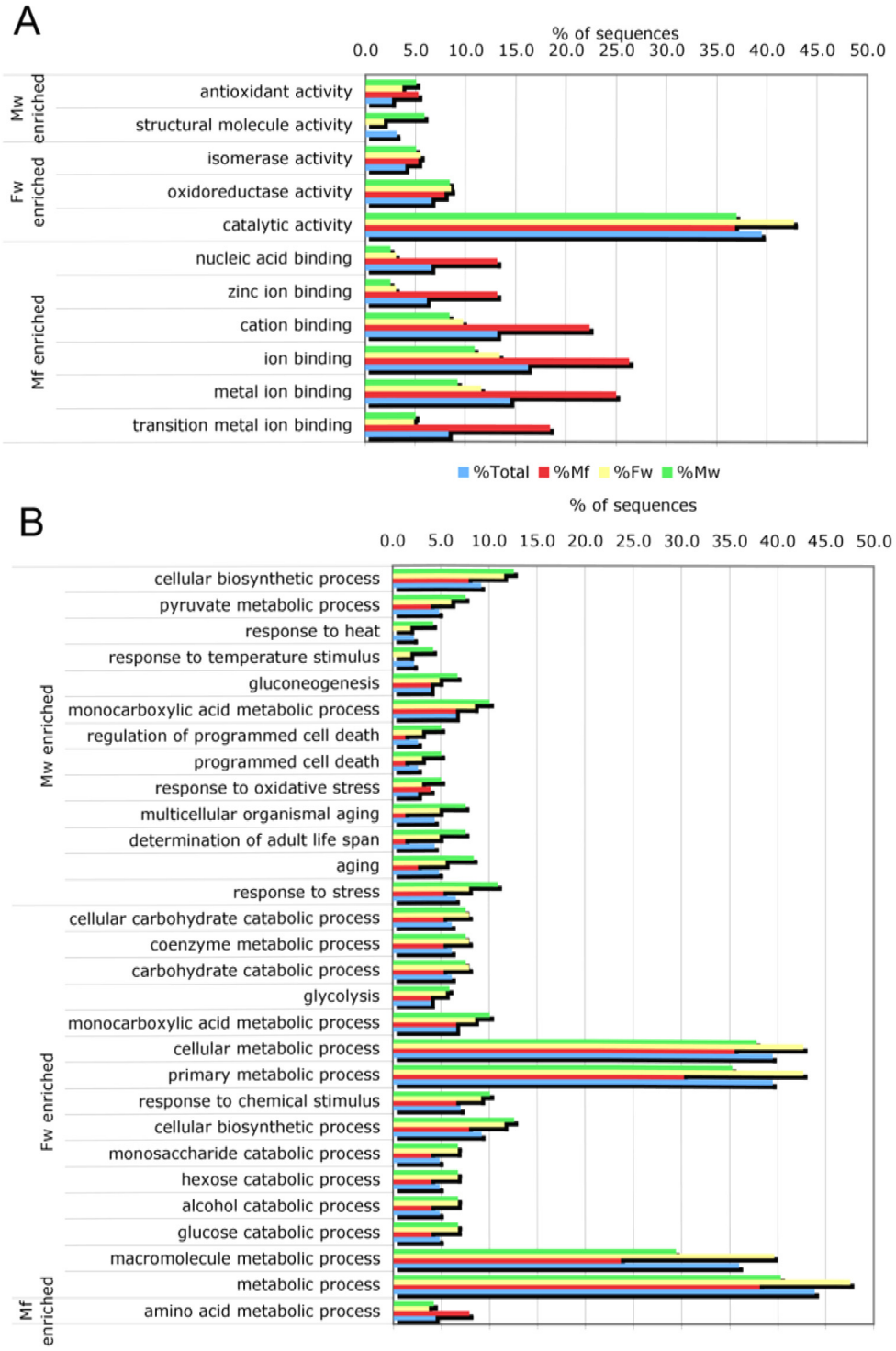

Figure 5. Gene Ontology terms concentrated on individual ESP from microfilariae, female and male *B. malayi* compared to the total protein set. A. Enriched molecular function terms. B. Enriched biological process terms.

Other proteins identified in all 3 stages/genders include potentially immunomodulatory proteins, including a homolog of MIF-1 (A8PJU3) [42], a galectin (GAL-1, A8PGM6), a cystatin-type cysteine proteinase inhibitor (CPI-2, O16159) [43] and leucyl aminopeptidase (LAP, A8QH34), a homolog of an ESP product that in *Acanthocheilonema viteae* (ES-62) is modified with N-Type glycans containing phosphorylcholine (PC) [44]. In contrast, the protein identified in [14] as harboring the PC moiety in *B. malayi*, a core-2/I-branching enzyme family protein (A8NPW6), was only found in adult stages together with another isoform of human MIF (MIF-2) (Q9NAS2) [45] and GAL-2 [14].

ESP from Mf

Taking into account the number of proteins identified, their abundance and the possible functions and processes in which they can be predicted to be involved, it is clear that the composition of ESP from Mf is quite distinct from adult ESP. This result is perhaps unsurprising, since adults and larvae may exhibit different physiological repertoires reflecting their different developmental stage and anatomical location (blood vs. lymph). Differences in ESP composition also suggest that there may be differences in the mechanisms of immune evasion between the stages.

Several proteins with no assigned function were identified in Mf-ESP, including the protein identified as antigen R1 (A8NW22), which was the most abundant. This protein seems to be preferentially expressed in Mf, although it was also identified in ESP from M and F. Although no function is annotated for this protein, recombinant R1 has been used in diagnostic IgG4 ELISAs with excellent success for the detection of *B. malayi* infection using serum from Mf(+) patients, with however significant positivity in Mf(-) patients [46].

Many Mf-ESP proteins with predicted function are associated with developmental processes and regulation of enzyme activity. Some may play a role in the immunology of the host-parasite relationship, including an endochitinase (P29030) and a serpin

(A8PJW0), which were the next most abundant proteins in Mf-ESP; both were only found at this developmental stage. Chitinases are essential for chitin degradation during molting of larval filariae [35]. P29030 was originally reported as an antigen recognized by MF1, a monoclonal antibody that mediates the transient clearance of Mf in jirds infected with *B. malayi* [47]. Immunization of gerbils with recombinant endochitinase induced partial protection against Mf, but did not reduce adult worm burdens, suggesting that this protein is crucial for Mf development but not for adult viability [48].

GO analysis revealed that protease inhibition was the most common functional class in the 'regulation of enzyme activity' category. Mf secreted a completely different set of protease inhibitors than adults. In particular, homologues of serine protease inhibitors (SPI) were abundant in Mf-ESP. This set included serpins, a class of proteins having a wide spectrum of functions at extracellular and intracellular levels in eukaryotic cells [49]. In mammals, serpins are involved in the regulation of fundamental processes, including coagulation, complement activation and inflammation [50,51], but their potential roles as modulators of host responses in lymphatic filariasis are uncertain [52]. The serpin identified as A8PJW0 is the predicted gene model for *B. malayi* serine protease inhibitor 2 (Bm-SPN-2, UniProt Accession No. O18656). Bm-SPN-2 induces a Th1 response as characterized by the *in vitro* production of IFN-γ but not IL-4 or IL-5 in murine T cells [53]. At least 14 serpins are predicted in the *B. malayi* genome, two of which (A8PHV4 and A8PHV1) were present in lower abundance compared to Bm-SPN2, and both of them only in Mf-ESP.

In addition to serpins, another abundant serine protease inhibitor in Mf-ESP is a putative trypsin inhibitor (A8P664). A8P664 contains a trypsin inhibitor-like cysteine rich domain (TIL) that can potentially inhibit peptidases belonging to families S1, S8, and M4. A8P664 shares 50% identity with a trypsin inhibitor from the gastrointestinal nematode *Ascaris suum*, but is less related to serine protease inhibitors from the filarial nematodes *Dirofilaria immitis* (Di-SPI-1) and *Onchocerca volvulus* (Ov-SPI-1, Ov-SPI-2) [54]. Bm-SPI-1, another inhibitor from *B. malayi* [54], was not found in any of the ESP and has no significant homology with A8P664. In *A. suum* and other gastrointestinal nematodes,

secretion of trypsin inhibitors has been proposed to interfere with the action of host digestive enzymes and with immunological effector mechanisms [55,56,57,58]. The Ov-SPI proteins seem to play a crucial role in nematode molting and in processes such as embryogenesis and spermatogenesis [54]. The roles of A8P664 may be different, as it is only found in Mf-ESP.

Another potential serine protease inhibitor present in relatively high abundance in adult and Mf-ESP is a homolog of the *O. volvulus* antigen Ov16, identified as A8Q2C4 [59]. A8Q2C4 was also identified in [14] in adult ESP as a homolog of proteins in the phosphatidylethanolamine-binding protein (PEBP) family, including a secreted 26-kDa antigen from the ascarid *Toxocara canis* [60] and a mouse PEBP with inhibitory activity against several serine proteases [61].

An intriguing finding in Mf-ESP is the presence of several zinc finger (ZnF) C2H2- type family proteins (A8QFZ3, A8PEN7, A8PLP4, A8QHJ5, A8QHP5, A8ND91). These proteins were classified in the GO analysis with the Zinc Ion binding term, a molecular function that was enriched in Mf-ESP compared to the total protein set of ESP. The most common role assigned to ZnF proteins is the control of gene transcription through binding to specific DNA segments [62]. In addition, ZnF motifs mediate RNA, protein and lipid binding [63,64]. Although no apparent function has been assigned to these nematode proteins as mediators of extracellular processes, the fact that all of them were found in a particular stage with relatively high levels of secretion in comparison to other known secreted proteins deserves further consideration.

Possible differences between Mf and adults in oxidative stress and nitric oxide evasion

Filarial nematodes deploy several mechanisms to detoxify reactive oxygen and nitric oxide derivatives produced by the host [65]. Based on our results, the different stages may use different mechanisms to overcome this type of stress. For example, gp29 (P67877), a glutathione peroxidase believed to act as a lipid hydroperoxidase, protecting parasite membranes from peroxidation caused by oxygen free radicals [66,67,68], was identified

in all 3 ESP sets. However, the markedly higher abundance of gp29 in adult compared to Mf ESP suggests that this enzyme may play a more important role in adult survival compared to Mf. In contrast, γ-glutamyl transpeptidase (γ-GT, O97392) [69], glutathione S-transferase (GST, A8Q729) [70] and Zn-Cu superoxide dismutase (SOD, Q6T8C4) [71,72,73] appeared with similar abundance in ESP from all three gender/stages of the parasite. Thus, mammalian-stage parasites may use common strategies to defuse free radicals in the host microenvironment by modulating the levels of glutathione, or by using the thioredoxin (TRX-1, A8Q921) system as a source of reducing equivalents [74,75]. In addition to its role in transferring the γ-glutamyl moiety of glutathione to an acceptor [76], *B. malayi* γ-GT can trigger autoimmunity against human γ-GT in lung epithelial cells and may play a role in the development of the local pulmonary pathology syndrome known as Tropical Pulmonary Eosinophilia [77].

Sex-specific secreted proteins

Several sex-specific secreted proteins were identified in this study, characterized by relatively low abundance. This list may be underpopulated due to the fact that some of the proteins identified in both stages could be misclassified as the result of transferring of proteins from male to females worms during insemination previous to the recovery of worms from the jird host, as is likely the case for major sperm protein 2 (MSP-2), expressed only in M [78] but found in both M and F-ESP. Other members of the MSP family were found only in M-ESP. In male *B. malayi* and other nematodes, MSP are expressed in the developing sperm and reproductive system and are essential for nematode sperm motility [79,80]. It is possible that proteins exclusively identified in F-ESP are released in uterine fluid, as some of them including the embryonic fatty acid binding protein (Bm-FAB-1, Q9GU91) and the papain family cysteine protease containing protein (A8NND7) have been reported to be expressed in the uterus as well as in developing gametes and embryos [79].

Conclusions

This proteomic analysis led to the identification of multiple components of the ESP of Mf, F and M *B. malayi*. In addition to the report of new identified ESP, the opportunity to compare the composition of ESP in all three stages/genders allowed us to propose different stage and gender specific related processes and to identify candidate proteins that may underlie stage specific strategies of immune evasion.

Acknowledgements

We thank Sylvie LaBoissière, Daniel Boismenu and Line Roy for their help with data analysis and the technical support of McGill University Genome Quebec Innovation Center. We also thank the NIH Filariasis Research Reagent Resource Center, College of Veterinary Medicine, University of Georgia, Athens, GA, for provision of infected jirds.

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Supplementary Data:

The following supplementary data associated to this article can be found at http://www.plosntds.org/article/info%3Adoi%2F10.1371%2Fjournal.pntd.0000326#s5

Table S1. Proteins identified in ESP from microfilaria, female and male *B. malayi*.

TP: Prediction of subcellular localization by TargetP, (M): Mitochondrion, (S): Secretory pathway (-): Any other location. **RC:** Reliability class, from 1 to 5, where 1 indicates the strongest prediction. **GO:** Gene Ontology. **EC:** Enzyme Commission number. **NQPCT:** Prorated Query Count Percentage values.

Table S2. List of previously secreted proteins identified by other methods in *B. malayi* and other parasitic nematode species.

Figure S1. Distribution of Molecular Function Gene Ontology terms (level 2) compared between stages/genders of *B. malayi*.

A. total set of ESP identified, B. ESP identified in microfilariae, C. ESP identified in females D. ESP identified in males.

Figure S2. Distribution of Biological Process Gene Ontology terms (level 2) compared between stages/genders of *B. malayi*.

A. total set of ESP identified, B. ESP identified in microfilariae, C. ESP identified in females D. ESP identified in males.

Connecting Statement I

In the first manuscript, *B. malayi* ESP from Mf and adult worms were characterized using a proteomic approach. Bioinformatic tools allowed us to predict that both classical and non-classical cellular secretory pathways are involved in the release of proteins from the parasite. The initial scope of the subsequent work aimed for the description of the anatomical pathways involved in protein release from the Mf. This was done to understand the processes involved in protein release from this developmental stage and particularly the contribution of the specialized ES apparatus. The development of methodologies for protein immunolocalization allowed us to conduct parallel experiments to determine the localization of the putative targets of ivermectin, the glutamate-gated chloride channels (GluCls). Our interest in this drug and its targets is primarily based on the lack of knowledge on the processes that prime the elimination of the Mf from the host despite its widespread use in mass drug administration programs for the control of filariasis. Preliminary evidence showed the lack of *in vitro* effects of the drug on Mf motility or viability suggesting that either the drug targets were not expressed at this stage or that the direct effect of the drug was exerted on a physiological process, such as protein release, that was associated with the measured variables. In the following chapter, I describe the anatomical patterns of ESP in Mf of *B. malayi* and the relationship of protein release from this stage with the activity of a muscle associated with the ES apparatus regulated by GluCls activity.

Chapter IV
(Manuscript II)

Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*

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Published in: *Proceedings of the National Academy of Sciences of the United States of America* (2010). 107(46): 20120 - 20125[2]

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Abstract

Ivermectin (IVM) is a broad-spectrum anthelmintic used in filariasis control programs. By binding to nematode glutamate-gated chloride channels (GluCl), IVM disrupts neurotransmission processes regulated by GluCl activity. IVM treatment of filarial infections is characterized by an initial dramatic drop in the levels of circulating microfilariae followed by long-term suppression of their production, but the drug has little direct effect on microfilariae in culture at pharmacologically relevant concentrations. We localized *Brugia malayi* GluCl expression solely in a muscle structure that surrounds the microfilarial excretory-secretory (ES) vesicle. This suggests that protein release from the ES vesicle is regulated by GluCl activity. Consistent with this hypothesis, exposure to IVM *in vitro* decreased the amount of protein released from microfilariae. To better understand the scope of IVM effects on protein release by the parasite, 3 different expression patterns were identified from immunolocalization assays on a representative group of 5 microfilarial ES products. Patterns of expression suggest that the ES apparatus is the main source of regulated ES product release from microfilariae as it is the only compartment that appears to be under neuromuscular control. Our results show that IVM treatment of microfilariae results in a marked reduction of protein release from the ES apparatus. Under *in vivo* conditions, the rapid microfilarial clearance induced by IVM treatment is proposed to result from suppression of the ability of the parasite to secrete proteins which enable evasion of the host immune system.

Introduction

Infections with filarial nematodes are not only major causes of long term disability, but also create economic hardship and social stigma in endemic areas. In recent years, very significant efforts toward elimination are in place for 2 of these diseases: lymphatic filariasis (LF), caused by infection with *Wuchereria bancrofti*, *Brugia malayi*, *B. timori* and *B. pahangi*, present in 83 countries with ~120 million people infected [1]; and onchocerciasis (river blindness) caused by *Onchocerca volvulus,* predominant in 22 countries in sub-Saharan Africa with ~18 million currently infected [2].

Filarial infections are chronic; adults and microfilariae (mf) can survive from years to decades in the mammalian host. Human infection starts with the release of infective larvae (L3) during feeding of the arthropod vector. L3 enter the host at the puncture site and migrate for maturation to lymphatic vessels (*W. bancrofti* and *Brugia* spp.), or to the subcutaneous and connective tissues (*O. volvulus*). After mating, female worms can release hundreds to thousands of mf per day. Mf from lymphatic filariae transit from lymph to the peripheral circulation; *O. volvulus* mf usually remain in the skin and lymphatics of connective tissue, where mf become available for ingestion by the vectors.

No vaccine is available; filariases are controlled through chemotherapy, although vector control and bed-nets are important adjuncts. Current programs for eradication of these diseases include mass drug administration (MDA) of a microfilaricide to interrupt transmission and diminish morbidity. Control of onchocerciasis since the late 1980's has primarily relied on MDA programs with ivermectin (IVM). By 2005, > 407 million treatments had been administered for this disease [3] (> 800 million by 2009). LF treatment includes annual, single-dose combinations of either IVM or diethylcarbamazine (DEC) with albendazole (ABZ). More than 1.9 billion treatments have been provided since 2000 through the Global Program for Elimination of LF (GPELF) [1,4].

IVM, a macrocyclic lactone endectocide, is considered a microfilaricide due to the fast mf clearance observed and the extremely prolonged suppression of their reappearance after

dosing [5]. Although kinetics of mf clearance vary depending on the species, most of the dramatic changes in the mf load take place during the first week of treatment. In LF, 100 – 200 µg/kg IVM reduces blood mf density to <5 % and to 32-39% of the initial mf load in Bancroftian and Brugian filariasis, respectively, 1 day after dosing [6,7]. In onchocerciasis, reduction in mf numbers 1 day after a dose of 150 µg/kg is ~60% of pre-treatment level [8]. One week after treatment, microfilaremia is reduced to 0% in Bancroftian filariasis and onchocerciasis and ~10% in Brugian filariasis; these reductions are sustained for at least 6 months.

In contrast to the unquestionable efficacy of *in vivo* IVM treatment and despite its extensive use, the pharmacological mechanism underlying rapid mf clearance remains elusive. *In vitro*, IVM effects on mf appear only at concentrations far above levels achieved during therapy for filariases [9]. In the model nematode *Caenorhabditis elegans* and intestinal parasitic nematodes, IVM affects pharyngeal pumping and motor activity at concentrations 0.1-10 nM [10,11,12]. Consistent with these effects, glutamate-gated chloride channels (GluCls), the primary nematocidal targets for IVM, are localized in the motor nervous system of these organisms, particularly in motor neuron commissures, lateral and sub-lateral nerve cords, amphids and pharyngeal neurons [13].

GluCls are members of the 'Cys-loop' family of ligand-gated ion channels (LGIC) which form pentameric structures composed of one or more subunit types [14]. GluCl gene diversity across the phylum Nematoda is significant, but the biological processes regulated by the activity of these channels have been investigated in few species. Neither their localization nor physiological roles can be simply extrapolated between phylogenetically distant nematode species. In *C. elegans*, for example, 6 GluCl genes have been identified as encoding 8 possible protein subunits formed by alternative splicing [14]. In contrast, homology searching for gene models that represent probable GluCl subunits in the genome of *B. malayi* reveals only four subunits, two of them (AVR-14A and AVR-14B) related to IVM-sensitive channels [15,16].

Although direct evidence is lacking, a synergistic effect between IVM and the host immune system has been proposed to explain the clearance of mf [9]. One possibility is that IVM disrupts filarial processes that result in the modulation of the host immune system. The immunomodulatory ability of filarial nematodes generates a host phenotype characterized by suppression of both Th1 and Th2 responses, impaired proliferation of T cells, increased production of the regulatory cytokine IL-10, and higher levels of IgG4 [17]. However, the molecular mechanisms that enable parasite evasion of host immune responses are incompletely understood. It appears that most immunomodulatory strategies used by these parasites are based on soluble mediators, especially proteins that modify or degrade cellular and/or molecular components of the host immune system [18].

Our goals in these experiments were to obtain a better understanding of the mechanism of action of IVM in mf, focusing on inhibition of the secretion by the parasite of immunomodulatory proteins. We conducted experiments to identify the anatomical location of GluCl expression in *B. malayi* mf. Localization was compared to the anatomy of the parasite excretory-secretory (ES) system. Finally, we directly measured the effects of IVM on protein secretion by mf.

Results

Identification of the putative IVM receptors in *Brugia malayi*: Cloning of AVR-14 subunits.

A homology search revealed 4 possible GluCl subunits in *B. malayi* [16]. Phylogenetic analysis and naming according to [19] allowed us to infer that these subunits are orthologs of the GLC-2, GLC-4, AVR-14A and AVR-14B subunits in *C. elegans* and other nematodes. Among them, only AVR-14A and AVR-14B are reported to form IVM-sensitive channels [14]. Since assembly and annotation of these subunits in the *B. malayi* genome database was ambiguous and incomplete, the full open reading frames for these subunits were assessed (see Materials and Methods and Figure S1).

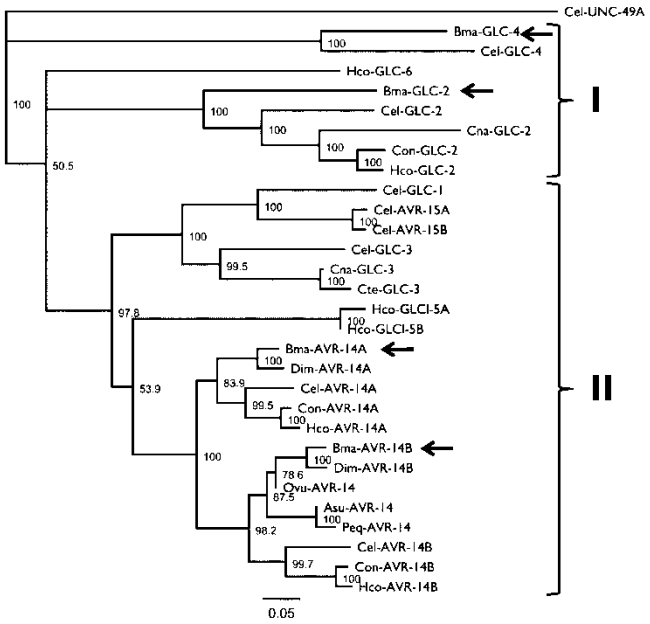

Figure 1. Boot-strapped (1000) Neighbour Joining (NJ) tree generated from a ClustalX alignment of the Bma-AVR-14A and B sequences and sequences of GluCls from other parasitic nematodes and *C. elegans*. Outgroup sequence for this tree was set with a γ-aminobutyric acid receptor subunit from *C. elegans* (Cel-UNC-49A).

I = non-sensitive or unknown sensitivity to IVM; II = sensitive to IVM.

A combination of PCR and 5' RACE was used to clone full-length sequences for 2 splice variants of the *Bma-avr-14* gene. These sequences encode 419 and 427 amino acids of the Bma-AVR-14A and Bma-AVR-14B subunits, respectively. Bioinformatic analysis predicts the characteristic features of the cys-loop LGIC gene family (Figure S2). These include a long N-terminal extracellular domain containing a disulphide-bond formed by two conserved cysteine residues separated by 13 amino acids, and four transmembrane regions (TM1–TM4) with a large cytoplasmic loop between TM3 and TM4 [20]. Phylogenetic analysis using NJ trees retained clustering of both Bma-AVR-14 subunits with their respective orthologs in nematodes (Figure 1).

Bma-AVR-14 subunits were more closely related to AVR-14 subunits from the filarial nematodes *Dirofilaria immitis* and *O. volvulus* than to those of other nematodes (Figure 1), with amino acid sequences 94 and 95% identical to their respective homologs in *D. immitis*. Our results agree with previous findings that the alternative splice patterns of filarial species differ from those of *C. elegans* and *H. contortus* [21]. Filarial *avr-14* transcripts were characterized by the presence of the SL1 sequence at the 5' end followed by a shared sequence containing the coding sequence for the N-terminal region of the two subunits and different C-terminal coding sequences. Filarial *avr-14A* transcripts lack the long UTR upstream of the poly-A tail characteristic of the *avr-14A* transcripts from *C.*

elegans and *H. contortus* (Figure S1 in Appendix 1). These differences reflect the considerable phylogenetic distance between filarial nematodes and the other two species [21].

Localization of GluCl subunits in *B. malayi* reveals association of muscle with the mf Excretory-Secretory vesicle.

To localize GluCl expression in mf, an antibody was raised against the peptide LRTKMILRREFS-cysteine, mapping to AA 239-250 of Bma-AVR-14A. Cross-reactivity of the affinity-purified antibody against the peptide ARVMLLLRREYS-cysteine, corresponding to the same region in Bma-AVR-14B, was determined by ELISA. The antibody has ~5-fold higher affinity for the Bm-AVR-14A subunit compared to the Bma-AVR-14B subunit. Comparison with the same region of the other two *B. malayi* GluCl subunits showed a comparable level of similarity to that between Bma-AVR-14A and B. We assume that the immunolocalization protocol employed here does not necessarily discriminate between the different GluCl subunits.

B. malayi GluCl subunits were localized by Confocal Laser Scanning Microscopy (CLSM), with counterstaining against nuclei and actin (Figure 2). Counterstaining distinguished the major anatomical features of *B. malayi* mf*,* including the oral opening, the nerve ring, the Excretory-Secretory (ES) apparatus, the inner body and anal pore. GluCl subunits were specifically and selectively localized to tissue surrounding the Mf ES vesicle. Ultrastructural observations have described this apparatus in *B. malayi* mf as consisting of a large vesicle that opens to the cuticle and a large excretory cell connecting to the vesicle by a cytoplasmic bridge [22]. Actin staining revealed that muscle tissue surrounds the ES vesicle and co-localizes with the GluCl signal (Figure 2B).

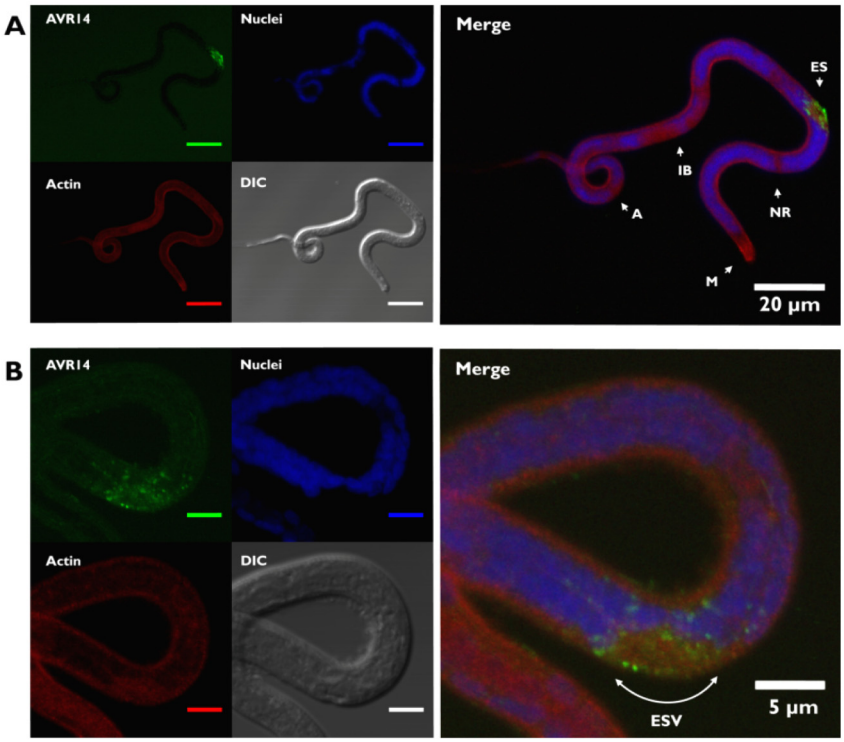

Figure 2. Immunolocalization of AVR-14 subunits in *B. malayi* mf by confocal laser scanning microscopy. (A) Bma-AVR-14 specific signal was detected in proximity to the Excretory-Secretory apparatus. Counter staining with DAPI and Phalloidin-Rhodamine distinguishes major anatomical features (A, anus; IB, inner body; ES, Excretory-Secretory apparatus; NR, nerve ring; M, mouth). (B) A closer examination of the signal reveals co-localization of Bma-AVR-14 subunits with a muscle structure surrounding the Excretory-Secretory Vesicle (ESV).

The presence of a muscle layer encompassing the ES vesicle suggests the possibility of neuromuscular regulation of the release of ES products. This implies that protein transit from the vesicle to the exterior pore of the ES apparatus is an active process. Under this assumption, we hypothesize that GluCls regulate the activity of this muscle and that IVM treatment of mf would lead to a decrease in protein release.

The mf-Excretory/Secretory Apparatus is the main source for protein secretion.

To clarify the contribution of the ES apparatus to protein release from mf and consequently the role of GluCl subunits in the processes that regulate it, a comparative analysis of the anatomical pathways involved in protein secretion was undertaken. Immunolocalization studies by CLSM of proteins known to be released from *B. malayi* mf, termed ES proteins, including translationally controlled tumor protein-like protein

(Bma-TCTP), macrophage migration inhibitory factor–1 (Bma-MIF-1), cysteine protease inhibitor–2 (Bma-CPI-2), venom allergen-like protein–1 (Bma-VAL-1) and triose phosphate isomerase (Bma-TPI), established three different localization patterns for ES proteins.

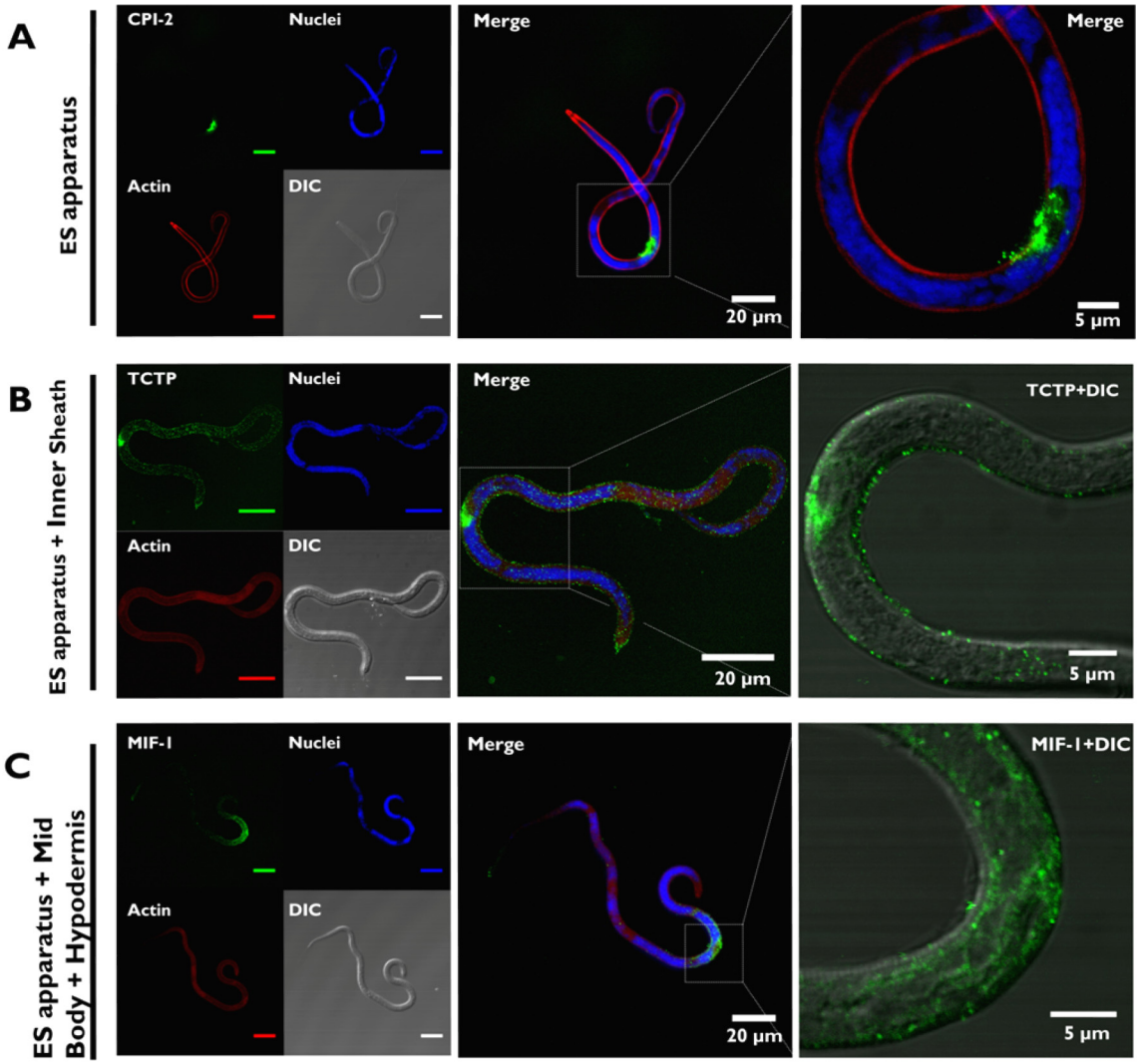

Figure 3. Immunolocalization studies of Excretory-Secretory (ES) proteins in *B.malayi* mf reveal 3 different expression patterns. (A) Protein localization exclusively in the ES apparatus. Representative staining is shown for CPI-2. Also exhibiting the same pattern, VAL-1, TPI and a fraction of mf stained with anti-TCTP (Figure S3 in Appendix 1). (B) Protein localization in either the ES apparatus or the mf inner sheath. This pattern was exhibited in another fraction of specimens stained for TCTP. (C) The third pattern, observed for MIF-1, consisted of prominent expression through the mid-body. Images on the right panels of B and C are presented as merged images of single planes showing signal presence in the ES-vesicle and differences in compartmentalization of TCTP and MIF-1 towards the parasite surface.

Bma-CPI-2, Bma-VAL-1 and Bma-TPI were exclusively found in the ES apparatus (Figure 3A). Localization was typically characterized by a strong signal in the ES vesicle and pore, suggesting that a mechanical force is involved in retaining proteins in this vesicle (Figure S3 in Appendix 1). In some specimens, the entire ES apparatus was labeled, showing the complete secretion path: ES cell – cytoplasmic bridge – vesicle – pore.

A different pattern was identified for Bma-TCTP (Figure 3B). In ~50% of the specimens stained with anti-Bma-TCTP, signal was found in both the ES apparatus and at the inner mf sheath surface. Localization in the sheath was characterized by punctate staining surrounding the whole body. Even for this protein, other mf were stained exclusively in the ES apparatus, similar to the first pattern. No staining in non-ES cells was observed, suggesting that this cell is the unique source of TCTP in the inner sheath and its accumulation on the parasite surface may occur after release from the uterus.

A third staining pattern was observed with Bma-MIF-1 (Figure 3C). This protein was found throughout the mid-body from the nerve ring to the inner body, including the ES cell, as well as in the ES vesicle, where a strong signal was observed. Compared to the TCTP staining pattern at the mf inner sheath, the MIF-1 signal was also high toward the lateral axis, but in a different layer which likely comprises the hypodermis and cuticle and, to a lesser extent, in the inner sheath (Figure 3B and 3C, right panel). This distribution suggests that the hypodermal cells in this area may be the main MIF-1 source for protein accumulation in the cuticle and sheath, but that secretion from the ES vesicle is a prominent source of the protein that accumulates in culture media containing mf.

It must be noted that the mf sheath has been shown to be impermeable to antibody penetration during immunostaining, requiring intensive permeabilization to allow antibodies to reach internal antigens. Thus, the mf sheath imposes an additional barrier to lateral protein release. These results suggest that the ES apparatus constitutes the major anatomical pathway for the release of ES products into the external environment.

IVM treatment of *B. malayi* mf reduces protein release.

To probe the relationship between GluCl-regulated muscle activity and protein release from the ES apparatus, the amount of protein released *in vitro* by mf was quantified in media containing IVM over 72 hr in culture (Figure 4A). ABZ was included as a positive control, as it has been shown to reduce protein secretion by the hookworm *Ancylostoma ceylonicum* and other nematodes through inhibition of microtubule polymerization (23) (Figure 4B).

A decrease in protein output was observed as soon as 24 h post-IVM exposure. As there were no significant differences among treatments in total protein in mf-somatic extracts at any time point; it can be inferred that the drop in protein secretion is not related to a simple decrease in mf viability, but instead is consistent with paralysis of the muscle associated with the ES vesicle (Figure 4A). Concentration-dependence of the IVM effect was only observed at 48-72 h time-point after exposure, suggesting that in addition to the rapid and consistent effect of IVM on mf protein secretion, there may be a secondary effect at higher IVM concentrations. Exposure to 0.1 µM IVM decreased protein secretion by 58% ($p<0.01$), 68% ($p<0.05$) and 42% ($p<0.05$) compared to non-treated controls at 24, 48 and 72 h, respectively.

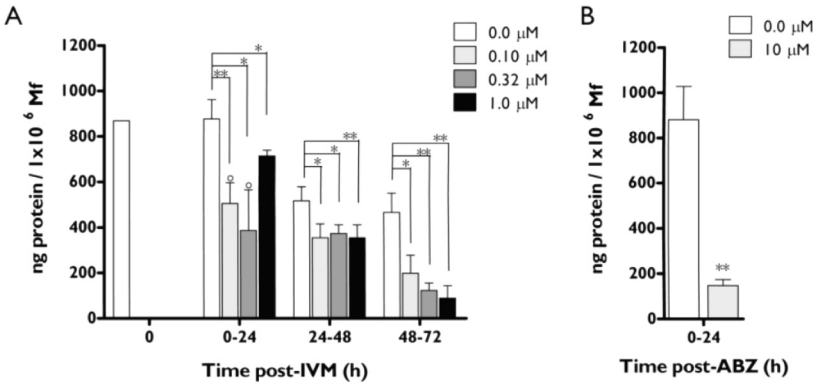

Figure 4. Effects of two anthelmintic drugs on protein release from *B.malayi* mf. (A) IVM reduces protein release *in vitro*. Mf (2.5 x 10^5 mf/ml) were incubated from 0 –72 h in RPMI 1640 with or without IVM. Media exchange was performed each 24 h (n=3) at 24, 48 and 72 h. (B) Protein release *in vitro* at 24 h from mf incubated with or without ABZ (10 µM). Mean ± SD; * = $p<0.05$, ** = $p<0.01$, control vs treatment; º = $p<0.05$, 1.0 µM IVM vs treatment.

Although limited by protein availability, preliminary analyses using two-dimensional difference gel electrophoresis (2D-DiGE) were done to detect changes in relative protein abundance in mf-ES products upon treatment with 0.1 μM IVM (additional information in the SI section in Appendix 1). Protein profiles and relative protein abundance were essentially unchanged, providing additional evidence that the ES apparatus is the main source for protein export.

Discussion

Despite the remarkable reduction in morbidity and profound interruption of disease transmission achieved by the MDA programs for the control of onchocerciasis and LF [1], there are concerns about the feasibility of eliminating filarial infections without the availability of a macrofilaricide in the short-term; additional concerns about the possible emergence of resistance to IVM, which forms a mainstay of both programs [23,24], exacerbate the need to attain a much better understanding of the pharmacology of existing antifilarial drugs, which remains poor [5]. Inadequate understanding of the basis for the action of IVM on these parasites impedes our ability to optimize its clinical use and to sustain the efficacy of the drug in the mid-to-long term. Generating new tools for detecting and monitoring filarial infections, including possible IVM-resistant parasite populations, and for discovering and developing new anthelmintics represent critical challenges, as current knowledge on the mechanism of action of IVM does not explain how it alters filarial viability and leads to mf clearance from the host [5].

IVM is a potent and generally very safe drug for the treatment of nematodes, and also infestations of ectoparasites of human and veterinary relevance [25]. This remarkably broad spectrum of action and record of mammalian safety is partly due to the absence of GluCls from vertebrates and the essential role of these channels in neurotransmission in some types of invertebrates [14]. Beyond this, the cascade of events following GluCl opening which leads to the observed effects of IVM treatment in filariases is still unclear.

In the present work, we assembled the full open reading frame sequences of two subunits of the *avr-14* GluCl of *B. malayi*. As no additional orthologs of IVM-sensitive channels were found in the *B. malayi* genome database, these proteins must represent the main putative target for IVM in this parasite based on data obtained in other species of nematodes [26,27,28]. Identifying their anatomical localization in mf provides new insights to explain the pharmacological consequences of treatment of filariases with IVM.

The localization of GluCl subunits observed in *B. malayi* has not been reported in other nematodes. The unique presence of GluCl immunoreactivity co-localizing with a muscle structure surrounding the ES vesicle allowed us to build a series of hypotheses about the role of these channels and the effect of IVM in mf. First, it is expected that glutamatergic synapses occur in this area and regulate the neuromuscular physiology that controls relaxation/contraction of this muscle and so protein secretion. Secondly, as no other sites of expression of GluCl subunits were observed, muscle activity in this area of mf must account for the mechanism of action of this drug against this stage. It has been commonly observed that IVM does not affect mf motility or viability at pharmacologically-relevant concentrations in culture [5,9], which is consistent with the apparent absence of GluCl expression in regions associated with somatic muscle function. Recently, it has been reported that IVM concentrations >1 μM affect mf motility [29]; this may be associated with drug effects on GABA-gated chloride channels, which are secondary targets for the ML class [30]. These effects may partially account for the concentration-dependence of the drug effect on secretion seen at the later time-point. Thirdly; the work done by the ES-associated muscle layer must be exerted on the ES vesicle, allowing secreted proteins to be pumped out through the ES pore. Like the pharynx in adult nematodes, the high internal turgor pressure in nematodes would tend to keep the ES pore closed, so that active contraction against this force would be needed to permit protein expulsion in a regulated fashion. Exposure of mf to IVM hyperpolarizes the muscle, resulting in its paralysis, thus preventing opening of the ES pore and inhibiting protein secretion.

Proteomic analysis of ES products has been limited by the very low amount of protein recovered from *in vitro* incubations. Overcoming this limitation using highly sensitive

approaches has lead to the identification of most of these proteins in *B. malayi* [31,32,33]. However, information about the anatomy and physiology of the protein secretory pathways in filarial nematodes has not been intensively investigated. The comparative analysis of the localization of ES proteins presented here aimed to dissect the sources and routes involved in the generation of these proteins. For this analysis, we selected proteins from the secretome with and without predicted N-terminal secretion signal peptide sequences. This allows a better understanding of the scope of the effect of IVM on overall protein release. Our results confirmed that, independent of the cellular secretory pathway involved (classical vs. non-classical), the role of the ES apparatus is central for the delivery of proteins from mf to the host environment.

This conclusion is supported by the fact that all the proteins accumulated in the ES vesicle. Although it could be argued that lateral secretion across the mf surface can also be a component for protein release, there seems to be no role for a mechanical force helping to deliver protein to the exterior as in the ES vesicle; the consistent inhibition of secretion of all ES proteins by IVM argues that a single, regulated pathway underlies at least the majority of protein secretion in mf. Coupled with the general impermeability to macromolecules exhibited by the mf sheath, this consideration allows us to infer that the ES vesicle pathway is the predominant route of protein secretion from mf.

Evidence for rapid changes in the host after IVM treatment that may be deleterious to mf has been presented elsewhere. These include the induction of nitric oxide derivatives in patients infected with *Loa loa* or *O. volvulus* and changes in the circulating levels of chemokines such as RANTES, IL-8 and IFN-γ that may affect recruitment of eosinophils [34]. The requirement of serum factors to promote *in vitro* cell-mediated cytotoxicity in combination with IVM has been shown in the *Dipetalonema (Acanthocheilonema) viteae* and *Litomosoides carinii* - *Mastomys natalensis* systems [35,36], supporting the argument that IVM treatment in human filariases leads to rapid disruption of the host-pathogen molecular negotiation via suppression of parasite protein secretion.

In support of these hypotheses, mf treatment with IVM *in vitro* resulted in a marked reduction in protein release. These observations were made in a time-frame that paralleled the reduction in circulating mf induced by IVM in patients with Brugian filariasis and at concentrations that are close to peak therapeutic levels [37]. It is therefore reasonable to infer that IVM treatment reduces protein release from mf *in vivo*. This hypothesis provides insights to explain how mf are rapidly cleared by IVM treatment.

In this context, our results support the proposal that the rapid clearance of mf following treatment with IVM is effectively the result of a direct or indirect interaction with the host immune system [9,38]. The proteins released *in vitro* by Bma-mf differ from those released by adults and are associated with modulation and evasion of the host immune system, among other potential functions [32,33]. As it is accepted that parasite protein secretion plays a fundamental role in host immunomodulation [18], it is reasonable to infer that inhibition of protein secretion would result in the inability of the parasite to neutralize pre-existing and induced host effectors at the local and systemic levels.

Our data do not address the lack of macrofilaricidal activity of IVM observed *in vivo*. The large size of the adult worms may render them relatively insusceptible to the actions of host immune effector cells and molecules, making the anti-secretory effects of the drug irrelevant for this stage. Infectious (L3) larvae and developing (L4) juvenile stages are targeted by IVM, at least in some filariases, and investigations on whether the observations made here on mf can be extended to L3 stages are underway.

Altogether, our results show that IVM targeting of the AVR-14 subunits from *B. malayi* mf *in vitro* leads to the disruption of protein release from the ES apparatus, the main pathway for protein delivery to the mammalian host. This finding provides additional insights on the mechanism of antifilarial action of IVM *in vivo* that can be valuable for drug and vaccine development. Finally, our data support a role of ES proteins in modulation and evasion of the host immune response by filarial nematodes.

Materials and methods

More detailed information on the materials and procedures is found in SI Methods (Appendix 1). All animal procedures were approved by the Animal Care Committee of McGill University and were in accordance with the guidelines of the Canadian Council on Animal Care. Infected Mongolian jirds (*Meriones unguiculatus*) were obtained from the Filariasis Research Reagent Repository Center (Athens, GA). Mf and adult *B. malayi* were recovered >120 days post-infection from the peritoneal cavity as described in [32].

Cloning and sequencing of Bma-AVR-14 subunits.

Partial sequences for two GluCls (AVR-14) were obtained from the *B. malayi* genome project (Bm1_00335 and Bm1_15450). Completion of the full coding sequences for these genes was achieved by following a strategy like that employed for *avr-14* from *D. immitis* [21].

Immunohistochemical analysis.

For immunohistochemical analysis, antibodies were obtained from external sources or raised against on-site produced His-tagged proteins. Mf were fixed, permeabilized and treated by an adaptation of the tube protocol for *C. elegans* [39]. Controls include observation of mf with omission of primary antibody and, for on-site produced proteins, with peptide- or protein-adsorbed primary antibodies.

***In vitro* treatment of *B. malayi* with IVM.**

Exposure of *B. malayi* to IVM was carried out after initial overnight incubation of mf in RPMI 1640. Mf were incubated for 72 h in volumes of 1 ml (250 x 10^3 mf/ml) in 24-well plates at 37º, 5% CO_2. RPMI 1640 containing 0, 0.1, 0.32 or 1.0 µM IVM was prepared by dilution of a 10 mM stock in dimethyl sulfoxide (DMSO). Control wells contained 0.1% DMSO. Media from each plate were collected and replaced with fresh media with or without drug every 24 h.

Protein determinations.

Protein determinations were performed with an EZQ protein quantitation kit (Invitrogen) with some modifications. 250 µl of either test medium or RPMI 1640 were spiked with 50 ng ovalbumin as an internal standard and concentrated to dryness in a speedvac. Samples were resuspended in 5 µl doubled distilled H_2O. The total sample was serially spotted in volumes of 2 µl on assay paper in the sampling plate. The assay paper was washed twice with MeOH for 5 min and then incubated with the EZQ reagent for 30 min. Final 2 min washes were performed 3 times with 10% MeOH, 7% acetic acid. Assay paper fluorescence was determined in a plate fluorimeter (FlexStation II, Molecular Devices) at 450 nm (excitation) and 610 nm (emission). Protein amounts were estimated by extrapolation from the standard curve, corrected according to the internal standard readings of RPMI 1640 alone.

Acknowledgements

We thank Sergei Dernovici for training and assistance during confocal image acquisition and Elke Küster-Schöck for advice on the 2D-DiGE experiments at the Cell Imaging and Analysis Network (CIAN) of McGill University. We also thank the NIH Filariasis Research Reagent Resource Center for provision of infected gerbils and Prof. Murray Selkirk for the Bma-VAL-1 antibody. This work was supported by NSERC, Canada Research Chairs and the FQRNT Centre for Host-Parasite Interactions.

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Connecting Statement II

In manuscripts I and II, we present evidence suggesting that the survival of the filarial parasite in the host is dependent on the continuous release of ESP. These products are predicted to be involved in immunomodulation along with a variety of other biological processes. Since helminth infections are typically restricted to a group of hosts and anatomical compartments therein, it may be possible to infer that both the ESP components presented to the host and their associated processes are the result of the parasite adaption to their specific niches. Determining which of these features are specific for each parasitic life form and which are part of a more generalized strategy for parasitism would require the identification and comparison of ESP from several nematode species. This is feasible in terms of current instrumental sensitivity and accessibility, but is limited by the lack of sequence information required for the database searching strategy in MS-base proteomic analysis. In manuscript III, the possibility of using transcriptomic next-generation sequencing (RNA-seq) information to overcome this limitation for the identification of ESP from the mouse parasitic nematode *Heligmosomoides polygyrus* is explored. ESP from this parasite have been shown to possess strong immunomodulator activities.

Chapter V
(Manuscript III)

Proteomic analysis of Excretory-Secretory Products of *Heligmosomoides polygyrus* assessed with next-generation sequence transcriptomic information

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Running title: *H. polygyrus*-derived excretory-secretory products

Accepted PLoS Neglected Tropical Diseases

Abstract

The murine parasite *Heligmosomoides polygyrus* is a convenient model to study immune responses and pathology associated with gastrointestinal nematode infections. The excretory-secretory products (ESP) produced by this parasite have potent immunomodulatory activity, but the protein(s) responsible has not been defined. Identification of the protein composition of *H. polygyrus* ESP has been hampered by the lack of genomic sequence information required for proteomic analysis based on database searches. To overcome this, a transcriptome next generation sequencing (RNA-seq) *de novo* assembly containing 33,641 transcripts was generated, annotated, and used to interrogate mass spectrometry (MS) data derived from 1D-SDS PAGE and LC-MS/MS analysis of ESP. Using the database generated from the 6 open reading frames deduced from the RNA-seq assembly and conventional identification programs, 209 proteins were identified in ESP including homologues of vitellogenins, retinol- and fatty acid-binding proteins, globin, and the allergen V5/Tpx-1-related family of proteins. Several potential immunomodulators such as macrophage migration inhibitory factor, cysteine protease inhibitors, galectins, C-type lectins, peroxiredoxin, and glutathione S-transferase were also identified. Comparative analysis of protein annotations based on the RNA-seq assembly and proteomics revealed processes and proteins that may contribute to the functional specialization of ESP, including proteins involved in signaling pathways and in nutrient transport and/or uptake. These results provide data that will help to illuminate molecular, biochemical, and in particular immunomodulatory aspects of host-*H. polygyrus* biology. The methods and analyses presented here are applicable to study biochemical and molecular aspects of the host-parasite relationship in species for which sequence information is not available.

Author summary

Gastrointestinal (GI) nematode infections are major causes of human and animal disease. Most of this morbidity is associated with their ability to establish chronic infections in the host, reflecting the deployment of mechanisms to evade and modulate the host immune response. The molecules responsible for these activities are poorly known. However, most of them can be found among the proteins released from the parasite as excretory-secretory products (ESP). The murine parasite *Heligmosomoides polygyrus* has served as a model to understand several aspects related to GI nematode infections. In this work, we aimed to identify the protein components of *H. polygyrus* ESP through a proteomic approach. However, the lack of genomic sequence information for this organism limited our ability to identify proteins by relying on comparisons between experimental and database-predicted mass spectra. To overcome these difficulties, we used transcriptome next-generation sequencing and several bioinformatic tools to generate and annotate a sequence assembly for this parasite. We used this information to support the protein identification process. In addition to the identification of 209 proteins, we recognized particular processes and proteins that define the functional specialization of ESP. This work provides valuable data to establish a path to identify and understand particular parasite proteins involved in the orchestration of immune evasion events.

Introduction

Gastrointestinal (GI) nematode infections are major causes of disease in both humans and animals. Infections with *Ascaris lumbricoides*, hookworms (*Necator americanus* and *Ancylostoma duodenalis*), *Trichuris trichiura* and *Strongyloides stercoralis* are highly prevalent in developing countries, affecting ~1 billion people and posing a burden estimated at ~2 M DALYs (Disability-adjusted life years) (http://apps.who.int/ghodata) [1]. GI nematodes can establish chronic infections, surviving in the host for considerable periods of time. This characteristic reflects the ability of these parasites to evade and modulate the host immune response from the early stages of infection while optimizing both feeding and reproduction [2,3]. As a result, in addition to their commonly associated effects on host physiology including malnutrition, growth stunting and anaemia, infection

with GI nematodes influences the development and/or severity of co-occurring infectious and immune-mediated diseases such as malaria or type 1 diabetes, respectively [4,5].

Infection with the nematode *Heligmosomoides polygyrus*, a natural GI pathogen of mice, has provided a convenient experimental model to understand the biology of GI nematodes and the pathology associated with chronic infections with this class of helminth parasites [6]. Primary infection with *H. polygyrus* induces a highly polarized Th2 immune response in mice; despite induction of this response, the parasite survives and establishes a chronic infection with the differentiation and activation of host cell types that mediate potent immunoregulatory mechanisms, such as regulatory T cells and alternatively activated macrophages (AAMΦs) [7,8]. Recent studies indicate that these regulatory responses, especially regulatory T cells, can be stimulated by treatment with *H. polygyrus* excretory-secretory products (ESP) [9,10,11,12]. These observations suggest that this fraction of the proteome contains many of the immunomodulatory factors responsible for evasion of the host immune response, but the proteins in ESP which mediate these effects remain largely unknown.

The use of mass-spectrometry based proteomics has overcome many limitations in the analysis and identification of helminth-derived proteins in ESP [13]. In general, these analyses achieve a remarkable sensitivity in protein identification if either genome, transcriptome or proteome sequence information is available to support the interrogation of experimentally obtained mass spectra with peptide matching algorithms in database search programs [14]. However, most of this sensitivity is lost when assignation is based on homology with proteins identified in other species [15,16,17], as is the case for *H. polygyrus* and almost all other relevant parasitic nematode species.

To better understand the molecular mechanisms that lead to the activation and modulation of the host immune response by GI nematodes, we used transcriptome next generation sequencing (RNA-seq) technologies and several bioinformatic tools to overcome the limitations in the proteomic analysis of ESP from *H. polygyrus*. Illumina sequencing (www. illumina.com) was employed to generate transcriptomic sequence data in a rapid

and cost-efficient way [18]. The transcriptome assembly was used to identify proteins in the ESP using an experimental proteomic approach.

Results

Protein identification of *H. polygyrus* ESP using protein homologs for peptide assignation

~100 µg protein of ESP was separated by SDS-PAGE. The entire lane was excised in 15 pieces, digested with trypsin and analyzed by LC-MS/MS. A preliminary protein identification attempt was performed on the complete MS data set (10227 spectra) using the protein sequences from nematodes in the Uniprot database (taxonomy ID:6231, September 17, 2010) as a search source for the peptide matching algorithms. After validation with Scaffold (v. 2_05_02), 20 proteins were assigned (95% probability) with a total number of assigned spectra ranging between 2 to 12 and between 2 to 7 unique peptides assigned per sequence. Nineteen of 20 identified proteins were homologs from nematodes other than *H. polygyrus* (Table S1).

A transcriptomic analysis of adult *H. polygyrus*

To provide a more suitable information source for the peptide assignation software and to increase the number of proteins identified in the *H. polygyrus* ESP, an RNA-seq analysis of this organism was carried out. Using the GAIIx platform from Illumina, ~24.7 million reads of raw data, amounting to >2.7 Gbp, were obtained from *H. polygyrus* poly-A selected mRNA. Initial assembly was performed after the removal of adapters, random hexamer primer sequences, and quality control trimming using Velvet 1.0.13 [19], generating 76,616 contigs. Final assembly with Oases 0.1.6 resulted in 33,641 total transcripts (isoforms) in 29,918 loci (3723 alternative splice events) (Table 1). These values do not include sequences < 100 bp, which were removed for downstream analysis.

Searching for protein homologs in the *H. polygyrus* assembly with BLASTx identified 18,816 (55.9%) transcripts sharing homology with proteins from *C. elegans* (E cut-off

$1x10^{-5}$) and 15,338 (45.6%) with proteins from *Brugia malayi* (Table 1). Only 4 sequences were found to return mice proteins as the first BLASTx output (E cut-off $1x10^{-15}$), indicating a low degree of host RNA contamination in the preparations.

Assembly Statistics		
Number of transcripts (Isoforms) >100 bp	33641	
Number of Loci >100 bp	29918	
Average lenght (SD)	561.0 + (566.2)	
Blastx (E> 10^{-5})		
C. elegans	18816 (55.9%)	
B. malayi	15338 (45.6%)	
nr	19196 (57.1%)	
	Total Transcriptome (33641 seqs)	**ESP subset (208 seqs)**
GO annotation		
Number of Seqs with at least 1 GO term (%)	14094 (41.9)	113 (54.3)
Total Number of terms (unique)	85884 (6647)	642 (306)
Cellular Part terms (unique)	17180 (764)	99 (52)
Number of Sequences (%)	8694 (25.8)	47 (22.6)
Molecular Function terms (unique)	25715 (1805)	210 (87)
Number of Sequences (%)	11671 (34.7)	107 (51.4)
Biological Process terms (unique)	42989 (4078)	333 (167)
Number of Sequences (%)	11296 (33.6)	89 (42.8)
InterPro Scan (IPS) annotation		
Seqs with at least 1 protein signature (%)	17342 (51.5)	158 (76.0)
Annotated Interpro Entries	4547	134
Domains	2204	70
Protein Families	1896	41
Seqs with annotated Interpro Entries (%)	12126 (36)	157 (75.4)
Seqs annotated with predicted domains (%)	15435 (45.9)	104 (50)
Seqs annotated with protein families (%)	5760 (17.1)	70 (33.7)

Table 2. Summary of the transcriptomic assembly and ESP subset annotations

		Transcriptome			ESP subset		
		GO code	**GO Term**	**Seqs (%)**	**GO code**	**GO Term**	**Seqs (%)**
Celular Component	1	GO:0016021	integral to membrane	1513 (4.5)	GO:0005737	cytoplasm	14 (6.7)
	2	GO:0005634	nucleus	1129 (3.4)	GO:0005576	extracellular region	6 (2.9)
	3	GO:0005737	cytoplasm	990 (2.9)	GO:0005615	extracellular space	6 (2.9)
	4	GO:0016020	membrane	743 (2.2)	GO:0016021	integral to membrane	4 (1.9)
	5	GO:0005829	cytosol	669 (2.0)	GO:0016020	membrane	4 (1.9)
Molecular Function	1	GO:0005515	protein binding	3782 (11.2)	GO:0005515	protein binding	43 (20.7)
	2	GO:0005524	ATP binding	1327 (3.9)	GO:0008233	peptidase activity	8 (3.8)*
	3	GO:0005488	binding	937 (2.8)	GO:0020037	heme binding	7 (3.4)*
	4	GO:0008270	zinc ion binding	643 (1.9)	GO:0008237	metallopeptidase activity	7 (3.4)*
	5	GO:0046872	metal ion binding	590 (1.8)	GO:0019825	oxygen binding	7 (3.4)*
Biological Process	1	GO:0009792	embryonic development ending in birth or egg hatching	2239 (6.7)	GO:0008340	determination of adult lifespan	28 (13.5)*
	2	GO:0002119	nematode larval development	1655 (4.9)	GO:0009792	embryonic development ending in birth or egg hatching	27 (13.0)
	3	GO:0040010	positive regulation of growth rate	1513 (4.5)	GO:0040010	positive regulation of growth rate	26 (12.5)*
	4	GO:0000003	reproduction	1311 (3.9	GO:0040011	locomotion	10 (4.8)
	5	GO:0040011	locomotion	992 (2.9)	GO:0000003	reproduction	10 (4.8)

Table 3. Most abundant GO terms mapped and annotated using Blast2GO in both the transcriptome and ESP datasets. (*) Indicates GO term enrichment in the ESP subset when compared to the transcriptome dataset (FDR<0.5).

Functional annotation using BLAST2GO allowed us to assign GO terms to 14,094 (41.9%) sequences; 764 different cellular component terms were assigned to 8694 sequences, 1805 molecular function terms to 11671 sequences, and 4078 biological process terms to 11,296 sequences (Table 1). The most frequently annotated GO terms within these three categories were "integral to membrane" (GO:0016021, 1513 sequences), "protein binding" (GO:0005515; 3782 sequences), and "embryonic development ending in birth or egg hatching" (GO:0009792, 2239 sequences) (Table 2 and S2).

Distribution of GO terms at level two shows that "binding" (GO:0005488, 49% of annotated sequences) and "catalytic activity" (GO:0003824, 32%) were the two major molecular function categories (Figure 1A). In the case of biological process, the most

represented categories at level two were “cellular process” (GO:0009987, 17%), “metabolic process”(GO:0008152, 13%), “multicellular organismal process” (GO:0032501, 10%), “developmental process” (GO:0032502, 10%), and “biological regulation” (GO:0065007, 10%).

Functional domains and protein families were assigned to *H. polygyrus* transcripts using InterproScan [20,21]: 17,342 (51.5%) sequences retrieved at least one protein signature, 2204 different functional domains were predicted in 15,435 (45.9%) sequences, and 1896 protein families in 5760 sequences (17.1%) (Table 1). A protein kinase-like domain (IPR011009) was found in 360 sequences (1.1%) and represents the most frequently found predicted domain in the annotated dataset. In the case of protein families, the P-type, K/Mg/Cd/Cu/Zn/Na/Ca/Na/H-transporter family (IPR001757) was the most abundant, found on 80 sequences (0.2%) (Table 3).

Use of transcriptomic data for assessing *H. polygyrus* ESP composition

The translation of the 6 ORFs of each transcript from the RNA-seq assembly was used as input for the matching algorithms in the protein identification software. Using this strategy, 209 proteins were identified with a total number of assigned spectra between 132 and 2 with 2 to 19 unique peptides assigned per sequence. It should be noted that 1 sequence appears twice as it was assigned to 2 different ORFs (Locus_541_Transcript_1/4_Confidence_0.692, frames 4 and 5) (Tables 4 and S3). Manual verification of peptide assignments showed that all the identified peptides group in a single ORF.

Annotations from the non-redundant list of ESP hits (208 proteins) were extracted from the full transcriptome data set for further analysis. 642 GO terms could be annotated to sequences from the ESP subset (54.8% of the identified sequences), identifying 52 different cellular component terms in 47 (22.6%) sequences, 87 molecular functions in 107 (51.4%) sequences, and 167 biological processes in 89 (42.8%) sequences (Table 1).

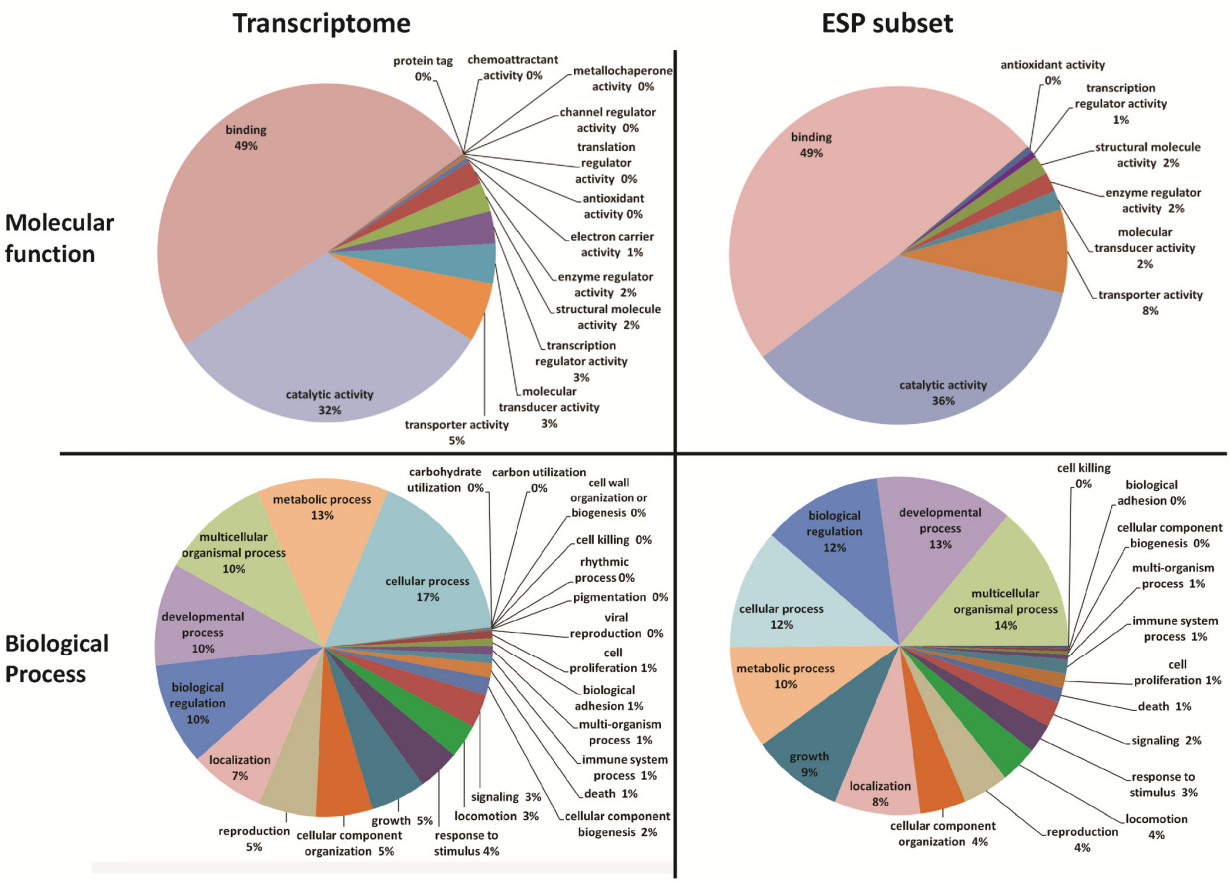

Figure 1. Distribution of Molecular Function and Biological Process Gene Ontology (GO) terms (level 2) for the transcriptome and ESP subset.

At Level 2, within the molecular function category, 8 of the 14 terms initially found in the full transcriptome dataset were also found in the ESP subset. The GO terms "binding" (GO:0005488, 49% of annotated sequences) and "catalytic activity" (GO:0003824, 36%) are the most abundant terms in this category. In the biological process category, 19 of 25 terms were found in the ESP subset. Although the proportion of annotated terms in the ESP subset was slightly different than in the whole transcriptome dataset, the terms "multicellular organismal process" (GO:0032501, 14%), "developmental process" (GO:0032502, 13%), "biological regulation" (GO:0065007, 12%), "cellular process" (GO:0009987, 12%), and "metabolic process"(GO:0008152, 10%) were the most represented terms in both (Figure 1, left panels).

	Transcriptome			Secretome		
	InterPro Accession	**Name**	**Seqs (%)**	**InterPro Accession**	**Name**	**Seqs (%)**
	Domains					
1	IPR011009	Protein kinase-like domain	360 (1.1)	IPR014044	CAP domain	25 (12.0)*
2	IPR013783	Immunoglobulin-like fold	288 (0.9)	IPR006026	Peptidase, metallopeptidase	6 (2.9)*
3	IPR015943	WD40/YVTN repeat-like-containing domain	235 (0.7)	IPR000436	Sushi/SCR/CCP	5 (2.4)*
4	IPR015880	Zinc finger, C2H2-like	223 (0.7)	IPR001747	Lipid transport protein, N-terminal	5 (2.4)*
5	IPR016024	Armadillo-type fold	176 (0.5)	IPR008753	Peptidase M13	5 (2.4)*
	Families					
1	IPR001757	ATPase, P-type, K/Mg/Cd/Cu/Zn/Na/Ca/Na/H-transporter	80 (0.2)	IPR001283	Allergen V5/Tpx-1-related	18 (8.7)*
2	IPR002198	Short-chain dehydrogenase/reductase SDR	69 (0.2)	IPR000718	Peptidase M13, neprilysin	8 (3.8)*
3	IPR001283	Allergen V5/Tpx-1-related	68 (0.2)	IPR001534	Transthyretin-like	5 (2.4)*
4	IPR020685	Tyrosine-protein kinase	61 (0.2)	IPR008632	Nematode fatty acid retinoid binding	3 (1.4)*
5	IPR020636	Calcium/calmodulin-dependent protein kinase-like	56 (0.2)	IPR009283	Apyrase	3 (1.4)*

Table 4 Five most abundant domains and families inferred from InterproScan in the transcriptome and ESP datasets. (*) Indicates enrichment of the domain or the family in the ESP subset when compared to the transcriptome dataset (FDR<0.5).

InterproScan hits assigned to the ESP subset predicted at least one protein signature for 158 (76.0%) sequences, identifying 70 functional domains in 104 (50.0%) sequences and 41 protein families for 70 (33.7%) sequences. The cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1 protein (CAP) domain (IPR014044) with 25 (12.0%) sequences identified, was the most abundant domain in the ESP subset. Associated with the CAP domain, the allergen V5/Tpx-1-related family (IPR001283) was the most prevalent found in the ESP subset (Table 3).

The proteins were organized according to the number of assigned spectra (Table 4), indicative of protein abundance [22]. The most abundant hits organized in this manner were delineated into 3 main groups according to their annotated features. The first group is the proteins predicted to contain the CAP domain belonging to the allergen V5/Tpx-1-related family. This group of proteins is described in the annotation tables as homologues of venom allergen-like proteins (VAL), *A. caninum* secreted proteins, or activation-associated secreted proteins (ASP). The second group is composed of globin homologues.

Proteins found within this group were annotated with the biological process GO term "oxygen transport" (GO:0015671) and the molecular function terms "heme binding" (GO:0020037), "oxygen transporter activity" (GO:0005344), and "oxygen binding" (GO:0019825). Although not predicted from the InterproScan in all these sequences, the globin-like domain (IPR009050) and globin family (IPR000971) were also annotated to some of these hits. The third group of most abundant proteins contains vitellogenin (Vtg) homologues. Most of these proteins are predicted to contain the characteristic Vtg open β-sheet (IPR15255, IPR 15817) domain as well as domains associated with lipid transport (IPR015819, IPR001747, and IPR015816) and GO terms associated with the molecular function of "protein binding" (GO:0005515) and the biological processes "embryonic development ending in birth or egg hatching" (GO:0009792), "determination of adult lifespan" (GO:0008340), and "positive regulation of growth rate" (GO:0040010) (Figure 1, right panels).

Enrichment analysis delineates functional specialization of ESP

GO terms enrichment analysis using GOSSIP [23] identified terms that were over-represented in the ESP subset compared to the total transcriptome dataset (Table S4). Using adjusted P-values to control False Discovery Rate (FDR; significance set at $p<0.05$) aas criteria for statistical significance, 14 terms within the biological process category and 8 within the molecular function category were enriched in the ESP subset.

In accordance with the number of globin homologues found in the ESP subset, the biological process term "oxygen transport" (GO:0015671) and its parent "gas transport" (GO:0015669) were enriched in the ESP subset. Consistent with the globins and their putative role in oxygen transport via heme prosthetic groups, the molecular function terms "oxygen binding" (GO:0019825), "oxygen transporter activity" (GO:0005344), and "heme binding" (GO:00200037), together with their parent terms, "iron ion binding" (GO:0005506) and "tetrapyrrole binding" (GO:0046906), were also enriched in this subset.

Protein name	Blast2GO Seq. Description	Accession numbers	P (%)	SC	UP	Closest Blastx Hit description	Closest Blast hit Specie	Closest Blast hit Accession Number	Blastx Hit eValue
L_272_T_4/4_C_0.700_3	venom-allergen-like protein family member (vap-1)	L_272_T_4_0.700_3	100	132	19	two-domain activation associated secreted protein ASP4 precursor	*Ostertagia ostertagi*	CAO00417.1	2E-89
L_566_T_2/6_C_0.476_3	globin	L_566_T_2_0.476_3 L_566_T_3_0.571_3 L_566_T_4_0.524_3 L_566_T_5_0.762_2 L_566_T_6_0.762_3	100	82	5	Globin-like host-protective antigen	*Trichostrongylus colubriformis*	P27613.1	3E-30
L_120_T_1/8_C_0.410_1	secreted protein asp-2	L_120_T_1_0.410_1 L_120_T_5_0.436_1 L_120_T_8_0.462_1	100	74	6	secreted protein 4 precursor	*Ancylostoma caninum*	AAO63576.1	3E-26
L_384_T_8/9_C_0.306_5	globin	L_384_T_8_0.306_5	100	73	10	Globin-like host-protective antigen	*Trichostrongylus colubriformis*	P27613.1	3E-55
L_2405_T_4/4_C_0.700_4	secreted protein asp-2	L_2405_T_4_0.700_4	100	53	11	secreted protein 6 precursor	*Ancylostoma caninum*	AAO63578.1	8E-20
L_2261_T_1/1_C_1.000_4	globin	L_2261_T_1_1.000_4	100	47	7	Globin-like host-protective antigen	*Trichostrongylus colubriformis*	P27613.1	4E-39
L_73_T_5/5_C_0.619_4	vit-2	L_73_T_5_0.619_4	100	46	10	C. briggsae CBR-VIT-6 protein	*Caenorhabditis briggsae*	XP_002634040.1	6E-37
L_94_T_3/4_C_0.429_4	C. elegans protein confirmed by T evidence	L_94_T_3_0.429_4 L_94_T_4_0.429_1	100	46	8	LYSozyme family member (lys-8)	*Caenorhabditis elegans*	NP_495083.1	5E-30
L_122_T_10/10_C_0.312_3	vitellogenin structural genes (yolk protein genes) family member (vit-2)	L_122_T_10_0.312_3	100	45	10	VITellogenin structural genes (yolk protein genes) family member (vit-1)	*Caenorhabditis elegans*	NP_509305.1	1E-45
L_2367_T_1/3_C_0.375_1	ll20 15kda ladder antigen	L_2367_T_1_0.375_1	100	44	17	DVA-1 polyprotein	*Dictyocaulus viviparus*	Q24702.1	2E-145
L_93_T_5/6_C_0.444_2	vitellogenin structural genes (yolk protein genes) family member (vit-2)	L_93_T_5_0.444_2	100	43	10	VITellogenin structural genes (yolk protein genes) family member (vit-2)	*Caenorhabditis elegans*	NP_001123117.1	4E-53
L_207_T_17/20_C_0.196_1	briggsae cbr-vit-5 protein	L_207_T_17_0.196_1	100	41	7	VITellogenin structural genes (yolk protein genes) family member (vit-4)	*Caenorhabditis elegans*	NP_508612.1	3E-35
L_290_T_3/3_C_0.833_3	briggsae cbr-vit-2 protein	L_290_T_3_0.833_3	100	35	4	C. briggsae CBR-VIT-2 protein	*Caenorhabditis briggsae*	XP_002644638.1	4E-32
L_211_T_5/5_C_0.765_5	vitellogenin structural genes (yolk protein genes) family member (vit-6)	L_211_T_5_0.765_5	100	30	7	Vitellogenin-6	*Oscheius sp.* (strain CEW1)	Q94637.1	8E-39
L_3_T_3/3_C_0.875_4	vitellogenin structural genes (yolk protein genes) family member (vit-2)	L_3_T_3_0.875_4	100	28	7	Vitellogenin-6	*Oscheius sp.* (strain CEW1)	Q94637.1	6E-31
L_2733_T_1/1_C_1.000_3	globin	L_2733_T_1_1.000_3	100	27	7	Globin-like host-protective antigen	*Trichostrongylus colubriformis*	P27613.1	5E-56
L_6303_T_1/1_C_1.000_1	ancylostoma-secreted protein 1 precursor	L_6303_T_1_1.000_1	100	27	2	ancylostoma-secreted protein 1 precursor	*Ancylostoma duodenale*	AAD13339.1	2E-04
L_927_T_1/2_C_1.000_6	---NA---	L_927_T_1_1.000_6 L_927_T_2_1.000_4	100	25	2				
L_160_T_5/5_C_0.733_4	secreted protein asp-2	L_160_T_5_0.733_4	100	23	7	secreted protein 5 precursor	*Ancylostoma caninum*	AAO63577.1	3E-38
L_407_T_1/2_C_1.000_5	vitellogenin structural genes (yolk protein genes) family member (vit-6)	L_407_T_1_1.000_5	100	23	3	Vitellogenin-6	*Oscheius sp.* (strain CEW1)	Q94637.1	4E-15
L_7106_T_1/1_C_1.000_2	acetylcholinesterase 2	L_7106_T_1_1.000_2	100	23	14	putative neuromuscular acetylcholinesterase	*Dictyocaulus viviparus*	AAS49411.1	6E-169

Table 5. Most abundant list of proteins based on the total number of spectra identified in the ESP from adult *H. polygyrus*. Protein name identifiers are derived from the original transcriptome assembly nomenclature (L=locus, T=transcript, C=confidence) from which the conceptual translation frame number was added to the identifiers. P(%)= Protein identification probability (%); SC= Number of spectral counts; UP= Number of unique peptides.

Two other groups of hierarchically-related enriched biological process terms were delineated for their association with Vtg homologues in the ESP subset. The first group comprises the term "determination of adult lifespan" (GO:0008340) and its parent "multicellular organismal process" (GO:0032501). The second group consists of "positive

regulation of growth rate" (GO:0040010) and parent terms "regulation of growth rate" (GO:0040009), "positive regulation of growth" (GO:0045927), and "regulation of growth" (GO:0040008).

In the molecular function category, two groups of enriched terms were associated with proteins of lower relative abundance. One group includes homologues of retinol and/or fatty acid binding protein as well as repetitive ladder antigens and Vtg homologues, which have the putative ability to bind and transport lipids and/or vitamin A. These proteins are annotated under the terms "retinol binding" (GO:0019841) and their parents, "retinoid binding" (GO:0005501), "isoprenoid binding" (GO:0019840), and "lipid binding" (GO:0008289). The other group is composed of certain proteases in the ESP subset, particularly several zinc metallopeptidase homologues. GO annotations in this group include the term "metallopeptidase activity" (GO:0008237) and the parent terms "peptidase activity acting on L-aminoacid peptides" (GO:0070011) and "peptidase activity" (GO:0008233).

In addition, the molecular function terms "intramolecular oxidoreductase activity" (GO:0016860) and "nucleoside diphosphate metabolic process" (GO:0009132) were also enriched in the ESP dataset. The first was associated with homologs of protein disulfide isomerase, triosephosphate isomerase (TPI) and macrophage migration inhibitory factor (MIF). The latter included homologs of nucleoside diphosphate kinases (NDPK), calcium activated nucleosidases, and ribonucleotide reductases (RNR).

Furthermore, Interpro domain enrichment analysis was performed using FatiGO [24] (Table S5). Likewise, adjusted P-values to control FDR were used as criteria for statistical significance ($p<0.05$). 23 domains and families were enriched in the ESP subset compared to the transcriptome dataset. Consistent with what was found in the enrichment analysis of GO terms, there was an enrichment of predicted families and domains associated with homologues of peptidases, globins, nucleosidases, glutathione-S-transferases, Vtg and retinol and/or fatty acid binding proteins. In addition, CAP domain (IPR014044) and its related allergen V5/Tpx-1-related family (IPR001283) and Ves

allergen (IPR002413), along with the transthyretin-like family (IPR0001534), were enriched in the ESP dataset.

Discussion

The ESP fraction of the proteome from parasitic nematodes is thought to contain many of the effector molecules that contribute in a direct or indirect way to establishment and survival within the host [25]. The *H. polygyrus*-mouse model is a convenient system for the study of human chronic gastrointestinal parasitism; potent immunomodulatory effects of ESP preparations from this parasite have been documented [9,10,11,12]. Specification of the protein composition of ESP is an important step toward compiling a comprehensive list of the proteins responsible for these effects. In addition, the transcriptomic analysis-based protein identification presented here highlights other aspects related to the biology of GI nematode infections that may illuminate new therapeutic strategies.

Proteomic analyses based on an RNA-seq assembly

Proteomic approaches involving mass spectrometry have been applied for the characterization of ESP in several helminth species [26]. Protein identification in this manner has typically been empowered by the availability of information resulting from genome sequencing projects. Our preliminary results exemplify how the lack of this type of information and the reliance on sequences of protein homologues from different nematode species severely limit protein identification of *H. polygyrus* ESP; these factors would similarly limit such analyses from other unsequenced species.

To overcome this limitation, we sequenced the transcriptome of *H. polygyrus* using Illumina technology to provide the peptide matching software with the resulting RNA-seq *de novo* assembly. Next-generation sequencing technologies applied to the study of parasitic nematode transcriptomes offer an efficient way to understand how these organisms orchestrate their biochemical and molecular processes within the host [27,28,29]. However, we show here that its potential includes the use of this information

to study specific aspects of the proteome. In particular, the *H. polygyrus* RNA-seq assembly was used as a reference for the identification of proteins present in the ESP.

Mass spectrometry-based proteomics has started to be exploited for the validation and/or correction of sequence datasets and associated annotations [30]. To a certain extent, this is the case for the present analysis. On the other hand, the overall output of the protein identification process is dependent on the searching space explored, in this case the 6 ORFs of the RNA-seq assembly. In addition to the sequence coverage, factors that may affect the quality of the *de novo* RNA-seq assembly include the performance of the assembly program as well as errors in individual reads during sequencing and genetic variation in the transcribed sequences, which complicates the recognition of sequence overlap during assembly [31]. How these factors and others (e.g., instrumental aspects of mass spectra acquisition) alter the final output has not been studied extensively. In practical terms, this imposes the need for further validation when using such a dataset for downstream analysis.

Proteins involved in functional specialization of the ESP

Comparison of frequencies and distribution of annotations provide a way to describe the degree of functional specialization of proteins in the ESP relative to the total transcriptome. GO terms enrichment analysis revealed how some of the components of the *H. polygyrus* ESP may be involved in processes associated with the transport and/or uptake of nutrients from the host as well as possible involvement in signalling pathways.

Globin homologues in the ESP were enriched in functional annotation categories related to oxygen and heme binding. Nematode globins are distantly related to those in vertebrates and are known or predicted to play a role in several processes, given their expression in different anatomical patterns and diversity in gene structure and amino acid sequence [32,33]. Although a more precise understanding of the multiple functions of nematode globins is needed, it can be expected that their role in oxygen transport and supply must be critical in the low oxygen conditions of the host microenvironment, where the adult *H. polygyrus* attaches to and coils around the duodenal villi [34]. In this context,

globin functions can vary from transport and delivery to oxygen sink depending on the affinity of oxygen binding. For example, the high oxygen affinity globin from *Ascaris suum* has been proposed to prevent toxic effects of oxygen for this parasite [33,35]. In addition, parasitic as well as free living nematodes are heme auxotrophs [36], and thus secreted globins may also participate as heme carriers for the supply of this prosthetic group required for many other biological processes.

Another group of enriched functions found in the ESP are related to binding of lipids and retinoids. Proteins associated with these functions are involved in the transport of these hydrophobic molecules as substrates for energy metabolism, membrane biosynthesis, and signalling [37]. Identified proteins in this group include homologues of nematode polyprotein allergens/antigens (NAR), fatty acid and retinol binding (FAR) proteins, and Vtg proteins. NAR and FAR proteins comprise classes of small (~14 kDa and ~20 kDa, respectively) lipid binding proteins from nematodes. NAR proteins bind both retinol and fatty acids; they are synthesized as repetitive polypeptides in tandem and are subsequently cleaved into multiple functionally similar proteins [38,39]. FAR proteins exhibit higher affinity for retinol than for fatty acids [40]. In addition to a role in the acquisition of small lipids from the host or the microbiota, their role as parasite secreted proteins has been proposed to be the sequestration or delivery of signalling lipids to host cells [37]. Their possible role in sequestering vitamin A from the host has been associated with the pathology of parasitic nematode infections. Among these are visual impairment caused by infections with *Onchocerca volvulus* [40] and vitamin A deficiency in patients infected with *A. lumbricoides*, possibly due to malabsorption [41]. Sequestration of vitamin A may also contribute to immunomodulation as it is required for host adaptive immunity and is involved in the differentiation of T-helper (Th) cells and B-cells. In particular, vitamin A deficiency leads to impaired intestinal immune responses, including antibody-mediated responses directed by Th2 cells [42,43].

Vtg proteins form a highly diverse family in the large lipid transfer protein (LLTP) superfamily. In addition to the ESP from *H. polygyrus,* these proteins have also been identified in ESP from other parasitic GI nematodes [44,45]. In *C. elegans,* Vtgs are

implicated in the delivery of nutrients to support embryonic development, hence the enrichment of biological process terms associated with growth regulation. They are secreted from the intestine to the pseudocoelomic space where they transit through the gonadal basal lamina and then through the sheath pores for receptor-mediated oocyte endocytosis [46,47]. Therefore, it is likely that their presence in ESP from parasitic GI nematodes is the result of egg release. However, the involvement of Vtg-like proteins in modulation of insect host immune responses [48,49,50] suggests a possible additional role in negotiation of the host-parasite interface.

Peptidase activity was another GO function enriched in the ESP protein set. Helminth proteases participate in the establishment, development, and maintenance of infection [51]. In *H. polygyrus,* developmental regulation of ES-proteases suggest possible roles in exsheathment, invasion of the mucosa, and immune regulation during the larval stages, and feeding and migration during the adult stage [52]. Nothing is known about the substrate specificities of the *H. polygyrus* ES proteases. However, by analogy to the proteolytic cascade required for haemoglobin degradation by hookworms [53], several components of which were also identified in *A. caninum* ESP [45], the identified aspartyl, cysteine, and metalloproteinases from *H. polygyrus* are predicted to participate in degradation of host proteins acquired during tissue feeding.

The identification of enzymes involved in nucleotide metabolism suggests a possible role of ESP in modulation of host signalling pathways. Regulation of local levels of extracellular nucleotides could affect the activity of host purinergic receptors, which mediate a variety of cellular responses, including elements of the immune system [54]. Enzymes involved in nucleotide metabolism have previously been identified in ESP from parasitic nematodes [55,56,57,58]. These include nucleoside diphosphate kinases, nucleosidases, and adenosine deaminases that participate in the formation of activators of purinergic receptors from ATP or UTP, such as AMP, UMP, adenosine, or inosine [54]. In addition, the homolog of ribonucleotide reductases in *H. polygyrus* ESP may contribute precursors for this pathway through the generation of deoxynucleotides from ribonucleotides.

In addition to proteins of interest based on comparison of GO annotation between datasets, homologues of ASP or VAL proteins were also highlighted for their abundance and number of isoforms identified. These proteins are characterized by the presence of the CAP domain (also known as SCP-like domain) and belong to the allergen V5/Tpx-1-related family of proteins, a group of evolutionarily related eukaryotic extracellular proteins whose function remains largely unknown [25,59,60]. Interpro terms associated with this domain and families were found to be enriched in the ESP dataset. Members of this family include cysteine-rich sperm proteins (CRISPs), insect venom allergens, and plant pathogenesis family-1 (PR-1) proteins. Reasons to suspect a role for these proteins at the nematode-host interface (including pathogenesis) include the rapid and specific release of *N. americanus* ASP-2 during the transition from larval to parasitic stages as well to their neutrophil chemoattractant activity [61,62], and the angiogenic effects of several *O. volvulus* ASPs [63].

Other possible immunomodulators

In addition to proteins highlighted on the basis of enrichment of functional annotation, other relevant proteins in *H. polygyrus* ESP include homologues of glycolytic and metabolic enzymes. Of particular interest are triosephosphate isomerase (TPI), fructose bisphosphate aldolase A (FBPA), and enolase (ENO), which have consistently been reported in nematode ESP [25]; this pattern suggests that their release cannot be simply due to worm death or damage during culture. While the function of these proteins remains obscure in the context of host-nematode relationships, there is evidence of the association of these enzymes with host cell surface components and their involvement in functions unrelated to glycolysis, including microbial pathogenesis and autoimmune disorders [64,65,66,67].

Possible immunomodulators also include a homologue of macrophage migration inhibitory factor (MIF), a parasite protein which mimics a mammalian cytokine, which has been reported in a many nematode ESPs. MIFs are usually associated with pro-inflammatory responses. However, in contrast to the mammalian cytokine, nematode MIF

acts in a Th2 environment to induce AAMΦs [25,68,69]. In addition, the cysteine protease inhibitor (CPI) homologue identified in *H. polygyrus* ESP may modulate immune responses to unrelated antigens by inhibition of antigen processing and presentation by antigen presenting cells [70,71] or by inhibition of T-cell proliferation, which may contribute to the state of cellular hypo-responsiveness characteristic of chronic parasitic nematode infections [72]. Also of interest are the previously characterized C-type lectins (CTL) from *H. polygyrus* [73] and galectin homologues identified in the ESP. Their role as immunomodulators is suggested by the involvement of these carbohydrate-binding proteins in a variety of immune functions [74,75,76,77] as well as the eosinophil attracting activity that has been reported for a galectin from *Haemonchus contortus* [78].

Finally, the presence of homologues of peroxiredoxin (PRX) and glutathione S-transferase (GST) in *H. polygyrus* ESP suggests a role for enzymes involved in detoxification of reactive oxygen species (ROS) released from the host [79,80]. Other roles for these enzymes may include the induction of AAMΦs, as shown for a helminth PRX, promotion of Th2 immune responses [81], and the involvement of GST in heme transport and detoxification [82,83].

Conclusions

This study employed next-generation sequencing and proteomic approaches to gain insights into the transcriptome of adult *H. polygyrus* and to use the dataset to identify protein components of the ESP. Comparison of functional annotation categories of the total transcriptome, which provides a picture of the total proteome, with those of the ESP subset allowed us to identify functions and associated proteins that may play a role at the host-parasite interface, where many events critical for success of the infection occur. The data presented here contribute to the identification of individual components that are responsible for the immunomodulatory activity that has been reported for *H. polygyrus* ESP. Moreover, methods and analyses presented here are useful for the study of biochemical and molecular aspects of nematode biology in other species for which sequence information is not available.

Materials and Methods

Parasites

H. polygyrus was maintained and propagated in male BALB/c mice (Charles River Laboratories, St. Constant, Canada) by oral gavage inoculation of 400-500 third-stage larvae (L3) as described [84]. Adult parasites were collected from the small intestine 21days post-infection under a dissection microscope. Worms were washed extensively with sterile endotoxin-free PBS (Invitrogen, Burlington, ON, Canada) containing 80 μg/ml gentamicin (Schering, Montreal, QC, Canada), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen), and 20 μg/ml polymyxin B (Sigma, St. Louis, MO). Mice were housed in the Animal Care Facility at the Research Institute of the McGill University Health Centre. Animal procedures were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals, Animal Resources, McGill University.

RNA extraction, cDNA library preparation and Illumina sequencing

For RNA extraction, ~1000 adult female and male worms free of host tissue were selected and extensively washed. After resuspension in 0.5 ml PBS, 3.0 ml Trizol (Invitrogen) were added to the worm suspension. Worms were homogenized with a polytron at maximum speed for 3 min with the tube positioned on ice. Following centrifugation at 12,000 x g for 10 min at 4 °C, the clear upper phase was collected and extracted with chloroform. After centrifugation at 12,000 x g for 10 min at 4°C, the upper aqueous phase was collected and RNA was precipitated with isopropanol. RNA was centrifuged at 12,000 x g for 10 min at 4 °C. The RNA pellet was washed with 75% ethanol, followed by centrifugation at 7500 x g for 5 min, and the RNA pellet was dissolved in water. The 260/280 ratio of the sample was >1.6. The RNA samples were stored at -70 C and until sequencing at the McGill University and Génome Québec Innovation Centre.

Total RNA quality was verified on an RNA chip using an Agilent 2100 Bioanalyzer and quantified using a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher). A cDNA library was prepared from 5 µg total RNA using the mRNA-Seq Sample Preparation Kit (Illumina), according to the manufacturer's recommendations. Quality of the library was verified on a DNA 1000 chip using the Agilent 2100 Bioanalyzer and quantified by picogreen fluorimetry. The library was subjected to 108 single-read cycles of sequencing on an Illumina Genome Analyzer IIx as per the manufacturer's protocol. Cluster generation was performed on a c-Bot (Illumina) with a single read cluster generation kit. Sequencing was performed using a 36 cycle sequencing kit v4.

ESP preparation

ESP were prepared using a modification of previously described methods [9]. Briefly, adult worms were collected as described above; viable worms were selected, washed, and cultured at a density of ~1000 worms per ml of serum-free RPMI 1640 medium (Invitrogen) supplemented with 2% glucose (Sigma) and antibiotics for 36 h at 37º C. The supernatant was harvested, centrifuged at 8000 x g for 10 min to remove eggs and debris, and concentrated using an Amicon centrifugal filter device with a 3 kDa cut-off (Millipore, Billerica, MA). ESP were stored at -80º C until used. The protein concentration in ESP preparations was determined with a Bradford Reagent kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

1D electrophoresis and band excision

ESP were resuspended in loading buffer containing 2-mercaptoethanol, and ~100 µg protein were separated by SDS-PAGE through a 3 cm gradient gel (7-15% acrylamide) as described [55]. Following gel staining with Coomassie Brilliant Blue G, the entire lane was subjected to automated band excision using the Picking Workstation ProXCISION (Perkin Elmer) to generate 15 bands per lane (5-7 pieces/line).

Tryptic digestion and Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) analysis

Proteins from gel bands were subjected to reduction, cysteine-alkylation and in-gel tryptic digestion in a MassPrep Workstation (Micromass, Manchester, UK) as previously described [55]. Twenty µl of the tryptic digest solution were injected on a Zorbax 300SB-C18 pre-column (5 x 0.3 mm, 5 µm) previously equilibrated with water containing acetonitrile (5%) and formic acid (0.1%) using the Micro Well-plate sampler and the IsoPump modules of an Agilent 1100 Series Nanoflow HPLC. Following washing for 5 min at 15 µl/min, the pre-column was back-flushed to a 75 µm i.d. PicoFrit column (New Objective, Woburn, MA) filled with 10 cm of BioBasic C18 packing (5 µm, 300 Å) by the acetonitrile gradient supplied by the Agilent series 1100 Nanopump to allow elution of the peptides towards the mass spectrometer at a flow rate of 200 nl/min as described [55]. Eluted peptides were analyzed in a QToF micro (Waters Micromass, Manchester, UK) equipped with a Nanosource modified with a nanospray adapter (New Objective, Woburn, MA). The MS survey scan was set to 1 s (0.1 s interscan) and recorded from 350 to 1600 *m/z*. MS/MS scans were acquired from 50 to 1990 *m/z*, scan time was 1.35 s, and the interscan interval was 0.15 s. Doubly and triply charged ions were selected for fragmentation with collision energies calculated using a linear curve from reference collision energies.

MS raw data were acquired on the Data Directed Analysis feature in the MassLynx (Micromass) software with a 1, 2, 4 duty cycle (1 sec in MS mode 2 peptides selected for fragmentation, maximum of 4 sec in MS/MS acquisition mode). MS/MS raw data were transferred from the QTOF Micro computer to a 50 terabyte server and automatically manipulated for generation of peaklists by employing Distiller version 2.3.2.0 (http://www.matrixscience.com/distiller.htmls) with peak picking parameters set at 5 for Signal Noise Ration (SNR) and at 0.4 for Correlation Threshold (CT). The peaklisted data were then searched by employing Mascot version 2.3.01 (http://www.matrixscience.com) and X! Tandem version 2007.01.01.1 (http://www.thegpm.org) against the 6 open reading frames (ORF) translation of the transcriptomic assembly (see below). Searches were

restricted to up to 1 missed (trypsin) cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, 0.5 mass unit tolerance on parent and fragment ions, and monoisotopic. Scaffold (version Scaffold_2_05_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [85]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [86]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Bioinformatics

Reads from Illumina sequencing were trimmed in a process that consisted of search and clipping for adapter sequences, elimination of the first 16 bases of the reads to remove random hexamers, and quality trimming using a Q20 threshold on the 3' end. The assembly was done with Velvet 1.0.13 with a kmer value set at 43 [19]. Oases 0.1.6 (http://www.ebi.ac.uk/~zerbino/oases/) was then used for final transcriptome assembly.

Loci generated from the Oases assembler were subjected to analysis by BLASTx and BLASTn to identify putative homologues in *C. elegans*, other parasitic nematodes, and organisms other than nematodes (e-value of ≤1e-05). Full assembly will be available at Nembase4 (http://www.nematodes.org/nembase4/) (Submission date: May 6th, 2011) [87].

Gene Ontology (GO) annotations were performed using BLAST2GO [88]. Mapping of GO terms was performed on the hits retrieved from the initial search with BLASTx for protein homologs against the NCBI non-redundant database with a minimum expected value of 1×10^{-3} and a high scoring segment pair cut-off of 33. The annotation algorithm was set with default parameters; pre-eValue-Hit-Filter of 1×10^{-6}, annotation cut-off of 55, and GO weight of 5. Identification of enriched GO terms in the secretome dataset

compared to the transcriptome was done by assessing *P* values from Fisher's exact tests applying robust false discovery rate (FDR) using the integrated framework Gossip [23].

InterProscan [20,21] searches were performed using the built-in feature of BLAST2GO using the conceptual translation from the longest ORF of each locus. Enrichment analysis of exported Interpro terms in the ESP vs. transcriptome datasets was also performed by assessing adjusted P values to control for FDR from Fisher's exact tests run using FatiGO [24] on the integrative online platform Babilomics (http://babelomics.bioinfo.cipf.es) [89].

Acknowledgements

Work on this project was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Canada Research Chairs (CRC) to T.G.G, the Canadian Institutes for Health Research (CIHR) (MOP-81169) to M.M.S., and the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT)-Centre for Host-Parasite Interactions. We also thank Robin N. Beech, Line Roy, Sylvie LaBoissière, and Louis Letourneau for their help with data acquisition and analysis as well as the technical support of the proteomics and sequencing platforms of McGill University Genome Quebec Innovation Centre.

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List of Supplemental Files

The following supplementary data associated to this article. A stable URLat www.plosntds.org will be provided with the publication of the article.

Table S1. Proteins identified in ESP from *H. polygyrus* through search on proteins from nematodes (taxonomy id: 6231) in the Uniprot database.

Table S2. *H. polygyrus* RNAseq assembly automated annotation.

Table S3. Proteins identified in ESP from *H. polygyrus* through search on conceptual translation from RNAseq assembly.

Table S4. Enrichment analysis of gene ontology terms in the ESP dataset when compared to the total transcriptome dataset.

Chapter VI
General Discussion

Helminth infections are still a major source of debilitating morbidity and disease particularly in low income countries [1]. Although recognition of these effects on health and wage earning ability has been translated into the implementation of mass drug administration programs to reduce morbidity and transmission of these diseases, the success of this strategy is threatened by current gaps in knowledge on several pharmacological aspects of these anthelmintics along with the need to understand and monitor drug resistance as well as to develop novel and better control strategies [2].

On the other hand, helminth infections offer an interesting area of research. Different from most microparasites that rely on fast reproduction times and antigenic variation, helminth parasites are able to induce and modulate the immune response of the host, so their infections can remain for considerable periods of times with little apparent damage to the host [3]. The study of the mechanisms that prime these responses might lead not only to the identification of vaccine and drug candidates, but also to the exploitation of these immunomodulatory properties to address other human autoimmune-related diseases.

Lymphatic filariasis is a terrible disease. Immunopathological evidence shows that although immunomodulation is the key and the rule for the parasite survival in the host, the undesirable pathologies are associated with the host ability to clear the larval stage of the parasite.

In this work, we identified 228 proteins released *in vitro* by microfilariae and adult female and male worms of *B. malayi.* The list of identified proteins includes potential immunoregulators as well as proteins having multiple functions that are necessary to complete other physiological and developmental parasite processes. In addition, we demonstrated that, in the microfilariae of *B. malayi, in vitro* treatment with ivermectin (IVM) suppresses the release of protein from the main anatomical pathway for proteins

delivery, the ES apparatus. Proper functioning of this apparatus is dependent on neuromuscular activity regulated by GluCls.

These results suggest that the continuous delivery of parasite proteins to the host is necessary for survival of the parasite. Our results also support previous findings suggesting that the interface between the filarial parasite and the host is the place where the infection outcome is decided.

It is also important to note that two additional reports on the identification of ESP from *B. malayi* appeared almost simultaneously [4,5,6]. We reported 59 of the 80 proteins identified by Hewitson et al. [6] from mixed-sex adults incubations. In addition, Bennuru et al [4] reported 90 out of the 835 proteins reported by us and 40 of proteins initially reported by Hewitson et al [6]. These estimates may vary to a small degree depending on counting criteria. Differences between analyses can be explained in terms of biological variation, but also may be due to differences in sample manipulation conditions and identification approach (Table 1). It is not possible to determine at this time which one of these studies provide better predictors of the *in vivo* interaction, mainly as there is no previous information showing the differences between *in vivo* worms and those incubated *in vitro*. However, taking into account proteins identified in all these independent reports, there is still a clear recognition of the importance of the release of immunomodulators as well as other putative immune evasion molecules.

Additionally, the ability to elucidate the composition of complex protein mixtures must be accompanied by a strategy for prioritization, if the analysis is based on comparisons with functional annotations with proteins from model organisms. One of the ways that our laboratory is currently trying to accomplish this is by comparing ESP from different nematodes to identify both generalized and specific survival strategies of parasitic nematodes. In manuscript III, we used transcriptomic next-generation sequencing (RNA-seq) technologies to interrogate mass spectrometry (MS) data derived from a proteomic analysis of the ESP from the mouse gastrointestinal (GI) parasitic nematode *Heligmosomoides polygyrus*. Use of RNA-seq *de novo* assemblies opened the possibility

to analyze with an accessible setting the ESP from any species regardless of the availability of information from genome projects or other sequencing efforts.

An additional advantage of gaining insights on both the transcriptome and ESP composition of *H. polygyrus* is the possibility to unlock the study of individual proteins from this parasite, which offers many advantages as a GI nematode model. In this proteomic identification, we identified 209 proteins that include a number of possible immunomodulators which are currently being studied in the laboratory of Prof. Mary Stevenson (The Research Institute of the McGill University Health Centre). Comparisons of the functional annotations between the transcriptome and ESP datasets also allowed us to highlight important essential processes such as signalling and nutrient transport and/or uptake that are suggested to occur at the host – parasite interface.

	Hewitson *et al*	**Moreno & Geary**	**Bennuru *et al.***
Year	2008	2008	2009
Identified Proteins	80	228	852
Samples	Adults in Co-coculture	Microfilariae (76), Male (119) & Female (160) worms	Microfilariae (540), Male (170) & Female (239) worms, L3 (5), L3 to L4 (25) moulting
Sample source	Infected Gerbils -Parasite life cycle in house	Infected Gerbils shipped to laboratory from Filariasis Research Reagent Resource Center (FR3)	Parasites shipped from FR3
Incubation conditions	8 days	4 days	7 days
Identification Approach	2D/MALDI-ToF/ToF and shotgun LC–MS/MS	1D/LC–MS/MS	microcapillary RP-LC/LC-MS/MS
Database search program (Database)	Mascot v2.1 (Bma genome at TIGR + ESTs)	Mascot v.2.1.04 (Bma genome at UNIPROT)	SEQUEST (Bma genome at TIGR)
Proteins *Wolbachia*	No	No	90
Comparison (1)		59 (37*)/80	40/80
Comparison (2)			90 (72*)/228

Table 6. A comparison of 3 proteomic analysis of ESP from *B. malayi*

Although more information is still needed to reach more general conclusions, comparison between the ESP composition of *B. malayi* and *H. polygyrus* suggests that this strategy might be useful in determining differences/similarities between parasitic lifestyles. At the protein homology level, proteins such as macrophage migration inhibitory factors, cysteine protease inhibitors, galectins, C-type lectins, peroxiredoxins, glutathione S-transferases, several glycolytic enzymes and others were identified in both sets as well as in several other reports of ESP identification in parasitic nematodes [8,9,10], indicating that these proteins and their functions may be part of a conserved strategy for parasite survival regardless of the host and/or anatomical landscape in which it resides.

Among the interesting differences, the presence of several zinc finger (ZnF) C2H2- type family proteins, a superoxide dismutase, a glutathione peroxidase in *B. malayi* ESP but not in *H. polygyrus* and other GI nematode ESP indicate that they might play a particular role related to the group of tissues that are associated with lymphatic filarial infections. Similarly in *H. polygyrus*, the presence of several homologues of vitillogenins along with a much higher number of homologues of globins and members of the allergen V5/Tpx-1-related family of proteins contrasts with the low to none number of isoforms identified in the *B. malayi* ESP dataset. Comparing these findings with other recent analyses [8,10] indicates this group of proteins may be characteristic hallmarks of GI infections.

Consequently, the functional annotations associated with the identified proteins exhibit a certain degree of variation. Preliminary statistical analysis shows that it might be possible to identify some particular over- or under- represented processes in each dataset with statistical significance[3]. The inclusion of new datasets might provide a more robust analysis and might reach appropriate statistical and biological significance. Current efforts in our laboratory are directed towards the elucidation of the ESP composition of the free living nematode *Caenorhabitis elegans,* the dog heartworm filarial nematode *Dirofilaria immitis,* the pig GI roundworm *Ascaris suum* and plant root-knot nematode *Meloidogyne incognita*.

[3] e.g. determination of adult lifespan (GO:0008340); multicellular organismal aging (GO:0010259); and aging (GO:0007568) were over represented and primary metabolic process (GO:0044238) was under represented in the *H. polygyrus* ESP dataset compared to the *B. malayi*. (Fisher's test FDR<0.05).

So far, no filarial or GI nematode ESP have been directly identified *in vivo*, although some of the products that we identified in *B.malayi* have subsequently been recognized as antigens of the host humoral responses in filariasis patients [11]. Identification of ESP products might also be advantageous for the development of novel diagnostic tools that can improve our ability to monitor parasite viability *in vivo*.

In conclusion, the results presented here expand current knowledge on the biology of secretory processes in parasitic nematodes. This provides an opportunity to understand from a holistic perspective the role of proteins released from filarial and GI nematodes in immune evasion events. Also, they constitute an important step forward towards a complete elucidation at the molecular level of this and other aspects of the host-parasite relationship. In addition, it is expected that the data presented here may help to define specific molecular hallmarks of the different parasitic nematode lifestyles. The findings related to the effect of IVM in filarial nematodes provide basic knowledge for current programs for the elimination of filariasis. The fact that this drug affects protein release indicates that certain of these secreted proteins and the processes associated with their release are suitable therapeutic targets for the control of parasitic nematode diseases.

It is my main expectation that this work would provide valuable data to guide the development of new therapies for parasitic diseases.
YM.

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Appendix 1
Supporting Information Manuscript II

SI Materials and methods

Parasites

All animal procedures were approved by the Animal Care Committee of McGill University – Macdonald campus and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Infected gerbils were obtained from the Filariasis Research Reagent Repository Center (Athens, GA). Mf and adult *B. malayi* were recovered >120 days post-infection from the peritoneal cavities of Mongolian jirds (*Meriones unguiculatus*) infected subcutaneously with 200–300 L3. Adult worms were washed several times in RPMI 1640 medium [supplemented with L-glutamine, 20 mM HEPES, 100 µg/ml penicillin, 100 units/ml streptomycin (Gibco), pH 7.2] (henceforth, RPMI 1640) and separated by gender according to size. Mf were obtained by washing the peritoneal cavity with 37° RPMI 1640. The washes were centrifuged 5 min at 1000×g to pellet mf, which were subsequently purified from host cells by passage through PD-10 columns equilibrated with pre-warmed RPMI 1640 as described (1).

Cloning and sequencing of Bma-AVR-14 subunits.

Partial sequences for two GluCls (AVR-14) were obtained from the *B. malayi* genome project (Bm1_00335 and Bm1_15450). Sequence alignment of the predicted genomic and coding sequences allowed us to initially infer that the two gene models are splice variants of the same gene. Completion of the full coding sequences for these genes was carried out by following a strategy like that employed for *avr-14* from *D. immitis* (2).

B. malayi total RNA was extracted from adult worms or mf using the RNeasy Microkit (Qiagen) according to the manufacturer's instructions for tissue samples. cDNA was obtained from reverse transcription reactions at 50° on 100 - 500 ng RNA in a total

volume of 20 µl with SuperScript III (Invitrogen) using either an oligo dT primer (12-18) (Invitrogen) or the 3'RACE adaptor from a 3'RACE kit (Bioline)(Table S1). PCRs were performed (SI Figure 2) with 1 µl of the RT reaction and 0.02 U/µl Phusion High-Fidelity DNA polymerase (New England Biolabs), 1X Phusion HF Buffer, 200 µM dNTPs, 0.5 µM each primer (Table S1) in a total volume of 20 µl on a Mastercycler epgradient thermocycler (Eppendorf) following a programme of initial denaturation at 98° for 1 min, followed by 35 cycles of denaturation at 98° for 30 sec, annealing at 50° for 30 sec, extension at 72° for 30 sec and a final extension step at 72° for 5 min. PCR products were visualized under UV light after electrophoresis through a 1% agarose gel. Selected bands were purified using a QIAquick gel extraction kit (Qiagen) and cloned into pJET1.2/blunt using the CloneJET PCR cloning kit (Fermentas). Recombinant clones were sequenced in both directions using pJET1.2 forward and reverse primers and assembly was performed in NTIvector v.10 (Invitrogen).

Antibodies

Immune sera against on-site produced C-terminal His-tagged Bma-MIF-1, Bma-TCTP and Bma-CPI-2 recombinant proteins were raised in rabbit, goat and guinea pig, respectively, under contract with commercial sources (Pacific Immunology for Bma-MIF-1 and Bma-TCTP, Harlan Bioproducts for Bma-CPI-2). Immune sera were subsequently purified by affinity chromatography on antigen-coupled Affi-Gel-10 beads (Biorad) or Sulfolink (Pierce) according to the instructions of the manufacturers. Affinity purified rabbit anti-human-TPI (Santa Cruz, sc-30145) raised against the full length human protein was cross reactive towards Bma-TPI and was also used in this study. Purified rabbit antibody against Bm-VAL-1 was a kind gift from Prof. Murray Selkirk (Imperial College, London).

A peptide with the sequence LRTKMILRREFS-cysteine, mapping to residues 239- 250 of Bma-AVR-14A, was synthesized, coupled to KHL and used to immunize rabbits

commercially (Antagene Inc). Immune serum was subsequently purified by chromatography through a peptide-coupled Sulfolink resin (Pierce).

Goat anti-rabbit IgG and anti-guinea pig IgG-Alexa Fluor 488, donkey anti-goat IgG Alexa Fluor 488 (Invitrogen), donkey anti-rabbit IgG HRP-linked (Amersham) were used as secondary antibodies for immunoassays.

Indirect Enzyme-linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed to estimate affinity of the GluCl antibody for different subunits of the channel. High binding polystyrene 96-well plates (Costar) were coated with 50 µl of a 12 µM solution of either LRTKMILRREFS-cysteine or ARVMLLLRREYS-cysteine in 50 µM sodium carbonate buffer, pH 9.6. After extensive washing with PBS – 0.1% Tween 20, unbound sites in the plate were blocked by incubation with blocking buffer [1% bovine serum albumin in PBS - 0.1% Tween 20] for 2 h. 100 µl of primary antibody diluted in blocking buffer was added to the plate in serial dilution and incubated for 2 hr. After extensive washes with PBS – 0.1% Tween 20, 100 µl of a 1:5000 dilution of anti-rabbit IgG-HRP in blocking buffer was added to each well and incubated for 1 h at room temperature. After incubation with secondary antibody, the wells were washed with PBS– 0.1% Tween 20 and 100 µl of a solution of 3,3',5,5'-Tetramethylbenzidine (Sigma, T0440) were added to the plate. The coloring reaction was stopped by adding of 100 µl of 2 N HCl 10 min after the addition of substrate. Absorbance values were subsequently determined at 445 nm in a plate reader (EL808, Biotek).

Immunohistochemistry

Mf were fixed and permeabilized by an adaptation of the tube protocol for *C. elegans* (3). Mf were collected by centrifugation at 1000 rpm x 5 min and washed once with warm PBS. Freeze-cracking was performed in fixing solution [4% (w/v) paraformaldehyde in PBS] by immersing the tube in liquid nitrogen for 2-3 min followed by thawing in a 37°C water bath (3 times). Mf were incubated in the fixing solution for 4 h at 4°C, collected by

centrifugation and washed several times with PBST [0.1% Triton X-100 in PBS]. Permeabilization was done by incubation overnight at 37°C in fresh 2-mercaptoethanol solution [5% 2-mercaptoethanol, 1% Triton X-100, 120 mM Tris, pH 7.0]. After extensive washing in PBST, worms were incubated for 12 h in collagenase solution [1000 U/ml collagenase type VII (Sigma), 1 mM $CaCl_2$, 0.1% Triton X-100, 100 mM Tris, pH 7.4] at 37°. Mf were washed with PBST and then incubated overnight with AbD solution [0.1% BSA, 0.1% sodium azide in PBST].

Incubations with primary affinity-purified antibodies diluted in AbD solution (5-16 µg/ml) were performed for 2 days at 4ºC. Removal of unbound antibodies was done by 3-4 successive centrifugation–resuspension steps in AbD and final incubation overnight at 4ºC. Incubation and removal of secondary antibodies (dilution 1:1000 or 1:3000) was performed the same way. Muscle tissue and nuclei were counterstained by incubation overnight with 100 nM rhodamine-phalloidin (Cytoskeleton) and 50 ng/ml DAPI (Sigma), respectively.

Specimens were washed in AbD as above and mounted in glycerol–PBS-DABCO 1%. Observations were performed on a Biorad Radiance 2100 confocal laser scanning microscope equipped with a Nikon E800 fluorescence microscope for confocal image acquisition and the LASERSHARP 2000 analyzing software package. Controls include observation of mf with omission of primary antibody and in the case of on-site produced proteins, with peptide- or protein-adsorbed primary antibodies. Pre-adsorption of the antibodies was performed by overnight incubation of 50 µg/ml of peptide (for anti-GluCl) or 5-40 µg/ml of protein (for anti-MIF-1, anti-TCTP and anti-CPI-2). Image processing was performed on ImageJ v1.42q (National Institutes of Health, USA).

In vitro treatment of *B. malayi* with IVM

After initial incubation overnight, parasites were incubated for 72 h in volumes of 1 ml in 24-well plates at 37º, 5% CO_2 . RPMI containing 0, 0.1, 0.32 or 1.0 µM IVM was used for drug treatments after dilution of a 10 mM stock solution dissolved in dimethyl sulfoxide (DMSO). Control wells contained 0.1% DMSO. Parasite densities were set at 250 x 10^3

mf/ml. Media from each plate were collected and replaced with fresh media with or without drug every 24 h. The medium in each well was treated as an independent sample; mf were recovered by centrifugation at 1000×g for 5 min, the supernatant was filtered on 0.22 µm membranes. Both the media and collected mf were stored at -80° until analysis.

For 2D-DiGE experiments, ESP were recovered from 3 independent incubations with or without 0.1 µM IVM. Media were collected each 24 h for 3 consecutive as mentioned above. The spent media were immediately passed through a 0.22 µm filter; all collected samples from each independent replicate were pooled before concentration for subsequent analysis. Proteins were concentrated to 1–1.5 ml in an Amicon Ultra 3000 MWCO (Millipore) and then precipitated with trichloroacetic acid (TCA, 20% final concentration) as described previously (1). Proteins were resuspended in 30 mM Tris-HCl pH 8.5 containing 7 M urea, 2 M thiourea and 4% CHAPS.

Protein determination

Protein determinations were performed with an EZQ protein quantitation kit (Invitrogen) with some modifications. For protein quantitation in ESP, 250 ul of either sample or RPMI were spiked with 50 ng of ovalbumin as an internal standard and then concentrated to dryness in a speedvac. Samples were resuspended in 5 µl doubled distilled water. The total sample was serially spotted in volumes of 2 ul on the assay paper in the sampling plate. The assay paper was washed twice with MeOH for 5 min and then incubated with the EZQ reagent for 30 min. Final 2 min washes were performed 3 times with 10% MeOH, 7% acetic acid. The assay paper was placed into the sampling plate and fluorescence was determined in a plate fluorimeter (FlexStation II, Molecular Devices) set at 450 nm for excitation and emission at 610 nm. Protein amounts were estimated from extrapolation from the standard curve, corrected according to the internal standard readings of RPMI alone.

2-Dimensional Difference Gel Electrophoresis (2D-DiGE)

0.5 µg of each protein sample was labeled with 40 pmol either Cy3 or Cy5, alternating the dyes to avoid labeling bias, on ice for 30 min in the dark as described (4). A pooled sample that was included as an internal standard in the gel runs was generated by combining an equal amount of each sample and labeling it with Cy2. Labeled samples were combined and randomized and diluted with rehydration buffer [1% IPG buffer (GE Healthcare), 7 M urea, 2 M thiourea, 2% DTT (w/v), 4% CHAPS (w/v), 0.002% bromophenol blue (w/v)] to 250 µl. For isoelectrofocusing, samples were actively rehydrated into IPG strips (pH 3–10 NL, 24 cm length, GE Healthcare) and focused with an Ettan IPGphorII Isoelectric Focusing System (GE Healthcare) using a step gradient protocol ranging from 30 to 8000 volts for approximately 26 h (30V 10 h, 500V 1 h, 1000V 1 h, 8000V 3 h, 800V 3 h).

IPG strips were rehydrated in10 ml equilibration buffer-1 [6 M urea, 5 mg/ml DTT, 30% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue (w/v), 100 mM Tris-HCl, pH 6.8] for 10 min followed by a second incubation with 10 ml equilibration buffer-2 [6 M urea, 45 mg/ml iodoacetamide, 30% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue (w/v), 100 mM Tris-HCl, pH 6.8] and proteins were separated on 4-16% SDS-PAGE gels (24 cm × 20 cm × 1 mm). The SDS-PAGE gels were run at 1W/gel for 60 min and then at 2W/gel at 25° until the bromophenol blue dye front reach the bottom of the gel (~16h). Proteins were visualized with a Typhoon 9400 (GE) fluorescence scanner. The Cy2, Cy3, and Cy5 labeled images for each gel were scanned at the excitation/emission wavelengths of 488/520 nm, 532/580 nm, and 633/670 nm, respectively. The images were imported and analyzed with DeCyder software v.7.0 (GE Healthcare).

Analysis consisted of initial spot detection and pair-wise comparisons of treated and untreated mf to the pooled internal standard on each gel. Calculation of individual protein abundance represented by normalized spot volumes (Cy3:Cy2 and Cy5:Cy2 ratios) was initially performed with the DeCyder differential in-gel analysis (DIA) module. Subsequently, the three gels were matched using the internal standard pool spot map with

the DeCyder biological variation analysis module (BVA). Protein spot matches were confirmed and in some cases adjusted manually using the spot editing features of DeCyder v 7.0. Comparative cross-gel statistical analyses of the spot maps were used to determine specific changes in abundance. Paired Student's t-test p-values for each normalized spot volume across the gels were calculated.

SI text 1

Determination of changes in protein abundance in excretory-secretory products from *Brugia malayi* microfilariae after incubation with 0.1 µM ivermectin.

To determine the effect of IVM on the relative abundance of specific proteins in mf-ESP, mf were incubated with or without 0.1 µM IVM. Media were recovered and exchanged each 24 h for 3 consecutive days, pooled, concentrated and analyzed using 2D-DiGE methods. Incubation with 0.1 µM IVM decreased by ~ 30% the amount of protein released by mf compared to unexposed controls in 3 independent replicates ($p<0.05$, paired t-test).

Analysis of protein maps using the DeCyder V. 7.0 software allowed the comparison of the normalized volume in 61 major protein spots from treated and untreated samples (Figure S5). As indicated in Table 1, there were no significant differences in any of the analyzed spots. This indicates that, although there is a drop in the amount of protein released following exposure of mf to IVM, the relative composition of proteins in ESP remains unchanged.

Taking into account the association of the IVM receptor with the muscle structure encompassing the ES-vesicle, it is expected that the effect of IVM on protein release would result in a drop in the relative abundance of proteins that are released through the ES-pore. The absence of changes in the protein composition of the ESP indicates that protein release through a different anatomical path is negligible compared to the release through the ES-apparatus. This result supports our proposal that the mf ES apparatus is the predominant anatomical path for protein release to the host environment.

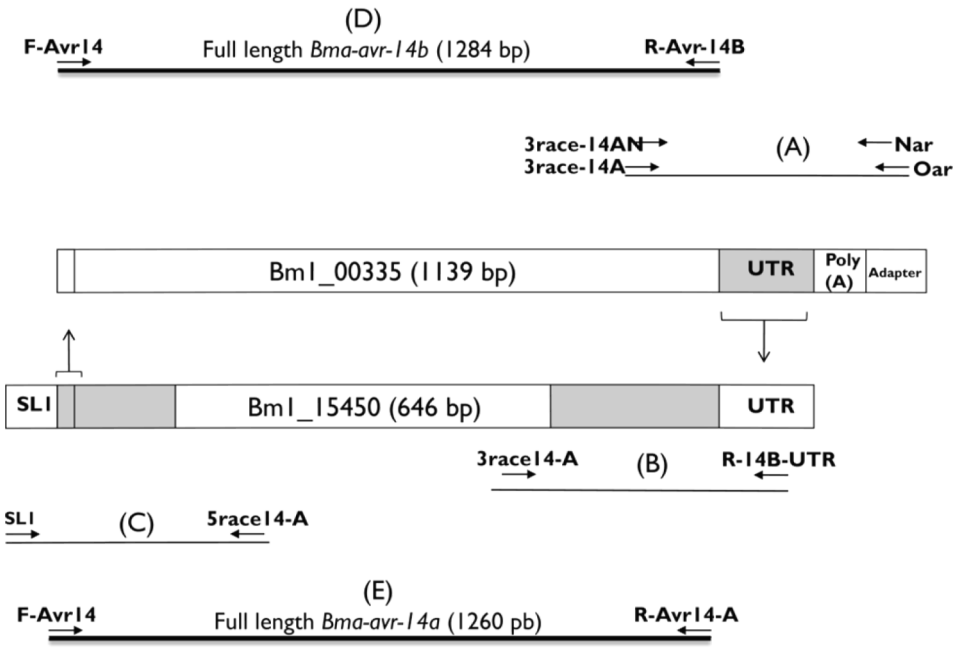

Figure S1 Strategy for cloning two splice variants of the *B. malayi avr-14* gene. Mining the *B. malayi* genome database revealed 2 partial sequences (Bm1_00335 and Bm1_15450) sharing homology with AVR-14 subunits from *C. elegans*. Sequence alignment of the predicted genomic and coding sequences showed that these gene models are splice variants of the same gene. The strategy for cloning the two full open reading frames consisted of: (A) assessing the sequence by cloning the amplified un-translated region (UTR) of the *avr-14* transcripts by 3'RACE using nested PCR with cDNA obtained from retrotranscription with a commercial adaptor (Bioline) and primers annealing to both the *avr-14b* (3race-14 A and AN) and the adaptor sequence (Nar and Oar). (B) The 5' region of *avr-14a* containing the stop codon was assessed by cloning and sequencing the PCR product obtained from cDNA using a forward primer designed on the reported sequence (3race14-A) and a reverse primer designed on the UTR of the *avr-14b* (R-14B-UTR). (C). The 3' end of the *avr-14* variants was found by cloning and sequencing the PCR product resulting from the amplification of cDNA using a forward primer annealing to the Splice Leader sequence (SL1) and a reverse primer designed on the *avr-14a* reported sequence. (D and E) The full coding sequences for *avr-14 a* and *b* were obtained by cloning and sequencing the PCR products resulting from the amplification of cDNA using a common forward primer (F-Avr14) and reverse primers specific for each subunit (R-Avr14 A and B).

Experiment	Primer name (source)	Primer Sequence
3' RACE for Bma-AVR-14B	3RACE adaptor (Bioline)	5'CCCTGTTCAAGCGCATCTGAGGTGAACCATGAACCGTGC TTTTTTTTTTTTTTTTTT-3’
	Oar (Bioline)	5’CAGTCGGTCCTGCAGGGCATCTGAGGTGAACCATGA-3’
	Nar (Bioline)	5’CAGTCGGTCCTGCAGGGCATCTGAGGTGAACCATGA-3’
	3race-14B	5'-TGGTTAAATAGATACCTGTGCGG-3'
	3race-14BN	5'-GTTGACCTCATTTCACGATTTGC-3'
3'RACE for Bma-AVR-14A	R-14B-UTR	5'-GCGGCCGCATTCATTTACCGCAACAAAACAG-3'
	3race-14A	5'-ATACCCGAACTTTGTGTGGTCATCG-3'
5' end of Bm-AVR-14A	SL1	5'-GGTTTAATTACCCAAGTTTGAG-3'
	5race-14A	5'-TTCCTCTCGAAACGTAAACTGTGCG-3'
Bma AVR-14 Full Length amplification	R-AVR-14B	5'-TCAATTCACATAATTCACATAGTA-3'
	R-AVR-14A	5'-TTAAATGAGATAAACAGCCCA-3'
	F-AVR-14	5'-ATGAATGGTTGTATGATTTGTTGG-3'

Table S1 Primer sequences employed for cloning the two splice variants of the *B. malayi avr-14* gene.

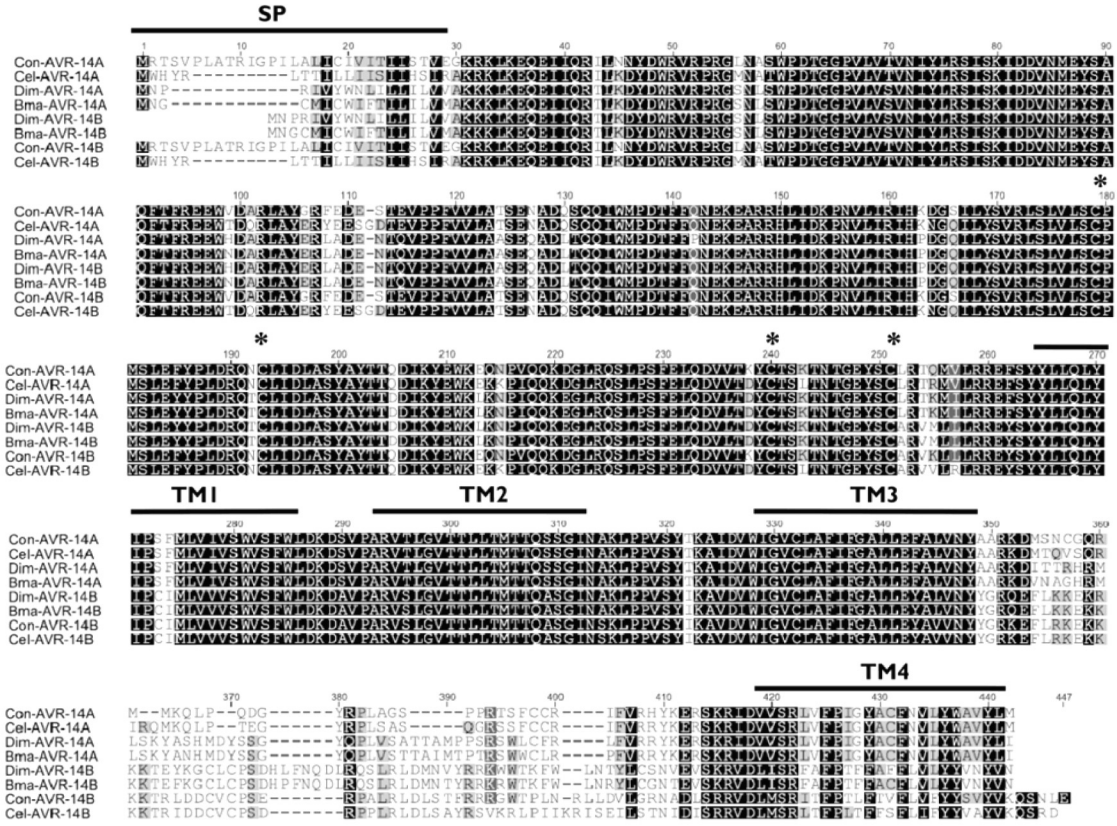

Figure S2 ClustalX alignment of AVR-14 subunit amino acid sequences from *B. malayi* (Bma), *D. immitis* (Dim), *C. elegans* (Cel) and *Cooperia oncophora* (Con). Characteristic features of the ligand-gated ion channel superfamily (LGIC) were predicted for Bma-AVR-14 subunits, including the presence of a signal peptide (SP), two pairs of cysteine residues in the N-terminal region, one pair characteristic of LGIC (*) and a second pair diagnostic for glutamate-gated chloride channels and glycine receptors (+). 4 transmembrane helices (TM 1 to 4) are predicted in the C-terminal regions.

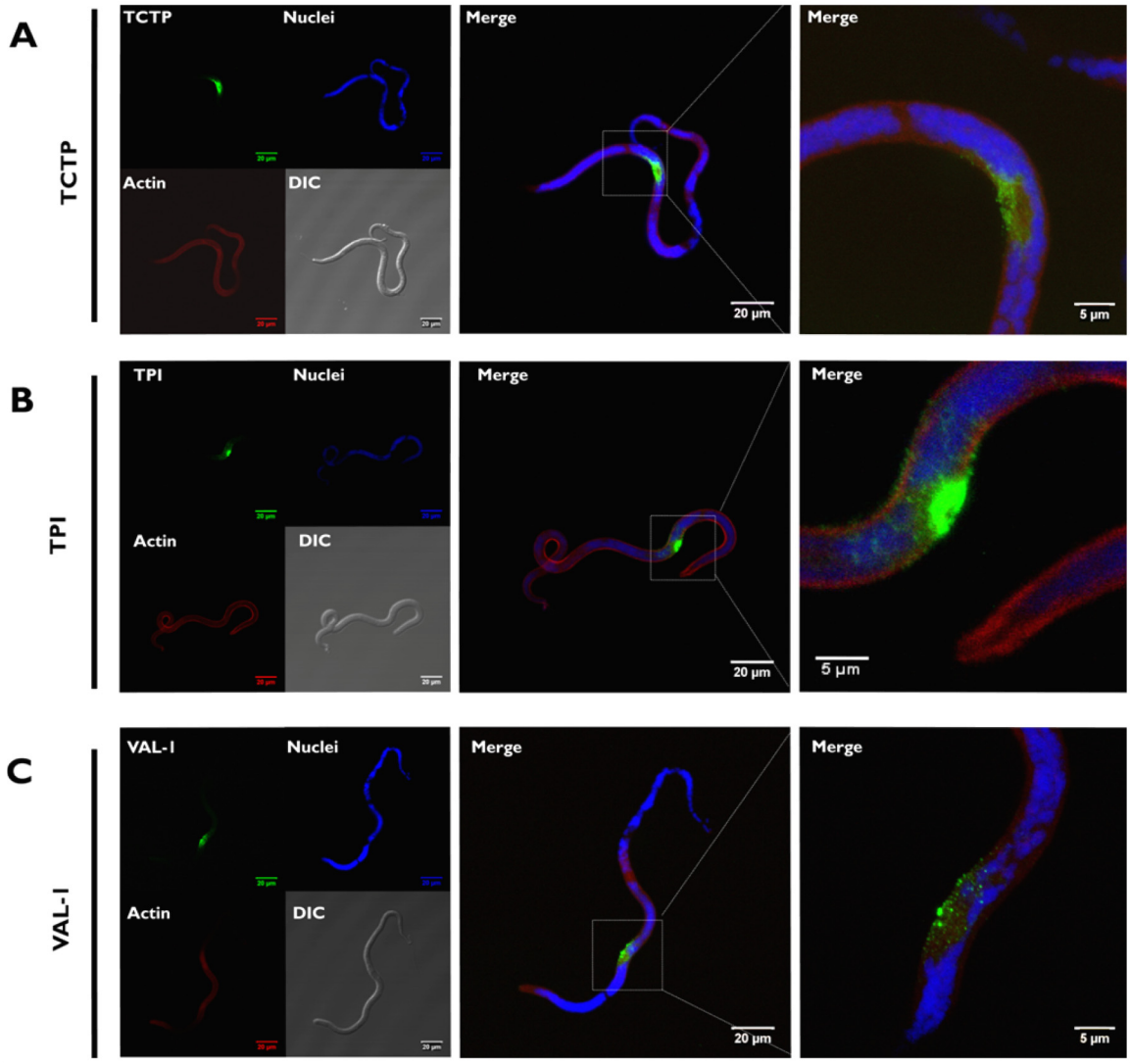

Figure S3 Immunolocalization in the Excretory-Secretory apparatus of *B. malayi* mf of 3 ES proteins. In addition to CPI-2, this localization pattern was also found with (A) TCTP, (B) TPI and (C) VAL-1.

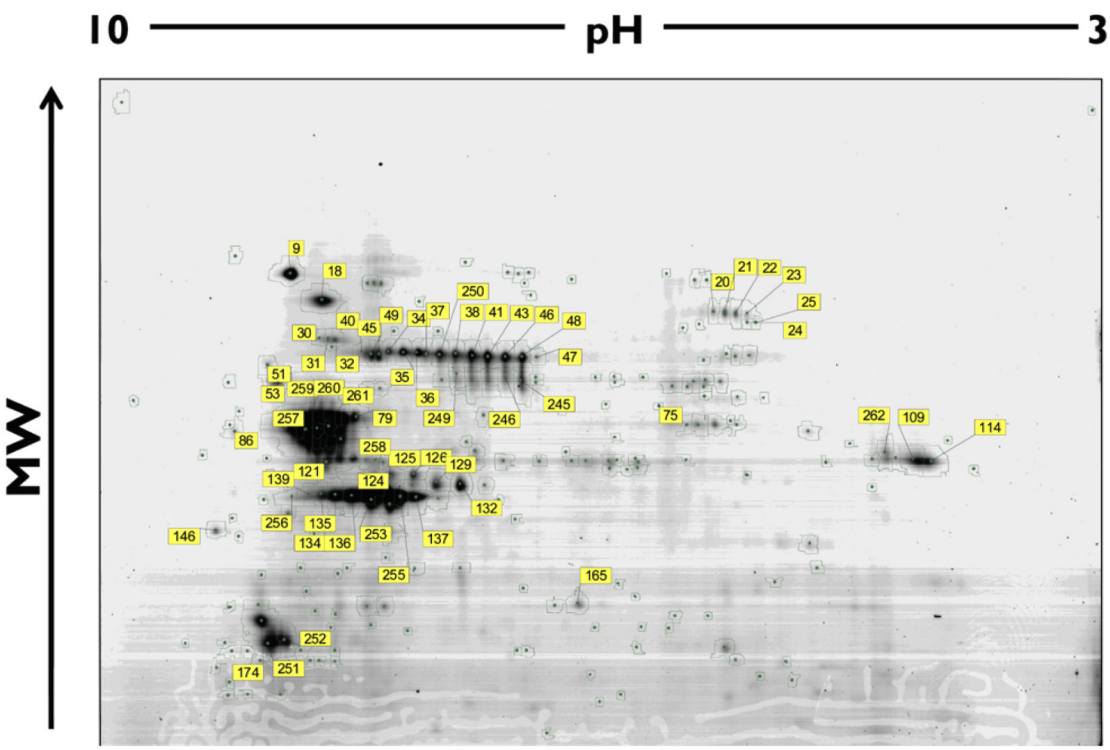

Figure S4 Master gel (Cy2 label) showing the protein map obtained by Difference Gel Electrophoresis analysis of Excretory-Secretory proteins from *B. malayi* microfilariae treated or untreated with 0.1 µM ivermectin. Labels represent the master numbers of the 61 spots analyzed.

	Master No.	Status	Appearance	Paired T-test	Paired Av Ratio
1	75	Confirmed	9 (9)	0.18	1.25
2	32	Confirmed	9 (9)	0.43	1.09
3	23	Confirmed	9 (9)	0.5	1.25
4	86	Confirmed	9 (9)	0.51	1.12
5	256	Confirmed	9 (9)	0.51	1.31
6	51	Confirmed	9 (9)	0.53	1.33
7	139	Confirmed	9 (9)	0.55	1.34
8	252	Confirmed	9 (9)	0.59	1.29
9	121	Confirmed	9 (9)	0.6	-1.1
10	53	Confirmed	9 (9)	0.6	1.27
11	251	Confirmed	9 (9)	0.6	1.32
12	49	Confirmed	9 (9)	0.61	1.51
13	174	Confirmed	9 (9)	0.63	1.27
14	79	Confirmed	9 (9)	0.64	1.41
15	45	Confirmed	9 (9)	0.65	1.37
16	134	Confirmed	9 (9)	0.65	1.39
17	218	Confirmed	9 (9)	0.66	1.18
18	24	Confirmed	9 (9)	0.66	1.45
19	245	Confirmed	9 (9)	0.73	1.24
20	40	Confirmed	9 (9)	0.73	1.33
21	137	Confirmed	9 (9)	0.73	1.41
22	38	Confirmed	9 (9)	0.74	-1.03
23	255	Confirmed	9 (9)	0.74	1.31
24	129	Confirmed	9 (9)	0.74	1.36
25	261	Confirmed	9 (9)	0.74	1.39
26	35	Confirmed	9 (9)	0.75	1.21
27	253	Confirmed	9 (9)	0.77	1.3
28	258	Confirmed	9 (9)	0.77	1.4
29	22	Confirmed	9 (9)	0.79	1.18
30	34	Confirmed	9 (9)	0.79	1.21
31	246	Confirmed	9 (9)	0.8	1.07
32	21	Confirmed	9 (9)	0.81	1.13
33	132	Confirmed	9 (9)	0.81	1.47
34	114	Confirmed	9 (9)	0.82	1.37
35	249	Confirmed	9 (9)	0.83	1.07
36	260	Confirmed	9 (9)	0.83	1.25
37	257	Confirmed	9 (9)	0.83	1.32
38	31	Confirmed	9 (9)	0.83	1.33
39	259	Confirmed	9 (9)	0.83	1.35
40	250	Confirmed	9 (9)	0.84	1.03
41	41	Confirmed	9 (9)	0.84	1.04
42	25	Confirmed	9 (9)	0.84	1.12
43	47	Confirmed	9 (9)	0.85	1.24
44	109	Confirmed	9 (9)	0.86	1.3
45	43	Confirmed	9 (9)	0.87	1.06
46	46	Confirmed	9 (9)	0.87	1.07
47	37	Confirmed	9 (9)	0.88	1.02
48	36	Confirmed	9 (9)	0.88	1.09
49	125	Confirmed	9 (9)	0.88	1.27

50	135	Confirmed	9 (9)	0.89	1.12
51	30	Confirmed	9 (9)	0.89	1.14
52	124	Confirmed	9 (9)	0.91	1.14
53	126	Confirmed	9 (9)	0.92	1.25
54	48	Confirmed	9 (9)	0.94	1.15
55	18	Confirmed	9 (9)	0.94	1.16
56	136	Confirmed	9 (9)	0.95	1.15
57	165	Confirmed	9 (9)	0.97	1.08
58	9	Confirmed	9 (9)	0.97	1.18
59	262	Confirmed	9 (9)	0.98	1.36
60	146	Confirmed	9 (9)	0.99	1.02
61	20	Confirmed	9 (9)	0.99	1.06

Table S2 Paired T-test values for 61spots analyzed by Difference Gel Electrophoresis.

References

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