Schistosoma mansoni-derived extracellular vesicles: Content, origins, and functions

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Abstract

Schistosomes are wide-spread and clinically significant parasites of humans in the tropics and sub-tropics. Trematodes of the genus *Schistosoma* require a freshwater snail intermediate host and colonise the circulatory system of their definitive mammalian host. The treatment of schistosomiasis, the disease caused by infection with schistosomes, currently relies on a single widely used drug, praziquantel (PZQ), causing concerns about the emergence of drug resistance. The development of new treatment options for the control of schistosomiasis is thus an urgent priority. An interesting avenue for the development of therapeutics is targeting the mechanisms through which parasites interact with their host. Host–parasite interactions have been shaped by millennia of co-evolution and are characterised by highly intricate molecular dialogues, involving the release of excretory/secretory products (ESPs) by the parasite. Amongst helminth ESPs, extracellular vesicles (EVs) are increasingly recognised as key mediators of host–parasite interactions, making them appealing targets for the development of new therapeutics. EVs include all types of secreted membrane-bound vesicles, of which exosomes (formed within multivesicular bodies) and microvesicles (formed by the outward budding of the plasma membrane) are the best characterised. We and others have reported the release of EVs by schistosomes and showed enrichment in putative effector molecules such as microRNAs (miRNAs) and proteins. Building on our previous work, this thesis focuses on the study of EVs secreted by the parasite *Schistosoma mansoni*.

First, using a lectin microarray approach, we assessed the diversity of surface-exposed glycans present on parasite-derived EVs and identified several lectins with strong binding affinity for *S. mansoni* EVs, suggesting the presence of multiple glycan structures. Despite a growing appreciation for the roles of glycans in EV biology, such as in interactions between EVs and target cells, limited information is available on schistosome EV glycomics and its role in infection. Interestingly, SNA-I, a lectin that recognises terminal sialic acid, was amongst the lectins displaying strong binding, suggesting sialylation in the EV sample, which was confirmed in additional experiments. This finding is of interest, as sialic acids play important roles in the context

of infection, such as by aiding immune evasion, and affecting target recognition and cell entry, but are not thought to be synthesised by helminths. Mass spectrometry analyses identified the sialoglycoconjugates associated with *S. mansoni* EVs as mammalian serum proteins, potentially revealing a novel immune evasion mechanism. Then, we investigated the route of EV secretion in adult worms using a lectin histochemistry and fluorescence *in situ* hybridization approach. Our results suggest the involvement of the tegument and the digestive and excretory systems in the release of EVs. This work addressed for the first time the origin of EV secretion in schistosomes. Lastly, we hypothesized that miRNAs contained in parasite EVs contribute to changes in host gene expression during infection. We performed a comparative transcriptomic analysis of intestinal lymphatic tissues extracted from infected and non-infected mice and report the differential expression of several genes of interest. This work provides new insights on the mechanisms of host-parasite interaction in schistosomiasis and provides foundation for the development of novel therapeutic interventions.

Résumé

Les schistosomes sont des parasites de l'homme très répandus et cliniquement significatifs dans les régions tropicales et subtropicales. Les trématodes du genre *Schistosoma* nécessitent un mollusque d'eau douce comme hôte intermédiaire et colonisent le système circulatoire de leur hôte définitif. Le traitement de la schistosomiase, la maladie causée par l'infection par les schistosomes, repose actuellement sur un seul médicament largement utilisé, le praziquantel (PZQ), ce qui suscite des inquiétudes quant à l'émergence d'une résistance au médicament. Le développement de nouvelles options thérapeutiques pour le contrôle de la schistosomiase est donc une priorité croissante. Une piste intéressante pour le développement de thérapies consiste à cibler les mécanismes par lesquels les parasites interagissent avec leur hôte. Les interactions hôte-parasite ont été façonnées par des milliers d'années de coévolution et sont caractérisées par des dialogues moléculaires très complexes, impliquant la libération de produits excrétoires/sécrétoires (excretory/secretory products; ESPs) par le parasite. Parmi les ESPs des helminthes, les vésicules extracellulaires (extracellular vesicles; EVs) sont de plus en plus reconnues comme d'importants médiateurs des interactions hôte-parasite, ce qui en fait des cibles intéressantes pour le développement de nouvelles thérapies. Les EVs comprennent tous les types de vésicules membranaires sécrétées, dont les exosomes (formés dans les corps multivésiculaires) et les microvésicules (formées par le bourgeonnement de la membrane plasmique) sont les mieux caractérisés. Nous avons précédemment rapporté la libération d'EVs par les schistosomes et avons montré un enrichissement en effecteurs putatifs tels que les microARNs (miRNAs) et les protéines. S'appuyant sur nos travaux précédents, cette thèse se concentre sur l'étude des EVs sécrétées par le parasite *Schistosoma mansoni*.

Tout d'abord, en utilisant une approche de puces de lectines, nous avons évalué la diversité des glycoconjugués présents à la surface des EVs et avons identifié plusieurs lectines ayant une forte affinité de liaison pour les EVs de *S. mansoni*, suggérant la présence de multiples structures de glycanes. Malgré une appréciation croissante des rôles des glycanes dans la biologie des EVs, par exemple, dans les interactions entre les EVs et les cellules cibles, peu d'informations sont

disponibles sur la glycomique des EVs sécrétées par les schistosomes ainsi que son rôle dans l'infection. Il est intéressant de noter que SNA-I, une lectine qui reconnaît l'acide sialique terminal, était parmi les lectines affichant une forte liaison, suggérant une sialylation dans l'échantillon d'EV, ce qui a été confirmé par des expériences supplémentaires. Cette découverte est intéressante, car les acides sialiques jouent des rôles importants dans le contexte de l'infection, notamment en aidant à l'évasion immunitaire et en affectant la reconnaissance des cibles et l'entrée dans les cellules, mais on ne pense pas qu'ils soient synthétisés par les helminthes. Les analyses de spectrométrie de masse ont identifié les sialoglycoconjugués associés aux EVs de *S. mansoni* comme étant des protéines sériques de mammifères, révélant potentiellement un nouveau mécanisme d'évasion immunitaire. Nous avons ensuite étudié la voie de sécrétion des EVs dans les vers adultes en utilisant une approche d'histochimie des lectines et d'hybridation *in situ* par fluorescence. Nos résultats suggèrent l'implication du tégument, ainsi que des systèmes digestif et excréteur dans la libération des EVs. Ce travail aborde pour la première fois l'origine de la sécrétion des EVs chez les schistosomes. Enfin, nous avons émis l'hypothèse que les miRNAs contenus dans les EVs du parasite contribuent aux changements de l'expression des gènes de l'hôte pendant l'infection. Nous avons réalisé une analyse transcriptomique comparative des tissus lymphatiques intestinaux extraits de souris infectées et non infectées et rapportons l'expression différentielle de plusieurs gènes d'intérêt. Ces travaux fournissent de nouvelles informations sur les mécanismes d'interaction hôte-parasite dans la schistosomiase et jettent les bases pour le développement de nouvelles interventions thérapeutiques.

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I must start by expressing my deepest gratitude to my supervisor, Prof. Timothy G. Geary, for his continuous support, both academic and personal. I feel extremely fortunate to have had the opportunity to work with Tim. He is the best mentor one can hope for, he is incredibly reassuring, devoted, trusting and trustworthy, but most importantly, he elevates people in a remarkably selfless way. I will always remember his humanity, his willingness to listen and give advice on just about anything, and his Hawaiian shirts. Tim is not only a mentor to me, but also a confidant and a friend. He has continued to guide me through my studies as well as through life and I would not be here without him.

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Thank you. Merci.

MD

Statement of Originality

The following aspects described in this thesis are considered contributions of original knowledge:

Manuscript I. Dagenais, M., Gerlach, J. Q., Wendt, G. R., Collins III, J. J.; Atkinson, L. E., Mousley, A., Geary, T. G., Long, T, 2021. Analysis of *Schistosoma mansoni* extracellular vesicles surface glycans reveals potential immune evasion mechanism and new insights on their origins of biogenesis. *Pathogens 10, 1401*.

In this manuscript, we profiled carbohydrate moieties present on the surface of adult *S. mansoni EVs* via lectin microarray. This report provides the first attempt at characterising adult *S. mansoni* EV glycome and is one of the very few studies investigating the glycan content of helminth EVs. We also report the presence of sialic acid residues on EVs secreted by adult *S. mansoni*. However, the origin of the sialylated content remains unclear, as helminths are generally regarded as being unable to synthesise sialic acid due to the apparent lack of enzymes required for its synthesis. Moreover, we labelled whole adult worms with lectins displaying high avidity for *S. mansoni* EVs and identified the tegument and the digestive and excretory systems as potential sources of EVs. Again, this represents the first study attempting to identify the origin(s) of EV secretion in schistosomes.

Manuscript II. Dagenais, M., Gerlach, J. Q., Geary, T. G., Long, T, 2022. Sugar coating: utilisation of host serum sialoglycoproteins by *Schistosoma mansoni* as a potential immune evasion mechanism. *Pathogens 11, 426*.

In this manuscript, we investigated the origin of the sialic acid residues previously identified at the surface of adult *S. mansoni* EVs and report that these sialylated molecules are in fact mammalian serum proteins. In addition, our data suggest that most sialylated EV-associated proteins do not elicit a humoral response upon injection into mice, or in sera obtained from infected animals. Given the involvement of sialic acid in immune evasion and cell adhesion, entry, and invasion, we hypothesise that the parasite might be exploiting host sialylated glycoconjugates to mask antigenic

sites, protecting EVs from removal from serum and aiding in cell adhesion and entry to exert their functions.

Manuscript III. Dagenais, M., Haçariz, O., Xia, J., Long, T. & Geary, T. G. Transcriptomic changes in Peyer's patches associated with *Schistosoma mansoni* infection in mice. *In preparation*.

In this manuscript, we investigated the transcriptomic changes in Peyer's patches 7-weeks post-infection with *S. mansoni*. We also explored the possibility that parasite EV-associated miRNAs may contribute to the regulation of gene expression in this tissue. This study provides a snapshot in time of the intestinal immune tissue transcriptome as affected by infection with the mesenteric helminth parasite *S. mansoni*. It provides valuable information to further understanding of the disease and the potential involvement of miRNAs secreted by the parasite in modulating host responses.

Appendix, Manuscript IV. Samoil, V., Dagenais, M., Ganapathy, V., Aldridge, J., Glebov, A., Jardim, A., Ribeiro. P, 2018. Vesicle-based secretion in schistosomes: Analysis of protein and microRNA (miRNA) content of exosome-like vesicles derived from *Schistosoma mansoni*. *Scientific Reports 8, 3286*

In addition to the work presented in the body of this thesis, we optimised an isolation protocol for adult *S. mansoni* EVs and characterised the protein and miRNA content of those EVs. Moreover, we confirmed the presence of parasite-derived miRNAs in EVs isolated from infected mouse sera, suggesting that schistosomes secrete EVs *in vivo*. This study provided evidence of EV secretion by these parasites as well as valuable information about their cargo.

Author Contribution

The design, execution and analysis of the experiments presented in this thesis were carried out by the author under the supervision of Drs. Timothy G. Geary and Thavy Long.

In the first manuscript, the author collected the extracellular vesicles and the parasites used throughout the manuscript. Dr. Jared Q. Gerlach contributed to the design and analysis of the lectin microarrays and the author conducted the free-sialic acid detection assays. The lectin histochemistry experiments were conducted by the author with the technical assistance of Drs. Angela Mousley and Louise E. Atkinson at the laboratory of Dr. Mousley (Queen's University-Belfast). Dr. George R. Wendt contributed to the fluorescence *in situ* hybridization experiments which were conducted with the author at the laboratory of Dr. James J. Collins (University of Texas Southwestern Medical Center).

The author conducted all the experimental work described in the second manuscript. Drs. Jared Q. Gerlach, Thavy Long, and Timothy G. Geary contributed to editing the manuscript, which was written by the author.

In the third manuscript, Drs. Jianguo Xia, Thavy Long, and Timothy Geary contributed to the design of the experiment. The author carried out the experimental work and the data analysis. Dr. Orçun Haçariz performed the raw read count for gene-expression.

The study presented in the Appendix was conducted under the supervision of Dr. Paula Ribeiro. The author contributed to the purification of extracellular vesicles, data analysis, and writing of the manuscript.

This thesis was written by the author with editorial contributions from Drs. Thavy Long and Timothy G. Geary.

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List of Abbreviations

3’-UTR: 3' untranslated regions

5’-UTR: 5' untranslated regions

AA: Arachidonic acid

AAM: Alternatively activated macrophage (also M2)

AbD: Antibody diluent

ACN: Acetonitrile

AdjP: Adjusted P value

ADP: Adenosine diphosphate

AGC: Automatic Gain Control

ALA: Alpha-linoleic acid

Arg1: Arginase-1

ATP: Adenoside triphosphate

ATPDase-1: ATP-diphosphohydrolase 1

BCA: bicinchoninic acid

BSA: Bovine serum albumin

***Bt*:** *Bos taurus*

Ca^{2+}: Calcium ion

CAA: Caragana arborescens Lectin

$CaCl_2$: Calcium chloride

Calsepa: Calystegia sepium lectin

CAM: Classically activate macrophage (also M1)

cAMP: Cyclic adenosine monophosphate

CCA: Circulating cathodic antigen

CCL: Chemokine (C-C motif) ligand

CD: Cluster of differentiation

CHU: Centre hospitalier universitaire

CID: Collision-induced dissociation

CLP: Chitinase-like protein

CLR: C-type lectin receptor

CO_2: Carbon dioxide

Ct: Cycle threshold

CXCL: Chemokine (C-X-C motif) ligand

CYP450: Cytochrome P450

DAPI: 4′,6-diamidino-2-phenylindole

DC-SIGN: Dendritic cell-specific ICAM-3-grabbing nonintegrin

DDAH: N.N-dimethylarginine dimethylaminohydrolase

DEG: Differentially expressed gene

DNA: Deoxyribonucleic acid

- **cDNA:** Complementary deoxyribonucleic acid
- **EV-DNA:** EV-enclosed DNA
- **mtDNA:** Mitochondrial DNA

dNTP: Deoxynucleoside triphosphate

DSA: Datura stramonium Lectin

DTT: Dithiothreitol

ECL: Enhanced chemiluminescence

eEF1: Eukaryotic elongation factor 1 complex

ELV: Exosome-like vesicle

EM: Electron microscopy

- **TEM:** Transmission electron microscopy

ER: Endoplasmic reticulum

ES: Excretory/secretory

ESCRT: Endosomal sorting complex required for transport

ESI-LC-MS/MS: Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric

ESP: Excreted/secreted product

EV: Extracellular vesicle

15k EV: EV sedimenting at 15k × g

120k EV: EV sedimenting at 120k × g

sEV: Small EV

EXOmotif: sEV-export sequence

CELLmotif: Cellular retention sequence

FA: formic acid

FBS: Fetal bovine serum

FcεRI: high-affinity IgE receptor

FDR: False discovery rate

***Fh*EV:** *Fasciola hepatica* EV

FISH: Fluorescence *in situ* hybridization

FITC: Fluorescein isothiocyanate

FoxO: Forkhead box proteins class O

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GlcNAc: N-acetyl-glucosamine

GNA: Galanthus nivalis agglutinin

GnRH: Gonadotropin-releasing hormone

GO: Gene ontology

GPI: Glycosylphosphatidylinositol

GSL: Glycophospholipid

GST: glutathione- S-transferase

H_2O_2: Hydrogen peroxide

HCD: Higher energy Collision-induced Dissociation

HGRPT: hypoxanthine-guanine phosphoribosyltransferase

HHA: Hippeastrum hybrid lectin

HIV: Human immunodeficiency virus

HRP: Horseradish peroxidase

HSP: Heat shock protein

IC: Immune complex

Ig: Immunoglobulin

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IGF: Insulin-like growth factor

IL: Interleukin

ILC2: Group 2 innate lymphoid cell

ILV: Intraluminal vesicle

INF: Interferon

iNOS: Inducible nitric oxide synthase

IP: Intra-peritoneal

ITIM: Immunoreceptor tyrosine-based inhibitory motif

KEGG: Kyoto Encyclopedia of Genes and Genomes

LA: Linoleic acid

LAP: leucine aminopeptidase

LC: Liquid chromatography

LEL: Lycopersicon esculentum lectin

LogFC: Log_2 fold-change

LOX: Lipoxygenase

LPS: Lipopolysaccharide

M1: Classically activate macrophage (also CAM)

M2: Alternatively activated macrophages (also AAM)

MAPK: Mitogen-activated protein kinases

MC: Mast cell

MDA: Mass drug administration

$MgCl_2$: Magnesium chloride

MGF: Mascot generic format

MHC: Major histocompatibility complex

***Mm*:** *Mus musculus*

MS: Mass spectrometry

MSMS: Tandem mass spectrometry

mTOR: Mechanistic target of rapamycin

MVE: Multivesicular body

MW: Molecular weight

MWCO: Molecular weight cutoff

NaCl: Sodium Chloride

NaN_3: Sodium azide

nanoLC/MSMS: Nanoscale liquid chromatography coupled to tandem mass spectrometry

NCBI: National Center for Biotechnology Information

NK: Natural killer

NLR: NOD-like receptor

NOD: Nucleotide-binding oligomerization domain

NPA: Narcissus pseudonarcissus lectin

NTD: Neglected tropical disease

ORA: Overrepresentation analysis

P: Probability value

PAMP: Pathogen-associated molecular pattern

PBS: phosphate buffered saline

DPBS: Dulbecco's phosphate buffered saline

PBST: PBS containing Tween 20

PBSTx: PBS containing triton-X100s

PC: Principal component

PCA: Principal component analysis

PCR: Polymerase chain reaction

qPCR: Quantitative polymerase chain reaction

qRT-PCR: Quantitative real-time polymerase chain reaction

PFA: Paraformaldehyde

PGE_2: Prostaglandin E-2

PHA-E: Phaseolus Vulgaris Erythroagglutinin

P.I.: Post-infection

PI3K-Akt: phosphatidylinositol 3-kinase-protein kinase B

PLA2: Phospholipase A2

PMT: Photomultiplier tube

POC-CCA: Point-of-care circulating cathodic antigen

PPM: Parts per million

PRR: Pattern recognition receptor

PS: Phosphatidylserine

PVDF: polyvinylidene difluoride

PZQ: Praziquantel

RAS: Reticular activating system
RCA-I: Ricinus communis agglutinin
RELM-α: Resistin-like alpha (also FIZZ or Retnla)
RFU: Relative fluorescence units
RISC: RNA-induced silencing complex
RLR: RIG-I-like receptor
RNA: Ribonucleic acid
lncRNA: Long non-coding RNA
miRNA: Micro RNA
mRNA: Messenger RNA
piRNA: Piwi-interacting RNA
rRNA: Ribosomal RNA
snRNA: Small non-coding RNA
tRNA: Transfer RNA
vtRNA: Vault RNA
RNAseq: RNA sequencing
RPMI: Roswell Park Memorial Institute medium
RT: Reverse transcription
SD: Standard deviation
SDS-PAGE: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
Siglec: sialic acid-binding immunoglobulin-like lectin
***Sm*:** *Schistosoma mansoni*
***Sm*EV:** *Schistosoma mansoni* EV
SmSP2 : Type 2 serine protease
SNA-I: Sambucus nigra lectin-I
STAT: Signal transducer and activator of transcription
SWAP: Schistosome soluble worm antigen preparation
TAMRA: Tetramethylrhodamine
TBS: Tris-buffered saline
TBST: Tris-buffered saline with Tween 20
TCEP: Tris (2-carboxyethyl)phosphine
TFA: Trifluoroacetic acid
TGF: Transforming growth factor
Th1: T helper 1
Th2: T helper 2
Th17: T helper 17
TLR: Toll-like receptor
TNF: Tumour necrosis factor
Treg: Regulatory T cell
Tris-HCl: Tris(hydroxymethyl)aminomethane hydrochloride
TRITC: Tetramethylrhodamine
TSP-2: Thrombospondin
UA: Uranyl acetate
VEGF: Vascular endothelial growth factor
WB: Western blot
WGA: Wheat germ agglutinin
WHO: World Health Organisation
YLDs: Years lived with disabilities

Introduction

Parasitic platyhelminths of the genus *Schistosoma* are wide-spread and clinically relevant to human health. Infection with *Schistosoma* spp causes schistosomiasis, a chronic disease affecting ~240 million people worldwide and resulting in significant morbidity and mortality [1]. The infective larval stage (cercaria) is released from freshwater snails. Cercariae infect human hosts by penetrating the skin and morphing into maturing larvae (schistosomulae), which enter the host's circulation and migrate to the hepatic portal system and mesenteries (*S. mansoni*, *S. japonicum*) or the venous plexus of the urinary bladder (*S. haematobium*). Larvae mature throughout this journey to eventually become adult male and female worms, which form pairs and produce large numbers of eggs [2]. Some eggs are released into the environment through faeces or urine, but many remain trapped in the liver and surrounding tissues, thereby causing tissue fibrosis and organ damage. Schistosomes have the ability to establish long-term chronic infections [2]. If left untreated, adult worms can survive within a mammalian host for many years [3]. Their outstanding longevity is attributable, in part, to their remarkable capacity to manipulate host immunity [4,5]. Treatment of schistosomiasis relies heavily on the efficacy of a single drug, praziquantel, which is administered to tens of millions of people each year, raising concerns of drug resistance [6]. It is thus critical to pursue the search for alternative schistosomicidal chemotherapies and develop new treatment options for the control of schistosomiasis.

In this context, there is a growing interest in better characterising and understanding the carefully orchestrated molecular dialogue employed by the parasites to modulate the host immune system, as it could lead to the identification of novel therapeutic strategies. Parasite excreted/secreted products (ESPs) are widely regarded as important players in the interactions between the parasite and the host, with increasing attention being paid to parasite-derived extracellular vesicles (EVs) in this regard. EVs encompass all types of secreted membrane-bound vesicles, of which exosomes (originating within multivesicular bodies and release upon fusion with the plasma membrane) and microvesicles (plasma membrane-derived) have received the most attention [7]. There is increasing evidence of vesicle-based secretion in parasitic infections, with

roles in both parasite-parasite inter-communication [8] and parasite-host interactions [9-11]. EVs are involved in cell-cell communication, transferring molecules from one cell to another via membrane vesicle trafficking, thus explaining the broad array of functional activities attributable to them.

Despite multiple reports of EV secretion in schistosomes, the origin(s) of EV secretion remains elusive. To date, the cargo of schistosome EVs has been partially characterised in various life stages [12-16], and has unveiled an enrichment of putative effectors such as proteins and microRNAs (miRNAs). The mechanisms by which host cells interact with parasite-derived EVs are not well characterised, and thus little is known about the functions of EVs during the course of infection. A better portrait of EV surface molecules could help unveil means by which host recipient cells interact with and take up schistosome EVs. There is growing interest for the carbohydrate composition of EVs, with evidence suggesting glycan-dependent EV uptake mechanisms [17-21]. Despite these findings, there is a flagrant lack of knowledge on carbohydrate moieties present on the surface of *S. mansoni*-derived EVs.

In contrast, EV-associated miRNAs have received considerable attention. miRNAs are a class of small non-coding RNAs which act as regulators of gene expression. Specifically, miRNAs bind target messenger RNA (mRNA) by complementary base-pairing, typically inducing mRNA degradation. Recent studies indicate that helminth-derived miRNAs can regulate gene expression in mammalian cells, suggesting that parasites may exploit this mechanism by secreting miRNA to shape the host into an environment favourable for their own development. Supporting this idea, we and others have previously detected schistosomal miRNAs in infected hosts [14,22], with Meningher *et al*. (2020) reporting the presence of the schistosomal miRNAs Bantam and miR-10 in gastrointestinal lymph nodes (Peyer's and mesenteric lymph nodes) of *S. mansoni*-infected mice [22]. However, the question of whether parasite-derived miRNAs actively regulate host gene expression *in vivo* remains unresolved.

Central hypothesis

This thesis is centered around *S. mansoni*-derived EVs, their content, their origins of secretion, and their functions. The central hypothesis of this project is that adult *S. mansoni* secrete

EVs which are involved in the interaction between the parasite and the host and the regulation of host gene expression, thereby contributing to the development of a permissive environment for parasite propagation.

Objectives

The aim of this thesis was 1) to explore the content of EVs secreted by adult *S. mansoni* parasites, 2) to investigate the origins of EV secretion in adult worms, and 3) to investigate EV functions *in vivo* as well as host response to infection with *S. mansoni*.

Chapter II (Manuscript I)

- Profile the major glycan motifs present on the surface of adult *S. mansoni*-derived EVs using lectin microarrays;
- Identify potential sources of EV secretion in adult *S. mansoni*.

Chapter III (Manuscript II)

- Determine the origin of the sialylated molecules identified in Chapter II;
- Investigate the immunogenic potential of EVs.

Chapter IV (Manuscript III)

- Carry out target searches of previously identified EV-associated *S. mansoni* miRNAs against mouse mRNAs;
- Investigate transcriptional changes in intestinal lymphoid tissue Peyer's resulting from infection with *S. mansoni;*
- Analyse and compare predicted targets and differentially expressed genes.

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Chapter II. Literature Review

1. Schistosomiasis

1.1 Epidemiology and distribution

Schistosomiasis, or bilharziasis, is a parasitic disease caused by blood-dwelling trematodes of the genus *Schistosoma*. There are 2 major forms of schistosomiasis: intestinal – which, in humans, mainly develops from infection with *S. mansoni* or *S. japonicum,* and less frequently with *S. mekongi, S. intercalatum*, or *S. guineensis* – and urogenital, which is caused by infection with *S. haematobium.* Additionally, there have been several reports of hybridisation occurring within and between human and animal schistosome species in West African countries, and more recently in Corsica [1-4]. Transmission has been reported in at least 78 countries [5]. According to estimates from 2017, schistosome infections were responsible for over 1,000 years lived with disabilities (YLDs) that year alone, with more than 140 million people infected worldwide [6]. This prevalence, however, is based on insensitive egg-detection methods, which under-represent active infection due to frequent false negative diagnosis [7-9]. The great majority of cases (> 90%) occur in Africa, most of which are found in the sub-Saharan area, but schistosomiasis transmission also occurs in South America, the Caribbean, the Middle East, Europe (Corsica), and Southeast Asia (Figure 1.1) [5,10-13]. Schistosomiasis is a disease intrinsically linked to poverty, predominantly transmitted in poor rural areas without access to safe drinking water and adequate sanitation system. In such settings, infection is highly prevalent in school-aged children and people with frequent contact with infested water, and can lead to growth stunting, anemia, cognitive impairment and decreased physical fitness [11,14]. For this reason, the World Health Organisation (WHO) prioritized schistosomiasis as one of the 20 neglected tropical diseases (NTDs). Control strategies employed to reduce or preclude transmission include vector control, chemotherapy, health education, and improved sanitation [5].

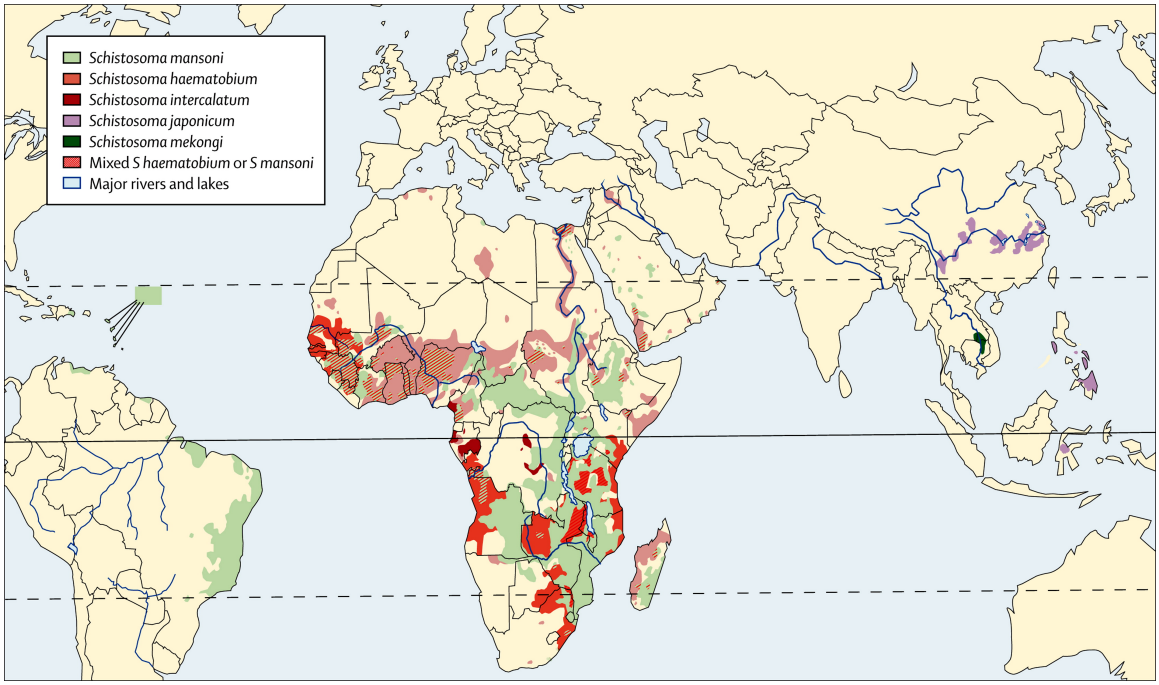

Figure 1.1: Global distribution of countries where human schistosomiasis is transmitted. Main foci: *S. mansoni* — much of sub-Saharan Africa, northeast Brazil, Surinam, Venezuela, the Caribbean, lower and middle Egypt, the Arabic peninsula; *S. haematobium* — much of sub-Saharan Africa, Nile valley in Egypt and Sudan, the Maghreb, the Arabian peninsula; *S. japonicum* — along the central lakes and River Yangtze in China; Mindanao, Leyte, and some other islands in the Philippines; and small pockets in Indonesia; *S. mekongi* — central Mekong Basin in Laos and Cambodia; *S. intercalatum* — pockets in west and central Africa.
Source: Modified from Gryseels et al (2006) [10] & Colley *et al*., 2014 [11]

1.2 Life cycle

Schistosoma species develop through a similar life cycle, requiring a freshwater snail intermediate host and a mammalian definitive host (figure 1.2, from [12]). The infectious larval stage of the parasite, the cercariae, emerge from the freshwater snail intermediate host and utilise environmental cues including motion, light-dark contrast, and chemical and thermal gradients to find a suitable mammalian definitive host [10,15,16]. Upon encounter with the definitive host, cercariae penetrate the skin by secreting acetabular gland mucus-like substances and proteases, shed their tails within 3-4 hours to become schistosomulae, enter the dermal circulation within the first two days, and migrate through the lungs and to the liver via systemic circulation. Young worms mature in the portal veins for 4-6 weeks before male-female pairs migrate to the mesenteric venules (*S. mansoni, S. japonicum*) or venules that drain the bladder (*S. haematobium*) [10], where

female worms produce hundreds of eggs per day. The average lifespan of adult worm is 3-5 years, but they can live as long as 30 years [10]. Each egg contains a miracidium larva that secretes proteolytic enzymes to assist its migration into the lumen of the intestine for excretion within host feces (*S. mansoni* and *S. japonicum*) or into the bladder for excretion with urine (*S. haematobium*); however, some eggs become entrapped within host tissues and often lead to significant pathology [10,17]. Eggs that are excreted into the environment remain viable for up to 7 days, and the miracidium hatches after the egg is in contact with water. The miracidium is then directed by light and chemical stimuli to the intermediate host, a freshwater snail of the *Biomphalaria* (*S. mansoni*), *Oncomelania* (*S. japonicum*) or *Bulinus* (*S. haematobium*) genera [10]. After penetrating the snail, the miracidia differentiate into multicellular mother sporocysts containing germ cells that divide asexually to produce many daughter sporocysts. The daughter sporocysts then migrate to the hepatopancreas, where their germ cells divide via asexual reproduction to produce cercariae [10]. Four to six weeks after infection, cercariae emerge from the snail and the life cycle repeats (Figure 1.2) [10,15,16].

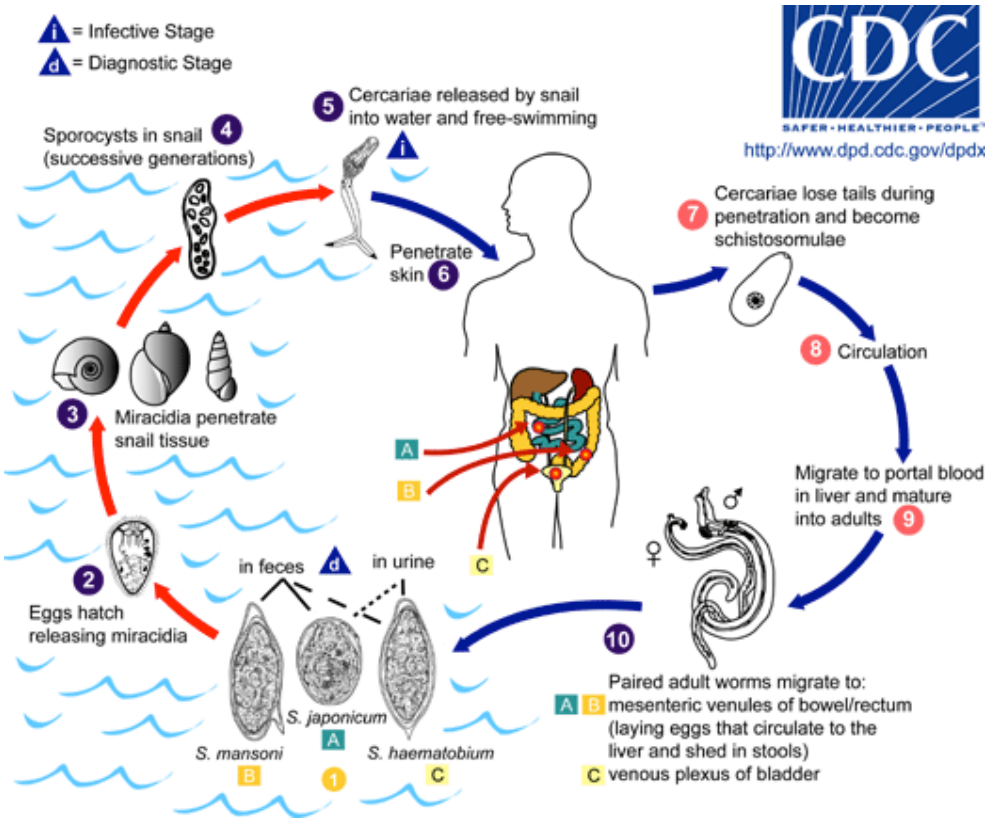

Figure 1.2: Life cycle of *Schistosoma* species. *Schistosoma* eggs are eliminated with feces or urine, depending on species 1. Under appropriate conditions the eggs hatch and release miracidia 2 which swim and penetrate specific snail intermediate hosts 3. The stages in the snail include two generations of sporocysts 4 and the production of cercariae 5. Upon release from the snail, the infective cercariae swim, penetrate the skin of the human host 6, and shed their forked tails, becoming schistosomulae 7. The schistosomulae migrate via venous circulation to lungs, then to the heart, and then develop in the liver, exiting the liver via the portal vein system when mature 8 9. Male and female adult worms copulate and reside in the mesenteric venules, the location of which varies by species (with some exceptions) 10. For instance, *S. japonicum* is more frequently found in the superior mesenteric veins draining the small intestine A, and *S. mansoni* occurs more often in the inferior mesenteric veins draining the large intestine B. However, both species can occupy either location, and are capable of moving between sites. *S. intercalatum* and *S. guineensis* also inhabit the inferior mesenteric plexus but lower in the bowel than *S. mansoni*. *S. haematobium* most often inhabits in the vesicular and pelvic venous plexus of the bladder C, but it can also be found in the rectal venules. The females (size ranges from 7–28 mm, depending on species) deposit eggs in the small venules of the portal and perivesical systems. The eggs are moved progressively toward the lumen of the intestine (*S. mansoni*, *S. japonicum*, *S. mekongi*, *S. intercalatum*/*guineensis*) and of the bladder and ureters (*S. haematobium*), and are eliminated with feces or urine, respectively 1.
Source: Center for Disease Control and Prevention. Schistosomiasis life cycle. Available at https://www.cdc.gov/parasites/schistosomiasis/biology.html Accessed on July 22, 2022. [12]

1.3 Schistosome morphology

Contrarily to most other trematodes, schistosomes are diecious organisms, with adult males and females exhibiting major morphologic differences (Figure 1.3). Mature males are short and stout and possess a gynecophoric canal in which the long and slender female resides. This pairing is essential for effective mating and is crucial for the stimulation of female growth and sexual maturation.

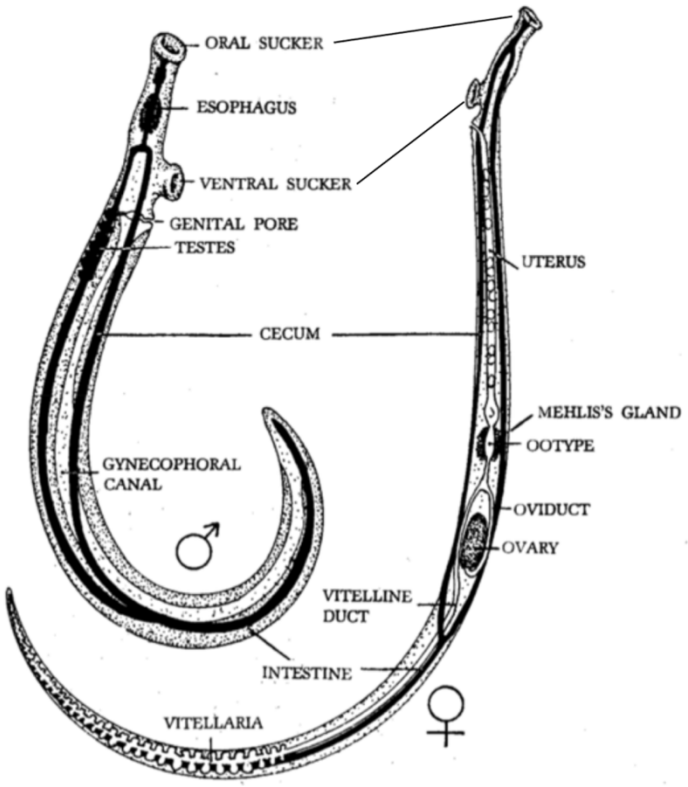

Figure 1.3: Overview of *S. mansoni* adult male and female anatomy.

Schistosomes are covered by a syncytial outer layer, called the tegument, which consists of a complex epithelial structure comprising multiple nucleated cell bodies (tegumental cells, also known as cytons) fused to a continuous cytoplasmic matrix (Figure 1.4, from [18]) [18]. Tegumental cell bodies are located beneath the body wall of the worm and are connected to the tegument syncytial cytoplasm by cytoplasmic projections. The tegument surface consists of a double outer bilayer which is overlayed by a secreted bilayer, the membranocalyx. The tegument is a major site of molecular interactions between the parasite and the host and it is believed to participate in several important functions such as acquisition of nutrients and other exogenous material [19-22], secretion of endogenous materials [23], osmoregulation [24].

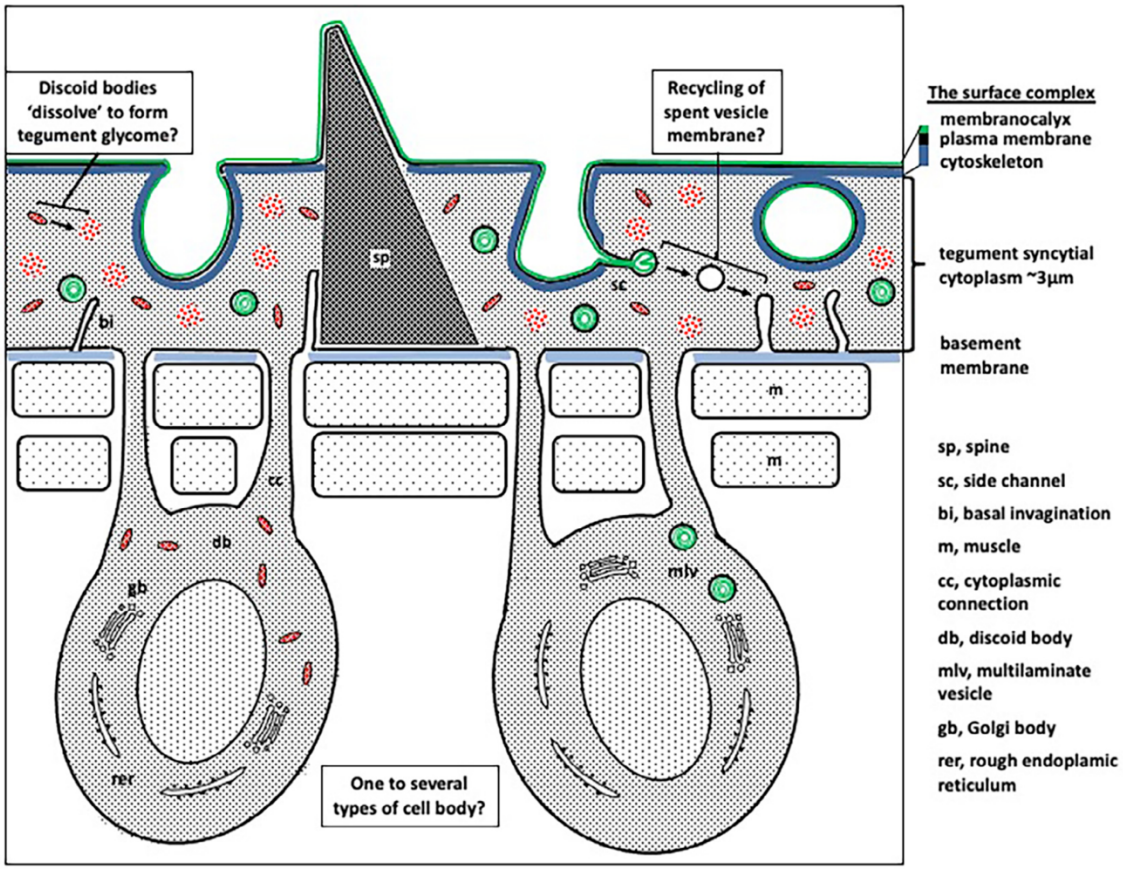

Figure 1.4: Schematic diagram of the *S. mansoni* male ventral tegument with underlying single layers of circular and longitudinal muscle fibres, and associated tegument cell bodies. The spaces between cell bodies are filled with interdigitated parenchyma cells. The layout in the female is similar, but with parenchyma largely replaced by vitellaria in the posterior third of the body. The male dorsal tegument is underlain by a single layer of circular fibres and three to four layers of longitudinal fibres making the corresponding cytoplasmic connections between cell bodies and tegument syncytium long and more tortuous. The male dorsal tegument is also lifted off the muscle layer at intervals by extensions of parenchymal cells to form tubercles, through which ciliated and non-ciliated sensory endings project. The two types of Golgi apparatus are speculatively shown in separate cell bodies but one to several cell types may exist.
Source: Wilson and Jones (2021) [18]

Blood flukes are equipped with two suckers, ventral (located on the ventral face of the mid-posterior end of the head) and oral (surrounds the opening of the mouth at the anterior end of the head region), which serve many functions, such as attachment to the host and feeding [25]. The alimentary tract of schistosomes is made up off the mouth, a short esophagus, and the absorptive gut, which consists of two intestinal caeca joining in the anterior end of the body and extending to the farthest extremity of the body to form a blind gut [26]. Blood cells ingested by the parasite are

lysed by digestive enzymes during their passage through the esophagus before entering the digestive gut, lined with gastrodermal cells. The blind-ended gut means that schistosomes regurgitate residual products of digestion through the mouth [26].

Platyhelminths also possess a protonephridial system, which is involved in excretion of metabolic wastes, xenobiotics, and immunomodulatory proteins, and performs osmoregulation [27,28]. The schistosome protonephridium consists of heavily ciliated cells called flame cells, branching and collecting tubules, and excretory tubules which connects to the outside by extending to the nephridiopore [27,28]. In *S. mansoni*, the nephridiopore is found at the posterior tip of the worm [27]. Flame cells are specialized excretory cells which filter interstitial fluid, allowing small molecules into the tubules, but restricting the passage of larger molecules.

1.4 Pathology of schistosomiasis

There are two major clinical forms of schistosomiasis: acute and chronic schistosomiasis. The symptoms associated with acute schistosomiasis, or Katayama fever, develop a few weeks after primary infection due to a systemic hypersensitivity reaction to migrating schistosomulae. Katayama fever due to *S. mansoni* and *S. haematobium* is common among tourists and migrants that visit an endemic region, but is rarely observed in people living in endemic regions, possibly due to *in utero* exposure as a result of maternal infection [29,30]. The characteristic symptoms include fatigue, malaise, fever, myalgia, non-productive cough and eosinophilia; spontaneous recovery is common within 2-10 weeks [10]. However, acute schistosomiasis due to infection with *S. japonicum* does occur in people living in endemic regions with a history of previous infections and can cause serious symptoms with persistent fever, cachexia, and organomegaly [10].

Chronic schistosomiasis, the most common form of the disease, develops as a consequence of the host's inflammatory response to eggs entrapped within host tissues, resulting in intestinal and hepatosplenic (*S. mansoni* and *S. japonicum*) or urogenital (*S. haematobium*) diseases. Many eggs are swept up in blood flow, accumulating in the liver, spleen, or urogenital systems. In addition, ectopic egg deposition may occur in the skin, lungs, cerebrospinal system, skeletal muscles, and adrenal glands. Eggs deposited in the liver or intestine often cause an obstruction of

these tissues. While trapped within host tissues, parasite eggs secrete proteolytic enzymes that stimulate a potent inflammatory response associated with eosinophilia and the formation of granulomas, giving rise to the most serious symptoms of disease [10,31]. Egg antigens induce a $CD4^+$ T helper cell response which orchestrates the formation of granulomatous lesions around tissue-trapped eggs. Granulomas are composed of a mix of cells, including macrophages, dendritic cells, B and T lymphocytes, eosinophils, basophils, and neutrophils, and collagen fibers [32,33]. Chronic infections with *S. mansoni*, *S. japonicum* and *S. mekongi* are associated with hepatic disease resulting from fibrosis within the portal tract of the liver portal, which can lead to obstructive vascular lesions, ascites and portal hypertension, ultimately resulting in oesophageal varices and death [11].

Intestinal schistosomiasis is due to the migration of egg through the intestinal wall, leading to mucosal granulomatous inflammation, microulcerations, and superficial bleeding mainly in the large intestine and rectum. Common manifestations include loss of appetite, abdominal pain and discomfort, and diarrhea [10]

In urinary schistosomiasis, eggs of *S. haematobium* induce granulomatous inflammation, ulceration and calcification of the bladder and ureteral wall, haematuria, dysuria, and proteinuria. Chronic infections with *S. haematobium* are associated with squamous bladder cancer [10].

Moreover, deposition in the reproductive organs of eggs from *S. haematobium* and *S. mansoni* (albeit to a lesser extent) cause genital schistosomiasis. Approximately 30% of women infected with *S. haematobium* develops genital schistosomiasis [34]. Common manifestations include lesions of the vulva, vagina, and cervix, which likely facilitate transmission of sexually transmitted infections, such as the human immunodeficiency virus (HIV) [10]. In addition, lesions to fallopian tubes and ovaries can lead to infertility. In men, lesions to the testes, epididymis, spermatic cord, and prostate commonly result in haemospermia [10].

1.5 Diagnosis

Traditionally, diagnosis of active schistosomiasis relies on the detection of viable eggs in urine (urogenital schistosomiasis), using filtration methods, or feces (intestinal schistosomiasis)

using the Kato-Katz technique [5,10,11]. Identification of the schistosome species is based on the size and morphology of the eggs; *S. mansoni* eggs are ovoid and laterally spined, *S. haematobium* and *S. intercalatum* eggs are ovoid and terminally spined, and *S. japonicum* and *S. mekongi* eggs are small, round, and laterally spined. This microscope-based diagnostic technique lacks sensitivity, especially for patients with a low worm burden [11]. Molecular techniques to detect the presence of schistosome DNA in urine, faecal, or blood samples increase sensitivity, but remain limited by the irregular egg distribution between samples [11]. Though detection of anti-schistosome antibodies is effective at diagnosing schistosomiasis in infected travellers, serological assays can cross-react with other helminth species and cannot be used for people living in endemic regions as they do not distinguish active infection from past exposure [11]. One promising alternative, the point-of-care Circulating Cathodic Antigen (POC-CCA) test, uses a rapid lateral flow strip and is based on the detection of CCA in urine using monoclonal antibodies, providing results within minutes [35,36]. CCA, however, is a genus-specific glycoprotein which does not discriminate between *Schistosoma* species [35].

1.6 Prevention and control

Control and prevention strategies are largely based on large-scale treatment of people at risk; access to clean water, improved water, sanitation, and hygiene (WASH); improved information, education, and communication (IEC); and snail control [37]. Currently, praziquantel (PZQ) is the only drug recommended by the WHO for the treatment of all forms of schistosomiasis and is used both in clinical settings and for public health interventions. Initially, preventative chemotherapy efforts were rather limited, but have scaled up significantly since 2005, when Merck pledged to donate 250 million doses of PZQ annually to enable mass drug administration (MDA) programs [38]. In February 2022, the WHO published updated guidelines on control and elimination of human schistosomiasis. The new guidelines recommend higher frequency of treatments and the expansion of treatment to more age groups and more risk groups [37]. The introduction of PZQ greatly improved treatment of individual uncomplicated infections. However, many endemic countries have no or irregular MDA programs; thus, only a small percentage of the millions of people who need PZQ currently receive the treatment because the distribution of the drug to people at risk can be challenging [37,39]. In addition, a major drawback of PZQ is its lack

of efficacy against juvenile stages of the parasite [40], which renders those who are treated at risk of immediate re-infection.

Nevertheless, an increase in PZQ distribution comes with greater risks of drug resistance. Resistance has not yet been observed in the field, although it has been induced in laboratory settings [41] and there have been reports of reduced PZQ efficacy [42,43]. PZQ being the only approved compound against all schistosome species, it is imperative to develop new treatment options for the control of schistosomiasis.

A *Schistosoma mansoni* Ca^{2+}-permeable transient receptor potential melastatin ion channel ($TRPM_{PZQ}$) was recently identified as a target of PZQ [44,45]. The effects of therapeutic doses of PZQ on schistosomes include massive calcium influx into the parasite, muscle contractions leading to spasmic paralysis, and disruption of the tegument [46]. *In vivo*, the efficacy of PZQ is, at least partly, dependant on the integrity of the host's immune system. One possibility is that alterations of the tegument results in the display of antigenic molecules, exposing them to recognition from host antibodies [47,48].

2. Immunobiology

2.1 Immunology background

Macrophages are found in virtually all tissues and maintain considerable functional and anatomical diversity. They are described as part of the mononuclear phagocytic system (MPS), which includes professional phagocytes and their bone marrow progenitors [49]. Macrophages are a component of the innate immune system and are distributed throughout the body to serve as sentinels for the immune response. These cells are important phagocytes, engulfing foreign material and apoptotic bodies, recycling waste products from dying cells. Thus, in addition to their essential immune functions, macrophages are key players in tissue development and homeostasis [50]. Macrophages can be activated by various stimuli; they express pattern recognition receptors (PRRs) that recognize specific pathogen-associated molecular patterns (PAMPs), which are conserved and necessary for the survival of many pathogens [51]. Several classes of PRRs have been described, notably Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like

receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [52]. PRRs maintain broad specificity due to their ability to detect a variety of molecules that share a similar structural motif [53-56]. Upon recognition of PAMPs through PRR ligation, macrophages may develop a variety of activation states. In general, activated macrophages are characterised into two broad subpopulations: classically activated (CAM, also known as M1) and alternatively activated (AAM, or M2) (Figure 1.5, from [57]) [57,58]. The M1 phenotype develops in response to cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor alpha (TNF-α) in combination with certain toll-like receptor (TLR) agonists (e.g., lipopolysaccharide; LPS), and is characterized by increased expression of inducible nitric oxide synthase (iNOS), as well as production of the inflammatory cytokines IL-1β, IL-6, IL-12, IL-23, TNF- α, and chemokine (C-X-C motif) ligand 9 (CXCL9). M1 macrophages are associated with pro-inflammatory responses and are crucial for protection against infections with many intracellular pathogens, including viruses, bacteria, and protozoan parasites [57-64]. Alternatively, M2 macrophages, which can be further divided as M2a, M2b, M2c, and M2d, can be polarised by various stimulatory molecules [57,65,66].

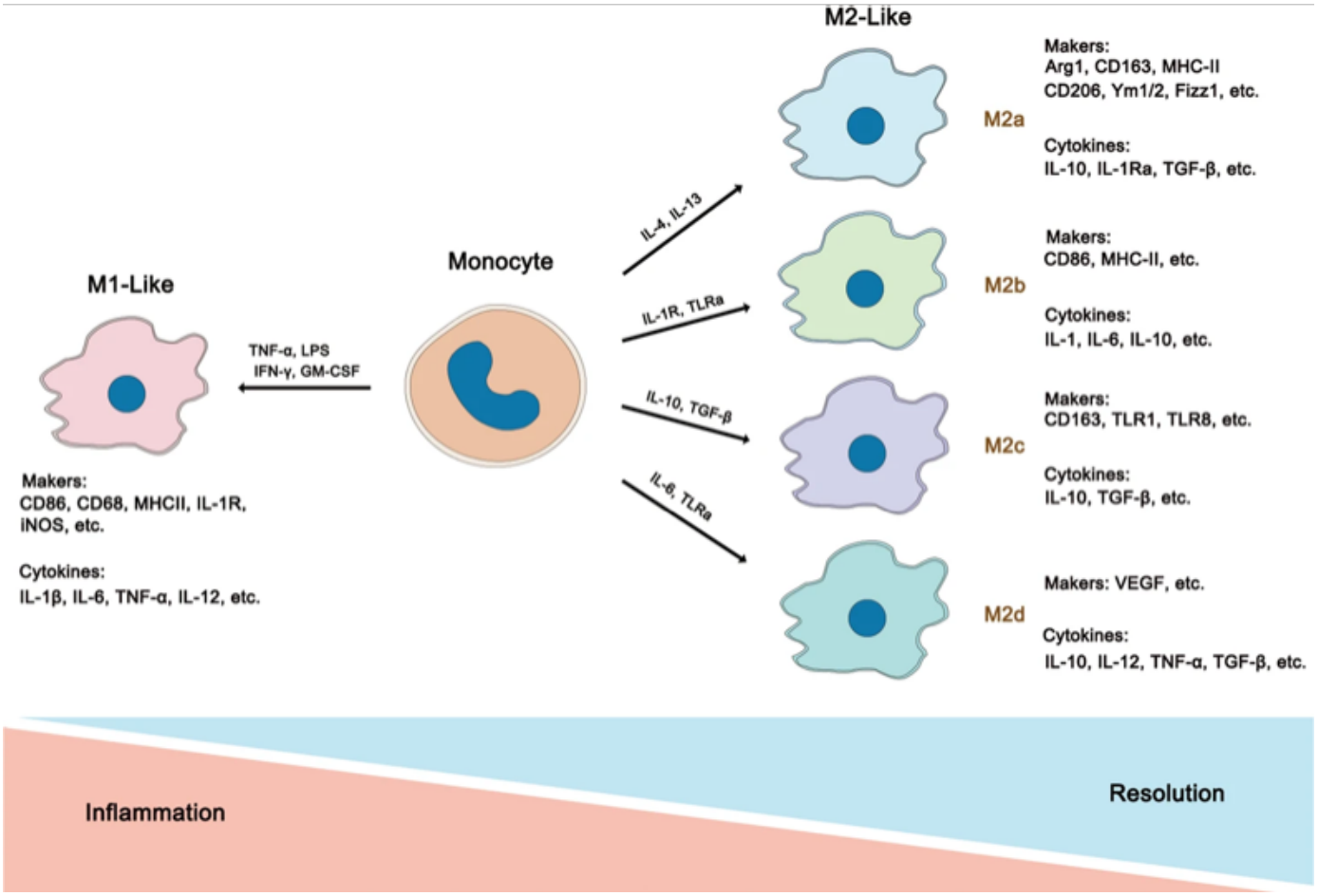

Figure 1.5: Overview of macrophage subsets. Macrophages can be roughly divided into two subtypes: M1-like and M2-like, while M2-like macrophages can be further differentiated into M2a, M2b, M2c, and M2d phenotypes depending on their different microenvironmental stimuli. All these phenotypes express different cytokines, chemokines, and receptors which give rise to their different functions. Generally, M1-like macrophages mainly induce proinflammatory responses and are usually associated with Th1 responses, while M2-like macrophages contribute trophism and tissue tolerance. Furthermore, M2a macrophages mainly mediate tissue repair and remodelling and Th2 responses; M2b macrophages are commonly responsible for immunoregulation; M2c mainly function in phagocytosis, and M2d participate in angiogenesis in tumours.
Source: Wang *et al*., 2020 [57]

M2a macrophages, known for their wound-healing properties, are induced via activation of the activating signal transducer and activator of transcription 6 (STAT6) pathway through IL-4Rα signalling by the cytokines IL-4 and IL-13 and are characterized by increased expression of arginase-1 (Arg1), resistin-like alpha (RELM-α, also known as FIZZ or Retnla), and chitinase-like 3 (Ym1) and 4 (Ym2) and secretion of the potentially regulatory cytokines IL-10 and IL-1 receptor antagonist (IL-1ra), and pro-fibrotic factors such as transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), and RELM-α [57,58,64,66]. The regulatory M2b subtype, which can arise from joint stimulation with immune complexes (IC) and TLR agonists or IL-1R agonists, expresses high levels of CCL1 and TNF superfamily member 14 (TNFSF14) and secretes the pro-inflammatory cytokines IL-1β and TNF-α the pleiotropic cytokine IL-6, which exhibits both pro- and anti-inflammatory properties, as well as large amounts of the anti-inflammatory cytokine IL-10 [57,64]. The M2c phenotype develops via STAT3 upon IL-10 stimulation of IL-10R and displays highly anti-inflammatory and pro-fibrotic properties by in turn releasing high amounts of IL-10 and TGF-β. M2c macrophages also express high levels of Mer receptor tyrosine kinase (MerTK), a surface receptor which recognises apoptotic cells, making them efficient apoptotic body phagocytes [64]. Finally, M2d macrophages, also known as tumour-associated macrophages (TAMs), arise from combined stimulation with TLR ligands and A2 adenosine receptor (A2R) agonists, or by IL-6 [57,63,67,68]. M2d cells secrete high levels of IL-10, TGF-β, and vascular endothelial growth factor (VEGF), which promotes angiogenesis, and low levels of IL-12, TNF-α, and IL-1β, and are known for inflammatory properties in neoplastic tissue and contribution to angiogenesis and tumour metastasis [60,63,64,69,70]. M2 macrophages are commonly enriched during chronic infections, such as infection with parasitic helminths [59,71-73]

Upon encountering their cognate antigen, lymphocytes of the acquired immune system (B and T cells) undergo clonal expansion to produce large numbers of antigen-specific lymphocytes

during infection [74]. Activated macrophages are key contributors to lymphocyte activation, presenting antigen in the context of major histocompatibility molecules (MHC) and providing the co-stimulation necessary for activation of T lymphocytes. More specifically, macrophages present antigen on MHC-II molecules to $CD4^+$ T lymphocytes that define the direction of the immune response through the secretion of cytokines. $CD4^+$ T helper (Th) lymphocytes are classified into phenotypic subsets based on the profile of cytokines they secrete (Figure 1.6, from [75]) [76]. The two most well-studied phenotypes are known as Th1 and Th2. Th1-polarized cells secrete INF-γ, IL-2 and IL-12, whereas Th2 polarized cells produce IL-4 and IL-13 [76]. The polarization of $CD4^+$ Th cells is based on the initial signals received from macrophages during the process of antigen presentation, establishing macrophages as key regulators of the immune response.

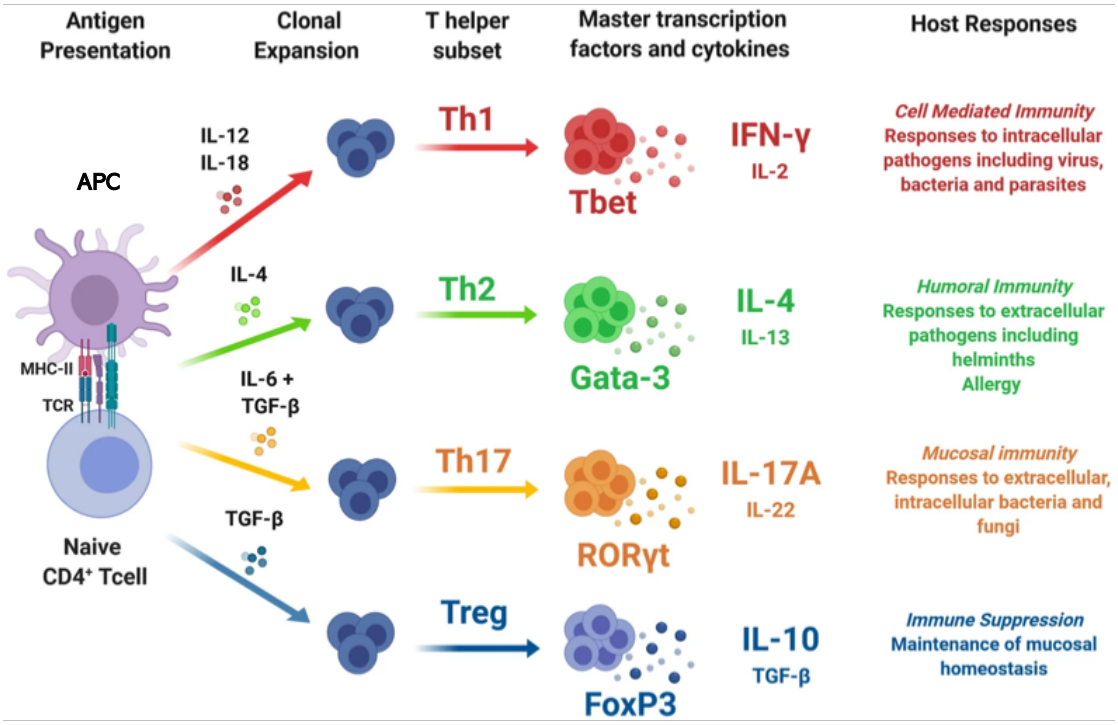

Figure 1.6: Overview of T helper subsets. Upon encountering foreign antigens, dendritic cells (highly specialised antigen presenting cells) process and present fragments of antigen to naïve $CD4^+$ T cells via major histocompatibility complex (MHC) class II molecules. During this process, specific cytokines drive differentiation and clonal expansion of $CD4^+$ T cells into functionally distinct T helper (Th) subsets. Each Th subset is associated with a master transcription factor, and secretes specific cytokines involved in coordinating different types of host immune response.
Source: modified from Corripio-Miyar *et al*., 2022 [75]

2.2 Immunology of *S. mansoni* infection

Infection with *S. mansoni* elicits a diverse host response, beginning after cercariae penetrate the skin. Invading cercariae are first identified by tissue resident macrophages that reside beneath the epithelial barrier. These macrophages secrete chemokines that recruit naïve mononuclear cells and neutrophils to the site of infection [77], and then migrate to the draining lymph nodes to deliver antigen for presentation to naive T helper (Th) cells [78]. Schistosomulae counter this response by inducing development of an immuno-suppressive environment, potentially via the secretion of an anti-inflammatory protein (Sm 16.8). Sm 16.8 suppresses lymphocyte proliferation and stimulates secretion of the anti-inflammatory IL-1ra [79]. Typically, the production of the key inflammatory factors IL-1α and IL-1β is part of the host response to injury and infection. IL-1ra competitively inhibits the binding of both cytokines to the IL-1 receptor, preventing them from stimulating inflammation [80]. Schistosomulae are also believed to stimulate the production of prostaglandin E-2 (PGE_2), which acts as a suppressor of T cell proliferation via an IL-10-dependent mechanism [81].

Parasite migration through the blood triggers a mixed systemic response, with signs of both Th2 [82] and modest Th1 differentiation (Figure 1.7, from [83]) [84]. The Th2 response is accompanied by production of antigen-specific IgE, which bind to Fc receptors on the membrane of circulating basophils. Antigen-driven cross-linkage of receptor-bound IgE causes degranulation of basophils and increased production of IL-4 [82]. At weeks 5-6 after infection, the parasites begin producing eggs that stimulate a dominant Th2 response [33,84], which dampens previously activated worm-specific immune responses [84]. The Th2 response peaks around 8 weeks post-infection (p.i) with a gradual increase in regulatory T cell (Treg) population. However, these dominant Th2 and Treg responses do not kill the parasites. Adult schistosomes typically survive 5-10 years in humans [85], but have been reported to live up to 40 years [86]. Clearly, these parasites have evolved to evade immunological attacks and thus establish chronic infections [14].

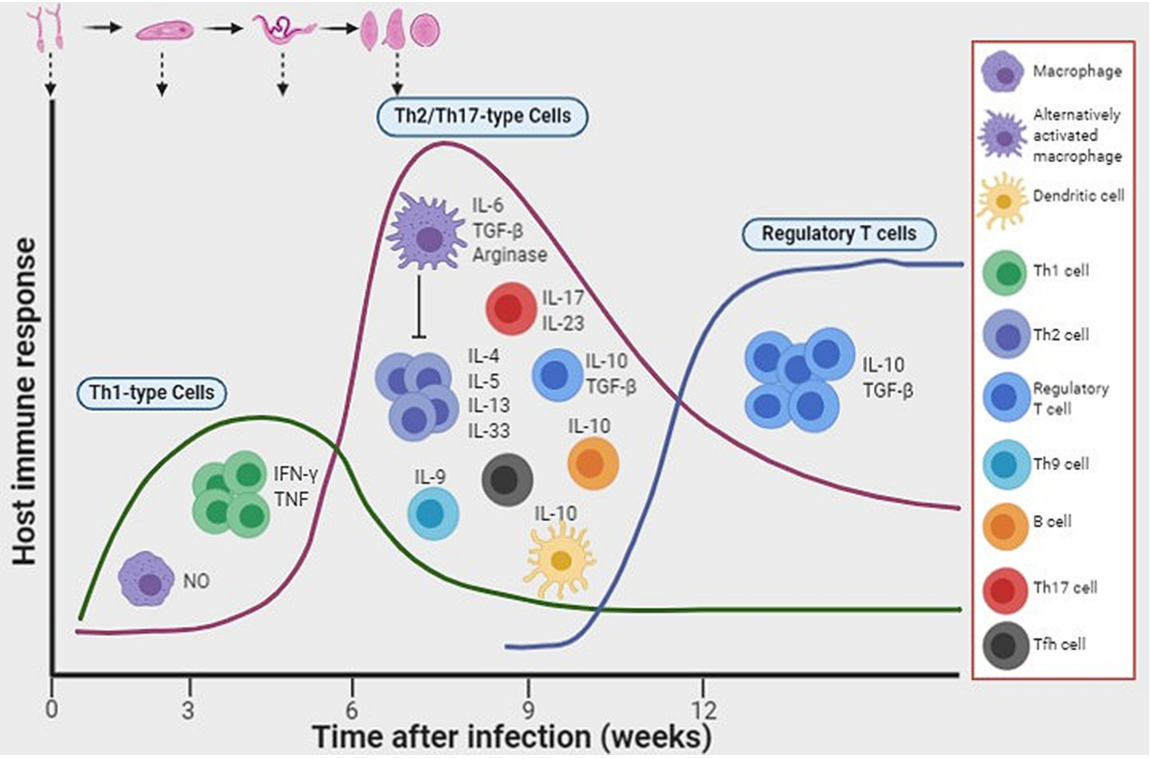

Figure 1.7: Induction of host immune responses after infection with schistosomes. Following infection with schistosomes, the early immune response that develops is a T helper 1 (Th1)-dependent cellular response. As the worms mature into adults and the females start to lay eggs, there is an increased production of interleukin-10 (IL-10) by dendritic cells, and a Th2 response ensues. In addition, B cells produce IL-10 in response to antigens derived from eggs and adult worms. Populations of regulatory T cells and alternatively activated macrophages also develop.
Abbreviations: NO, nitric oxide; IFN-γ, interferon-γ; TNF, tumor necrosis factor; TGF-β, transforming growth factor-β; Tfh, follicular helper T cells.
Source: Molehin 2020 [83]

2.3 Immunomodulation

Schistosomes employ several mechanisms to evade the immune response, which ultimately promotes the survival of both parasite and host [17]. Indeed, pathologies associated with schistosomiasis are due to damage inflicted by the host response to parasite eggs that become trapped in tissues [87]. Serological assays, however, show the presence of both anti-worm and anti-egg antibodies. Although live worms successfully evade the host immune response, the finding that efficacy of PZQ is severely decreased in the absence of a functional immune system suggests that dead or dying worms may be immunostimulatory [87]. Whether the success of live worms results from a failure to activate or directly suppress the host response remains to be determined. Paradoxically, schistosomes' proper development, reproduction [88-91] and excretion of parasitic

eggs [92] require the presence of functional $CD4^+$ T cells.

The first indication that parasite development may be linked to the host immune response was the observation that patients co-infected with *S. mansoni* and human immunodeficiency virus (HIV) shed significantly fewer eggs than those who were seronegative for HIV [92]. In HIV positive patients, the number of eggs shed was directly proportional to the number of circulating $CD4^+$ lymphocytes, suggesting that the presence of $CD4^+$ T cells promotes egg excretion [92]. This idea was supported using immunodeficient mice that lack mature B and T lymphocytes. Schistosome growth and sexual maturation were both impaired in these mice [88], but adoptive transfer of $CD4^+$ T cells restored worm development [93]; however, the contribution of $CD4^+$ T cells may be indirect, because repeated stimulation of the innate response restored parasite development in the absence of $CD4^+$ T cells [89,90]. Repeated stimulation of a PRR was previously shown to induce tolerance in macrophages via decreased transcription of inflammatory cytokines [94]. Similarly, parasite development was restored by either the absence of TNF, a Th1-associated cytokine produced by activated macrophages, or administration of IL4, a cytokine produced by Th2-polarized $CD4^+$ T-cells [90]. Collectively, these observations suggest that a shift in macrophage phenotype (from M1 to M2) is required for optimal schistosome development, and that the secretion of IL4 by $CD4^+$ Th-cells may trigger this phenotypic change.

Chronic infections generally result in the suppression of the innate, the adaptive, or both immune responses [95-99], and schistosomiasis is no exception. Indeed, patients chronically infected with *S. haematobium* [100,101] or *S. mansoni* [102] present blunted T cell responsiveness, not only to parasite antigens, but also after polyclonal stimulation [103]. Polyclonal stimulation of T cells by activators such as anti-CD3/CD28 antibodies is widely employed to stimulate all T cells of a population regardless of their antigen specificity. Interestingly, T cells isolated from a schistosome-infected host that are specific for antigens not expressed by schistosomes become non-responsive prior to the onset of egg production [91]. Ferragine *et al*. reported that loss of T cell responsiveness was attributable to a specific reduction in the ability of $CD11b^+$ monocytes, and not $CD11c^+$ dendritic cells, to stimulate T cells in a contact-dependent manner [91]. $CD11b^+$ is an integrin expressed primarily by monocytes, granulocytes, and macrophages [104]. In this case, however, $CD11b^+$ cells likely are macrophages, due to their antigen-presenting capacity. These findings support the idea that schistosomes exploit the host immune system by altering $CD4^+$ T cell and macrophage function, as both cell types were demonstrated to influence parasite

development during pre-patent infection [91].

The precise mechanisms by which schistosomes alter the host immune response for the establishment of chronic infections remain unknown. Thus far, most studies have focussed on proteins of the parasite excretory/secretory (ES) milieu, as well as antigens from the surface of the worm or egg [78,79,105,106]. Among the scientific community, there is a growing interest in the immunomodulatory potential of extracellular vesicles (EVs), although the potential contribution of helminth-derived EVs in immune evasion remains largely unexplored.

3 Extracellular vesicles

3.1 Background

Long regarded as little more than cellular debris, believed to function as "trash cans" of cells, extracellular vesicles (EVs) are now the subject of intense research. EVs are small vesicles enclosed by a phospholipid bilayer and are released by all cellular organisms, including bacteria and archaea, spanning all three domains of life [107]. Since the early 2000s, several subtypes of EVs have been described, with an impressive heterogeneity of properties and functions [108-110]. There are two main classes of EVs, distinguishable by their route of biogenesis (Figure 1.8, from [111]). Exosomes are formed as intraluminal vesicles (ILVs) by inward budding of multivesicular bodies (MVBs) or amphisomes and are released following fusion of the endosomal membrane with the plasma membrane [108,112,113] in a well-defined mechanism in mammals involving the endosomal sorting complex required for transport (ESCRT) [114]. Whilst ESCRT is central to exosome secretion, ESCRT-independent pathways have also been reported. Such pathways include instances of ceramide microdomain-dependant exosome biogenesis [115] and a pathway requiring members of the tetraspanin protein family (e.g., CD63) [116]. There may also be hybrid mechanisms, in which the various biogenesis systems act in concert [117]. Recent evidence points to the involvement of the endoplasmic reticulum [118] and the nuclear envelope [119], suggesting a variety of potential cellular origins for exosomes. The other type of EVs encompasses vesicles originating from direct outward budding of the plasma membrane, generally known as ectosomes.

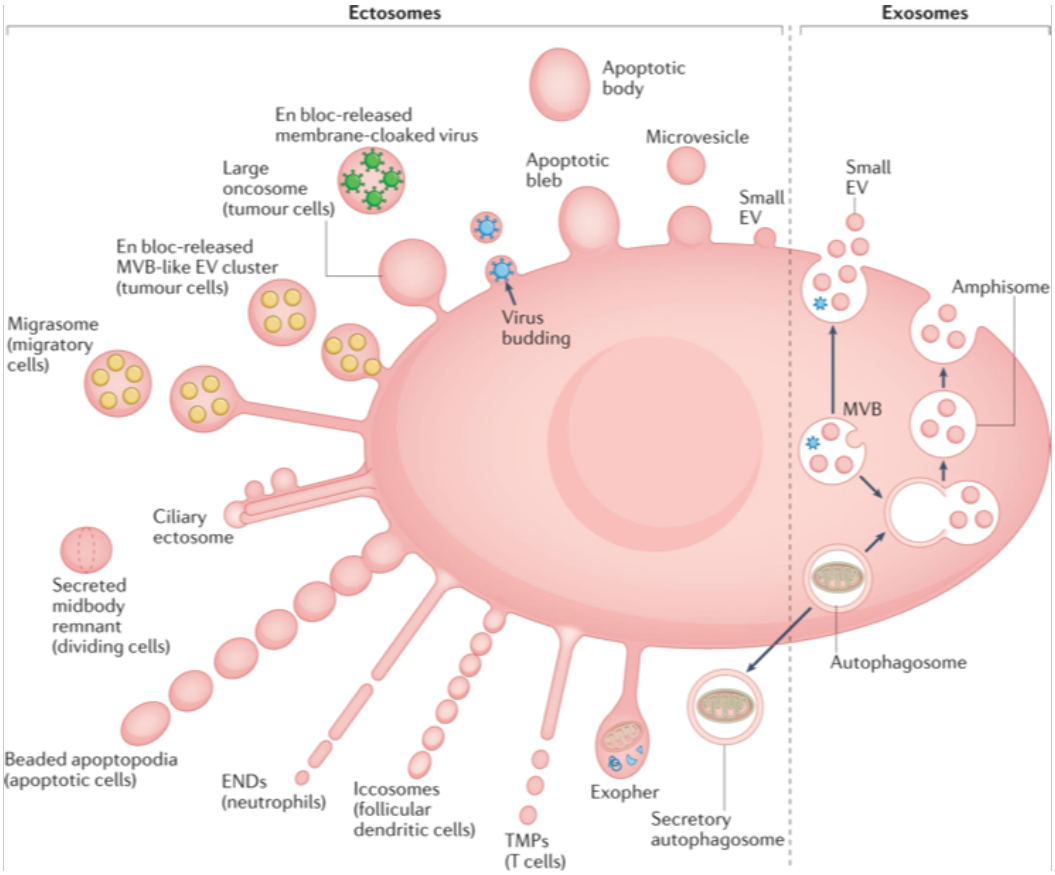

Figure 1.8: Overview of Extracellular vesicle (EV) subtypes. EVs are heterogeneous, phospholipid membrane-enclosed structures. Two main types of EV are distinguished based on their biogenesis, known as exosomes and ectosomes. Exosomes are small EVs of endosomal origin released by the exocytosis of multivesicular bodies (MVBs) and amphisomes. Amphisomes are formed by the fusion of autophagosomes and MVBs. By contrast, ectosomes are generated by plasma membrane budding and blebbing. Of note, some ectosomes may also carry endosomal cargo components. Ectosomes include small-sized EVs (such as small ectosomes and arrestin domain-containing protein 1-mediated microvesicles), medium-sized microvesicles and the larger-sized apoptotic bodies. Viruses can also bud from the plasma membrane or can be released from MVBs. *En bloc*-released virus clusters represent a novel type of large EV similar to the *en bloc*-released MVB-like EV clusters produced by tumour cells. Oncosomes are large EVs produced by tumour cells. Long protrusions of migrating cells give rise to EVs such as migrasomes, which detach from the end of the long retraction fibres of migrating cells. Secreted midbody remnants are released upon completion of cytokinesis by dividing cells. A special type of ectosome, known as ciliary ectosomes, are shed from the plasma membrane of cilia. Beaded apoptopodia release apoptotic vesicles during apoptosis. Neutrophils rolling on the vascular endothelium leave behind elongated neutrophil-derived structures (ENDs), which later round up. Cytoplasts are large remnants of neutrophils undergoing non-lytic NETosis (not shown). Follicular dendritic cells have long filiform processes from which a beading mechanism gives rise to iccosomes. In the immune synapse, T cell microvilli are fragmented by a similar beading process to give rise to EVs known as T cell microvilli particles (TMPs). Exophers are large vesicles hanging at the end of a stalk that contain damaged organelles and protein aggregates. Secretory autophagosomes are also released

by cells. Of note, in the extracellular space, non-EV nanoparticles, such as exomeres, supermeres and T cell-derived supramolecular attack particles, are also present (not shown). These nanoparticles are distinguished from EVs by their smaller size and by the lack of a phospholipid bilayer membrane surrounding them. The biogenesis of non-EV nanoparticles remains to be explored.
Source: Buzás 2022 [111]

Specific molecular markers for the different biogenetic routes are lacking, hindering identification of EVs based on their biogenesis. Instead, operational terms have been proposed to distinguish EVs based on their biochemical and biophysical properties [109]. The notable heterogeneity of EVs is due to the different biogenesis pathways in addition to the extreme variety of their cellular origin, the activation status, and functional state of those cells. Importantly, these also include vesicles released as a result of different cell death mechanisms, such as necroptosis, apoptosis, and pyroptosis (Table 1.1, modified from [111]). EVs are found in bodily fluids, including saliva, blood, ascites, breast milk, cerebrospinal fluid, and urine [120-123], where they contribute to a variety of physiological and pathological processes. Additionally, EVs are found in high concentrations in tissue culture supernatants [124-126]. The classification of EV subtypes remains challenging and inconsistent, due in part to the lack of universal subtype-specific markers and their overlapping size and density, which most isolation procedures are based on [127]. The most common types of EVs are small EVs (of either endosomal or plasma membrane origin) and microvesicles.

Table 1.1: Size-based categories of extracellular vesicles
Source: Modified from Buzás 2022 [111]

Property	Small EVs	Medium EVs	Large EVs
Diameter	~ 50 – 150 nm	~ 200 – 800 nm	≥ 1,000 nm
Biogenesis	Endosomal (exosomes) but some small EVs can be derived from the plasma membrane (ectosomes)	Plasma membrane-derived ectosomes	Plasma membrane-derived ectosomes (some of which may carry endosomal small EVs)
Examples	Exosomes, small ectosomes [128], ciliary ectosomes [129], arresting domain-	Microvesicles, FDC-derived iccosomes, T cell microvilli particles [131], elongated	Apoptotic bodies, large oncosomes [134], beaded apoptopodia [135], migrasomes [136], exhophers [137], *en bloc*-release virus clusters [138],

	containing protein 1-mediated microvesicles [130]	neutrophil-derived structures [132], secreted midbody remnants [133]	*en bloc*-released MVB-like EV clusters [139], secretory autophagosomes [108], cytoplasts [140]

EV, extracellular vesicle; FDC, follicular dendritic cell; MVB, multivesicular body.

3.2 General composition and content of extracellular vesicles

On-line databases, such as ExoCarta (www.exocarta.org) [141] and Vesiclepedia (www.microvesicles.org/) [142], comprising EV-associated proteins, RNAs, and lipids have been made easily accessible.

3.2.1 Proteins composition

Proteomic studies of EVs from a variety of cells, *in vivo* and *in vitro*, and from a variety of organisms, have yielded extensive lists of proteins abundant in different EV subsets. Some proteins commonly found in EV samples are considered to be general EV markers. These include tetraspanins (CD9, CD63, CD81, CD89), 14-3-3 proteins, proteins involved in membrane vesicle trafficking such as the Endosomal Sorting Complex Required for Transport (ESCRT-3) binding proteins Alix and Tsg101, and heat shock proteins [143], as well as the cytoskeletal proteins β-actin, cofilin, myosin and tubulin. In addition, the metabolic proteins glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase 1 (ENO1) are common EV markers [144-151]. Many EVs also carry MHC class I and II proteins, which supports the idea that they may contribute to antigen presentation to naïve lymphocytes [152-155].

Importantly, EVs have been shown to carry a variety of cytokines and chemokines, either as internal cargo [156] or bound to the surface [157]. Unsurprisingly, the selection of soluble mediators present in cytokine-containing EVs is highly dependent upon the cellular origin and activation state of the parent cell. Encapsulation of cytokines into vesicles confers protection from enzymatic degradation and potentially allows for their delivery to distant cells [111].

However, the diversity of isolation methods used highly influences the subtypes and homogeneity of EVs recovered and thus affect the resulting protein profile, limiting comparison between studies. Although protein profiles may be representative of the cellular origin and biogenesis pathway of EVs and thus may be characteristic of different EV subtypes, no single

protein marker is unique to EVs [158].

3.2.2 RNA content

In the extracellular milieu, RNA has been found circulating as free RNA, bound to protein complexes, or encapsulated within EVs. Several types of RNA have been reported to localise inside EVs, including messenger RNA (mRNA), mRNA fragments, long non-coding RNA (lncRNA), and various small non-coding RNAs (snRNAs), such as microRNA (miRNA), piwi-interacting RNA (piRNA), ribosomal RNA (rRNA), and fragments of transfer RNA (tRNA), Y RNA, and vault RNA (vtRNA).

mRNAs found in EVs are typically no longer than 700 nucleotides (nt) [159,160] and can remain functional and be translated once in the target cell [161,162]. EVs are enriched in 3'UTR mRNA fragments, which contains miRNA-binding sites [160]. It has thus been proposed that they may compete with recipient cell RNA for binding of regulatory miRNAs, therefore contributing to RNA stability and translation [160].

miRNAs are commonly described as integral components of EVs. Approximately 22 nt in length, miRNAs function as regulators of gene expression. Specifically, miRNAs are bound by argonaut proteins (Ago), recruited to the RNA-induced silencing complex (RISC), and their seed sequence (roughly nucleotides 2-7 from the miRNA 5′-end) base-pairs with complementary sequences of mRNA, inducing mRNA degradation or translation repression by interfering with ribosomal binding [163]. Typically, base-pairing occurs in the 3'UTR region of mRNAs; however, interaction with other regions such as coding sequences, 5'UTR, and gene promoters, has also been observed [163,164]. The presence of RISC complex proteins in EVs has been inconsistently reported by different groups [165-167], which raises many questions on the functions and requirements of EV-associated miRNAs.

The RNA cargo of EVs does not mirror parental cell RNA population; instead, specific RNA populations are enriched in EVs. This selection is not merely based on size exclusion, as specific subsets of miRNAs are commonly found to be preferentially included in EVs [168,169], suggesting that miRNAs are actively sorted for inclusion as EV cargo [158]. In this context, the recent discovery of small EV (sEV)-export sequences (EXOmotifs) and cellular-retention sequences (CELLmotifs) represents a major breakthrough in elucidating the underlying sorting mechanisms, though the complete process remains elusive [170]. EXOmotifs are 4-7 nt in length with high G+C content and their presence strongly correlates with miRNA enrichment in sEVs

compared to cellular content. In contrast, cellular retention of miRNAs shows strong association with the presence of G+C low CELLmotifs. These localisation sequences appear to be cell-specific and to be preferentially found in the 3' half of the miRNA, away from the regulatory seed sequence [170].

3.2.3 DNA

Despite early reports of EV-enclosed DNA (EV-DNA) [171], comparatively little attention has been given to this topic. Nevertheless, single-stranded and double-stranded DNA have been detected in EVs derived from various cell types [172-175] and DNA transfer has been demonstrated, with EV-DNA localised in the cytosol and the nuclei of the target cell [173], with reports suggesting it can integrate into the genome of recipient cells [176]. Also of interest, Guescini *et al*., reported that EVs derived from astrocytes, glioblastoma cells and myoblasts contain mitochondrial DNA (mtDNA) [177,178]. It is difficult to estimate what proportion of EVs contain DNA, and the significance of EV-DNA cargo is still unclear, but roles have been proposed in cellular homeostasis [179-181] and regulation of inflammatory responses [182-184].

3.2.4 Lipids

Similarly, despite being an essential component of EVs, there is limited knowledge about EV lipidomics. Although lipid composition varies depending on sources and EV subtypes, analyses of the lipid composition of EVs have identified certain lipids that are generally enriched among EVs. Notably, EVs are enriched in cholesterol, sphingolipids, ceramide, glycosphingolipids, and phosphatidylserine (PS) compared to their parent cells [115,185,186]. A study has consistently observed a higher content in cholesterol and sphingomyelin in exosomes from various cell lines than in microvesicles and apoptotic bodies originating from the same cells [187], which is consistent with reports of exosomes being less sensitive to detergents than other EV subtypes [188]. Cholesterol and long saturated fatty acids provide structural rigidity to lipid membranes by increasing intermolecular interactions between neighbouring phospholipid tails, resulting in a tightly packed lipid arrangement. This also provides resistance to physicochemical changes. The distinct lipid composition of EVs confers different characteristics and contribute to their stability in different extracellular environments [158]. As with other EV components, lipids are not arbitrarily included, rather, they are selected for inclusion [158,189].

3.2.5 Glycans

An important yet until recently completely overlooked component of EVs are carbohydrates (glycans). Glycans are carbohydrate-based polymers which can conjugate proteins (glycoproteins) or lipids (glycolipids) or exist as free oligosaccharides or as polysaccharides. Glycans are a vastly diverse group of biological compounds, both in terms of structure and function, and are involved in a plethora of biological functions. Long recognised for their structural and energy storage functions, glycans are increasingly regarded as important players in recognition, adhesion, and signalling [190,191]. It is estimated that 50% of human proteins are glycosylated [192] and many diseases are associated with abnormal glycosylation [193,194], emphasising the importance of glycans in the molecular make-up of cells. Despite that, the field of glycomics emerged long after genomics, transcriptomics, and proteomics due to the wide array of glycan structures, properties, and conjugates, thus preventing the development of a single standardised approach for analysis [195]. In addition, the lack of template and sequence relationship between glycans and their protein conjugates makes it impossible to determine the glycome based on the genome [191,196].

Different types of protein glycosylations have been described. N-linked glycosylation occurs on the nitrogen atom of a carboxyamide of asparagine residues in the consensus sequence Asn-X-Ser/Thr, where X is any amino acid except proline, and is initiated in the endoplasmic reticulum (ER) by the co-translational on-bloc addition of a 14-mer precursor oligosaccharide. This is then trimmed down, and the protein is transferred to the Golgi, trimmed further, forming a high-mannose oligosaccharide, or being remodeled by the sequential addition of various monosaccharides, forming a multitude of possible complex oligosaccharide structures. O-linked glycosylation, however, is characterised by the incremental addition of sugars and occurs in the cis-Golgi compartment following protein folding and N-glycosylation. It can occur on the oxygen atom of the hydroxyl group of any serine or threonine residue, and, to a lesser extent, hydroxyproline and hydroxylysine. Many proteins contain multiple glycosylation sites, which can be occupied by various structures, adding to the incredible complexity of glycobiology.

Conversely, glycolipids represent an entirely separate class of glycoconjugates. In animals, glycosphingolipids (GSLs), characterised by their ceramide backbone, represent the vast majority of glycolipids [197]. Ceramides can be glycosylated with either glucose or galactose while in the ER and then be extended in a sequential manner by glycosyltransferases once it reaches the Golgi.

GSLs can contain carbohydrates chains between one and six monosaccharides, but the addition of sialic acid residues can lead to larger, branched glycans. Prediction of glycolipid abundance is impossible and can therefore only be determined experimentally [191].

Recent interest for the EV glycome led to the identification of generally conserved sugar moieties in EVs derived from mammalian cells, and the observation that, although there are cell-specific differences in glycosylation, EV carbohydrate profiles differ from that of their parent cells [198]. Amongst commonly enriched glycan epitopes in EVs are high mannose, complex N-linked glycans, α-2,6 sialic acids, and poly N-acetyllactosamines, as determined by lectin microarray. In addition, EVs showed reduced binding to lectins recognising blood group antigens and N-acetyl-D-galactosamine compared to their cell of origin, suggesting the exclusion of such epitopes [198,199]. There is mounting evidence suggesting roles for carbohydrate moieties in the interaction between EVs and target cells, either through charge-based effects, direct glycan binding, or the joint action of both. Experimental alterations to EV glycosylation, in particular the removal of terminal sialic acid, affect cellular uptake and biodistribution of EV [191,200-202]. Degradation of O-linked glycans also results in altered biodistribution of EVs in mice [203].

The content of EVs is highly dependent on multiple factors, such as cellular origin, environmental factors, and the activation state of the producing cell. In addition, a given cell may release a heterogeneous population of EVs depending on the site of secretion (e.g., basal or apical side) [160], and on different subtypes of EVs (the same cell may release multiple subgroups). It has also been proposed that a given cell may be populated by a variety of MVBs with distinct content [204,205].

3.3 EV uptake and biological functions

EVs have been implicated in various aspects of health and disease, including but not limited to homeostatic processes, e.g., removal of unnecessary molecules, cell maturation, and adaptation to environmental variation, the regulation of pregnancy and delivery [206], and the establishment of conditions favourable for tumour growth [207,208]. These functions are exerted via interactions with recipient cells. EVs act as intercellular messengers, delivering signals from one cell to another, and can affect cellular function via various mechanisms. As the many EV subtypes with their distinct characteristics, which undoubtedly impact their cellular interaction and subsequent

effector functions, are only starting to be appreciated, our understanding of specific EV uptake mechanisms remains limited [112]. General EV interaction and uptake mechanisms are depicted in Figure 1.7. EVs can affect target cell intracellular signalling cascades initiated through ligand-receptor interaction following direct contact between an EV and a cell, by direct fusion with the plasma membrane of a target cell, thereby releasing their cargo into the cytoplasm of the cell, or target cells may internalise EV via endocytosis. EVs represent important elements of cell signalling and their function is highly dependent on their components and encapsulated cargo molecules. The variety of these molecules and potential interaction mechanisms are responsible for the impressive array of functions attributable to EVs.

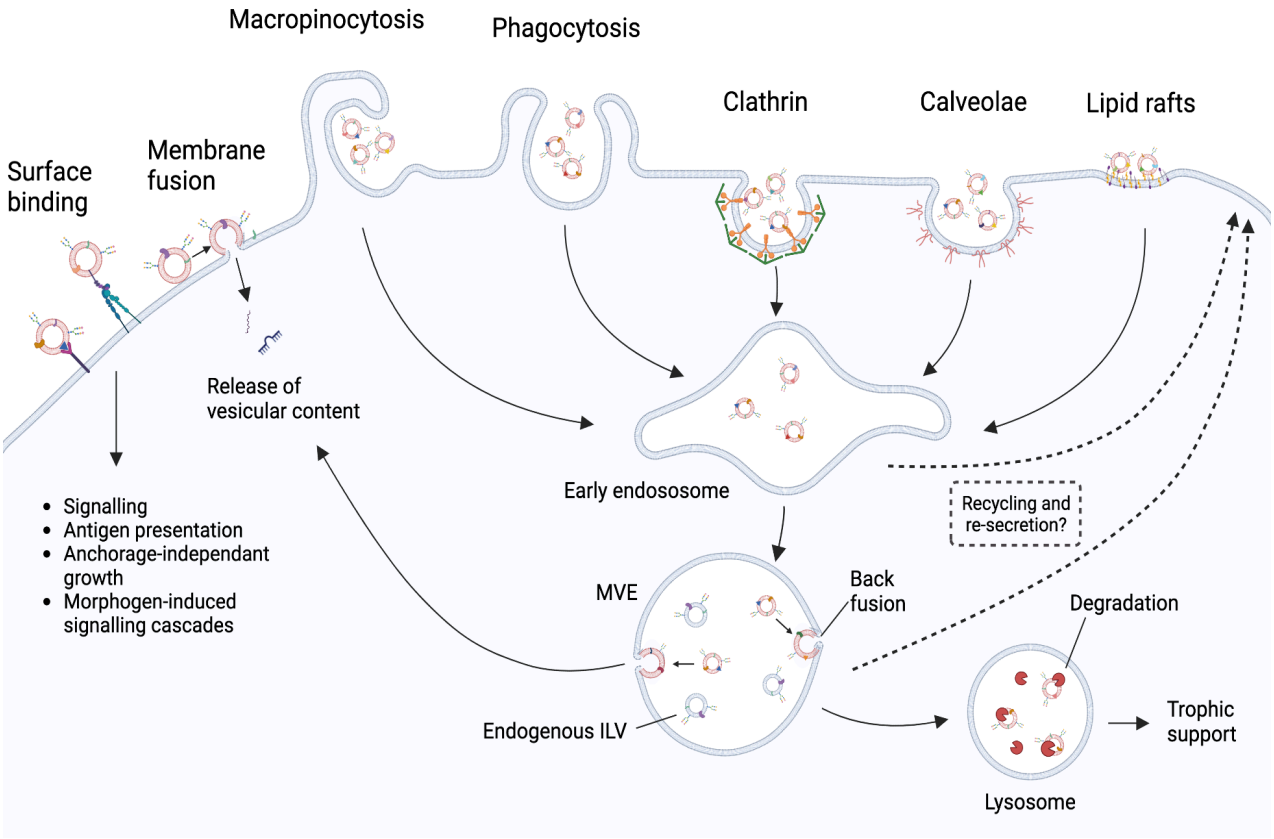

Figure 1.9: Mechanisms of exosome interaction with target cells. Once EVs reach a recipient cell, they may bind a cell surface receptor, initiating intracellular signalling pathways. Target cells may also internalise EVs via multiple pathways, targeting exogenous EVs to the canonical endosomal pathway, reaching MVEs where they are likely to mix with populations of endogenous ILVs. Fusion of MVEs to lysosomes will result in degradation of EVs and recycling of their components by the recipient cell. Alternatively, it is possible that EVs docked to the limiting membrane of MVEs or to the plasma membrane may discharge their cargoes into the cytoplasm of the target cell by fusion. Though this mechanism remains poorly characterised, it would be critical for

delivery of EV-derived molecules such as miRNA. It is also possible that endocytosed EVs may be redirected to the plasma membrane and re-secreted to the extracellular environment.
Source: created with BioRender (https://app.biorender.com/), modified from van Niel *et al*., 2018 [209].

EVs maintain a variety of functions within a given organism but have also been reported to participate in inter-organism, inter-species, and even inter-kingdom communication. Notably, there is accumulating evidence for their contribution to host-parasite interactions during infections [210-212]. For instance, malaria-derived EVs were identified from malaria-infected human and murine hosts. In these instances, the level of circulating EVs was correlated with clinical signs of disease, which suggests that EVs may contribute to the development of pathology in a host [213-216]. Supporting this idea, EVs isolated from the plasma of malaria-infected mice potently induced inflammation via TLR-dependent activation of macrophages [217], and increased production of inflammatory cytokines [218]. Moreover, inhibition of EV formation protected mice in a murine model of cerebral malaria [219]. Moreover, *Plasmodium berghei* releases EVs capable of inhibiting T-cell responses [220]. Similarly, EVs isolated from *Toxoplasma gondii*-infected macrophages induced pro-inflammatory responses both *in vitro* and *in vivo* [221]. Apicomplexa are not the only protozoan parasites for which EVs are important. Kinetoplastids, including *Trypanosoma* and *Leishmania* species, also produce and release EVs that are suggested to induce the development of a permissive environment for parasite survival [222-226]. *Leishmania* EVs promote an anti-inflammatory phenotype in monocytes and monocyte-derived dendritic cells, favouring Th2 polarisation and exacerbating disease [225].

EVs also mediate communication between individual parasites within a host. *In vitro*, *Plasmodium falciparum*-infected erythrocytes release vesicles that contain both host- and parasite-derived proteins. These vesicles are subsequently acquired by other infected erythrocytes, where they contribute to the initiation of gametocytogenesis [218,227].

Comparatively little is known about helminth-derived EVs as they are a more recent field of study. In the recent years, however, a number of parasitic helminths have been found to secrete EVs [228-243] . This may explain the observation that some proteins lacking the classical signal peptides necessary for secretion, including enolase, are indeed found in the secretory milieu of helminth parasites [244]. As more helminth-derived EVs have their molecular content characterized and their function studied, there is increasing experimental evidence supporting the role of

parasitic helminth EVs in host manipulation [73,236,245-247]. The characterization of these vesicles and how they may confer or modify intra- and extracellular signalling may prove beneficial in the development of novel diagnostic tools as well as identification of putative drug and/or vaccine targets [248].

3.4 Helminth EVs

3.4.1 Origin of secretion

EV secretion in parasitic helminths was first reported in 2012 in the trematodes *Echinostoma caproni* and *F. hepatica* [240] and has since been reported for many species of parasitic helminth, including *S. mansoni* [228-230], and, though most research has focused on the adult stage [228,234-237,249-251], EV release has also been described for various larval stages [229,238,239]. This is supported by the fact that proteins involved in microvesicle formation as well as those making up the ESCRT machinery are conserved in helminths [252]. The content (primarily proteins and miRNAs) of EVs from an increasing number of helminths are being characterised. However, the cellular origin(s) and route(s) of EV secretion remain largely uncharacterised for most species. Thus far, evidence points to the gut as a site of EV secretion for adult stages of the nematode *Heligmosomoides polygyrus* [236] and *Haemonchus contortus* L4 larvae [235], while both the gut and the secretory pore are possible sources of EVs in *Ascaris suum* [239]. Similarly, the excretory-secretory pore is a likely site of EV secretion in *Brugia malayi* microfilariae [249]. In platyhelminths, on the other hand, the syncytial outer coat called the tegument appears to be involved in the release of EVs. Platyhelminths are characterised by a tegument, which both protects them and plays roles in nutrient acquisition. A study on adult *F. hepatica* reported localisation of RAL-A (one of two markers used for small EVs) in the tegumental syncytium and sub-tegumental cells. The same group also identified the protonephridial system through the excretory pore as the source of small EVs (EVs sedimenting at 120k × g) and gastrodermal epithelial cells lining the gut as the source of large EVs (EVs sedimenting at 15k × g) [251]. Importantly, Bennett *et al* described a novel EV secretion mechanism from *F. hepatica* gastrodermal cells, whereby the cells undergo progressive thinning of the apical membrane until its rupture, releasing cellular contents such as secretory vesicles into the gut lumen [253]. However, the origin of EV secretion in schistosomes remains elusive.

Other important questions to address include the composition and biological functions of helminth-derived EVs. Given the plethora of immunoregulatory and immunosuppression roles attributed to EVs, the prospect that they may be involved in the extensive molecular dialogue between the parasite and the host is extremely appealing, particularly in the context of helminth infections, which are associated with immune down-modulation and in which parasitic worms regularly display significant longevity.

A major determinant of EV function is their cargo. Whilst contents of helminth EVs are comparable to mammalian EVs, including the same general classes of macromolecules, some notable differences have been observed.

3.4.2 Helminth EV composition and cargo

The protein fraction is by far the best characterised component of helminth EVs. Biogenesis-related content, such as proteins required for MVB formation, in particular members of the ESCRT pathway and accessory proteins, are also found in helminth EVs. For instance, EVs from *F. hepatica* contain proteins of the ESCRT pathway as well as other proteins involved in vesicular trafficking [251]. Likewise, the endosome-specific tetraspanin CD63, which is commonly used as a mammalian EV marker, has also been identified in EVs from *F. hepatica* and the nematode *Trichinella spiralis* [234,254]. Other tetraspanins have been detected in EVs from various other helminths, including the nematode *H. polygyrus* and the platyhelminths *Echinococcus multilocularis*, *Opisthorchis viverrine*, and *Schistosoma* species [228,236,255-257]. Heat shock proteins, GAPDH, and enolase have also served as markers for schistosome EVs [228,258]. Whilst there does not appear to be a universal helminth-specific protein marker, proteins from the EF-hand family were consistently detected in EVs from cestodes and trematodes, whereas M13 metallopeptidases were found in nematode EVs [259]. In addition, known immunomodulatory proteins, such as members of the SCP/TAPS family, have been identified in EVs from several nematodes, namely *Trichuris muris*, *Nippostrongylus brasiliensis*, *Teladorsagia circumcincta*, and *H. polygyrus* [236-238,260]. The protein cargo of EVs is likely to play a role in host-parasite interactions.

Like their mammalian homologues, helminth EVs also contain small RNAs, such as miRNAs, which are regarded as key effectors of host modulation, capable of regulating host gene

expression. Recent studies suggest that EV-encapsulated helminth miRNAs can be transferred to host cells, where they can induce gene silencing with major potential for immunomodulation. Parasite-derived miRNAs have been found in host biofluids and tissues. The *S. mansoni* miRNAs miR-10 and miR-Bantam were detected in Th cells derived from mesenteric lymph nodes and Peyer's patches of infected mice during chronic infection with *S. mansoni*, suggesting EV delivery of those miRNAs in the lymphatic tissues draining the site of infection and potentially regulating gene expression in those cells [261].

Few studies have focused on the lipid components of helminth EVs. *H. polygyrus* EVs were reported to be enriched in ether phospholipids, including plasmalogens, which are believed to increase membrane rigidity and resistance and, although sphingomyelinases and ceramides were detected, their level appeared to be significantly reduced compared to mammalian EVs [262]. Sphingomyelinases were also identified as part of the molecular makeup of *F. hepatica* EVs [234,251]. Further lipidomic studies are needed to determine the components and roles that helminth EV lipids may play in the context of infection. Importantly, it has been suggested that, since parasitic helminths do not synthesise fatty acids [263], worm culture conditions may greatly affect EV lipid composition, and so extra care must be taken when investigating this question [264].

Similarly, the number of studies that have focused on helminth EV glycomes is extremely limited. Glycan structures coating the surface of EVs have sparked interest as regulators of EV uptake, making them potentially key players in the host-parasite interaction as they are presumably responsible for delivering parasite-derived molecules to host cells. Comparison between *F. hepatica* EV glycans and tegument glycans revealed a different profile of carbohydrate moieties and removal of EV surface glycans by treatment with glycosidases blocked internalisation by a macrophage cell line [251]. This finding supports a requirement for these molecules in cellular uptake, though this may also be due to a disruption in surface charge, altering stability of EVs [264]. Surface N-ligand and glycolipid-glycan analysis of EVs secreted by *S. mansoni* schistosomulae revealed the presence of ligands for dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN; CD209), including LewisX motifs (Galβ1-4(Fucα1-3)GlcNAc) [265]. LewisX is a well-known immunogenic glycan motif conjugated to glycoproteins, proteoglycans, or glycolipids, and a known component of the schistosome glycome and ES products [266,267].

Blocking DC-SIGN greatly reduced EV uptake by human monocyte-derived dendritic cells (moDCs) [265], reenforcing the requirement for EV glycosylation for interaction with host cells. In addition to aiding target recognition or cell adhesion and cell entry/invasion, glycans also play roles in immune evasion. For instance, carbohydrate structures can mask antigenic sites or protect from attacks by proteases or glycosidases. Therefore, it is possible that EV glycans may also participate in parasite evasion of the host immune system by shielding EVs, allowing them to travel to and interact with their target cells, where they can exert their function.

3.4.3 Host-manipulation effects of trematode EVs

Despite the growing number of studies reporting vesicle-based secretion in various helminth species, few have focused on the functional aspect of EVs. Functions have been proposed for EVs secreted by various nematodes, cestodes, and trematodes (reviewed in [268,269]). This section will focus on potential functions and effects described for trematode EVs.

Helminth parasites are characterised by complex life cycles, each stage occupying one or more anatomical niches, thus interacting with various types of cells and tissues throughout the life cycle. As such, studies have investigated interactions between parasite EVs and their surrounding non-immune cells. In 2012, a study reported internalisation of *E. caproni* EVs by rat intestinal epithelial cells *in vitro*, suggesting that they could modulate functions of these cells [240]. Subsequently, human intrahepatic biliary epithelial cells were shown to take up *Fasciola gigantica* EVs *in vitro*, which resulted in increased levels of reactive oxygen species and induction of autophagy and damage and repair processes in recipient cells [270]. EVs secreted by adult *S. japonicum* were internalised by murine liver cells [231] and egg-derived EVs by hepatic stellate cells [271], transferring their miRNA cargo into recipient cells. *S. japonicum* egg EV-associated miRNAs were linked to inhibition of hepatic stellate cells *in vitro* and the attenuation of liver fibrosis *in vivo* [271]. In the same genus, adult *S. mansoni*-derived EVs were internalised by monocytic and human umbilical vascular endothelial cell lines, resulting in differential expression of genes associated with intravascular parasitism, including vascular endothelial contraction, coagulation, arachidonic acid metabolism and immune cell trafficking and signalling [272].

In addition, there is increasing evidence for uptake of trematode EVs by host immune cells. For instance, *S. japonicum* EVs were reported to increase production of several M1 and M2 markers, such as iNOS, TNF-α, IL-12, IL-10, and Arg-1, in the murine macrophages cell line

RAW264.7, promoting a mixture of M1 and M2 macrophages [233,258]. Similarly, *S. japonicum* EVs are taken up by other peripheral blood immune cells *in vitro* as well as *in vivo*, and *S. japonicum* miRNAs were shown to associate with Argonaut proteins once in a recipient cell, suggesting that parasite miRNAs can be functional in the host. Transfection of RAW264.7 cells with the *S. japonicum* EV-associated miRNAs miR-125b and bantam led to increased proliferation. Interestingly, reduction in host monocyte populations has been associated with reduced worm burden and egg deposition, supporting the hypothesis that parasite-derived EVs have immunomodulatory properties and play crucial roles in parasite survival and proliferation [258]. The uptake of *S. mansoni* schistosomula EVs by mouse DCs resulted in increased expression of IL-6, IL-10 and IL-12 and of costimulatory molecules CD80 and CD86, which are required for co-stimulation of CD4 T cells [265]. *F. hepatica* EVs are also capable of modulating DCs, inducing increased secretion of TNF-α, and expression of CD80, CD86, and resulting in a DC phenotype which supresses IL-2 secretion from T cells [273]. Parasite EVs also interact directly with lymphocytes. Meningher *et al* demonstrated that primary murine Th cells can take up adult *S. mansoni* EVs, internalising their miRNA content. Importantly, the authors reported the detection of *S. mansoni* miRNAs in Th cells isolated from the gut-associated lymphoid tissues Peyer's patches and mesenteric lymph nodes [261], suggesting that parasite-derived EVs make their way to neighbouring lymph nodes to carry out their immunomodulatory functions. Moreover, it has been speculated that *S. mansoni* EVs may be responsible for the targeting of bioactive lipids to eosinophils, which may play critical roles in tissue repair and fibrosis during infection [274].

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Chapter III. Manuscript I

Analysis of *Schistosoma mansoni* extracellular vesicles surface glycans reveals potential immune evasion mechanism and new insights on their origins of biogenesis

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Abstract

Parasitic helminths are master manipulators of host immunity. Their strategy is complex and involves the release of excreted/secreted products, including extracellular vesicles (EVs). The protein and miRNA contents of EVs have been characterised for many parasitic helminths but, despite reports suggesting the importance of EV surface carbohydrate structures (glycans) in the interactions with target cells and thus subsequent effector functions, little is known about parasite EV glycomics. Using lectin microarrays, we identified several lectins that exhibit strong adhesion to *Schistosoma mansoni* EVs, suggesting the presence of multiple glycan structures on these vesicles. Interestingly, SNA-I, a lectin that recognises structures with terminal sialic acid, displayed strong affinity for *S. mansoni* EVs, which was completely abolished by neuraminidase treatment, suggesting sialylation in the EV sample. This finding is of interest, as sialic acids play important roles in the context of infection by aiding immune evasion, affecting target recognition, cell entry, etc., but are not thought to be synthesised by helminths. These data were validated by quantitative analysis of free sialic acid released from EVs following treatment with neuraminidase. Lectin histochemistry and fluorescence *in situ* hybridisation analyses on whole adult worms suggest the involvement of sub-tegumental cell bodies, as well as the digestive and excretory systems, in the release of EVs. These results support previous reports of EV biogenesis diversity in trematodes and potentially highlight new means of immune modulation and evasion employed by schistosomes.

1. Introduction

Schistosomiasis is caused by infection with parasitic trematodes in the genus *Schistosoma*, which have a complex life cycle that includes freshwater snails as intermediate hosts and mammalian definitive hosts. Despite mass drug administration programs with praziquantel, schistosomiasis is estimated to affect ~ 240 million people worldwide [1] and to still be responsible for the loss of 1.43 million disability-adjusted life years [2].

Owing to their ability to evade and modulate host immune responses [3,4], adult schistosomes can survive within their mammalian host for many years [5]. In this regard, their excreted/secreted products (ESPs) are of particular interest, as parasite-secreted molecules are instrumental for host-parasite interactions and thus represent promising targets for diagnostic and vaccine antigen discovery [4,6-8]. Significant advances have been made in the characterisation of the secretomes of *Schistosoma* species [9-11], but it is only recently that the release of extracellular vesicles (EVs) by schistosomes has been described [8,12-16], suggesting such vesicles as a route of ESP secretion.

EVs include all types of secreted membrane-bound vesicles, of which exosomes (formed within multivesicular bodies) and microvesicles (formed by the outward budding of the plasma membrane) are the best characterised [17]. Despite mounting evidence for vesicle-based secretion in schistosomes, the specifics of EV release remain elusive and the origin(s) of EV secretion is still unclear. There is increasing evidence for the release of EVs in parasitic infections, with roles in both parasite-parasite inter-communication [18] and parasite-host interactions [7,19,20]. EVs transfer molecules from one cell to another via membrane vesicle trafficking, thus explaining the broad array of functional activities attributable to them. However, little is known about the mechanisms of parasite-derived EV uptake by host cells and therefore how their effects are transduced *in vivo*. This is due, at least in part, to the lack of knowledge regarding schistosome-derived EV surface molecules. A growing body of evidence suggests a requirement for various carbohydrate moieties, on the surface of both cells and vesicles, for EV uptake by recipient cells [21-25]. Despite increasing appreciation for the role of glycans in EV biology, little information is available regarding carbohydrate structures present on the surface of adult *S. mansoni*-derived EVs. Glycoproteins secreted by helminth parasites are immunogenic and represent appealing

components of vaccine preparations. A better understanding of the glycans present on vesicles secreted by the parasite could lead to the identification of novel biomarkers for the development of superior diagnostic tools as well as new targets for prevention and therapy of schistosomiasis.

We previously characterised the protein and miRNA composition of adult *S. mansoni*-derived EVs [12]. Here, we profiled the major glycan motifs present on the surface of adult *S. mansoni*-derived EVs using lectin microarrays. Interestingly, SNA-I, a lectin that recognises structures with terminal sialic acid residues, displayed strong affinity for *S. mansoni* EVs, which was validated by quantitative analysis of free sialic acid released following treatment with neuraminidase, suggesting sialylation in the EV sample. The presence of sialic acid in *S. mansoni* EV samples is of interest, as sialic acids play important roles in infection by, for example, aiding immune evasion, and affecting target recognition and cell entry [26,27], but are not thought to be synthesised by helminths [28]. Moreover, our lectin histochemistry and fluorescence *in situ* hybridisation (FISH) studies suggest possible roles for the tegument, the gut and the excretory system in the secretion of EVs, indicating the presence of a heterogeneous EV population.

2. Materials and Methods

2.1 Parasites

Biomphalaria glabrata snails infected with the *S. mansoni* Puerto Rican strain PR1 were maintained the Institute of Parasitology of McGill University. *Schistosoma mansoni* adult worms were obtained by perfusion of female CD1 mice 7 weeks after infection via tail exposure to approximately 150 cercariae. Mice were housed in the animal facility at the Small Animal Research Unit of McGill University as per the McGill University Animal Care Committee (Permit # 2019-8138). Briefly, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and adult *S. mansoni* worms harvested by cardiac perfusion. For histochemistry, worms were washed in sterile PBS and fixed immediately (as described below), whereas for EV isolation, worms were maintained in RPMI-1640 medium supplemented with 100 U penicillin, 100 mg/mL streptomycin, and 10% EV-depleted FBS. EV depletion was performed by ultracentrifugation of FBS under sterile conditions for 18 h at 120,000 × g and 4 °C, followed by filtration through 0.22 μm hydrophilic PVDF Durapore membranes (EMD Millipore; SVGV01015) as described [29].

FBS was of the highest grade and lowest endotoxin level (Life Technologies; Ref: 16000). Adult male and female worm pairs were maintained in 6-well plates for 72 h at a density of approximately 20 worms (10 males and 10 females)/well in 10 ml culture medium at 37 °C. Conditioned medium was collected after 48 and 72 h. Worms remained active during this period of incubation, with no apparent change in morphology or movement.

2.2 Isolation of adult *S. mansoni* extracellular vesicles

EVs were isolated as described [12]. Briefly, parasite-conditioned culture medium was collected and centrifuged at increasing speeds: 300 × *g*/15 min, 700 × *g*/15 min and 3000 × *g*/15 min to remove larger debris, and the supernatant was centrifuged at 12,000 × *g* for 45 min. Supernatants were filter-sterilised using a 0.22 μm hydrophilic PVDF Durapore membrane as above and centrifuged at 100,000 × *g* for 2 h using a Beckman-Coulter SW-48 rotor on a Beckman-Coulter ultracentrifuge. The pellet was re-suspended in sterile GIBCO® Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies) and loaded onto a sucrose step gradient (25%, 30% and 35%), then centrifuged at 120,000 × *g* for 18 h using a SW-48 rotor. Following centrifugation, the 30% sucrose fraction was collected and washed by diluting 4–5-fold with sterile DPBS and EVs were re-pelleted by centrifugation in an SW-28 rotor at 100,000 × *g* for 2 h. The pellets were washed once again by re-suspension in DPBS and centrifugation in a SW-41 rotor at 100,000 × *g* for 2 h. Finally, pellets were re-suspended in 1.0 ml DPBS, transferred to 1.5 ml Beckman-Coulter ultracentrifuge tubes and centrifuged in an Optima TL100 tabletop ultracentrifuge (Beckman-Coulter) for 1.5 h at 100,000 × *g* using a TLA-100.3 rotor. Exosomes were quantified based on protein concentration using a Pierce BCA Protein Assay Kit (ThermoFischer Scientific) and absorbance measured at 562 nm using a Synergy H4 Hybrid plate reader. The pelleted material was snap-frozen and stored at –80°C.

2.3 Soluble worm antigen preparation

Schistosome soluble worm antigen preparation (SWAP) was prepared from ~30 adult worms collected as described above. Worms were freeze-thawed in 150 μL lysis buffer (1X DPBS, 0.5% Triton X-100) with cOmplete™ Mini Protease Inhibitor Cocktail (Roche, Laval, QC,

Canada), homogenised using a pestle, and centrifuged at 4000 × g for 30 min at 4 °C. The supernatant was collected and used as SWAP. SWAP concentration was determined using a Pierce BCA Protein Assay Kit as above.

2.4 Profiling of fluorescently-labeled EVs with lectin microarrays

Microarrays (v2.4.0) comprised of 50 unique plant and fungal lectins were printed by a non-contact method onto Schott Nexterion® H (Schott, Mainz, Germany, Cat. No. 1070936) substrates with a Scienion SciFlexArrayer S3 piezoelectric spotter (Scienion, Berlin, Germany) as previously described [30]. Lectins and their specificities are presented in Supplementary Table 1. Microarrays were washed with PBS containing 0.05% Tween 20 (PBST) three times and once with PBS, centrifuged dry (450 x g, 5 min) and stored at 4 °C with desiccant until use.

Aliquots of EVs were thawed over ice and immediately labeled with the lipophilic dye PKH26 (Sigma Aldrich, Dublin, Ireland) as previously reported [31]. All steps were carried out at room temperature and in the dark. Following labeling, excess dye was removed from EVs by centrifugal filtration in a 500 µL, 100 kDa MWCO spin device (Amicon, EMD-Millipore, Cork, Ireland). The centrifugal filter was pre-washed by centrifugation with PBS containing 0.1% BSA before the entire final volume of the EV labeling mixture was added. An additional 400 µL PBS was added prior to centrifugation for 15 min, 8000 × *g*, at room temperature. Finally, an additional 400 µL PBS was added to the spin device prior to the final centrifugation at 8000 × *g* in which the final volume was reduced to approximately 50 µL. Concentrated EVs were removed by pipetting and the bottom of the spin device was rinsed with an additional 20 µL PBS which was added to the recovered volume. EVs were used immediately after labeling.

Lectin microarray interrogation of EV surface glycosylation was carried out in the dark at 23°C for 40 min with gentle inversion (approximately 4 rpm) as previously described [32]. Microarray slides were incubated with PKH26-labelled EVs diluted in buffer containing 20 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ (pH 7.4) with 0.025% Tween 20 (TBST) for 40 min at 23°C. Titrations with labeled EVs were carried out at 5, 2.5, 1.25, and 0.63 µg/mL. Subsequent lectin microarray interrogations were performed in 12 replicates, 4 from one EV

isolation sample (SmEV1 A-D) and 8 from pooled EV samples (SmEVc A-H), at 3 µg/mL which provided the best signal to noise ratio and fewest artefacts. Following incubation, arrays were unpacked under TBST, washed once in TBST for 5 min with gentle shaking and then briefly rinsed with TBS before being centrifuged dry (450 × *g*, 5 min). Microarrays were imaged immediately at 532 nm in an Agilent G2505B microarray scanner at 100% PMT. Data extraction was carried out in GenePix Pro (v6.0, Molecular Devices, San Jose, CA, USA), processing was carried out in Excel (v2013, Microsoft, Redmond, WA), and analysis performed with Excel, HCE 3.5 (http://www.cs.umd.edu/hcil/hce/) and Morpheus (Morpheus, https://software.broadinstitute.org/morpheus).

2.5 Neuraminidase treatment

Neuraminidase treatments employed a broad-spectrum α2-3,6,8,9 neuraminidase A (New England Biolabs P0722, Ipswich, MA, USA). For microarray experiments, no detergents or reducing agents were used in the enzymatic digestion and hydrolysis was carried out on PKH26-labeled EVs in the dark at 37 °C for 1.5 h. For Western blots and free sialic acid detection assays, 50 µg of proteins (EV samples or SWAP) were incubated with 40 units (2 µL) neuraminidase for 1.5 h at 37 °C according to the manufacturer's protocol and assayed immediately. 10 µg fetuin (New England Biolabs P0722, Ipswich, MA, USA) was used as a control sialylated glycoprotein.

2.6 Free sialic acid detection assay

Free sialic acid was quantified according to the manufacturer's instruction using a Sialic Acid Assay Kit (abcam ab83375, Cambridge, UK), which uses an enzyme coupled reaction in which oxidation of free sialic acid creates an intermediate that reacts stoichiometrically with a probe, generating a product which is detected by measuring fluorescence (Ex/Em = 535/587 nm) using a Synergy H4 Hybrid plate reader.

2.7 Lectin- and immunofluorescence microscopy of parasites

Dextran-labelled parasites were prepared as previously described *[33]*. Briefly, ~50 freshly perfused worms were immersed in 100 µl of a 5 mg/ml solution of biotin-TAMRA-dextran (Life

Technologies D3312) dissolved in ultrapure water. Worms were gently vortexed for 3 min, and then submerged in 10 ml fixative solution (4% formaldehyde in PBSTx (PBS + 0.3% triton-X100)) to stop labeling. The fixative solution was discarded and replaced with 10 ml fresh fixative solution to dilute residual dextran. The worms were subsequently fixed for 4 hr in the dark with mild agitation. Parasites were subsequently washed with 10 ml fresh PBSTx for 10 min and then labelled with FITC-conjugated lectins as detailed below. In other experiments, adult *S. mansoni* worms were flat-fixed in 4% paraformaldehyde (PFA) in PBS; freshly perfused male and female worms were separated, placed between two microscope slides and further flattened under additional weight while submerged in 4% PFA in the dark for 4 hr at 4 °C. Following fixation, worms were washed in antibody diluent (AbD; 0.1% BSA, 0.1% NaN_3, 0.5% Triton x-100 in 1X PBS) and incubated with biotinylated lectins (SNA-I, DSA, or RCA-I; 1:100 in AbD) (Biolynx B-1305, B-1185, B-1085, Brockville, ON, Canada) for 3 days at 4 °C. Parasites were then washed in AbD overnight and incubated with fluorescein (FITC)-conjugated anti-biotin (abcam ab53469, Cambridge, UK) diluted 1:500 in AbD for 3 days at 4 °C. Worms were again washed in AbD overnight and counterstained overnight with tetramethylrhodamine (TRITC)-conjugated phalloidin (Millipore Sigma, Oakville, ON, Canada) (1:100 in AbD) at 4 °C, after which parasites were washed in AbD overnight and mounted on glass microscope slides with Vectashield® anti-fading solution (Vector Laboratories). Specimens were viewed using a Leica TCS SP5 confocal scanning microscope or a Nikon A1R MP confocal scanning microscope.

2.8 Fluorescence *in situ* hybridisation (FISH) lectin co-labelling

Fluorescence *in situ* hybridisation was performed as described [33-35]. To label the cytoplasm of tegumental cells by FISH, riboprobes recognising the tegument-specific markers *calpain*, *gtp-4*, *annexin*, and *npp-5* were pooled as described by Wendt et al., 2018. Male and female parasites were separated by incubation (2–3 min) in 0.25% ethyl 3-aminobenzoate methanesulphonate (Sigma-Aldrich A5040) in PBS. Relaxed worms were fixed for 4 h in the dark in 4% formaldehyde in PBSTx (PBS + 0.3% triton-X100) with gentle agitation. Following the fixation process, parasites were dehydrated in methanol and rehydrated in 1:1 methanol:PBSTx followed by incubation in PBSTx. Rehydrated worms were then bleached under bright light for 1 h in formamide bleaching solution (0.5% formamide, 0.5% saline-sodium citrate buffer, and

1.2% H_2O_2), rinsed with PBSTx and briefly post-fixed for 10–15 min in 4% formaldehyde in PBSTx. Samples were hybridised overnight with the 4 riboprobes at 52 °C. Worms were then washed in AbD and incubated with fluorescein-conjugated lectins (SNA-I or DSA) (Vector Laboratories, FL-1301-2 and FL-1181-2, Burlingame, CA, USA); 4 µg in 500 µL AbD) overnight at 4 °C. FISH-labeled parasites were exposed to either FITC-conjugated SNA-I or DSA as described above, counterstained with DAPI (1 mg/ml), cleared in 80% glycerol, and mounted on slides with Vectashield (Vector Laboratories). Specimens were viewed using a Nikon A1R MP confocal scanning microscope.

3. Results

3.1 Sialic acid residues are detected on *Schistosoma mansoni*-derived EVs.

Using lectin microarrays, we identified several lectins with various binding specificities (Table 1; see Supplemental Table S1 for full list of lectins and specificities) that exhibited strong adhesion with EVs, including SNA-II, DSA, LEL, Calsepa, NPA, GNA, HHA, WGA, SNA-I, RCA-I and CAA (Figure 1a).

Table 1. Lectins exhibiting strong adhesion with *Schistosoma mansoni* EVs with specificity information for each.

Lectin	Organism	Common name	Specificity
SNA-II	*Sambucus nigra*	Sambucus lectin-II	Gal/GalNAc
DSA	*Datura stramonium*	Jimson weed lectin	GlcNAc
LEL	*Lycopersicum eculentum*	Tomato lectin	GlcNAc-β-(1,4)-GlcNAc
Calsepa	*Calystegia sepium*	Bindweed lectin	Man/Maltose
NPA	*Narcissus pseudonarcissus*	Daffodil lectin	α-(1,6)-Man
GNA	*Galanthus nivalis*	Snowdrop lectin	Man-α(1,3)-
HHA	*Hippeastrum hybrid*	Amarylis agglutinin	Man-α(1,3)-Man-α(1,6)-
WGA	*Triticum vulgaris*	Wheat germ agglutinin	NeuAc/GlcNAc
SNA-I	*Sambucus nigra*	Sambucus lectin-I	Sialic acid-α-(2,6)-Gal(NAc)
PHA-E	*Phaseolus vulgaris*	Kidney bean erythroagglutinin	biantennary, bisecting GlcNAc,β-Gal/Gal-β-(1,4-)GlcNAc
RCA-I/120	*Ricinus communis*	Castor bean lectin	Gal-β-(1,4)-GlcNAc
CAA	*Caragana arborescens*	Pea tree lectin	Gal-β-(1,4)-GlcNAc

These results suggest the presence of a wide range of glycan structures on these vesicles. Interestingly, SNA-I, a lectin that recognises structures with α2-6-linked terminal sialic acid,

displayed strong affinity for *S. mansoni* EVs, which treatment with a broad-spectrum neuraminidase (also "sialidase"; enzymes that cleave sialic acid) completely abolished (Figure 1b; Table 2), suggesting the presence of oligosaccharides with terminal sialic acid residues in the EV sample. WGA, a lectin that recognises both N-acetyl-glucosamine (GlcNAc) and sialic acid residues on proteins, also demonstrated a sharp decrease in intensity after EVs were treated with neuraminidase. Importantly, SNA-II and RCA-I, which have affinity for structures which are frequently terminated with sialic acids (those with distal N-acetyl-galactosamine and/or galactose, especially in Type I and Type II N-acetyllactosamine configurations) demonstrated sharp increases in signals following neuraminidase treatment (Figure 1b; Table 2), which is consistent with the exposure of these structures following removal of terminal sialic acids.

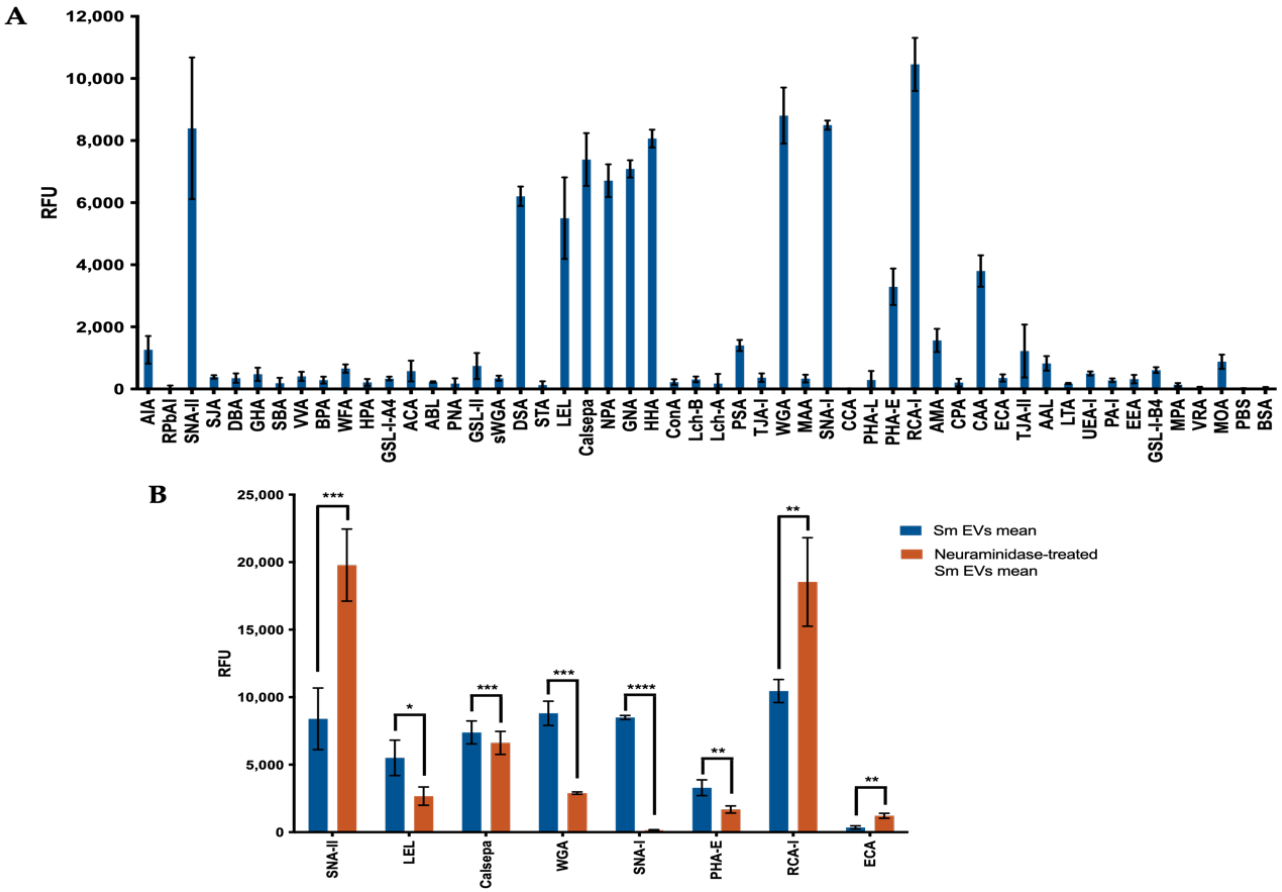

Figure 1. Lectin microarray. Microarray slides were incubated with PKH26-labelled EVs and imaged at 532 nm in an Agilent G2505B microarray scanner at 100% PMT (**a**) Lectin microarray response generated for *S. mansoni* EVs. (**b**) Significant effect of neuraminidase treatment on lectin-binding at the surface of EVs. Data subjected to total intensity mean normalisation, n = 4, +/- SD. Statistics were performed using a paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.00001$.

Table 2. Summary of the effect of neuraminidase treatment on lectin affinity.

Difference[1]	*P* value	Significance[2]	+/-?[1]	Lectin	Top ligands
11386.58	0.000285	***	Up	SNA-II	Gal/GalNAc
2829.56	0.028967	*	Down	LEL	GlcNAc-β-(1,4)-GlcNAc
775.70	0.000462	***	Down	Calsepa	Man/Maltose
5911.46	0.001086	***	Down	WGA	NeuAc/GlcNAc
8346.68	0.000001	****	Down	SNA-I	Sialic acid-α-(2,6)-Gal(NAc)
1610.86	0.003570	**	Down	PHA-E	biantennary, bisecting GlcNAc,β-Gal/Gal-β-(1,4-)GlcNAc
8081.83	0.009170	**	Up	RCA-I	Gal-β-(1,4)-GlcNAc
858.01	0.002417	**	Up	ECA	Gal-β-(1,4)-GlcNAc oligomers

[1] Numbers in red show an increase in lectin affinity (Up) following incubation with neuraminidase, whereas numbers in green represent a decrease in lectin affinity (Down) following enzymatic digestion.
[2] Statistics were performed using a paired t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.00001$.

We reasoned that if terminal sialic acid residues were indeed present on the surface of EVs, not only would hydrolysis of these structures by a broad-spectrum neuraminidase prevent SNA-I binding, but would also result in the release of free sialic acid. We thus validated sialylation of EVs by quantitative analysis of sialic acid released following treatment with neuraminidase and observed a consistent increase in free sialic acid concentration as a result of enzymatic digestion (Figure 2).

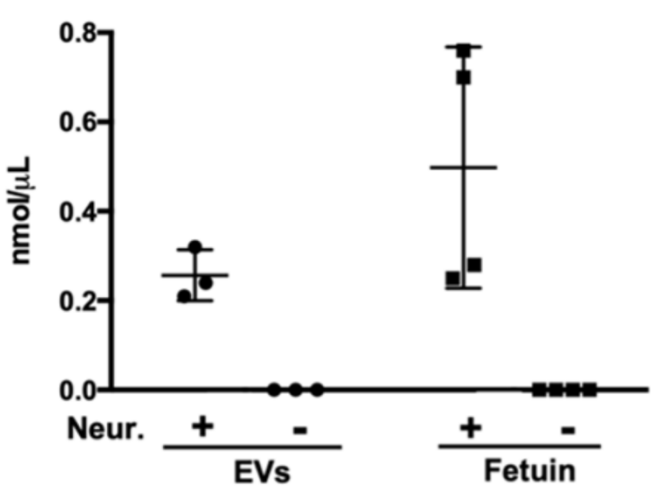

Figure 2. Treatment of *S. mansoni* EVs with neuraminidase (Neur.) results in the release of free sialic acid residues. EVs were incubated with a broad-spectrum neuraminidase, after which samples were assayed for the presence of free sialic acid residues. Fetuin was used as a control sialylated glycoprotein. +/- SD.

Microarray profiles were nearly identical between technical replicates from two independent samples (*Sm*EV1 and *Sm*EVc; Figure 3A). We compared our analysis to the EV

microarray profile of another trematode, *Fasciola hepatica*, obtained by de la Torre-Escudero *et al* [21]. A two-dimensional hierarchical clustering of scale-normalised lectin microarray profile data for all technical replicates of the two trematode genera highlighted the different abundances and/or combinations of carbohydrate moieties present on the surface of their respective EVs (Figure 3A). The four mannose-binding lectins which displayed strong affinity with *S. mansoni* EVs, Calsepa, NPA, GNA and HHA maintained a comparably strong signal with *F. hepatica* EVs, suggesting a similar abundance of high-mannose glycans for both trematodes (Figures 3A, B). RCA-I and CAA, with specificity for terminal galactose, also had similar affinity for both EV populations (Figures 1A, B). Overall, *F. hepatica* EVs had a slightly wider variety of lectin affinities than *S. mansoni* EVs, but had very low-level interactions with SNA-I (Figure 3), suggesting minimal sialylation in the *F. hepatica* samples, if any.

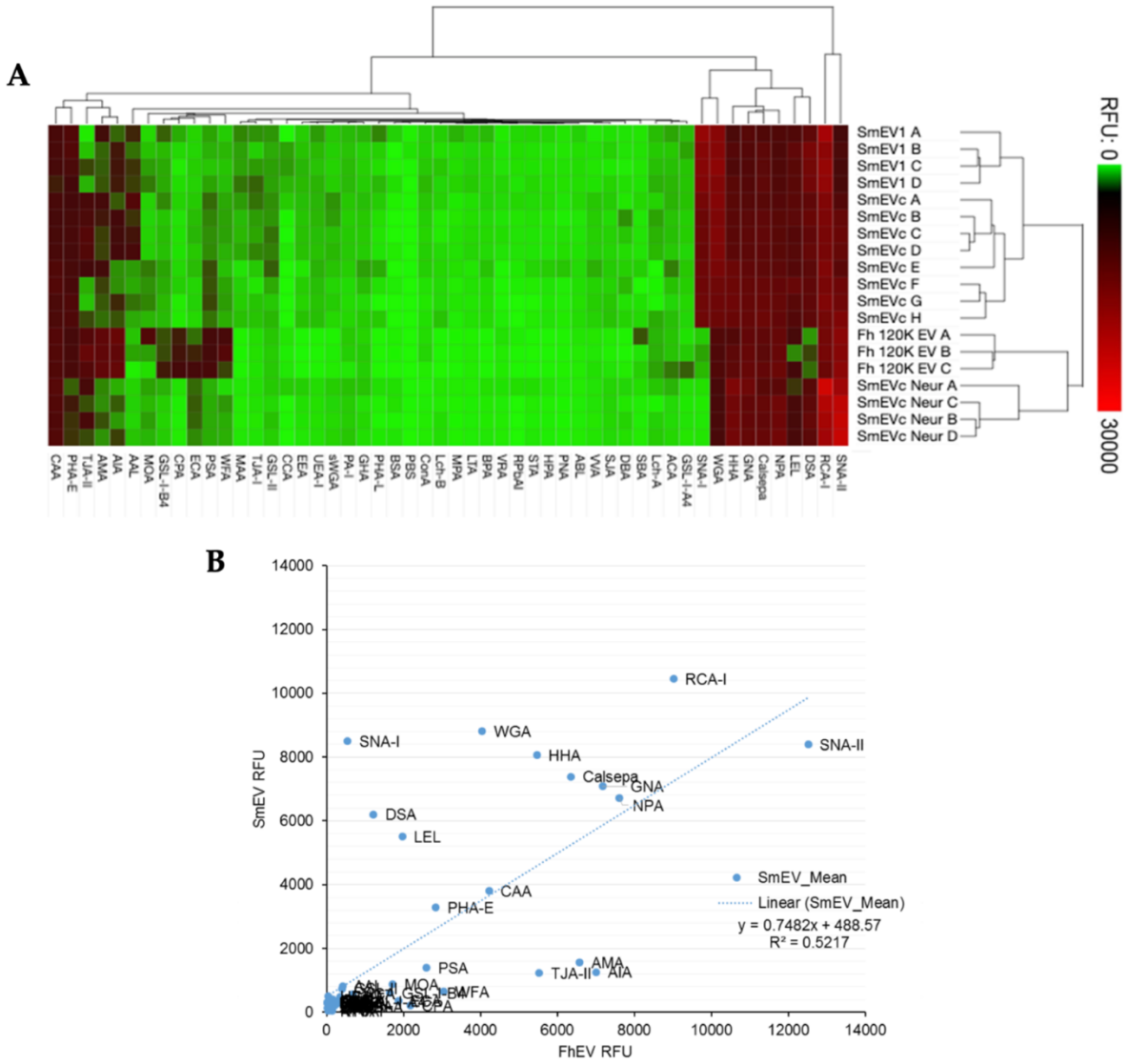

Figure 3. Comparison of *Schistosoma mansoni*-derived EVs and *Fasciola hepatica*-secreted EVs lectin microarray profiles. (a) Heat map and two-dimensional hierarchical clustering of scale-normalised lectin microarray profile data for all twelve *Sm*EV technical replicates, 4 from one EV isolation sample (SmEV1 A-D) and 8 from pooled EV samples (SmEVc A-H), and three *Fh*EV replicates (*Fh* 120k EV A-C) obtained by de la Torre-Escudero *et al*. (2019). Data depicted in heat map was scaled to fit a 0–30,000 RFU window and clustered by average linkage, Euclidean distance method (b) Regression analysis comparing *S. mansoni* EV and *F. hepatica* EV lectin-binding profiles.

3.2 Lectin immunocytochemistry and *in situ* hybridisation reveal sub-tegumental cells as a potential source of EVs in *Schistosoma mansoni*

To identify worm structure(s) as possible sources of EVs, we performed histochemistry on whole adult worms with three lectins that exhibited strong affinity for *S. mansoni* EVs (DSA,

RCA-I and SNA-I) (Figures 4–6). We observed strong labelling of distinct cell bodies immediately below the body wall muscle by SNA-I (Figure 4) and less intense labelling of similar cellular structures by DSA (Figure 5A). We identified these cells as tegumental cell bodies by performing FISH-lectin co-labelling experiments (Figure 7, Figure 8), using a combination of markers previously shown to specifically label definitive tegumental cells (*calp*, *gtp-4*, *npp-5*, *annexin*) [33]. Indeed, 100% of FISH-labelled tegumental cells were positive for SNA-I (Figure 7) and DSA labelling (Figure 8). A few non-tegumental cells at the same level within the worm were also SNA-I positive (possibly tegumental progenitor cells; Figure 7A). Interestingly, we also noted SNA-I nuclear labeling, not only in tegumental cell bodies, but also in nuclei at the same level within the worm (Figure 7B).

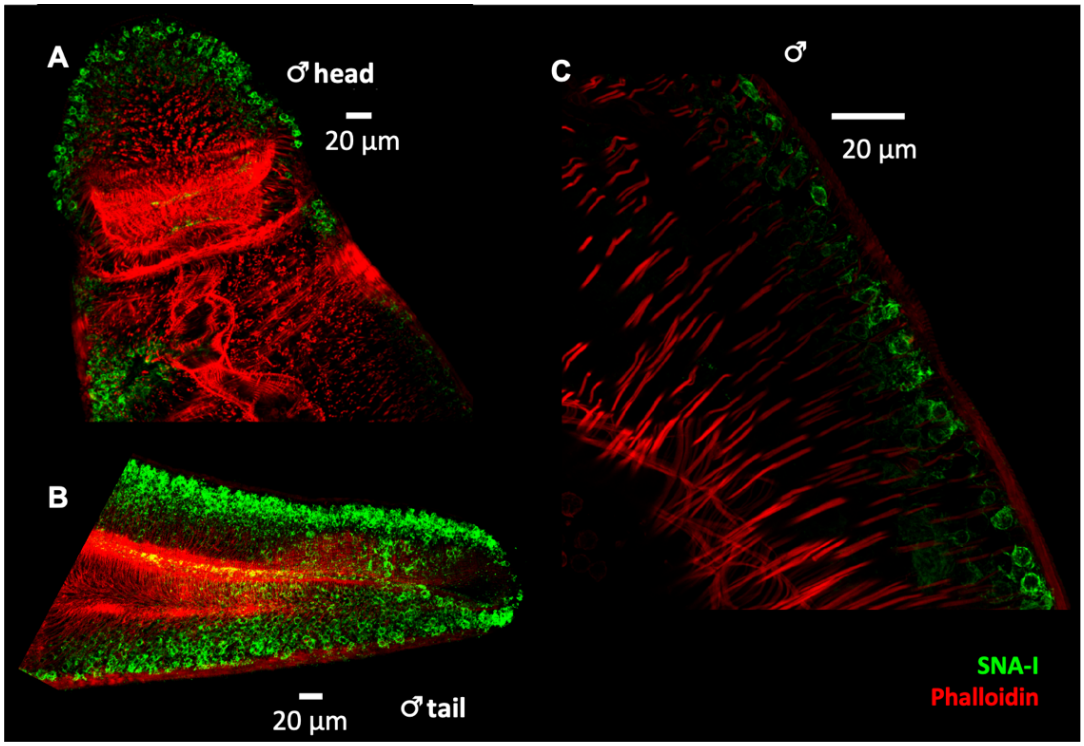

Figure 4. Whole worm labelling with SNA-I lectin. Flat-fixed adult worms were labelled with SNA-I (green) and fluorescent phalloidin (red) and imaged by confocal microscopy (a) Transverse plane through the anterior portion of a male worm and (b) maximum intensity projection of z-stacks acquired at the tail of a male worm. (c) Higher magnification of a transverse plane through the side of the worm.

Higher magnifications of DSA-labelled tegumental cells revealed a granular pattern of DSA localisation (Figure 5B), which could indicate the presence of intraluminal vesicle (ILV)-filled multivesicular bodies (MVBs; Figure 5C). In addition, DSA strongly labelled the gut of the

parasite (Figure 5D, Figure 8), marking the digestive system as a potential route of EV secretion in adult worms.

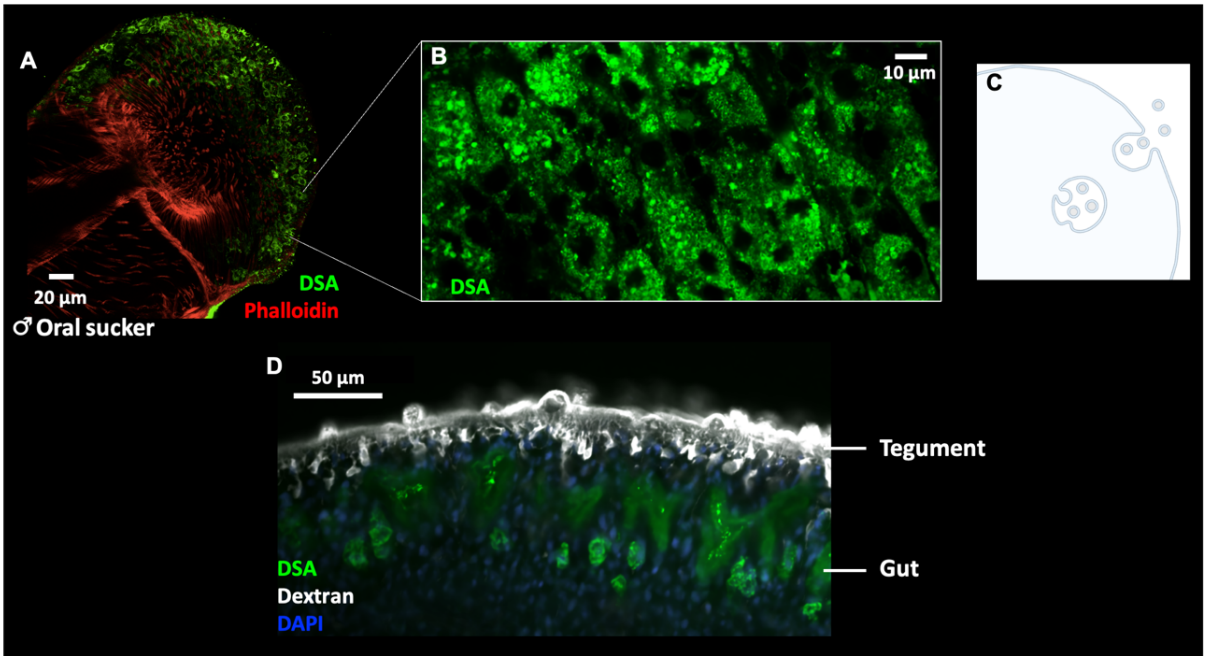

Figure 5. Whole worm labelling with DSA. (a, b) Flat-fixed adult worms were labelled with DSA (green) and fluorescent phalloidin (red) and imaged by confocal microscopy (a) Transverse plane through the oral sucker of a male worm and (b) Higher magnification of cells labelled with DSA show a granular labelling pattern. (c) Cartoon depicting the biogenesis of exosomes, via the formation of intraluminal vesicles within multivesicular endosomes (MVEs) and the subsequent fusion of MVEs with the plasma membrane, thereby releasing exosomes into the extracellular space. (d) Cross-section of a male worm labelled with fluorescent dextran (tegument), DAPI (nuclei) and DSA (green) shows clear labelling of the parasite gut with the lectin.

Finally, RCA-I labelled various structures within the worm, including potential tegumental cells and what appear to be the excretory pore and excretory tubules (Figures 6A–B), which might implicate the excretory system in the release of at least a subset of EVs. RCA-I also labels the esophageal glands (Figure 6C).

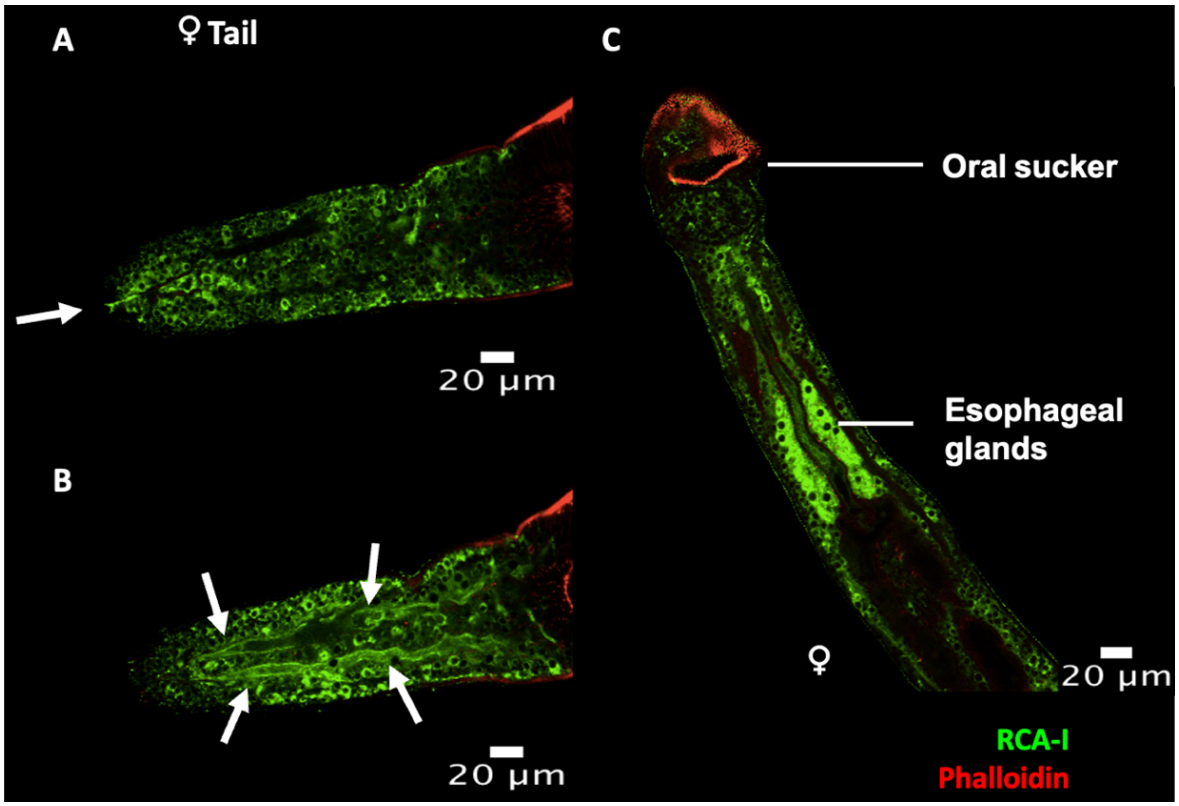

Figure 6. Whole worm labelling with RCA-I. (a – c) Flat-fixed adult worms were labelled with RCA-I (green) and fluorescent phalloidin (red). (a, b) Transverse plane acquired at different levels through the tail of a female worm, arrows indicate (a) the excretory pore and (b) the excretory tubules. (c) Image of the anterior region of a female worm.

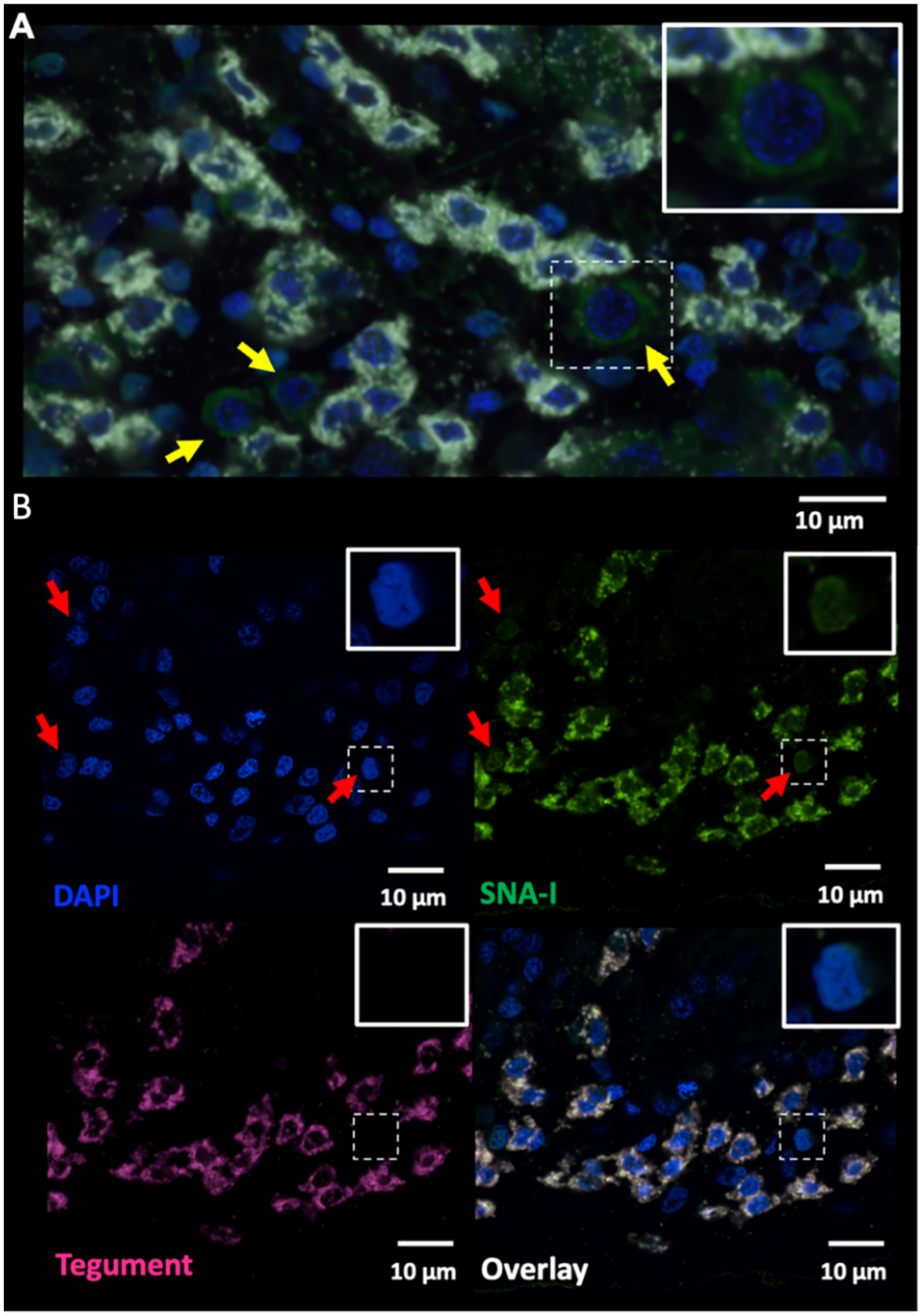

Figure 7. All definitive tegumental cells are SNA-I positive. Fluorescence *in situ* hybridisation with tegument markers (*calpain, gtp-4, annexin, and npp-5*; white), and SNA-I (green) co-labelling. 100% of tegumental cells are also positive for SNA-I labelling, SNA-I also labelled (a) a few non-tegumental cells at the same level within the worm (yellow arrows inset shows enlargement of an SNA-I^{+} non-tegumental cell) and (b) some nuclei (red arrows; insets)

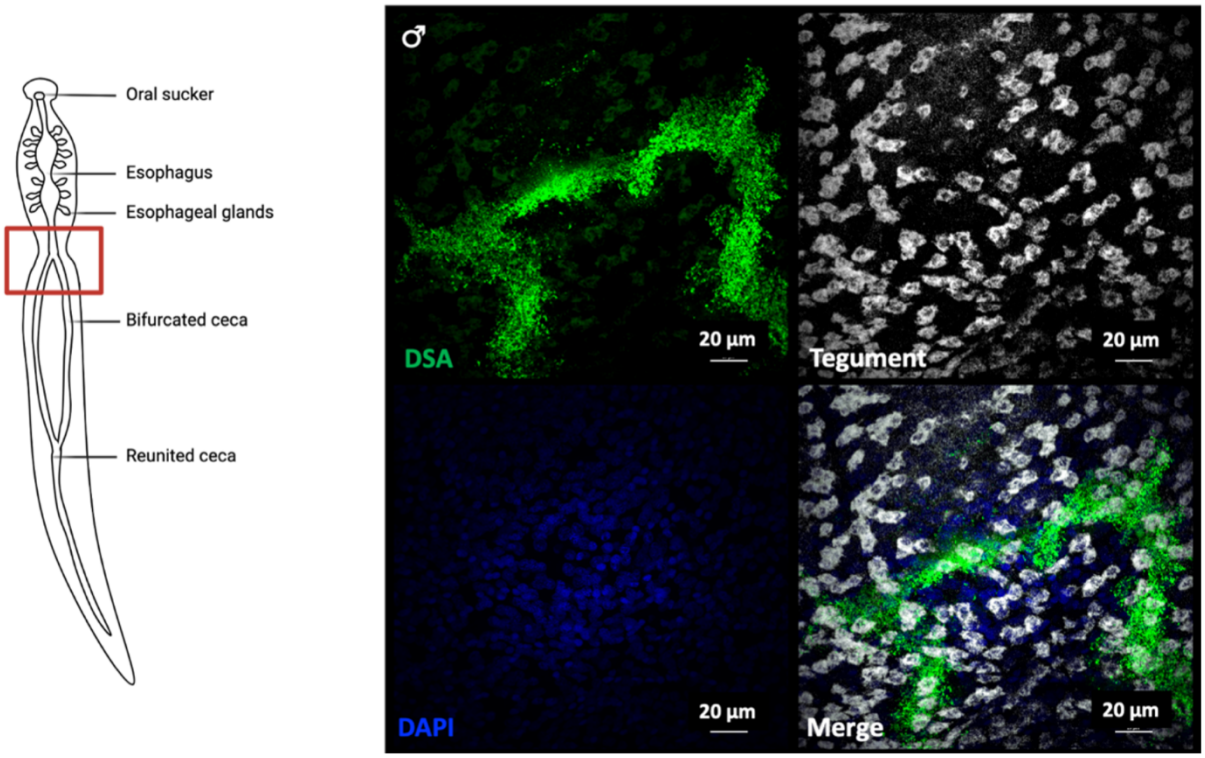

Figure 8: DSA labels definitive tegumental cells and the gut of the parasite. Adult worms were labelled by fluorescence *in situ* hybridisation with tegument markers (*calpain, gtp-4, annexin, and npp-5*), after which they were incubated with fluorescent DSA lectin and imaged via confocal microscopy. Dorsal view of a male worm, showing the bifurcated intestinal ceca, right above the ventral sucker.

4. Discussion

There is growing interest for understanding the role of EVs in the context of parasitic infections. Many groups have studied the protein, miRNA, and even lipid composition of different populations of parasite EVs, providing important information regarding pathways of EV biosynthesis and potential functions [8,12,16,36-39]. Despite mounting evidence pointing to the importance of the glycan composition of EVs [21-23,40], only one study on schistosome EV glycomics has been published [25]. Glycan structures coating the surface of EVs are likely to be functionally important to host-parasite interactions, as they are presumably responsible for delivering parasite-derived molecules to target cells. In this study, we profiled various carbohydrate moieties present on the surface of adult *S. mansoni* EVs via lectin binding. Comparison with lectin profiling of *F. hepatica* EVs [21] revealed a comparable abundance of high-mannose sugars, as well as the presence of terminal galactose on EVs from both trematodes.

Our results also suggest the presence of sialic acid residues on adult *S. mansoni*-derived EVs. Helminths are generally regarded as unable to synthesise sialic acids due to their apparent lack of enzymes required for sialic acid synthesis [28,41]. However, evidence of sialylation has been found in different helminth glycoprotein preparations, such as the *Echinococcus granulosus* hydatid cysts [42], *Taenia crassiceps* metacestodes [43], and *F. hepatica* tegument [44], but these findings have systematically been attributed to the presence of host glycans. The origin of sialic acid in our samples remains unclear (parasite, host, or EV-depleted fetal bovine serum; FBS), but the presence of host or bovine sialylated molecules on *S. mansoni* EVs, despite the many washes and a density sedimentation step, could be relevant, given that *in vivo*, EVs are released in host blood and would be exposed to similar blood proteins. We previously found no evidence of murine or bovine miRNAs in our EV samples [12], suggesting that minimal contamination of schistosome EVs with host or bovine EVs occurred during our EV isolation protocol.

Coating with host glycoproteins could confer EVs protection from host immune effectors, allowing them to travel undetected and interact with target cells to exert their function. Indeed, previous lectin microarray analyses on mammalian EVs revealed an enrichment in specific glycan features, including α2-6 linked sialic acid, compared to the plasma membrane of the producing cells [23]. Additional reports have also linked sialic acid residues and sialic acid receptors to EV uptake [22,24,45] and others have observed the impact of EV desialylation on biodistribution *in vivo* [40], further highlighting the importance of EV glycosylation state. *Fasciola hepatica* EVs, on the other hand, displayed low-level interactions with SNA-I [21], suggesting minimal sialylation. This discrepancy, however, could be explained by the life cycle differences between these parasites; adult *S. mansoni* are found in the blood whilst *F. hepatica* reside in the bile duct. Alternatively, the discrepancy may be due to the fact that liver fluke EVs were collected from parasites cultured without serum [21].

Moreover, given their avidity for *S. mansoni* EVs, we employed SNA-I, DSA and RCA-I in lectin immunofluorescence studies to locate site(s) of EV secretion in adult parasites. SNA-I and DSA both label sub-tegumental cell bodies in adult *S. mansoni*. In addition, we noted SNA-I labelling in a small number of non-definitive tegumental cells as well as some SNA-I positive nuclei at the same level within the worm. Significantly, we observed distinct granular structures

in DSA-labelled tegumental cells, perhaps representing future exosomes encapsulated within MVBs, although it is important to note that the size of EVs is below the level of resolution of the confocal instruments used. Besides accumulating within tegument cells, DSA also strongly labelled the gut of the parasite. Lastly, RCA-I labelled various regions of the worm, possibly including tegumental cells, although co-localisation experiments have not been conducted in this case. RCA-I also accumulated in what appears to be the excretory pore and the excretory tubules and around the esophageal glands. Together, these results may indicate the presence of multiple EV sub-populations and the involvement of the tegument and the digestive and protonephridial (excretory) systems in the release of EVs from adult schistosomes. A previous study on adult *F. hepatica* identified the excretory system as the source of small EVs (EVs sedimenting at 120k × *g*; 120k EVs) and gastrodermal epithelial cells lining the gut as the source of large EVs (EVs sedimenting at 15k × *g*; 15k EVs) [21]. The same group also reported localisation of RAL-A (one of two markers used for 120k EVs) in the tegument syncytium and sub-tegumental cells [21]. It is important to consider that the localisation studies performed by de la Torre-Escuerdo et al. were done using antisera raised against markers for specific EV sub-populations (120k and 15k EVs) as their protein content differed, but that lectin microarray profiles of the 120k and 15k EVs were nearly identical. Comparable EV sub-populations (120k and 15k) have also been identified in adult *S. mansoni* [38]; it is thus fair to expect that lectin profiling could be similar between our EV samples and larger 15k EVs. That being said, it is possible that different EV sub-populations have different origins within the parasite, as observed in *F. hepatica*, and that our EV population of interest may only be released by one of the identified structures in our lectin histochemistry experiments.

To better understand the role of EVs in the course of infection, we must start with unravelling their mechanism of action, including how they travel within the host to reach their target cells and how they interact with these cells. EV surface-coating molecules such as glycans are highly likely to be key players in these important steps. Thus, their characterisation is crucial and could lead to the development of novel therapeutic interventions. In addition, glycans present on parasite-derived EVs may be used as biomarkers for the development of superior diagnostic tools.

Author Contributions

Conceptualisation, M.D., J.Q.G., T.G.G. and T.L.; formal analysis, M.D., J.Q.G.; investigation, M.D., J.Q.G. and G.R.W.; resources, L.A., A.M., J.J.C.; writing—original draft preparation, M.D.; writing—review and editing, J.Q.G., G.R.W., J.J.C., L.A., A.M., T.G.G., T.L.; visualisation, M.D., J.Q.G.; supervision, T.G.G., J.Q.G., L.A., A.M., T.L.; project administration, T.G.G., T.L.; funding acquisition, J.J.C., T.G.G., T.L. All authors have read and agreed to the published version of the manuscript.

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Supplementary Material

No	Lectin	Organism	Common name	Specificity
1	AIA Jacalin	*Artocarpus intergrifolia*	Jack fruit lectin	Gal (sialylation tolerant)
2	RPbAI	*Robinia pseudoacacia*	Black locust lectin	Gal, GalNAc
3	SNA-II	*Sambucus nigra*	Sambucus lectin-II	Gal/GalNAc
4	SJA	*Sophora japonica*	Pagoda tree lectin	β-GalNAc
5	DBA	*Dolichos biflorus*	Horse gram lectin	GalNAc
6	GHA	*Glechoma hederacea*	Ground ivy lectin	GalNAc
7	SBA	*Glycine max*	Soy bean lectin	GalNAc
8	VVA	*Vicia villosa*	Hairy vetch lectin	GalNAc
9	BPA	*Bauhinia purpurea*	Camel's foot tree lectin	GalNAc/Gal
10	WFA	*Wisteria floribunda*	Japanese wisteria lectin	GalNAc/sulfated GalNAc
11	HPA	*Helix pomatia*	Garden snail lectin	α-GalNAc
12	GSL-I-A4	*Griffonia simplicifolia*	Griffonia lectin-I A4	GalNAc
13	ACA	*Amaranthus caudatus*	Amaranthin	Sialylated/Gal-β-(1,3)-GalNAc
14	ABL	*Agaricus bisporus*	Edible mushroom lectin	Gal-β-(1,3)-GalNAc, GlcNAc
15	PNA	*Arachis hypogaea*	Peanut lectin	Gal-β-(1,3)-GalNAc
16	GSL-II	*Griffonia simplicifolia*	Griffonia /Bandeiraea lectin-II	GlcNAc
17	sWGA	*Triticum vulgaris*	Succinyl WGA	GlcNAc
18	DSA	*Datura stramonium*	Jimson weed lectin	GlcNAc
19	STA	*Solanum tuberosum*	Potato lectin	GlcNAc oligomers
20	LEL	*Lycopersicum eculentum*	Tomato lectin	GlcNAc-β-(1,4)-GlcNAc
21	Calsepa	*Calystegia sepium*	Bindweed lectin	Man/Maltose
22	NPA	*Narcissus pseudonarcissus*	Daffodil lectin	α-(1,6)-Man
23	GNA	*Galanthus nivalis*	Snowdrop lectin	Man-α(1,3)-
24	HHA	*Hippeastrum hybrid*	Amarylis agglutinin	Man-α(1,3)-Man-α(1,6)-
25	ConA	*Canavalia ensiformis*	Jack bean lectin	Man, Glc, GlcNAc
26	Lch-B	*Lens Culinaris*	Lentil isolectin B	Man, fucose dependent
27	Lch-A	*Lens Culinaris*	Lentil isolectin A	Man, fucose dependent
28	PSA	*Pisum sativum*	Pea lectin	Man, fucose dependent
29	TJA-I	*Trichosanthes japonica*	TJA One	Sialic acid-α-(2,6)-Gal(NAc)
30	WGA	*Triticum vulgaris*	Wheat germ agglutinin	NeuAc/GlcNAc
31	MAA	*Maackia amurensis*	Maackia agglutinin	Sialic acid-α-(2,3)-Gal(NAc)
32	SNA-I	*Sambucus nigra*	Sambucus lectin-II	Sialic acid-α-(2,6)-Gal(NAc)
33	CCA	*Cancer antennarius*	California crab	9-O -acetyl-sialic acid
34	PHA-L	*Phaseolus vulgaris*	Kidney bean leukoagglutinin	tri-/tetra-antennary β-Gal/Gal-β-(1,4)-GlcNAc
35	PHA-E	*Phaseolus vulgaris*	Kidney bean erythroagglutinin	biantennary, bisecting GlcNAc,β-Gal/Gal-β-(1,4-)GlcNAc
36	RCA-I/120	*Ricinus communis*	Castor bean lectin	Gal-β-(1,4)-GlcNAc
37	AMA	*Arum maculatum*	Lords and Ladies agglutinin	Gal-β-(1,4)-GlcNAc
38	CPA	*Cicer arietinum*	Chickpea lectin	Complex glycopeptides
39	CAA	*Caragana arborescens*	Pea tree lectin	Gal-β-(1,4)-GlcNAc
40	ECA	*Erythrina cristagalli*	Cocks comb/coral tree lectin	Gal-β-(1,4)-GlcNAc oligomers
41	TJA-II	*Trichosanthes japonica*	TJA Two	Fuc-α(1,2)Gal(NAc)-β(1,4)
42	AAL	*Aleuria aurantia*	Orange peel fungus lectin	α-(1,6 and 1,3)-linked Fuc
43	LTA	*Lotus tetragonolobus*	Lotus lectin	α-(1,3)-linked Fuc
44	UEA-I	*Ulex europaeus*	Gorse lectin-I	α-(1,2)-linked Fuc
45	PA-I	*Pseudomonas aeruginosa*	Pseudomonas lectin	α-Gal, Gal derivatives
46	EEA	*Euonymous europaeus*	Spindle tree lectin	α-Gal
47	GSL-I-B4	*Griffonia simplicifolia*	Griffonia /Bandeiraea lectin-I	α-Gal
48	MPA	*Maclura pomifera*	Osage orange lectin	α-Gal
49	VRA	*Vigna radiata*	Mung bean lectin	α-Gal
50	MOA	*Marasmius oreades*	Fairy ring mushroom lectin	α-Gal
51	PBS	N/A	N/A	N/A
52	BSA	N/A	N/A	N/A

Table S1. Lectin array v2.4.0 print list with coarse specificity information for each lectin.

Connecting Statement I

In this first manuscript, the terminal carbohydrate structures of *Schistosoma mansoni* EVs were profiled by lectin microarray. Three of the lectins exhibiting strong adhesion to EVs were used to localise sites of EV release from adult *S. mansoni* by lectin histochemistry and the identity of these structures was confirmed by coupling lectin histochemistry and *in situ* hybridisation experiments. These localisation experiments highlighted sub-tegumental cell bodies, as well as the digestive and excretory systems, as potential players in the release of EVs.

Interestingly, SNA-I, one of the lectins displaying high avidity for *S. mansoni* EVs, recognises structures with terminal sialic acid residues, suggesting the presence of sialic acid residues on schistosome EVs. The occurrence of sialylated molecules was validated by additional testing. This finding led to several more questions since helminths are generally regarded as unable to synthesise sialic acid. In the following chapter, I thus sought to investigate the origin of sialic acid present in our samples. Sialic acid-terminated glycans play important roles in infection by, for example, aiding immune evasion and affecting target recognition and cell entry. I therefore explored the hypothesis that they may be involved in masking antigens on EVs, thereby contributing to immune evasion.

Chapter IV. Manuscript II

Sugar coating: Utilisation of host serum sialoglycoproteins by *Schistosoma mansoni* as a potential immune evasion mechanism

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Abstract

Parasitic helminths resort to various mechanisms to evade and modulate their host's immune response, several of which have been described for *Schistosoma mansoni*. We recently reported the presence of sialic acid residues on the surface of adult *S. mansoni* extracellular vesicles (EVs). We now report that these sialylated molecules are mammalian serum proteins. In addition, our data suggest that most sialylated EV-associated proteins do not elicit a humoral response upon injection into mice, or in sera obtained from infected animals. Sialic acids frequently terminate glycans on the surface of vertebrate cells, where they serve important functions in physiological processes such as cell adhesion and signaling. Interestingly, several pathogens have evolved ways to mimic or utilise host sialic acid beneficially by coating their own proteins, thereby facilitating cell invasion and providing protection from host immune effectors. Together, our results indicate that *S. mansoni* EVs are coated with host glycoproteins, which may contribute to immune evasion by masking antigenic sites, protecting EVs from removal from serum and aid in cell adhesion and entry to exert their functions.

1. Introduction

Schistosomiasis, a chronic disease affecting ~240 million people worldwide and resulting in significant morbidity and mortality, is caused by infection with parasitic platyhelminths of the genus *Schistosoma* [1]. The infective stage is a short-lived larva (cercaria), which is released from freshwater snails. Cercariae infect human hosts by penetrating the skin and morphing into maturing larvae (schistosomula), which enter the host's circulatory system and migrate to the hepatic portal system and mesenteries (*S. mansoni*, *S. japonicum*) or the venous plexus of the urinary bladder (*S. haematobium*). Larvae mature during the migration to eventually become adult male and female worms, which pair up and produce large numbers of eggs [2]. Eggs are destined to be released into the environment through faeces or urine, but many remain trapped in the liver and surrounding tissues, thereby causing tissue fibrosis and organ damage. A particularity of schistosomes is their ability to establish long-term chronic infections [2]. If left untreated, adult worms can survive within a mammalian host for many years [3]. Their longevity is attributable, in part, to their remarkable capacity to manipulate host immunity [4,5]. There is a growing need to better characterise and understand the specific and carefully orchestrated molecular dialogue employed by the parasites to modulate the host immune system, as it could lead to the identification of novel diagnostic and therapeutic targets. Parasite excreted/secreted products (ESPs) are widely regarded as key players in these host–parasite interactions with increasing attention being paid to vesicle-based secretion. We, and others, have previously reported the release of extracellular vesicles (EVs) in schistosomes [6-13]. The contents of EVs have been partially characterised in eggs [13], larvae [7], and adult schistosomes [6,8,14,15], showing enrichment in putative effectors such as proteins and microRNAs (miRNAs). It is only recently, however, that attention has been given to the carbohydrate structures (glycans) present on the surface of schistosome EVs [10,16]. There is increasing appreciation for the roles of glycans in EV biology, with reports suggesting the importance of carbohydrate moieties in interactions between EVs and target cells [10,17-20]. Despite these findings, limited information is available on schistosome EV glycomics and its role in infection [10,16].

Recently, we reported the presence of sialic acid residues on the surface of EVs released from adult *Schistosoma mansoni* in culture [16]. Moreover, we observed the bright labelling of sub-tegumental cells bodies by SNA-I, a plant-derived lectin that recognises glycan molecules with terminal sialic acid residues [16]. Sialic acids are a family of nine-carbon carboxylated sugars,

which are common terminal monosaccharides of vertebrate glycans. They have mainly been identified in the animal kingdom, from the echinoderms onwards, with lower animals, including helminths, assumed to lack sialic acids [21-23]. We performed mass spectrometry analyses on sialylated EV and worm proteins to determine the origin of the sialylated molecules, and identified them as mammalian serum proteins. Given that, in vivo, worms reside in host venules and are thus exposed to blood proteins, this finding could indicate a novel immune evasion mechanism employed by the parasite. Incorporation of sialylated host glycoconjugates by *S. mansoni* is of great interest, as sialic acids play important roles in infection by, for example, aiding immune evasion and affecting target recognition and cell entry. Finally, we tested the antigenic profile of EV proteins and found that sialylated glycoproteins (sialoglycoproteins) did not elicit a humoral response in mice, supporting our hypothesis that sialic acid residues contribute to antigen masking.

2. Materials and Methods

2.1 Parasites

Adult *S. mansoni* were obtained by cardiac perfusion of infected female CD1 mice 7 weeks after infection as per approval from the McGill University Animal Care Committee (Permit # 2019–8138). Parasites were cultured as previously described [6,16]. Briefly, worms were washed 3 times in sterile RPMI-1640 for 15 min and subsequently maintained in RPMI-1640 medium supplemented with 100 U penicillin, 100 mg/mL streptomycin, and 10% EV-depleted FBS (Life Technologies, Burlington, ON, Canada; Ref: 16000). EV depletion was performed in advance by ultracentrifugation of FBS under sterile conditions for 18 h at 120,000 × *g* and 4°C, followed by filtration through 0.22 μm hydrophilic PVDF Durapore membranes (EMD Millipore, Billerica, MA, USA; SVGV01015) [24]

2.2 Isolation of adult *Schistosoma mansoni* extracellular vesicles

EVs were isolated as previously described [6,16]. Briefly, parasite-conditioned culture medium was collected after 48 and 72 h of culture and centrifuged at increasing speeds, 300 × g/15 min, 700 × g/15 min, and 3000 × g/15 min, to remove larger debris, following which the supernatant was centrifuged at 12,000 × g for 45 min. Supernatants were filter-sterilised using a 0.22 μm hydrophilic PVDF Durapore membrane (EMD Millipore; SVGV01015) and centrifuged

at 100,000 × g for 2 h using a Beckman-Coulter SW-48 rotor on a Beckman-Coulter ultracentrifuge. The pellet was re-suspended in sterile cell culture grade GIBCO®® Dulbecco's Phosphate-Buffered Saline (DPBS; Life Technologies) and loaded onto a sucrose step gradient (25%, 30% and 35%), then centrifuged at 120,000 × g for 18 h using an SW-48 rotor. Following centrifugation, the 30% sucrose fraction was collected and washed by diluting 4–5-fold with sterile DPBS, and EVs were re-pelleted by centrifugation in an SW-28 rotor at 100,000 × g for 2 h. Pellets were washed by re-suspension in DPBS and centrifuged in an SW-41 rotor at 100,000 × g for 2 h. Finally, pellets were re-suspended in 1.0 mL of DPBS, transferred to 1.5 mL Beckman-Coulter ultracentrifuge tubes and centrifuged in an Optima TL100 tabletop ultracentrifuge (Beckman-Coulter, Mississauga, ON, Canada) for 1.5 h at 100,000× g using a TLA-100.3 rotor. EVs were quantified based on protein concentration using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Burlington, ON, Canada) and absorbance-measured at 562 nm using a Synergy H4 Hybrid plate reader. The pelleted material was snap-frozen and stored at −80 °C until use.

2.3 Neuraminidase treatment

All neuraminidase treatments were carried out using a broad-spectrum α2–3,6,8,9 Neuraminidase A (New England Biolabs P0722, Ipswich, MA, USA). Proteins (50 μg EV samples) were incubated with 40 units (2 μL) neuraminidase overnight at 37 °C according to the manufacturer's protocol and assayed immediately. 10 μg fetuin (New England Biolabs P0722, Ipswich, MA, USA) was used as a control sialylated glycoprotein.

2.4 Lectin-probed Western blot assay and extraction of reactive bands

Samples were mixed with Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris HCl) and incubated at 95°C for 5 min. Aliquots were resolved by electrophoresis through 4–15% Mini-PROTEAN®® TGX™ Precast Protein Gels (Bio-Rad, Mississauga, ON, Canada) and transferred to a PVDF membrane according to standard protocols. Five μg fetuin (New England Biolabs P0722, Ipswich, MA, USA) was used as a control sialylated glycoprotein. Blots were blocked overnight at 4 °C in blocking buffer (1X Tween 20 (TBST), 0.1% Tween 20, 5% BSA) and probed with biotinylated SNA-I (1:200 in PBS; Biolynx B-1305, Brockville, ON, Canada), after which they were incubated with streptavidin–horseradish peroxidase (1:5000 in blocking buffer; abcam ab7403, Cambridge, MA,

USA). Blots were developed using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) following the manufacturer's protocol and imaged with a ChemiDoc MP Imaging System (Bio-Rad). For mass spectrometry analysis, freshly run gels were rinsed 3 times for 5 min with 100 mL of deionised water and stained by immersion in SimplyBlue™ SafeStain (Life Technologies, Burlington, ON, Canada) for 1 hr at room temperature with gentle shaking. The staining solution was discarded and the gel washed with 100 mL of water for 2 h. The two most prominent bands that lost reactivity with SNA-I in neuraminidase-treated samples were excised in the neuraminidase-treated group and de-stained with 30% ethanol. Bands were rinsed in ultrapure water and processed and analysed by MS/MS at the Plate-forme protéomique CHU de Quebec as described below.

2.5 Lectin pulldown

Streptavidin-conjugated resin beads (Novagen 69203–3, San Diego, CA, USA) were incubated with 250 µg biotinylated lectin SNA-I for 1 h on a rotating incubator at 4 °C. Beads were washed 10X with PBS in Pierce™ Spin Columns (ThermoFisher Scientific, 69705). Lectin-beads were incubated with lysed EVs. Briefly, 155 µg EVs were lysed in PBS, 0.1% Triton X-100, diluted 10-fold in PBS and centrifuged at 3000 × g for 3 min. The supernatant was collected, added to the SNA-I-conjugated beads and incubated overnight at 4 °C on a rotating incubator. Beads were washed 10 times with PBS followed by 5 washes with 50 mM ammonium bicarbonate (pH 7.4) and sent to the CHU de Quebec Research Center for mass spectrometry analysis.

2.6 Protein digestion

Protein digestion and mass spectrometry experiments were performed by the Proteomics platform of the CHU de Quebec Research Center, Quebec, Canada. Proteins in bands extracted from gels were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 126 nM of modified porcine trypsin (sequencing grade, Promega, Madison, WI) at 37 °C for 18 h. Digestion products were extracted using 1% formic acid and 2% acetonitrile followed by 1% formic acid and 50% acetonitrile. The recovered extracts were pooled, vacuum-centrifuge-dried and then resuspended in 10 µL of 0.1% formic acid, and 5 µL was analysed by mass spectrometry. On beads, protein digestion was carried out using 0.1µg modified porcine trypsin (sequencing grade, Promega, Madison, WI) in 50 mM ammonium bicarbonate for

5 h at 37 °C. Digestion was stopped with 5% formic acid (FA), and peptides were eluted from the beads with 60% acetonitrile (ACN) 0.1% FA. Tryptic peptides were desalted on Stage tips (Empore C18, 3 M Company), vacuum-dried, and then resuspended in LC loading solvent (2% ACN, 0.05% trifluoroacetic acid (TFA)).

2.7 Mass spectrometry

Half of the amount of each sample was analysed by nanoLC/MSMS using a Dionex UltiMate 3000 nanoRSLC chromatography system (Thermo Fisher Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA,USA) equipped with a nanoelectrospray ion source. Peptides were trapped at 20 µL/min in loading solvent (2% ACN, 0.05% TFA) on a 5 mm × 300 µm C18 pepmap cartridge (Thermo Fisher Scientific) during 5 min. Then, the pre-column was switched online with a 50 cm × 75µm-internal-diameter separation column (Pepmap Acclaim column, ThermoFisher), and the peptides were eluted with a linear gradient from 5–40% solvent B (A: 0.1% FA, B: 80% ACN, 0.1% FA) in 30 min, at 300 nL/min (60 min total runtime). Mass spectra were acquired using a data-dependent acquisition mode using Thermo XCalibur software version 4.1.50. Full-scan mass spectra (350 to 1800 m/z) were acquired in the orbitrap using an AGC target of 4e5, a maximum injection time of 50 ms, and a resolution of 120 000. Internal calibration using lock mass on the m/z 445.12003 siloxane ion was used. Each MS scan was followed by acquisition of fragmentation MSMS spectra of the most intense ions for a total cycle time of 3 s (top speed mode). The selected ions were isolated using the quadrupole analyzer with 1.6 m/z windows and fragmented by Higher energy Collision-induced Dissociation (HCD) with 35% of collision energy. The resulting fragments were detected by the linear ion trap at a rapid scan rate with an AGC target of 1e4 and a maximum injection time of 50 ms. Dynamic exclusion of previously fragmented peptides was set for a period of 30 sec and a tolerance of 10 ppm.

2.8 Database searching

MGF peak list files were created using Proteome Discoverer 2.3 software (Thermo). MGF files were then analysed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search a contaminant database and Uniprot Bos taurus (37885 entries, UP000009136), Mus musculus (63738 entries, UP000000589), and Schistosoma mansoni (16227 entries,

UP000008854) databases assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified in Mascot as variable modifications. Two missed cleavages were allowed.

2.9 Criteria for protein identification

Scaffold (version Scaffold_4.8.4, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. A false discovery rate of 1% was used for peptide and protein. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides in two independent experiments.

2.10 Soluble worm antigenic preparation

SWAP was prepared as previously described [16]. Approximately 30 freshly perfused adult worms were freeze–thawed in 150 μL lysis buffer (1X DPBS, 0.5% Triton X-100) with cOmplete™ Mini Protease Inhibitor Cocktail (Roche, Laval, QC, Canada), homogenised using a pestle, and centrifuged at 4000 × g for 30 min at 4 °C. The supernatant was collected and used as SWAP. SWAP concentration was determined using a Pierce BCA Protein Assay Kit as above.

2.11 Mouse infection and vaccination

CD1 mice were obtained as described above. Naïve CD1 mice received an intra-peritoneal (IP) injection of 200 μL cell culture grade PBS (Thermo Fisher Scientific) with or without 15 μg EV protein and received the same immunization regimen 2 weeks later. The experimental group and control group comprised 5 mice each. In both cases, mice were euthanised, and serum collected from blood was obtained by cardiac puncture.

2.12 Immunogenicity Western blot assays

To characterise immunogenic components, purified EVs or SWAP were mixed with Laemmli buffer [25] and incubated at 95°C for 5 min. Aliquots were resolved by electrophoresis through a 10% acrylamide SDS-PAGE gel and transferred to a nitrocellulose (or PVDF) membrane according to standard protocols. Antigenic EV proteins were detected using control uninfected mouse serum, vaccinated mouse serum (see below), or serum from infected mice obtained 2, 4, or 6 weeks post-infection. Goat anti-mouse IgG-HRP conjugate (Santa Cruz Biotechnology, Dallas, TX, USA, sc-2354, Lot L0312) was used as the secondary antibody. Blots were developed using Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific) following the manufacturer's protocol.

3. Results

3.1. Lectin-probed Western blots and mass spectrometry analysis of reactive bands

We previously reported that several lectins exhibit strong adhesion to adult S. mansoni EVs, including the sialic-acid-binding SNA-I [16]. Treatment of EV samples with a broad-spectrum neuraminidase markedly reduced SNA-I binding compared to the strong signal observed with untreated control samples following gel electrophoresis (Figure 1), supporting our previous findings and suggesting the presence of sialylated molecules in our EV samples [16]. To determine the origin of sialic acid residues (parasite or host), we sought to identify sialylated proteins. First, we excised two of the most prominent bands (roughly 55 kDa and 100 kDa) in the neuraminidase-treated group, which strongly interacted with SNA-I in the control group (Figure 1) for analysis by mass spectrometry. We found a mixture of S. mansoni proteins and mammalian serum proteins in each of these bands, potentially suggesting a host origin of sialic acid. The mammalian serum glycoproteins included alpha-2-macroglobulin, albumin, alpha-1-antiproteinase, and Inter-alpha-trypsin inhibitor heavy chain H3 (Table 1), all of which are sialylated or have known sialylated variants [26-30]. Similar protein profiles were found in size-matched bands from untreated samples (data not shown), indicating that neuraminidase treatment does not affect protein content.

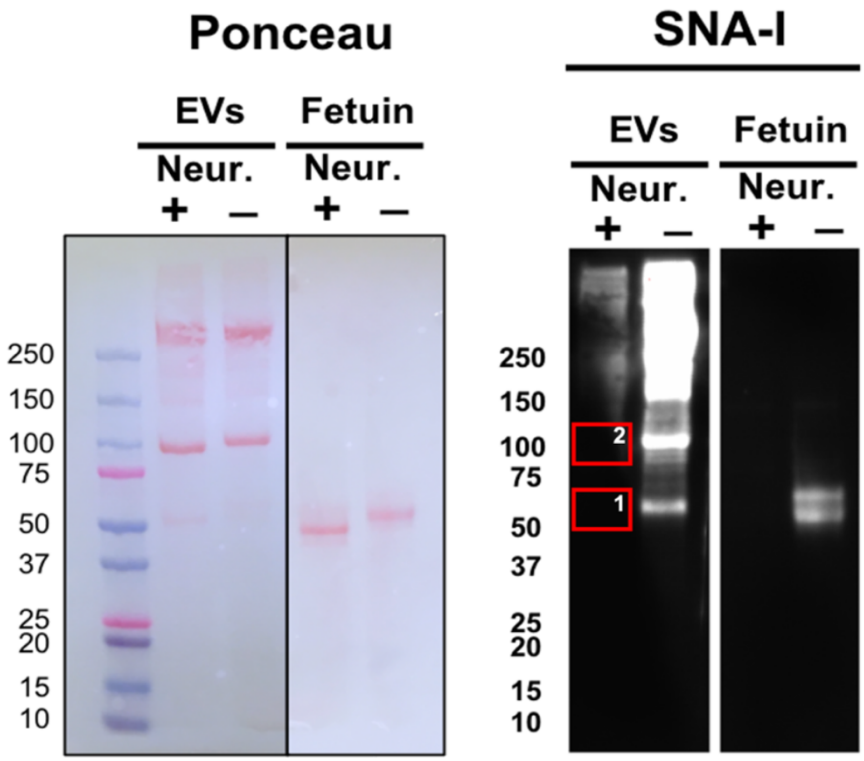

Figure 1. Treatment of *S. mansoni* EVs with neuraminidase (Neur) reduces SNA-I binding. EVs were incubated with a broad-spectrum neuraminidase, after which samples were resolved by electrophoresis, transferred to a PVDF membrane, and probed with SNA-I. Fetuin was used as a control sialylated glycoprotein. Ponceau-stained PVDF membrane and SNA-I-probed membrane. Two gel sections (corresponding to boxes 1 and 2) were excised and analysed by mass spectrometry.

		Total Spectrum Count	
Accession Number	**Identified Proteins**	**Band 1**	**Band 2**
A0A140T897	Albumin [*Bt*]	17	233
P34955	Alpha-1-antiproteinase [*Bt*]	7	62
F1MM86 (+1)	Complement component C6 [*Bt*]		13
Q7SIH1	Alpha-2-macroglobulin [*Bt*]	4	8
A0A3Q1MU98 (+1)	Complement component C9 [*Bt*]	4	
A0A3Q1NJR8	Antithrombin-III [*Bt*]		7
A0A3Q1LQ21 (+1)	Inter-alpha-trypsin inhibitor heavy chain H3 [*Bt*]		6
G3X6N3	Beta-1 metal-binding globulin [*Bt*]	3	2

Table 1. The most abundant proteins detected in excised bands 1 and 2. Bands were analysed by mass spectroscopy, and MS spectra were searched against the *Schistosoma mansoni, Mus musculus*, and *Bos taurus* Uniprot databases. *Bt, Bos taurus*.

3.2. Identification of glycosylated proteins

To confirm that EV-associated proteins that bind SNA-I are indeed mammalian proteins, we performed a lectin pull-down assay on EV lysate. Lysed EVs were incubated with biotinylated SNA-I-coated beads, and MS spectra were searched against the Schistosoma mansoni, Mus musculus, and Bos taurus Uniprot databases. This experiment pulled down a total of 20 mammalian proteins, 15 of which were bovine serum proteins (Table 2). These include alpha-2-macroglobulin, alpha-1-antiproteinase, clusterin, hemoglobin, bovine complement proteins, and alpha-2-HS-glycoprotein (Table 2). The five other proteins identified, actin, talin, glyceraldehyde-3-phosphate dehydrogenase, vitronectin, and filamin, have all previously been identified in exosomes from various species [6,31-34].

Table 2. Proteins detected in the SNA-I pull-down experiment.

UniProt Accession Number	Identified protein	Total spectrum count	# unique peptides
A2MG_BOVIN	Alpha-2-macroglobulin [*Bt*]	65	38
A1AT_BOVIN	Alpha-1-antiproteinase [*Bt*]	46	18
F1MJK3_BOVIN	Uncharacterized protein LOC506828 [*Bt*]	13	10
CLUS_BOVIN	Clusterin [*Bt*]	10	9
A0A3Q1LK49_BOVIN	Inter-alpha-trypsin inhibitor heavy chain H2 [*Bt*]	10	9
HBBF_BOVIN	Hemoglobin fetal subunit beta [*Bt*]	10	8
A0A3Q1M2B2_BOVIN	Complement C3 [*Bt*]	7	6
A0A3Q1LVV7_BOVIN	Fibrinogen alpha chain [*Bt*]	6	6
FETUA_BOVIN	Alpha-2-HS-glycoprotein [*Bt*]	6	6
A0A3Q1LQ21_BOVIN	Inter-alpha-trypsin inhibitor heavy chain H3 [*Bt*]	6	6
ACTB_BOVIN	Actin, cytoplasmic 1 [*Bt*]	6	5
A0A3Q1MLQ7_BOVIN	Talin 1 [*Bt*]	6	5
A0A3Q1LKN2_BOVIN	Thyroglobulin [*Bt*]	6	4
A0A140T881_BOVIN	Apolipoprotein E [*Bt*]	3	3
A0A3Q1LGY9_BOVIN	Angiotensinogen [*Bt*]	3	3
APOA1_BOVIN	Apolipoprotein A-I [*Bt*]	3	3
A2AP_BOVIN	Alpha-2-antiplasmin [*Bt*]	3	3
G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase [*Bt*]	3	3
Q3ZBS7_BOVIN	Vitronectin [*Bt*]	2	2
B7FAU9	Filamin, alpha [*Mm*]	2	2

MS spectra were searched against the *Schistosoma mansoni, Mus musculus*, and *Bos taurus* Uniprot databases. *Bt*, *Bos taurus*; *Mm*, *Mus musculus*.

3.3. Sialylated EV proteins do not elicit a humoral response in the murine host

To investigate our hypothesis that EV-associated mammalian sialoglycoconjugates contribute to antigenic escape (e.g., antibody recognition), we assessed the antigenic profile of *S. mansoni* EV proteins by Western blot analysis (Figure 2) and compared these findings with the profile of sialylated proteins (Figure 1). We probed EV lysate with sera from mice vaccinated with

S. mansoni EVs and detected a robust response to a cluster of three proteins with MW 50–70 kDa (Figure 2A). We next tested if *S. mansoni* infection induced similar antibody responses to EV proteins by probing EV lysate with serum from infected animals at 2, 4, and 6 weeks post-infection (Figure 2C–E). Interestingly, a different profile of EV proteins was recognised by serum from infected animals, with the most reactive proteins ranging between 30 and 55 kDa (Figure 2C–E). For comparison, Schistosome soluble worm antigenic preparation (SWAP) proteins were also probed with serum from infected mice 6 weeks post-infection, which revealed a similar band at 30 kDa, but otherwise displayed a different immunogenic profile (Figure 2G). To test the capacity of anti-EV antibodies to recognise schistosomal proteins, SWAP proteins were probed with immunised mouse serum (Figure 2F), generating one immunoreactive band at ~10 kDa and a fainter band at ~8 kDa.

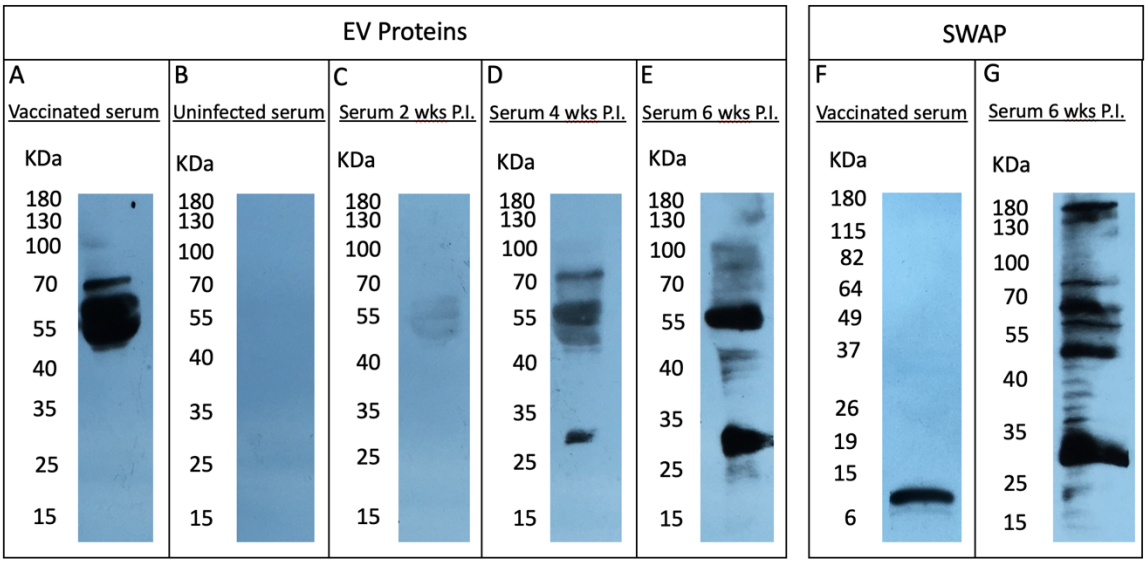

Figure 2. Antigenic profile of *S. mansoni* EV proteins. *S. mansoni* EVs and SWAP were prepared as detailed in the Methods section. The antigenicity of EV proteins was tested on EVs separated by SDS-PAGE and performing Western blot (WB) analysis with (A) serum from mice vaccinated with EVs, (B) serum from uninfected mice, or serum from infected mice collected at (C) 2 weeks, (D) 4 weeks, or (E) 6 weeks post-infection (P.I.). SWAP proteins were probed using serum from (F) vaccinated mice or (G) infected (6 weeks P.I.).

Results shown in Figure 1B suggest that, with the exception of two bands at lower molecular mass (~55 and 100 kDa), the majority of sialylated EV-associated proteins migrate with molecular mass ≥ 150 kDa. These results greatly contrast with the antigenic profile observed at 2, 4, and 6 weeks post infection (Figure 2A,D,E), in which the immunoreactive proteins are generally

no larger than 100 kDa. It thus appears that sialylated EV proteins induce limited or no antibody production.

4. Discussion

The treatment of schistosomiasis relies almost exclusively on the efficacy of a single drug, praziquantel, which is administered to tens of millions of people each year, raising concerns of drug resistance [35]. It is thus imperative to develop new treatment options for the control of schistosomiasis. An interesting avenue for the development of therapeutics is targeting the mechanisms through which parasites interact with their host. Host–parasite interactions have been shaped by thousands of years of co-evolution and are characterised by highly intricate molecular dialogues, involving the release of ESPs, such as EVs [14,36]. Helminth EVs are increasingly recognised as key mediators of host–parasite interactions, making them appealing targets for the development of new therapeutics. Thus far, helminth EV characterisation studies have mainly focused on the protein, miRNA, and lipid composition of different EV subsets, leaving the glycan content largely unexplored [6,9,14,37-39]. Surface carbohydrate moieties are likely required for EVs to exert their functions as they presumably are responsible for establishing contact with target cells. In fact, there is mounting evidence for a requirement of various glycan structures on both cell and EV surfaces for vesicle uptake by recipient cells [10,17-20]. We previously profiled the carbohydrate moieties coating the surface of adult *S. mansoni* EVs and reported the presence of sialic acid residues [16]. Orthologues of enzymes required for sialylation have not been found in the genomes of helminths and, for this reason, worms are generally considered unable to synthesise sialic acids [21,22]. Here, we investigated whether sialylated glycoconjugates were exogenous (host- or culture-medium-derived) or endogenous to the parasite.

Our mass spectrometry analyses revealed that the majority of EV proteins bound by the sialic-acid-specific lectin, SNA-I, were bovine serum proteins, suggesting that the sialylated components originate from the EV-depleted Fetal Bovine Serum (FBS) used for parasite culture. Previous reports of sialylation in various helminth glycoprotein preparations have systematically been attributed to contamination with host glycoconjugates [40-42]. However, it is important to consider that the presence of bovine sialylated serum proteins in our *S. mansoni* EV samples, despite the many washes and the density sedimentation step of our EV isolation protocol, could be relevant, given that in vivo, worms reside in host venules and are thus exposed to similar blood

proteins. In addition, previous analysis of our samples revealed high abundance of *S. mansoni* proteins and no evidence of murine or bovine miRNAs [6], suggesting minimal contamination of *S. mansoni* EVs with host or bovine EVs occurring during our EV isolation protocol, if any. A possible interpretation of these results is that *S. mansoni* EVs are coated in vivo with host glycoproteins, which may serve to protect EVs from removal from serum and aid in cell adhesion and entry to exert their functions. Our findings might thus be indicative of a novel immune evasion and/or immunomodulation mechanism employed by the parasite, in which schistosomes EVs are coated with exogenous host-sialylated molecules. Our results suggest that most sialylated EV-associated bovine proteins do not elicit antibody production in mice under these conditions, which is consistent with the idea that the coating of EVs by sialoglycans could contribute to immune evasion by masking antigenic sites.

Sialic acids, predominantly terminating the ends of many vertebrate glycans, play key roles in cell communication and cell adhesion, and are important regulators of the immune system [23,43]. Sialoglycans have been shown to affect immune responses in many ways. Sialic acids are believed to serve as self-markers, aiding in the discrimination between self and non-self in order to prevent autoimmune activity [23]. In addition, sialic acids have the ability to mask recognition sites via electrostatic repulsion and/or steric hindrance [43,44]. In that context, aberrantly high expression of sialoglycans is a long-recognised tumour evasion strategy and is a common characteristic of tumour cells, protecting malignant cells from recognition and thus removal by immune cells [45,46]. It was initially suggested that the thick layer of sialoglycans on the surface of tumour cells concealed surface antigens, preventing immune recognition. However, increasing evidence suggests that sialic acids exert their immunomodulatory functions via various mechanisms, such as interactions with sialic-acid-binding immunoglobulin (Ig)-like lectins (Siglecs), which are expressed on the majority of leukocytes and are characterised by an N-terminal Ig domain that recognises sialylated glycans [47]. Siglecs also often contain at least one immunoreceptor tyrosine-based inhibitory motifs (ITIMs) at their C-terminus, which, once activated, inhibit cellular activation [48]. Hence, most sialic acid–Siglec interactions result in dampening of immune responses and inhibition of immune cells and are thus believed to contribute to the distinction between self and non-self [49]. Moreover, surface sialic acids prevent complement activation by recruiting and binding the complement control molecule factor H, obstructing C3b binding, and thus blocking activation of the alternative complement pathway [49,50]. The highly

negative charge resulting from hypersialylation of cell surfaces also likely affects cellular interactions. For example, charge repulsions are believed to hinder the formation of immunological synapses between tumour cells and natural killer (NK) cells, preventing NK-cell-driven cytotoxicity [47].

Importantly, many pathogens have evolved the ability to coat themselves with sialic acid, aiding in evasion of the host immune response and the ability to interact with and invade host cells [51,52]. Examples include some viruses [53], bacteria [54], pathogenic fungi [55], and protozoa [52]. While some microorganisms, such as fungi and some bacteria, are capable of de novo synthesis of sialic acid, other bacteria and protozoa have to rely on acquisition from exogenous sources [49,56]. Pathogens which acquire sialic acids from the environment (e.g., the host) do so via a number of ways. Many pathogenic bacteria, for instance, express a sialidase, which cleaves terminal sialic acid from host sialoglycans, or a trans-sialidase, which hydrolyse sialic acids and directs their transfer onto bacterial structures [54,57]. However, there are examples of sialic-acid-utilising bacteria lacking sialidase-encoding genes [58], which are hypothesised to be reliant on sialidase activity either from the host [59,60] or other sialidase-encoding bacteria from the same niche [61].

Trypanosomes are well-known examples of trans-sialylation, whereby the parasites acquire host sialyl residues using trans-sialidase enzymes, allowing them to transfer terminal sialic acids from host glycoconjugates onto the terminal galactose residue of their own asialoglycoconjugates (non-sialylated glycoconjugates). This “theft” of sialic acid is well documented in *Trypanosoma cruzi*, in which this trans-sialidase activity confers protection to the parasite, and aids adhesion to and invasion of host cells [52,62-65].

Our results suggest that schistosomes might employ a similar strategy for co-opting host sialic acid, differing, however, by their apparent use of sialylated host serum proteins as opposed to carbohydrate residues alone. The underlying mechanisms remain unknown and are subjects deserving of further investigation. It could prove interesting to examine whether *S. mansoni* EVs interact with Siglecs, which could contribute to their internalisation as is the case for some sialic acid-coated viruses that utilise Siglec-1 binding on the surface of macrophages to invade host cells [53,66]. It will also be valuable to investigate the effects of sialic acid removal on schistosome EV cell entry and effector functions.

This work provides the first indication of the use of sialic acid by parasitic helminths, offering critical insights into parasite biology as well as a potential new avenue for the

development of therapeutics. It will be important, however, to confirm that this phenomenon is also observed in vivo as our observations are based on in vitro culture. Further experimental approaches are required to understand the mechanism of sialic acid acquisition and the roles of this coating strategy.

Author Contributions

Conceptualization, M.D., J.Q.G., T.G.G and T.L.; formal analysis, M.D.; investigation, M.D.; writing—original draft preparation, M.D.; writing—review and editing, M.D., J.Q.G., T.G.G., T.L.; visualization, M.D.; supervision, T.G.G., T.L.; project administration, T.G.G. and T.L.; funding acquisition, T.G.G. and T.L. All authors have read and agreed to the published version of the manuscript.

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Connecting Statement II

In manuscript II, I investigated the origin of the sialylated glycoconjugates present on *S. mansoni* EVs and determined that they are in fact post-translational modifications of mammalian serum glycoproteins. The presence of mammalian sialoglycoproteins on *S. mansoni* EVs could represent a novel immune evasion and/or modulation mechanism employed by the parasite, whereby the serum glycoproteins coat EVs, shielding them from detection by the host. Consistent with this hypothesis, I found that most sialylated EV-associated bovine proteins do not elicit antibody production in a mouse vaccination model.

In addition to the important questions addressed in manuscripts I and II, namely the composition and the origin of biosynthesis of adult *S. mansoni* EVs, an equally important question to address is the physiological role they may play in the context of infection. We sought to address this question in manuscript III. In addition, the broad approach used in this final manuscript allowed us to explore general changes caused by infection with *S. mansoni*, providing valuable information on the biology of schistosomiasis.

Chapter V. Manuscript III

Transcriptomic changes in Peyer's patches associated with *Schistosoma mansoni* infection in mice

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Manuscript in preparation

Abstract

Schistosomes are of great public health significance, infecting several hundreds of millions of people worldwide. These parasitic trematodes rely heavily on their remarkable ability to manipulate host biology in order to survive and thrive within their host. Whilst the underlying mechanisms employed by the parasite in that regard are not well characterised, extracellular vesicles (EVs) secreted by the parasite are increasingly regarded as key players in these interactions. Schistosome EVs have been shown to be internalised by mammalian cells *in vitro*, causing changes in gene expression. The considerable potential of EVs as mediators of cell communication lies in their architecture and molecular makeup; the lipid bilayer protects the cargo from degradation, allowing delivery of a variety of parasite-derived macromolecules to potentially distant recipient cells where they can act in a concerted manner. The molecular cargo of *S. mansoni* EVs includes miRNAs, some of which have been detected in T helper cells isolated from Peyer's patches of infected mice. It is thus tempting to speculate that parasite-derived EVs reach the gastrointestinal lymphoid tissues of their host, where they are internalised by immune cells and partake in the regulation of host gene expression via miRNA silencing of host mRNAs. Using RNA sequencing, we carried out a transcriptomic analysis of mouse Peyer's patches 7 weeks post-infection with *S. mansoni* and identified over 1,000 differentially expressed genes (DEGs) in infected *vs* control samples. In addition, we performed miRNA target prediction for previously identified *S. mansoni* EV-associated miRNAs via a computational approach, yielding over 5,000 potential murine mRNA targets which we then matched to DEGs. This study provides valuable insight into the biology of schistosomiasis and highlights the challenges associated with computational predictions of miRNA-mRNA interactions *in vivo*.

1. Introduction

Schistosomes are parasitic platyhelminths in the genus *Schistosoma* that cause the chronic disease schistosomiasis, resulting in significant morbidity and mortality, and affecting hundreds of millions of people worldwide. Infection occurs when cercariae, infective larval stages released from infected vector freshwater snails, penetrate a mammalian host's skin, morph into schistosomulae and make their way into the host's circulatory system. Maturing parasites then migrate to the venous plexus of the urinary bladder (*S. haematobium*) or the hepatic portal system and mesenteries (*S. mansoni, S. japonicum*), where pairs of female and male worms release hundreds of eggs daily [1]. Whilst many eggs make their way out of the body and into the environment through urine or faeces, many are trapped in surrounding tissues. The eggs are highly immunogenic, leading to the formation of granulomas, and causing tissue fibrosis and organ damage. If left untreated, adult schistosomes can survive up to several decades in human hosts.

No vaccines are available for schistosomiasis and treatment relies heavily on the efficacy of a single drug, praziquantel, raising concerns of drug resistance. New treatment and prevention methods are thus crucial for the control of the disease. However, it is not without challenges, as the parasite's ability to modulate and evade the host immune response greatly complicates elimination efforts. This *tour de force* is achieved, at least in part, by the release of excretory/secretory products (ESPs), such as extracellular vesicles (EVs).

EVs comprise all secreted membrane-bound vesicles, of which microvesicles (also known as ectosomes; formed by outward budding of the plasma membrane) and exosomes (formed within multivesicular bodies) are the most studied. We and others have studied the composition of *S. mansoni*-derived EVs, and have reported in them a variety of macromolecules, including proteins [2,3], microRNAs (miRNAs) [3,4], and glycans [4,5]. MiRNAs are a class of small non-coding RNAs, approximately 22 nt in length, which act as regulators of gene expression. Specifically, miRNAs are involved in post-transcriptional gene silencing by binding target messenger RNA (mRNA) by complementary base-pairing and subsequently inducing mRNA degradation. Recent studies indicate that parasite-derived miRNAs can regulate gene expression in mammalian cells, suggesting that parasites employ miRNA secretion to shape the host into an environment favourable for their own development. Encapsulation of miRNAs into EVs could confer protection to their fragile cargo and allow for its long-distance delivery to specific target cells in the host. We have previously detected schistosomal miRNAs in infected host plasma [2] and Meningher *et al*.

(2019) reported the presence of the schistosomal miRNAs Bantam and miR-10 in gastrointestinal lymphoid tissue (Peyer's patches and mesenteric lymph nodes) of *S. mansoni*-infected mice [6]. The question of whether parasite-derived miRNAs actively regulate host gene expression *in vivo* remains unresolved.

A better understanding of the parasite's survival strategies and molecular interactions with its host will provide valuable insights to support the development of novel control strategies.

Here, based on our previous characterisation of the miRNAome of adult *S. mansoni* EVs [2], we performed target searches of parasite-derived miRNAs against mouse mRNAs and pathways analysis on the predicted targets. In addition, we carried out a transcriptomic analysis of Peyer's patches, investigating transcriptional changes resulting from infection with this parasite, using an *in vivo* model and compared our results with the miRNA target predictions.

2. Materials and methods

2.1 Experimental design

Naïve mice were infected experimentally with *S. mansoni*. Their Peyer's patches were harvested 7 weeks post-infection (P.I.), and their transcriptomes sequenced and compared to Peyer's patches from age-matched uninfected control mice. Based on a previous study reporting the presence of *S. mansoni* miRNA in Peyer's patches [6], we sought to investigate whether some of the differential expression of genes in this tissue could be attributed to transcription regulation by parasite miRNAs. To do so, we ran target prediction analyses on previously identified miRNAs associated with adult *S. mansoni* EVs and compared the predictions with the results of our transcriptomic analysis.

2.2 miRNA target prediction

Previously identified miRNA sequences from adult *S. mansoni* EVs [2] were retrieved and miRNA IDs were obtained from miRBase (Release 22.1) [7]. The 29 most abundant identified parasite miRNAs were included in miRNA target prediction analyses.

miRNA target prediction to mouse 3' UTRs and their orthologs was performed using the TargetScan software package v. 7 (http://www.targetscan.org), implemented as a standalone workflow under iPortal [8] and openBIS [9], using default parameters [10] as previously described [11]. All predicted genes with a weighted context ++ score ≤ −1.8 and targeted at least twice were used in further analyses, a more stringent threshold adapted from described protocols [11,12]. Kyoto Encyclopedia of Genes and Genomes **(**KEGG) enrichment analyses were performed using ExpressAnalyst (https://www.expressanalyst.ca) [13] and results were plotted using the visualization packages ggplot2 and UpSetR in R. KEGG pathways with enrichment FDRs < 0.05 were considered to be significantly enriched. FDR was calculated based on nominal P-value from the hypergeometric test.

2.3 Experimental infection

Our study comprised two experimental groups, 6 non-infected and 6 mice infected with *S. mansoni* 7 weeks P.I.; Peyer's patches were assayed for each experimental group.

Biomphalaria glabrata snails infected with *S. mansoni* Puerto Rican strain PR1 were maintained at the Institute of Parasitology of McGill University. 40-day-old female CD1 mice were infected via tail exposure to approximately 150 cercariae and housed in the animal facility at the Small Animal Research Unit of McGill University as per McGill University Animal Care Committee Permit # 2019-8138. Seven weeks P.I., mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and adult *S. mansoni* worms flushed by cardiac perfusion and counted. Peyer's patches were harvested from each mouse and immediately placed in TRIzol™ (ThermoFisher Scientific 15596026, Burlington, ON, Canada). Control age-matched female CD1 mice were mock perfused prior to tissue harvesting. Tissue samples were stored at – 80°C until processed.

2.4 RNA isolation, cDNA library preparation and transcriptome sequencing

Tissue samples were thawed on ice and homogenized using a PowerGen 125 homogenizer (Fisher Scientific FS-PG125) equipped with Omni International PowerGen Omni Tip™ Plastic Homogenizing Probes (Fischer Scientific 15-340-146). Total RNA from all tissue samples was isolated using the phenol/chloroform extraction protocol provided by the TRIzol reagent

manufacturer. Total RNA (six samples/experimental group) was sent to the Génome Québec Innovation Centre, Canada for cDNA library preparation and subsequent sequencing in the Illumina NovaSeq 6000 sequencing system using S4 PE100.

2.5 Counting reads and analysis of differential gene expression

Sequence quality was checked with FASTQC (version 0.11.3) and adapter specific sequences were removed with Trim Galore (version 0.6.5) (https://www.bioinformatics.babraham.ac.uk/projects/). The genome sequence and gene annotation information of *Mus musculus* (Mus_musculus.GRCm39.104) were obtained from ENSEMBL (https://www.ensembl.org/). Reads were aligned to the *M. musculus* genome with HISAT2 (version 2.1.0) [14] and sorted alignment files were generated by SAMtools (version 1.7) [15] Raw read counts were obtained using HTSeq (version 0.9.1) [16] where the intersection-strict mode was applied.

The raw read counts were uploaded to ExpressAnalyst (https://www.expressanalyst.ca), a web-based tool for comprehensive gene expression profiling, for downstream analysis [13]. Low variance (15%) and low abundance counts (<4) were filtered, and the data was normalized by Trimmed Mean of M-values. Sample distribution was demonstrated by principal component analysis. The edgeR method [17] was used to identify differentially expressed genes between the infected and non-infected groups (adjusted P value <0.05 and $\log_2$ fold change >1). Further analyses including Volcano plot and gene enrichment were performed using the same tool.

3. Results

3.1 Predicted targets in murine host genome

miRNAs previously found in adult *S. mansoni*-derived EVs [2] were used to predict targets in the host genome. A total of 5,434 *M. musculus* genes were predicted to be targeted by *S. mansoni* EV-associated miRNAs (Table S1) and were used for downstream Gene ontology and KEGG enrichment analyses using ExpressAnalyst [13], which revealed many (>80) significantly overrepresented pathways (FDR < 0.05). Notable overlap was observed between these KEGG terms and those obtained by Duguet *et al.* from miRNA target predictions on seven different

helminth-host associations (*Ascaris suum*/*Sus scrofa*, *Haemonchus contortus*/*Ovis aries, Trichuris muris*/*M. musculus*, *Heligmosomoides polygyrus*/*M. musculus*, *Caenorhabditis elegans*/*M. musculus*, *C. elegans*/*S. scrofa*, *C. elegans*/*O. aries*) (Fig 1) [11]. Twenty-five terms were shared by all eight associations, including signaling pathways regulating pluripotency of stem cells, T cell receptor signaling pathway, focal adhesion, and MAPK, Wnt, mTOR, Ras, Rap1, FoxO, and ErbB signaling pathways. The full list is available in Table S2. In addition, the PI3K-Akt signaling pathway, T helper (Th) 1 and Th2 cell differentiation and Th17 cell differentiation, and GnRH signaling pathway were all predicted to be targeted in the mouse by *S. mansoni* miRNAs (Fig 2). Other overrepresented pathways include signaling pathways regulating pluripotency of stem cells, tight junction, adherens junction, focal adhesion and inflammatory mediator regulation of TRP channels. The comprehensive list of overrepresented pathways predicted to be targeted by *S. mansoni* miRNAs and their statistical significance is available in Table S3.

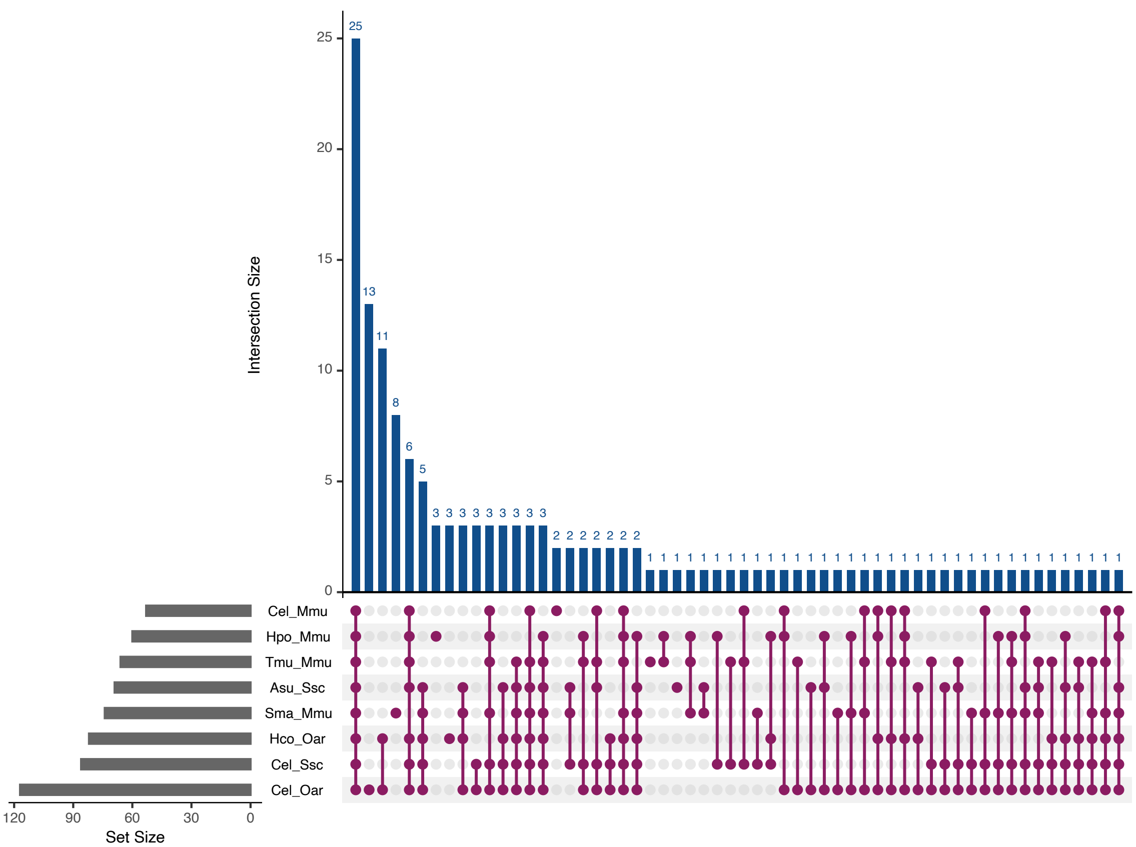

Figure 1: Predicted host biological pathways targeted by helminth miRNAs across helminth-host associations. This plot was generated with all significantly overrepresented pathways (adj. $p < 0.05$) across 8 helminth-host associations. The intersection size represents the number of unique

KEGG terms shared between the different association datasets; connected dots illustrate the distribution of predicted terms across datasets.

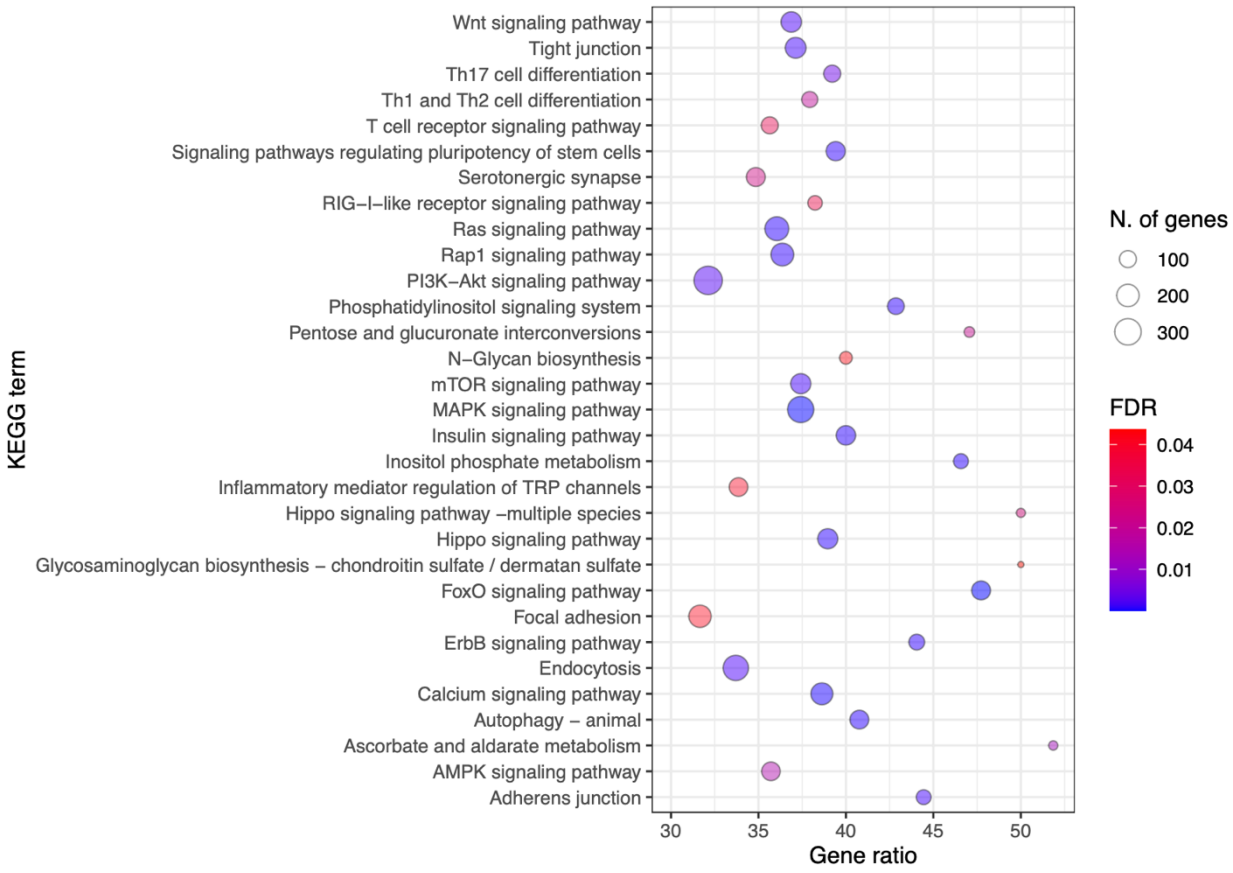

Figure 2. miRNA target predictions KEGG analysis. Selection of significantly enriched host pathways. N. of Genes = number of predicted genes. Gene ratio is the percentage of total predicted genes in the given KEGG term. Enrichment false discovery rate (FDR) is calculated based on nominal P-value from the hypergeometric test.

3.2 Experimental infection and transcriptomic changes

Six infected mice and 6 age-matched control mice were euthanized at 7 weeks P.I. Between 30 and 64 worms were recovered from infected mice, with a mean of 47 (SD = 12.5) (Fig 3A). RNAseq analysis of each sample provided around 45 million reads, and approximately 95% of these reads were mapped to the mouse genome. Principal component analysis (PCA) showed a marked distinction between infected and control animals (Fig 3B). The three mice with the highest worm burden also displayed the highest level of separation, though more replicates would be

required to confirm this correlation. Analyses revealed a total of 1,361 differentially expressed genes (DEGs), with 915 upregulated genes and 446 genes displaying downregulated expression compared to control mice; log fold-change (LogFC) values ranged from -4.98 for cytochrome P450, family 2, subfamily f, polypeptide 2 gene (Cyp2f2) to 10.67 for chitinase-like 3 gene (chil3). Of the 446 downregulated genes, 119 were predicted miRNA targets, whilst 193 of the 915 upregulated transcripts were predicted to interact with parasite miRNAs. LogFC values were plotted against their adjusted P values (adjP) and represented in a volcano plot (Fig. 3C). The comprehensive list of all DEGs and their statistical significance is available in Table S4.

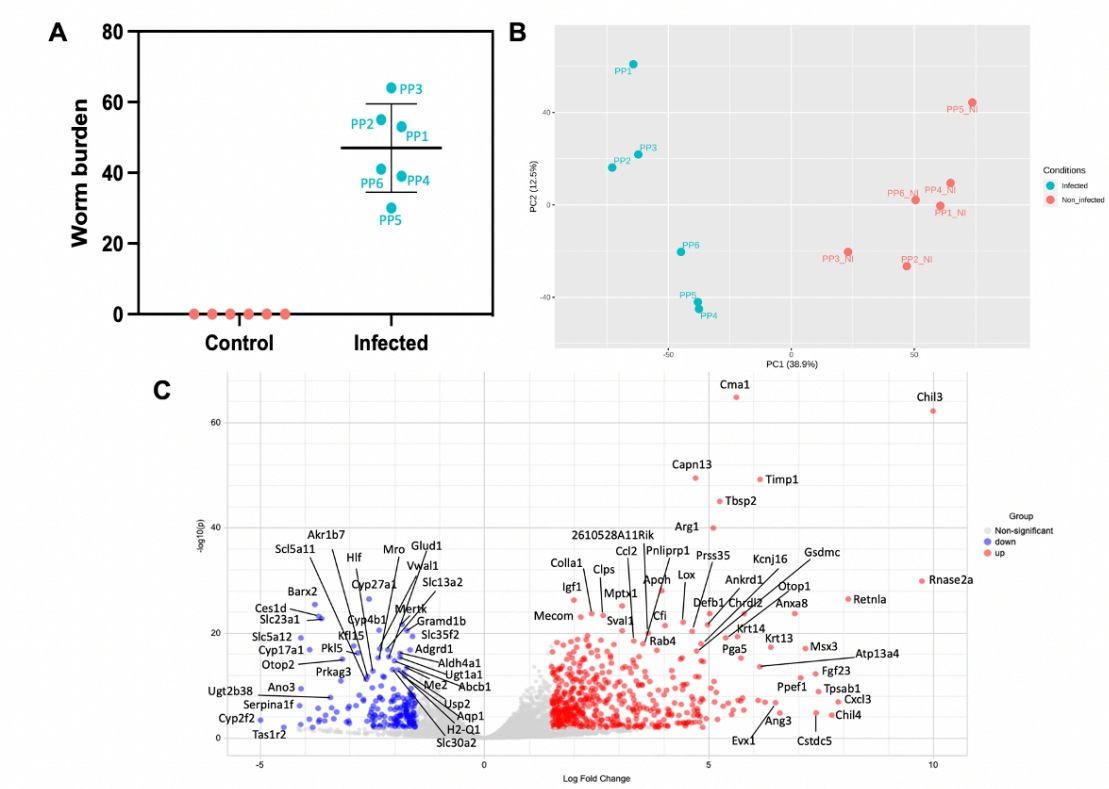

Figure 3: Differential Expression analysis of Peyer's patches from mice infected with *S. mansoni* 7 weeks P.I. vs. non-infected control mice. (A) Number of adult worm pairs recovered post-euthanasia at 7 weeks P.I.. (B) Principal Component Analysis (PCA). Principal components were calculated and the first two ranked by ratio of explained variance are shown (PC1 and PC2). (C) Volcano plot showing differential gene expression where $\log_2$ fold change in gene expression is plotted on the X-axis and $-\log_{10}$ adjusted *P* value is plotted on the Y-axis. Significantly downregulated genes are represented in blue (adj$P < 0.05$ and $\log_2$ fold change > -1.5), and significantly upregulated genes are represented in red (adj$P < 0.05$ and $\log_2$ fold change > 1.5).

3.3 Gene ontology (GO) and KEGG pathway analysis

Interrogation of the KEGG and GO databases with significant DEGs used the Express Analyst tools and revealed several overrepresented terms. Figure 4 shows networks of significantly enriched KEGG terms of DEGs processed by overrepresentation analysis (ORA). Many of these terms are associated with metabolism and the immune system. Top significantly overrepresented molecular functions, biological process, and cellular component terms (GO; AdjP < 0.5) are represented in Figures 5A, 5B, and 5C, respectively. Full lists are available in Table S5.

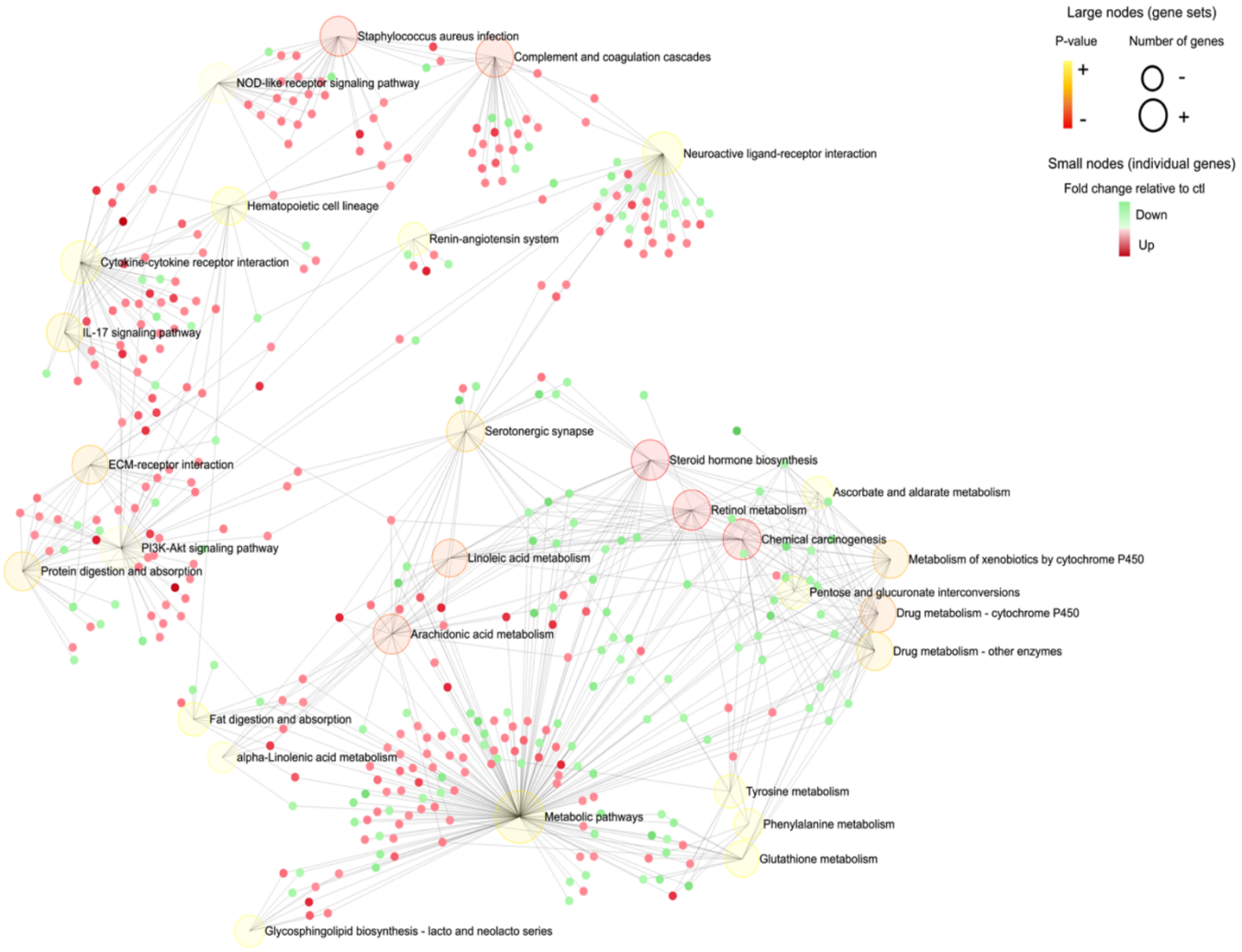

Figure 4: KEGG enrichment network of significantly differentially expressed genes. Significantly differentially expressed genes were subjected to overrepresentation analysis (ORA). The nodes are coloured according to p-value.

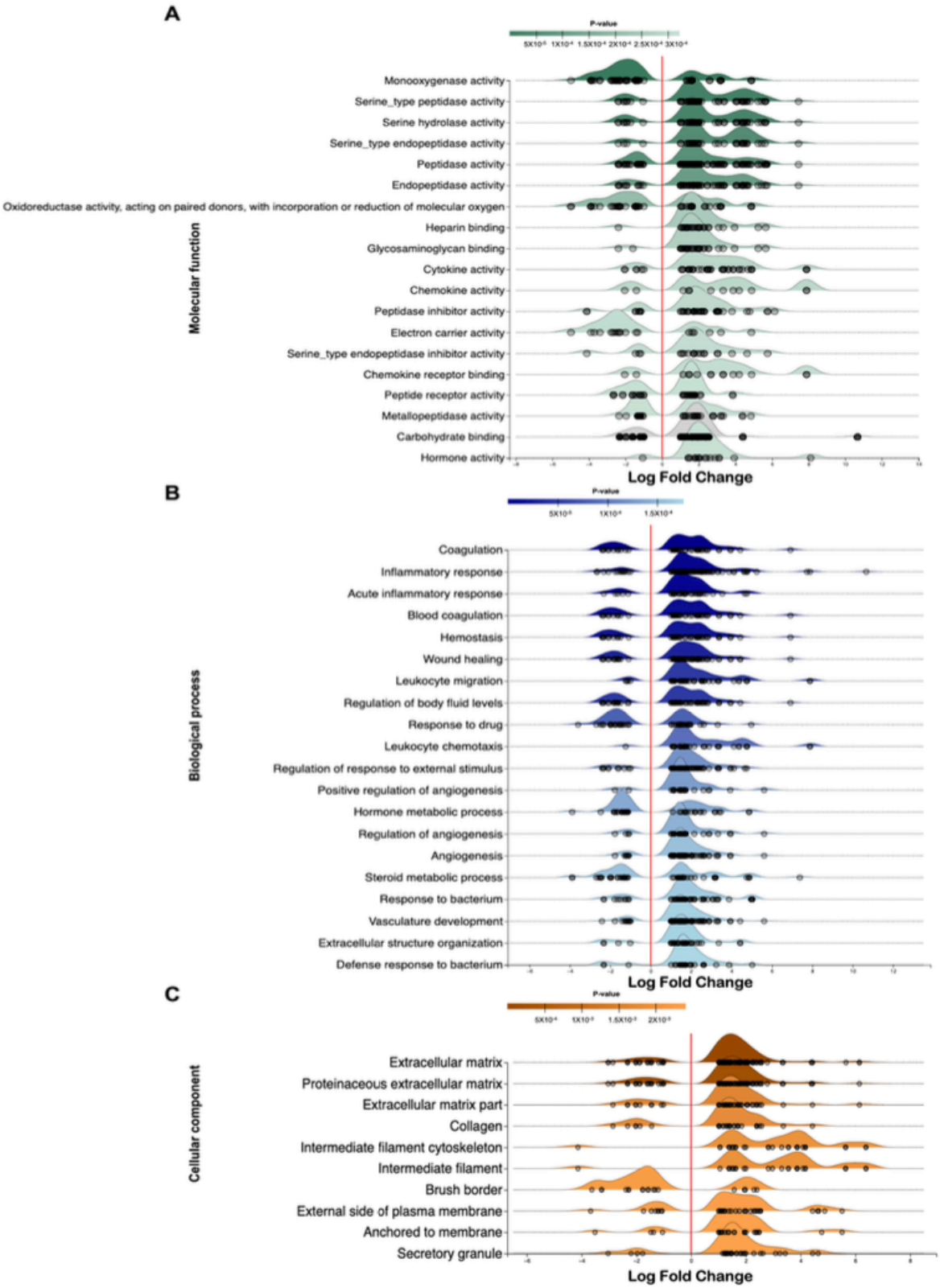

Figure 5. Gene ontology (GO) analysis of differentially expressed genes in Peyer's patches of *S. mansoni*-infected mice. Density ridgeline plot of fold-change distribution of enriched pathways.

The top overrepresented (A) molecular function, (B) biological process, and (C) cellular components GO terms identified are shown.

4. Discussion

The continuing high prevalence of schistosomiasis underscores the current challenges in parasite control and infection prevention and calls for new control approaches. An improved understanding of the disease and the molecular interactions between the host and the parasite that enable infection will provide new opportunities for the development of such innovations. This study delves into the role of *S. mansoni* EV-associated miRNAs in the regulation of host gene expression *in vivo* and investigates transcriptomic changes occurring within gastrointestinal lymph nodes of infected mice, providing a wealth of data and valuable information on immunological and other physiological changes occurring in response to infection.

There is accumulating evidence of host modulation by parasitic helminth miRNAs [18-23]. Our target predictions for the 29 most abundant adult EV-associated miRNAs yielded potential interactions with 5,434 *M. musculus* transcripts, the large number of predicted targets attributable in part to the pleiotropic properties of miRNAs. KEGG pathway analysis revealed over 80 overrepresented pathways, most of which were common to one or more of the seven helminth-host associations explored by Duguet *et al*. (2020)[11], with 25 pathways being shared by all eight associations. These include several immune-relevant pathways, many of which, as pointed out by Duguet *et al.* (2020), relate to various facets of T cell development, activation, and differentiation. Pathways such as T cell receptor signaling, Th1 and Th2 cell differentiation, and Th17 cell differentiation, but also PI3K-Akt signaling (key to T cell development) [24] and MAPK, which is central in the regulation of T-cell activation and differentiation [25], were prominent. Likewise, the Ras signaling pathway is crucial for T cell development, differentiation, and proliferation [26]. These predictions are interesting as Th cells internalise *S. mansoni* EVs *in vitro* [6].

Meningher *et al*. (2019) reported the presence of Sma-miR-10 and Sma-miR-Bantam in Th cells isolated from Peyer's patches of infected mice, suggesting the possibility of miRNA-mediated regulation of host gene expression in those cells by the parasite. Therefore, in the present

study, we investigated transcriptional changes in Peyer's patches associated with infection with mature egg-laying schistosomes.

Our analysis revealed 1,361 DEGs, of which 915 were upregulated and 446 downregulated. Schistosomes are well known for their remarkable ability to modulate the host immune response, shaping it into an environment favourable for the establishment of a chronic infection, and contributing to their considerable longevity in the human host. Early infection, starting with cercarial penetration, is characterised by a dominant Th1 response, which steers towards a Th2 response at the onset of egg production (~ 5 weeks P.I. in mice), peaking at ~ 8 weeks P.I. and steadily declining to give rise to a regulatory phenotype (Treg) [27-30]. A Th17-type immune response has also been reported to participate in the response to schistosomes and appears to be a driver of severe pathology in both humans and in the mouse infection model [31,32].

In this study, samples were collected during the dominant Th2 phase (7 weeks P.I.), which was reflected in our results. Transcripts for various Th2-associated cytokines, including IL-4, 5, 13 and 25 (IL-17E) were upregulated in infected mice. We found chitinase-like 3 (*chil3*, which encodes Ym1 in mice) to be the most upregulated transcript in infected mice, with a Log2(FC) of >10. This finding is consistent with previous reports of increased expression of chitinase 3-like protein (YKL-40 in humans) in patients infected with *S. haematobium* [33] and *S. japonicum*, with serum levels correlating with the stage of hepatic fibrosis in *S. japonicum* [34]. Ym1 is secreted by Th2-activated antigen-presenting cells (APCs), mainly alternatively activated macrophages (AAMs, also known as M2), and neutrophils [35-38]. The Th2 response is characterised by AAMs, which result from activation with Th2 cytokines and display a highly characteristic expression profile; Chil3 is one of the most highly expressed genes in AAMs in mice. Other markers of AAMs include resistin-like alpha (Retnla; also RELMα or FIZZ1) and arginase 1 (Arg1), both of which also had increased expression with Log2(FC) = 8.10 and 5.10, respectively. Independently of infection, RELMα, Arg1, and Ym1 are also associated with tissue injury as they play crucial roles in tissue repair and remodeling and fibrosis [36,39]. This is consistent with tissue injury caused by eggs trapped in host tissue, or as they migrate through the Peyer's patches lymphoid tissues to reach the gut lumen [40]. In addition, transcript levels for chitinase-like 4 (*chil4*; encodes Ym2), resistin-like gamma (RELMγ or Retnlγ), resistin-like beta (RELMβ or Retnlβ) and intelectin 1 (itln1) were also upregulated in infected mice. These molecules, expressed by intestinal epithelial

cells and induced by Th2 cytokines, [36] further reflect the establishment of a polarised type 2 immune environment. Expression of Ym1 and 2, RELMα, Arg1, RELMγ, and Itln1 is regulated by Th2 cytokines and high levels of these proteins are characteristic of many helminth infections [35,36,41]. Ym1 and Ym2 are highly homologous (~95%) secreted chitinase-like proteins (CLPs) and their secretion by AAMs and endothelial cells is strongly induced by Th2 stimuli. Though enzymatically inactive, CLPs bind chitin and are believed to regulate several aspects of helminth infection [35,42], allergy [43], wound healing [44,45], and cancer [46]. Originally described as an eosinophil chemokine, the chemotactic properties of Ym1 are debated and the extent of its function remains unclear [36,37,43,47,48]. Besides chitin, CLPs bind other sugars [37]. In addition, several other binding partners have been identified for Ym1 and 2, such as 12/15-lipoxygenase (12/15-LOX; encoded by LOX15) [49], and Interleukin-13 receptor subunit alpha-2 (IL-13Rα2) [50], both of which were upregulated in infected mice. 12/15-LOX is in the lipoxygenase family and is involved in the metabolism of fatty acids by catalysing the oxidation of polyunsaturated fatty acids into hydroperoxides, generating bioactive metabolites. Important roles in oxidative and inflammatory responses have been described for 12/15-LOX, which has a variety of substrates, including arachidonic acid (AA), linoleic acid (LA), and alpha-linoleic acid (ALA) [51]. AA, LA, and ALA metabolism were all overrepresented pathways in our KEGG analysis of DEGs. Studying these interactions could help unveil the role of CLPs in helminth infections.

Likewise, many metabolic pathways were altered in infected mice. This might be attributable in some degree to the entrapment of eggs in host tissues such as liver and intestine, and the subsequent inflammatory granulomatous reaction directed against the eggs, which results in significant tissue injury. Consequently, the host may suffer reductions in fitness and metabolic alterations [52], which may, at least partly, explain the metabolic changes we observed in Peyer's patches. Many genes associated with retinol metabolism were also downregulated, which is consistent with previous reports of vitamin A deficiency in patients infected with *S. mansoni* [53-55].

In particular, many DEGs were associated with AA metabolism. We note the upregulation of transcripts of enzymes in the phospholipase A2 (PLA2) family. AA is normally found as part of the plasma membrane. Following stimuli such as inflammatory reactions, PLA2 enzymes catalyse the hydrolysis of membrane glycerophospholipids, releasing AA, which is then converted

into bioactive metabolites promoting inflammatory cascades via the action of various downstream enzymes [56]. Amongst important pathways for AA metabolism are the lipoxygenase (LOX) and the cytochrome P450 (CYP450) pathways. Several transcripts of enzymes belonging to the LOX pathway, which is responsible for the conversion of AA into leukotrienes and lipoxins, were upregulated in infected mice. These are likely part of the host response to infection. Leukotrienes play crucial roles in innate and adaptive immune responses and can enhance the schistosome killing abilities of neutrophils and eosinophils in a complement-dependent manner, and lipoxins have anti-inflammatory properties and play roles in the transition from the M1 to the M2 phenotype [57,58]. On the other hand, we observed the downregulation of many transcripts from the CYP450 pathway, which is involved in the metabolism of AA into hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids.

An interesting possibility is that some of the observed metabolic changes are due to the immune status of the host. Different immune phenotypes are characterised by different metabolic profiles. For instance, fast-acting pro-inflammatory classically activated macrophages (CAMs) are reliant on glycolysis, whereas long-lived AAMs favour fatty acid oxidation and oxidative glucose metabolism [59]. These effects, however, are not limited to immune cells and impact metabolism systemically. Studies have highlighted this causality in obesity-associated inflammation and insulin-resistance and type 2 diabetes [59-61]. Alternatively, type 2 cytokines are associated with insulin sensitivity and can induce important systemic metabolic alterations [39,59]. Similarly, helminth infections are often accompanied by metabolic alterations and, although some of these changes are immunologically driven, some may be better explained by tissue injury, nutritional deficiency, or other factors such as changes in the gut microbiota [39,62,63].

Consistent with previous reports of mastocytosis, several mast cell (MC) markers were highly upregulated in infected mice. Helminth infections are typically characterised by an increase in MC populations [64]. Specifically, intestinal schistosomiasis is associated with prolonged recruitment of MCs in the hepatic and intestinal regions [65]. MCs are believed to contribute to the anti-parasitic response by degranulation, whereby Th2 cytokines induce B-cell secretion of helminth-specific IgE antibodies which bind to the FcεRI receptor on the surface of MCs. Parasite antigens then bind IgE antibodies, cross-linking Fc receptors and causing the release of MC granule proteases, cytokines, and growth factors which participate in the modulation and

polarization of the innate and adaptive immune response [64-66]. Though MCs have been linked to parasite clearance in some helminth infections, particularly intestinal nematodes [67], MC accumulation in schistosomiasis has been reported to correlate with susceptibility to infection, but their role remains unclear [66,68-70].

Interestingly, many genes associated with taste transduction and signaling were differentially expressed. Several studies have reported the expression of taste signaling elements in cells from nongustatory tissues [71-74]. Nongustatory cells expressing G protein-coupled taste receptors and/or their downstream signaling elements have been termed solitary chemosensory cells (SCCs) and have been found in the airway, urethra, trachea, and gingival epithelium [72,74-76]. Taste signaling molecules are also expressed in intestinal tuft cells and some populations of immune cells [71,77-83]. It is likely that some intestinal epithelial cells, including tuft cells, were collected along with lymphatic tissue during the tissue harvest procedure. Tuft cells play important roles in the establishment of type 2 immunity [84] and have been implicated in the immune response to parasite infection, from detection of parasitic worms to the initiation of a type 2 response [77,78,85]. Tuft cells act as sentinels of gastrointestinal nematode infection; detection of parasites leads to their production of IL-25, which induces proliferation of tissue-resident group 2 innate lymphoid cells (ILC2s; important producers of type 2 cytokines) and cytokine production [78,85]. Thus, tuft cell activation of ILC2s is a key driver of Th2 cell polarisation and worm expulsion in the context of gastrointestinal helminth infection [78,85]. The role of tuft cells during intestinal schistosomiasis is unknown, but it would be worthwhile to study their interaction with schistosome eggs, as it is possible that they contribute to egg excretion.

Of the 446 downregulated transcripts, 119 (~27%) were predicted targets of *S. mansoni* EV-associated miRNAs. Conversely, 193 of the 915 upregulated transcripts (21%) were predicted to be targeted by these miRNAs. If, instead, we perform the analysis following previously described target prediction threshold (weighted context ++ score ≤ -1.0) [11], 236 of the downregulated genes (53%) and 398 of the upregulated genes (44%) are identified as predicted miRNA targets. This illustrates the difficulty of accurately generating miRNA target predictions based on algorithms which yield many false positives and false negatives due to inherent biases in nucleotide composition, length, and conservation in the 3' UTR of genes [86]. Computational target predictions thus require experimental validation. Many unknowns limit the reliability of

predictions, as we strive to understand mechanisms for cargo selection and packaging of miRNAs into EVs, and the conditions required for their integration into a functional pathway once inside the target cell [86]. Predicting miRNA-mRNA interactions is thus challenging, especially in an *in vivo* setting, where additional factors influence these interactions, such as proximity to recipient cells, EV clearance rate, and interaction specificity between the various host cells targeted by EV subpopulations (i.e., different EV subpopulations may interact with different target cells).

Our study represents a snapshot in time of intestinal immune tissue at 7-weeks P.I. with the mesenteric helminth parasite *S. mansoni* and provides valuable information to further our understanding of the disease. Priorities for future research include analysis of transcriptomic changes in other tissues of relevance for schistosome-host interactions and analysis of these changes over time as the immune response to the parasite develops and matures.

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Supplementary material

Table S1. miRNA target predictions (additional file).

Table S2. Predicted host biological KEGG pathways targeted by helminth miRNAs across helminth-host associations.

Term	Sma_ Mmu	Asu_ Ssc	Hco_ Oar	Hpo_ Mmu	Tmu_ Mmu	Cel_ Mmu	Cel_ Oar	Cel_ Ssc
Pathways in cancer	x	x	x	x	x	x	x	x
Axon guidance	x	x	x	x	x	x	x	x
MAPK signaling pathway	x	x	x	x	x	x	x	x
Calcium signaling pathway	x	x	x	x	x	x	x	x
Proteoglycans in cancer	x	x	x	x	x	x	x	x
Signaling pathways regulating pluripotency of stem cells	x	x	x	x	x	x	x	x
Ras signaling pathway	x	x	x	x	x	x	x	x
Wnt signaling pathway	x	x	x	x	x	x	x	x
MTOR signaling pathway	x	x	x	x	x	x	x	x
Hippo signaling pathway	x	x	x	x	x	x	x	x
Rap1 signaling pathway	x	x	x	x	x	x	x	x
Insulin signaling pathway	x	x	x	x	x	x	x	x
FoxO signaling pathway	x	x	x	x	x	x	x	x
Focal adhesion	x	x	x	x	x	x	x	x
Autophagy	x	x	x	x	x	x	x	x
Adrenergic signaling in cardiomyocytes	x	x	x	x	x	x	x	x
ErbB signaling pathway	x	x	x	x	x	x	x	x
Thyroid hormone signaling pathway	x	x	x	x	x	x	x	x
T cell receptor signaling pathway	x	x	x	x	x	x	x	x
Chronic myeloid leukemia	x	x	x	x	x	x	x	x
Gastric cancer		x	x	x	x	x	x	x
Cushing syndrome		x	x	x	x	x	x	x
Melanogenesis		x	x	x	x	x	x	x
Breast cancer	x	x	x	x	x	x	x	x
Hepatocellular carcinoma		x	x	x	x	x	x	x
Prostate cancer	x	x	x	x	x	x	x	x
Pancreatic cancer	x	x	x	x	x	x	x	x
Glioma	x	x	x	x	x	x	x	x
Choline metabolism in cancer	x	x	x	x	x	x	x	x
Neurotrophin signaling pathway	x	x	x	x		x	x	x

Sphingolipid signaling pathway	x		x	x	x	x	x	x
Regulation of actin cytoskeleton	x		x	x	x	x	x	x
AMPK signaling pathway	x	x	x	x	x		x	x
CAMP signaling pathway	x	x	x	x	x		x	x
GnRH signaling pathway	x				x	x	x	
Adherens junction	x	x	x	x	x		x	x
Melanoma	x	x	x		x	x	x	x
Colorectal cancer	x	x	x		x	x	x	x
Cholinergic synapse		x	x		x		x	x
Phosphatidylinositol signaling system	x			x	x	x	x	x
Insulin resistance	x	x	x		x		x	x
Glutamatergic synapse	x	x	x	x			x	x
Phospholipase D signaling pathway	x			x	x	x	x	x
Prolactin signaling pathway	x	x	x		x		x	x
Tight junction	x	x	x	x			x	x
Longevity regulating pathway	x	x	x		x		x	x
PI3K-Akt signaling pathway	x	x	x				x	x
GABAergic synapse	x	x	x				x	x
Long-term potentiation	x	x	x				x	
Non-small cell lung cancer	x		x		x		x	x
Small cell lung cancer	x	x			x		x	x
Glycosaminoglycan biosynthesis	x			x	x		x	x
Regulation of lipolysis in adipocytes	x	x	x				x	x
Endocytosis	x					x	x	x
Dopaminergic synapse	x	x	x				x	
Morphine addiction	x	x	x				x	
Inositol phosphate metabolism	x			x			x	x
Notch signaling pathway		x		x			x	
Th1 and Th2 cell differentiation	x			x			x	
Th17 cell differentiation	x	x						x
Peroxisome	x			x	x			
Retrograde endocannabinoid signaling			x				x	
Aldosterone-regulated sodium reabsorption	x						x	x
P53 signaling pathway	x	x						x
Serotonergic synapse	x							x

RIG-I-like receptor signaling pathway	x	x						
Metabolic pathways								
MicroRNAs in cancer								
EGFR tyrosine kinase inhibitor resistance	x							
Inflammatory mediator regulation of TRP channels	x							
Endocrine resistance	x							
Nicotine addiction	x							
Bile secretion								
Hepatitis B	x	x	x				x	x
Toxoplasmosis	x	x		x		x	x	x
Chagas disease	x	x	x		x	x	x	x
Basal cell carcinoma	x			x	x	x	x	x
Human papillomavirus infection			x		x	x	x	
Hepatitis C			x					
Endocrine and other factor-regulated calcium reabsorption							x	
Measles	x							
Pentose and glucuronate interconversions	x							
Porphyrin and chlorophyll metabolism	x							
Human cytomegalovirus infection								
Ascorbate and aldarate metabolism	x							
Oxytocin signaling pathway		x	x	x	x	x	x	x
Parathyroid hormone synthesis, secretion and action		x	x	x	x	x	x	x
AGE-RAGE signaling pathway in diabetic complications		x	x	x			x	x
Arrhythmogenic right ventricular cardiomyopathy		x			x	x	x	x
Dilated cardiomyopathy		x			x	x	x	x
Endometrial cancer	x		x		x	x	x	x
CGMP-PKG signaling pathway		x	x				x	x
Ubiquitin mediated proteolysis				x	x		x	x
Relaxin signaling pathway		x	x				x	x
Long-term depression		x			x		x	x
Renin secretion		x	x				x	
Cell cycle				x	x		x	x
Insulin secretion		x	x				x	x

Renal cell carcinoma			x		x		x	x
C-type lectin receptor signaling pathway				x		x	x	
Platelet activation			x				x	x
Protein processing in endoplasmic reticulum			x	x				x
Apelin signaling pathway			x				x	x
Hypertrophic cardiomyopathy					x	x		x
ECM-receptor interaction					x		x	x
TNF signaling pathway					x			x
Oocyte meiosis			x				x	
TGF-beta signaling pathway				x	x			
HIF-1 signaling pathway			x				x	
B cell receptor signaling pathway			x				x	
Gap junction							x	x
Circadian entrainment			x				x	
Central carbon metabolism in cancer			x				x	
Fluid shear stress and atherosclerosis			x				x	
Inflammatory bowel disease							x	x
Gastric acid secretion							x	x
Fc epsilon RI signaling pathway			x				x	
SNARE interactions in vesicular transport				x				x
Estrogen signaling pathway			x				x	
Type II diabetes mellitus			x				x	
Cellular senescence							x	
Mitophagy				x				
Chemokine signaling pathway						x		
Progesterone-mediated oocyte maturation			x					
Aldosterone synthesis and secretion							x	
Hedgehog signaling pathway							x	
Fc gamma R-mediated phagocytosis							x	
Glyoxylate and dicarboxylate metabolism				x				
MRNA surveillance pathway							x	
JAK-STAT signaling pathway			x					
Amphetamine addiction							x	
Mucin type O-glycan biosynthesis		x						

Transcriptional misregulation in cancer	x	x	x				x	x
Salivary secretion		x					x	x
Leukocyte transendothelial migration		x					x	
Acute myeloid leukemia							x	
Bacterial invasion of epithelial cells			x	x	x	x	x	
Thyroid cancer			x				x	
Circadian rhythm			x	x		x	x	
One carbon pool by folate				x				
Synaptic vesicle cycle					x		x	
Steroid biosynthesis					x			
Pertussis						x		
Proximal tubule bicarbonate reclamation							x	
Pancreatic secretion							x	
Thyroid hormone synthesis							x	
Carbohydrate digestion and absorption							x	
Ferroptosis							x	
Amyotrophic lateral sclerosis	x						x	

Table S3. Overrepresented pathways predicted to be targeted by *S. mansoni* miRNAs (additional file).

Table S4. List of DEGs in Peyer's patches (additional file).

Table S5. GO and KEGG pathway analysis of DEGs (additional file).

Chapter VI. General Discussion and conclusion

Schistosomiasis is a debilitating parasitic disease caused by infection with platyhelminths of the genus *Schistosoma*. Based on estimates from 2017, schistosomes are responsible for over 140 million human infections and over 1,000 YLDs worldwide [1]. Schistosome infections lead to chronic morbidities which are associated with chronic inflammation, anemia, nutritional deficiencies, impaired child growth and development, and cause severe pathologies of the liver and bladder [2,3]. Schistosomiasis is a disease of poverty, affecting mainly rural communities, particularly in Sub-Saharan Africa, where most of the cases are detected [4]. No vaccine is available against schistosomiasis and chemotherapy relies almost exclusively on the efficacy of a single drug, PZQ. Growing concerns about pockets of sub-optimal drug efficacy and the prospect of resistance emphasises the urgent need for the development of novel therapies [5-9]. A better understanding of the molecular dialogue employed by the parasites to manipulate the host could lead to the identification of therapeutic targets for the control of schistosomiasis. In this regard, the secretome of the parasite is of particular interest, as ESPs are instrumental for host-parasite interactions and represent promising targets for diagnostic and vaccine antigen discovery. In particular, helminth EVs have been receiving increasing attention for their potential as host modulators. EVs contain a variety of macromolecules and thus can affect recipient cells via various mechanisms, such as by initiating intracellular signalling cascades through ligand-receptor interactions by direct contact between an EV and a cell, by direct fusion with the plasma membrane of a target cell, releasing their cargo into the cytoplasm, or by endocytosis. The functions of EVs are highly dependent on their molecular composition, and a better characterisation of *S. mansoni* EV content will help decipher their biological roles. The cargo of schistosome EVs has been partially characterised in different life stages [10-14], revealing an enrichment of putative effectors such as proteins and microRNAs (miRNAs). There is growing appreciation for the importance of EV surface carbohydrates, as evidence suggests they are key to cellular interaction and uptake. A better portrait of EV surface glycans could thus help unveil mechanisms by which host recipient cells interact with and take up schistosome EVs [15-19]. Despite these findings, very little is known about helminth EV glycomics, let alone schistosome EVs.

Using lectin microarrays (**manuscript I**), we observed that several lectins with varying binding specificities displayed affinity for EVs secreted by adult *S. mansoni* in culture, indicating the presence of a variety of glycan motifs. Unexpectedly, SNA-I, a lectin which binds terminal sialic acid, was amongst the lectins with the highest measured binding intensity, suggesting the presence of glycoconjugates with terminal sialic acid on the surface of adult *S. mansoni* EVs. This finding was unexpected, given the current dogma that helminths are unable to synthesis sialic acid, due to the apparent lack of orthologues of enzymes required for its synthesis in helminth genomes [20,21]. We thus conducted additional tests to confirm its presence, combining the use of a broad-spectrum neuraminidase with lectin binding and detection of free sialic acid. Treatment of EVs with neuraminidase consistently abolished SNA-I binding and lead to detectable levels of free sialic acid in the sample supernatant.

We then sought to determine whether sialic acid found in our EV samples was endogenously or exogenously sourced (host or EV-depleted FBS; **manuscript II**). Sialylated molecules were extracted via lectin pulldown and analysed by mass spectrometry, identifying them as bovine serum proteins. The presence of bovine serum sialoglycoproteins despite the depletion of EVs from FBS prior to its use and the density-based EV isolation protocol used could mean that host serum glycoproteins are in fact associated with *S. mansoni* EVs. This finding is potentially highly significant, given the numerous roles in cell adhesion and immune regulation which have been described for sialic acid [22,23], and that *in vivo*, adult worms inhabit host venules and are thus bathed in host blood proteins. Notably, sialic acids can mask antigenic sites, prevent complement activation, and are believed to serve as self-markers, aiding in the discrimination between self and non-self in order to prevent autoimmune activity [22,24,25]. We hypothesise that adult *S. mansoni* EVs are coated with host glycoproteins which may shield them, preventing their removal, and aid in their internalisation by recipient cells. To investigate our hypothesis that host sialoglycoproteins contribute to antigen masking, we compared the antigenic profiles of *S. mansoni* EV proteins and that of EV-associated sialylated proteins and, under our experimental conditions, it appears that sialylated bovine serum proteins induce limited or no antibody production. These results are consistent with roles in immune evasion and might represent a novel mechanism by which schistosomes evade and/or modulate their host's immune response. Several

infectious agents, ranging from viruses to protozoa, utilise sialic acid to coat themselves, aiding in evasion of the host immune response and the ability to interact with and invade host cells [26,27]. Some are capable of *de novo* synthesis, while others rely on exogenous sources, e.g., their host [24,28]. *Trypanosoma cruzi* is a well-documented example of trans-sialylation, whereby trans-sialidase enzymes encoded by the parasite allow the cleavage and transfer of host terminal sialic acids onto their own glycoconjugates, shielding the parasite from immune recognition and contributing to adhesion to and invasion of host cells [27,29-32]. Although it will be important to validate these findings with additional experimental approaches, our work provides the first indication of the use of sialic acid by parasitic helminths, and describes new ways by which pathogens might utilise sialic acid as part of their infection strategy. This represents yet another example of exploitation of host molecules by parasites and offers critical insights into parasite biology as well as a potential new avenue for the development of therapeutics.

Despite multiple reports of EV secretion in schistosomes, their mechanism of biogenesis and origin(s) of secretion have yet to be determined. A better understanding of how EVs are released will help identifying ways to disrupt their secretion, which will likely impede the parasite's ability to modulate the host and thus establish successful infections. To investigate the route(s) of EV secretion in adult *S. mansoni,* we selected three lectins with high binding affinity for our EVs (SNA-I, DSA, and RCA-I) and carried out whole worm lectin histochemistry (**manuscript I**). These lectins localised primarily in sub-tegumental cell bodies, as confirmed by FISH-lectin co-labelling. In addition, DSA also accumulated in the gut and RCA-I localised in esophageal glands and the excretory tubules and excretory pore. Together, these observations suggest that the tegument as well as the digestive and protonephridial systems may participate in the release of EVs. These results are consistent with previous reports implicating gastrodermal epithelial cells and the tegument in the release of EVs by the related trematode *F. hepatica* [15,33]. Although more work is needed to reach more specific conclusions, our findings provide new insights regarding vesicle-based secretion in schistosomes.

Another major component of EVs are miRNAs, which are positioned as key effectors of EV function given their ability to regulate gene expression. Through complementary base-pairing, EV-associated miRNAs can interact with specific mRNA transcripts in the recipient cell, reducing mRNA stability and leading to downregulation of protein expression. We have previously

identified a number of miRNAs as part of the cargo of *S. mansoni* EVs (**manuscript IV**) [12]. Using computational miRNA target prediction tools, we sought to identify mouse mRNAs which could be targeted by the most abundant adult *S. mansoni* EV-associated miRNAs (**manuscript III**). This search yielded many potential targets and KEGG analysis returned several overrepresented pathways, many of which were common to other helminth-host associations [34]. Meningher *et al* reported the presence of schistosome miRNAs in Th cells isolated from gut-associated lymph nodes (Peyer's patches and mesenteric lymph nodes) of infected mice. The same group also observed downregulation of specific transcript levels in Th cells following incubation with live *S. mansoni* worms using a trans-well system *in vitro* [35]. However, the question of whether parasite-derived miRNAs actively regulate host gene expression *in vivo* remains unresolved. To address this question, we sequenced the transcriptome of Peyer's Patches harvested from mice 7 weeks post-infection with *S. mansoni* and identified over 1,300 DEGs with 915 upregulated and 446 downregulated transcripts. Of the downregulated genes, 119 were predicted targets of *S. mansoni* miRNAs, compared to 193 for the 915 upregulated transcripts. It is important to consider that the EV population is likely heterogeneous, not only in size and cargo, as previously suggested [36], but also in destination and functions. Therefore, it is possible that not all of the miRNAs used in our target prediction analysis reach the intestinal lymphoid tissue or do so in too low of a concentration to result in significant changes in gene expression. Computational target predictions also bring challenges, as the multiple prediction algorithms available rely on distinct analytical methods and often offer varying results. Furthermore, the lack of real consensus on value thresholds can greatly impact predictions as we have seen when utilising previously reported thresholds [34,37], which led to 10,376 predicted targets *vs* 5,434 under our conditions. Accurately predicting miRNA-mRNA interactions is thus challenging, especially in an *in vivo* setting, where additional factors influence these interactions (e.g., EV clearance rate, proximity to recipient cells, etc.). Nevertheless, it is plausible that the differential expression of some of the DEGs are the result of *S. mansoni* miRNA silencing. Whilst downregulation of predicted targets does not prove *in vivo* interactions, it does provide a set of hypotheses for future testing, and experimental verification of these molecular interactions could provide new insights into host-parasite interactions. Importantly, our transcriptomic data allow us to glance into the biology of *S. mansoni* infection, which will be useful to guide future research.

The results detailed herein provide novel and important insights into the biology of *S. mansoni* and its host modulation strategies, specifically related to vesicle-based secretion by adult worms. We provided new information about the biochemistry of adult schistosome EVs, identified a previously undescribed potential immune evasion mechanism, explored the source of EV release, and delved into the gene regulating functions of EVs in the context of infection. These findings offer critical bases for future research on host-schistosome interaction and the mechanisms employed by the parasite to evade and modulate the host. Therefore, the knowledge acquired throughout this thesis may lead to new avenues for the development of new therapeutics.

Many questions remain: Are EVs coated with serum sialoglycoproteins passively or as an active and specific process? At which point do host glycoconjugates become associated with parasite EVs? In the trematodes *S. mansoni* and *Fasciola hepatica*, evidence points to the tegument, digestive and excretory systems as sources of EVs [33,38-40]. Thus, one possibility is that host sialoglycoconjugates are digested by the worms and incorporated into EVs before their release into host blood. However, lectin histochemistry of whole adult worms with SNA-I, a lectin which recognises structures with α2-6-linked terminal SA, strongly labelled sub-tegumental cell bodies of the parasite [38], suggesting uptake of SA into those cells. Consequently, it is plausible that schistosomes might acquire serum glycoproteins via the tegument. The schistosome tegument is the primary interface between the parasite and host blood and plays a crucial role in the uptake of nutrients from the surrounding environment [41]. Indeed, the tegument contributes to schistosome feeding by mediating the uptake of nutrients such as glucose [42,43], amino acids [44-46], and other metabolites via several tegumental transport systems [41]. Though these molecules are significantly smaller than glycoproteins, their import through the tegument is indicative of the absorptive nature of the schistosome surface. However, the underlying mechanisms remain elusive. Could schistosomes have receptors on sub-tegumental cell surfaces that recognize sialic acid as a signal for endocytosis? Do EVs emanate from sub-tegumental cells? Future research should aim to address these outstanding questions and investigate the involvement of SA in cellular interaction and EV uptake. Additionally, future work should prioritise the validation of miRNA targets and the analysis of transcriptomic changes in other tissues of relevance for schistosome-host interactions and analysis of these changes over time as the immune response to the parasite develops and matures.

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Appendix. Manuscript IV.

Vesicle-based secretion in schistosomes: Analysis of protein and microRNA (miRNA) content of exosome-like vesicles derived from *Schistosoma mansoni*

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Abstract

Exosomes are small vesicles of endocytic origin, which are released into the extracellular environment and mediate a variety of physiological and pathological conditions. Here we show that *Schistosoma mansoni* releases exosome-like vesicles *in vitro*. Vesicles were purified from culture medium by sucrose gradient fractionation and fractions containing vesicles verified by western blot analyses and electron microscopy. Proteomic analyses of exosomal contents unveiled 130 schistosome proteins. Among these proteins are common exosomal markers such as heat shock proteins, energy-generating enzymes, cytoskeletal proteins, and others. In addition, the schistosome extracellular vesicles contain proteins of potential importance for host-parasite interaction, notably peptidases, signaling proteins, cell adhesion proteins (e.g., integrins) and previously described vaccine candidates, including glutathione- S-transferase (GST), tetraspanin (TSP-2) and calpain. *S. mansoni* exosomes also contain 143 microRNAs (miRNA), of which 25 are present at high levels, including miRNAs detected in sera of infected hosts. Quantitative PCR analysis confirmed the presence of schistosome-derived miRNAs in exosomes purified from infected mouse sera. The results provide evidence of vesicle-mediated secretion in these parasites and suggest that schistosome-derived exosomes could play important roles in host-parasite interactions and could be a useful tool in the development of vaccines and therapeutics.

1. Introduction

Schistosomiasis is a major parasitic disease, affecting >200 million people in 74 countries, the majority in sub-Saharan Africa [1]. The disease is caused by blood-dwelling flukes of the genus *Schistosoma*, primarily *S. mansoni*, *S. haematobium* and *S. japonicum*. Schistosomes have a complex life cycle that requires a snail as an intermediate host. The infective stage of the parasite is a small, short-lived larva (cercaria), which is released by infected snails into fresh water and rapidly invades a human host by penetration through the skin. Shortly after penetration, the cercaria transforms into an immature parasitic larva (schistosomulum), which enters the circulation and migrates towards the hepatic portal system and mesenteries (*S. mansoni, S. japonicum*) or the venous plexus of the urinary bladder (*S. haematobium*). Larvae grow during migration, eventually maturing into adult male and female worms, which become tightly coupled and produce large numbers of eggs. The pathology associated with chronic schistosomiasis is due to the eggs. Many eggs leave the body in feces or urine, but some are trapped in tissues and induce a granulomatous immune response, leading to progressive tissue fibrosis and organ damage. Deaths due to schistosomiasis have been estimated at >200,000 per annum in Africa alone. There is no vaccine for schistosomiasis and chemotherapy relies heavily on a single drug (praziquantel), raising concerns about drug resistance [2] which highlights the need for more research into new vaccine and drug targets.

In this regard, the secretome is of particular interest. Molecules secreted by parasites play major roles in shaping host interactions and are promising targets for vaccine or chemotherapeutic intervention. Most studies of helminth excretory/secretory products (ESP) have focused on proteins. ESP proteome analyses have been reported for a variety of nematode species [3-5] as well as flatworms, including the liver fluke *Fasciola hepatica* [6,7] and several life stages of *S. mansoni*, *S. japonicum* and *S. bovis* [8-14]. Parasitic worms also secrete microRNAs (miRNA) [15-19], which could play important roles in modulating host immune responses. Secreted miRNAs are increasingly recognised as mediators of cell communication [20,21] and their regulatory roles in the immune system are well established [22].

Despite these advances, and the considerable efforts made to identify schistosome ESPs, very little is known about the mechanisms by which these molecules are released. Many proteins secreted by helminths are unlikely to be released through the classical secretory pathway, for example metabolic enzymes that lack a signal peptide, cytoskeletal proteins, heat shock proteins,

and membrane proteins. An alternative export mechanism for these proteins is through membrane-bound vesicles [23,24]. Extracellular secretory vesicles can be formed by outward bud- ding of the plasma membrane or formed intracellularly and subsequently released to the outside. The best characterised of these vesicles are exosomes, which are small (60–150 nm), typically cup-shaped when observed by transmission electron microscopy (TEM) and are produced from multivesicular intermediates of the endocytic pathway [24]. There is growing evidence that many proteins secreted by parasites are enclosed within exosome-like vesicles (ELVs) [19,25,26]. Recent studies of *S. japonicum* [27] and *S. mansoni* [28,29] suggest that schistosomes may use extracellular vesicles as a mechanism of secretion, but the molecular content of these vesicles has only partly been characterised.

Exosomes have attracted considerable interest because of their roles as mediators of intercellular communication, particularly within the immune system and in disease states such as cancer [24,30]. Exosomes are powerful agents of cell communication because of the many ways they can interact with target cells; they can bind to cell surface receptors, indirectly changing cell behavior, they can fuse with the target cell membrane, releasing their contents into the cytosol, and/or they may be taken up and internalised into phagosomes [31]. Exosomes also provide an effective mechanism for delivery of parasite molecules to a host cell, either to modulate the immune response or some other host activity that is beneficial to the parasite. Studies primarily in *Leishmania* [25,32,33] and *H. polygyrus* [19] have shown that parasite-derived extracellular vesicles (EVs) can be taken up by host (mammalian) cells in culture; they induce changes in gene expression in host cells and have strong immunomodulatory properties, both *in vitro* and in infected animals. *Leishmania* exosomes may be the principal mechanism by which the parasite delivers virulence factors to the host cell [25]. These studies clearly implicate EVs as important mediators of the host-parasite interaction and underscore the need for further research into their mode of action.

Here we show that *S. mansoni* release ELVs and describe the proteins and miRNAs contained within these vesicles. The results help to elucidate the mechanism by which proteins and miRNAs are secreted and provide new insight into the role of secreted products in schistosome infections.

2. Methods

2.1. Parasites

Schistosoma mansoni adults were obtained from CD1 mice after a six week experimental infection by perfusion [34]. Mice were maintained in the animal facility at the Small Animal Research Unit (SARU) on the Macdonald Campus of McGill University (Montreal, Canada) according to the McGill University Animal Care Committee (Permit # 2001–3346). Briefly, mice were euthanised by CO2 asphyxiation and cervical dislocation and *Schistosoma mansoni* adults harvested from mesenteric venules and thoroughly washed with Phosphate-Buffered Saline (PBS), and maintained in RPMI-1640 medium supplemented with 100 U penicillin, 100 mg/mL streptomycin, and 10% exosome-depleted Fetal Bovine Serum. Exosome-depletion was performed in advance by ultracentrifugation of FBS under sterile and apyrogenic conditions for 18 h at 120,000 × g and 4 °C as described [35]. FBS was of the highest grade and lowest endotoxin level (Life Technologies; Ref: 16000). Adult male and female worm pairs were maintained in 6-well plates for 72 h at a density of 20 worms (10 males and 10 females)/well in 10 ml culture medium at 37 °C. Conditioned medium was collected after 48 and 72 h. Worms remained active during this period of incubation, with no apparent change in morphology or movement. All experiments and protocols were approved by the McGill University Animal Care Committee according to the high standards established by the Canadian Council on Animal Care.

2.2. Extracellular vesicle isolation.

ELVs were purified by differential centrifugation followed by membrane filtration and sucrose density ultracentrifugation as described [35,36]. Briefly, after 48 and 72 h in culture, the parasite conditioned culture medium was collected and centrifuged first at low speed (300 × g/ 20 min), then at 3,000 × g (20 min) to remove larger debris, and the supernatant was centrifuged at 12,000 × g for 45 min. Supernatants were filter-sterilised using a 0.22 μm hydrophilic PVDF Durapore membrane (EMD Millipore; SVGV01015) and centrifuged at 100,000 × g for 2 h using a SW-41 rotor on a Beckman-Coulter ultracentrifuge. The pellet was re-suspended in sterile cell culture grade PBS (Life Technologies) and fractionated on a discontinuous 10–50% sucrose gradient (1.03–1.23 g/cm3), using a SW-28 rotor at 120,000 × g for 18 h. Alternatively, exosomes were purified by loading the re-suspended pellet onto a sucrose step gradient (25%, 30% and 35%)

[36], then centrifuged at 120,000 × g for 18 h as above. Following centrifugation, the 30% sucrose fraction was collected, diluted 4-5 fold with sterile PBS and vesicles were repelleted by centrifugation in an SW-28 rotor at 100,000 × g for 2 h. The pellets were washed once by re-suspension in PBS and centrifugation repeated at 100,000 × g. For the last spin, the pellets were re-suspended in 1.0 ml PBS, transferred to 1.5 ml Beckman-Coulter ultracentrifuge tube and centrifuged in an Optima TL100 tabletop ultracentrifuge (Beckman-Coulter) for 1.5 h at 100,000 × g using a TLA-100.3 rotor. Exosomes were quantified based on protein concentration using a Pierce BCA Protein Assay Kit (ThermoFischer Scientific). The pelleted material was snap-frozen and stored at −80 °C.

2.3. Western blot assays

Purified ELVs (30 μg total protein) were treated with lysis buffer (Invitrogen) supplemented with 50 mM Tris (2-carboxyethyl)phosphine (TCEP) and a cocktail of protease inhibitors (Sigma-Aldrich) for 15 min at room temperature, followed by heating for 15 min at 65 °C. Proteins were resolved on a 4–20% Tris–glycine gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane according to standard protocols. Proteins were detected using goat anti-rabbit enolase antibodies (Santa Cruz Biotechnology; sc-7455). Secondary antibodies were a goat anti-rabbit IgG HRP-conjugate from Millipore (EMD Millipore).

2.4. Electron microscopy (EM)

EM analyses of *S. mansoni* ELVs were performed at the Facility for Electron Microscopy Research of McGill University using standard protocols34. Briefly, exosomes were fixed in 2% paraformaldehyde in PBS for 4 h, deposited on Formvar-carbon coated EM grids, washed in PBS, treated with 1% glutaraldehyde and then washed with water. Samples were stained with a solution of 4% uranyl oxalate, pH 7, then contrasted and embedded in a mixture of 2% methyl cellulose/4% uranyl acetate, pH 4 (9:1 v/v, methyl cellulose-UA) and finally placed in methyl cellulose-UA for 10 min on ice to increase the contrast. Grids were observed in a FEI Tecnai 12 transmission electron microscope operated at 80 kV. Images were acquired with a digital AMT XR80C CCD Camera System.

2.5. Proteomics analysis

For protein identification purified ELVs (200 µg total protein) were dissolved in a MS-compatible protein solubilizer (Invitrosol LC/MS Protein Solubilizer, Invitrogen) according to the manufacturer's protocol and diluted with 5 volumes 50 mM ammonium bicarbonate, pH 8.5. Samples were heated to 60 °C for 30 min and sequentially reduced with 5 mM DTT (60 °C/30 min), alkylated with 5 mM iodoacetamide (20 °C in the dark/30 min) and finally treated with a small amount of DTT (10 µl 10 mM DTT) at 20 °C for 30 min to neutralise iodoacetamide. Proteins were quantified with a Pierce BCA Protein Assay Kit and samples were treated with Promega Gold Mass Spectrometry Grade Trypsin (1:50 enzyme:substrate ratio) and incubated for 14–16 h at 37 °C with shaking. Digestions were stopped with 1% trifluoroacetic acid (TFA) (Sigma-Aldrich) and peptides were purified with a C18 Zip-Tip (Millipore) and the peptide eluate was dried under vacuum. Peptides were reconstituted in 10 µl 0.1% formic acid before analysing by LC-MS/MS. For analysis, peptides were fractionated on a 360 μm ID x 4 mm C18 trap column prior to separation on a 150 µm ID x 10 cm nano-LC column (Jupiter C18, 3 µm, 300 Å, Phenomex) and sequenced using an ESI-LC-MS/MS system, LTQ-Orbitrap Elite hybrid mass spectrometer with a nanoelectrospray ion source (ThermoFisher, San Jose, CA) coupled with an Eksigent nano-LC 2D pump (Dublin, CA). Peptides were separated using a linear gradient of 5–40% acetonitrile (0.2% for- mic acid) for 53 min and a flow rate of 600 nl/min. MS survey scans were acquired in profile mode at a resolution of 60,000 at *m*/*z* 400. MS/MS spectra were acquired in the ion trap using collision-induced dissociation (CID) for multiply charged ions exceeding a threshold of 10,000 counts. Three independent LC-MS/MS experiments were performed.

2.6. Protein data analyses

MS data were acquired using Xcalibur (v. 2.0 SR1). Peak lists were then generated with Mascot distiller (v. 2.1.1, Matrix Science) and MS processing was achieved using the LCQ_plus_zoom script. Database searches were performed against the *S. mansoni* proteome dataset available at the National Center for Biotechnology Information (NCBI) and the mouse proteome (*Mus musculus*) to test for the presence of host proteins, using MassMatrix (v. 2.4.2). The following search parameters were consistently applied: trypsin digestion with two missed cleavages allowed, cysteine carbamidomethylation as a fixed modification, methionine oxidation

as variable modification, 0.8 Da tolerance for fragment ion masses, 10 parts per million mass tolerance for precursor ion masses and a minimum peptide length of 6 amino acids. A decoy reversed sequence database was included in every search. Peptide matches were considered significant if they exceeded the threshold statistical scores defined by MassMatrix [37,38] and the false discovery rate was <5% ($p < 0.05$), as determined by decoy database searching. Functional annotation of protein sequences was done with Blast2GO software [39] against the entire non-redundant NCBI nr database using default parameters. Annotation was augmented with Annotation Expander (ANNEX) [40] and InterPro database scans were performed within Blast2Go to retrieve additional GO terms associated with functional domains.

2.7. Illumina RNA sequencing

S. mansoni-derived exosomes were purified from worm-conditioned culture media as described above and the RNA contained within the vesicles was isolated using a Norgen Total RNA extraction kit (Thorold, Canada), according to the manufacturer's protocol. Total RNA was extracted from adult male and female pairs using the same kit. All subsequent steps were performed at LC Sciences, LLC (Houston, Texas, USA) using their standard protocols. Briefly, *S. mansoni* ELV (1000 worm pairs – 20 ng of miRNA) and whole worm (400 worm pairs – 100 ng of miRNA) small RNA libraries were generated, reverse-transcribed (RT) and the resulting cDNA constructs were purified using an Illumina TruseqTM Small RNA Preparation kit. ELV and whole worm cDNA libraries were used for cluster generation on a Cluster Station fluidics device and then sequenced on an Illumina GAIIx instrument, according to vendor's instructions. Raw sequencing reads (40 nt) were obtained using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8), following real-time sequencing image analysis and base-calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). A proprietary pipeline script, ACGT101-miR v4.2 (LC Sciences), was used to analyse the data. Starting with raw sequencing reads, a series of digital filters (LC Sciences) were employed to remove reads <15 nt and unmappable sequences (low quality reads, adaptors and other "impurities" due to sample preparation and sequencing chemistry). The remaining sequences with lengths between 15 and 32 nt were mapped to *S. mansoni* and related Platyhelminth (flatworm) miRNAs available in the latest release of miRBase (v.20) (http://mirbase.org) (Accessed September 18, 2015). Mapping was also performed against other small RNAs in the RFam database (http://rfam. janelia.org), against

repetitive sequences in RepBase (http://www.girinst.org/repbase) and the latest assemblies of the *S. mansoni* genome and transcriptome available at the *S. mansoni* GeneDB78,79 (Accessed September 28, 2015). For comparisons between ELV and whole worm samples, the number of read counts from each sample was tracked during mapping and normalised to account for differences in library size. Normalisation was achieved by dividing the counts by a scaling factor that reflected the size of each library. Scaling factors were calculated according to a median count method80 as outlined by LC Sciences.

2.8. Isolation of ELV RNA from mouse serum

Serum was collected from individual *S. mansoni*-infected mice 6–7 weeks post-infection and from age-matched uninfected controls. Exosomes were isolated with an ExoQuick Exosome Precipitation kit (SBI System Biosciences), starting with 500 µl serum per purification according to the recommendations of the manufacturer. The resulting exosomal pellets were resuspended in sterile lysis buffer and the RNA contained within the vesicles was isolated by column purification as specified in the kit protocol. A constant amount (1011 copies) of a spike-in synthetic RNA oligonucleotide (5′- CGUAUCGAGUGAUGUCACGUA- 3′) was added at the time of exosomal lysis, prior to RNA purification, and then carried through all additional steps. The spike-in RNA does not match any known miRNA in miRBase and was used for normalisation of qRT-PCR data as described [17].

Taqman qRT-PCR. Five highly abundant schistosome-derived miRNA sequences (Sma-Bantam, Sma-miR-71a, Sma-miR-36-3p, Sma-miR-125a and Sma-miR-125b) were selected for PCR amplification. At least 4 mismatches distinguished these miRNAs from the closest vertebrate miRNA homologue identified in miRBase, as deter- mined by BLASTn alignments. A stem-loop method of miRNA amplification was used throughout the study. Target-specific stem-loop RT primers were synthesised and re-folded as described [41]. RT was performed according to standard protocols in a 20 µl reaction containing 0.5 mM dNTPs, 200 units MMLV reverse transcriptase (Life technologies) (or no enzyme in the -RT control tubes), 1 × First strand RT buffer, 10 mM DTT, 20 units RNase out inhibitor, 5 nM RT stem-loop primer and either synthetic miRNA or serum-derived exosomal RNA as the template. qPCR amplifications were performed in a total volume of 20 µl containing Luminaris Probe qPCR colorless master mix (Thermo Scientific), 2 µl

fresh RT product, 1.5 μM sequence-specific forward primer, 0.7 μM universal reverse primer and 0.8 μM hydrolysis TaqMan probe. Primer and probe concentrations are considered optimal for stem-loop qRT-PCR of miRNAs [41]. Reactions were performed in replicates of 2–4 in an Applied Biosystems 7500 Fast thermocycler (Life Technologies) according to the following cycling protocol: 2 min at 50 °C, 10 min at 95 °C, followed by 60 cycles of denaturation at 95 °C/15 s, annealing at 58 °C/45 s and extension at 70 °C/30 s. Quantitative qRT-PCR was first performed with synthetic miRNAs to optimise reaction conditions. Amplification efficiencies were calculated from the slopes of serial dilution curves (where Efficiency (E)=10 (−1/slope)) and were determined to be approximately 90–100% for all five targets and the spike-in control. For qRT-PCR of serum-derived exosomal samples, we used a fixed volume (5 μl) of RNA corresponding to approximately 100 μl serum and RT was performed with two different stem-loop primers added simultaneously and at the same concentration (5 nM), one targeting the specific miRNA of interest and the other targeting the spike-in RNA used for data normalisation. RT reactions were split in half and used for parallel qPCR amplification of the target miRNA and spike-in control, using appropriate primers. A complete list of all the primers and TaqMan probes used in these experiments are described in Table S1. Synthetic miRNA sequences and oligonucleotides (stem-loop RT primers, forward primers, universal reverse primer) and hydrolysis TaqMan probes were purchased from Life Technologies (Foster City, USA).

qPCR data analysis. A relative ΔCt method was used to compare qPCR data from schistosome-infected mice and uninfected controls. The method was adapted for the analysis of circulating miRNAs from serum and other biological fluids, where conventional data normalisation based on a housekeeping gene is not feasible. Ct values were first "median normalised" to the spike-in oligo as described [17,42] to correct for sample-to-sample variation and differences in RT efficiency. The normalised Ct (Ctn) values were then used to calculate the ΔCtn for each sample. This was done by subtracting a median Ctn of all the uninfected control samples (background amplification) from the Ctn of the test (infected) sample. Fold-change relative to the uninfected control was calculated from the formula $2^{-\Delta Ctn}$. The median Ctn values for the uninfected controls varied between 53 and 60 for the four miRNA targets. Samples where there was no detectable amplification of the target miRNA were arbitrarily assigned a maximum Ct value of 60 for data calculations.

3. Results

3.1. Purification of ELVs

ELVs were initially purified from culture medium by differential centrifugation, followed by filtration and sucrose density ultracentrifugation (Fig. 1) to establish the sucrose concentration required for the sedimentation and enrichment of the *S. mansoni* ELVs. Purification was monitored by Western blot analysis using an anti-human enolase antibody, a common exosomal marker, which is highly conserved with *S. mansoni* enolase (75% sequence identity). Fractions exhibited distinct protein profiles, with the 20, 25, 35 and 40% sucrose fractions containing diverse proteins, possibly corresponding to protein aggregates, smaller vesicles or plasma membrane-derived vesicles (Fig. 2A). A predominant anti-enolase reactive band was recovered from the 25%, 30% and 35% sucrose fractions (Fig. 2B), consistent with the presence of exosomes [36]. Vesicles recovered from the 30% sucrose fraction were tested with antibodies against exosomal markers that are highly conserved in *S. mansoni* (anti-HSP70, anti-tubulin, anti-elongation factor 1; sequence identities 38–98% compared to mouse homologues) and all showed immunoreactive bands of the expected size (not shown). Subsequent purifications were done by overlaying the 0.2 μm-filtered 100,000 × g pellet directly onto a 30% sucrose cushion followed by ultracentrifugation [36]. EM analysis showed that the purified cup-shaped vesicles were ~100 nm in diameter (Fig. 2C), consistent with exosomes. Our experimental analysis cannot rule out the possibility that other types of secretory vesicles of similar shape and density may be present.

3.2. Proteomics studies

LC-MS/MS analysis was repeated on three separate preparations of purified vesicles and candidate proteins identified by searching against the *S. mansoni* NCBI genome database. These analyses identified 130 *S. mansoni* proteins, including 125 known proteins and 5 annotated as hypothetical (Table 1). Matching peptides are shown in Table S2. The detected proteins include many homologues of common exosomal markers [43] (Table 1). We note, in particular, energy-generating enzymes often associated with exosomes (e.g., enolase, pyruvate kinase, GAPDH, phosphoglycerate kinase 1), heat shock proteins (HSP70), cytoskeletal proteins (actin, tubulin, fimbrin), 14-3-3 proteins, tetraspanin, histones, vesicular traffic proteins (e.g., Rab proteins, dynein) and translational elongation factor eEF1. Their presence in the schistosome vesicles gives

further confidence that these are exosomes. Importantly, proteomics data confirmed the presence of enolase.

The proteins in Table 1 were compared to previously reported schistosome ES proteins. *S. mansoni* secretome studies have been done mainly on larval stages or eggs [8,11,12,14] but a comprehensive dataset is available for adult *S. japonicum* [9], which was used for this comparative analysis. The *S. japonicum* secretome lists approximately 100 proteins, of which about half are "atypical" (or non-secretory) proteins that lack a signal peptide [9]. We found ~50% of these atypical secretory proteins (Table 1), in particular heat shock proteins, enolase, GAPDH, GST, 14-3-3 proteins and a fatty acid binding protein. These are all present in the purified vesicles and were previously described as some of the most abundant secreted proteins in *S. japonicum* and *S. mansoni* larvae. These results suggest that a significant proportion of protein secretion in schistosomes occurs through vesicles.

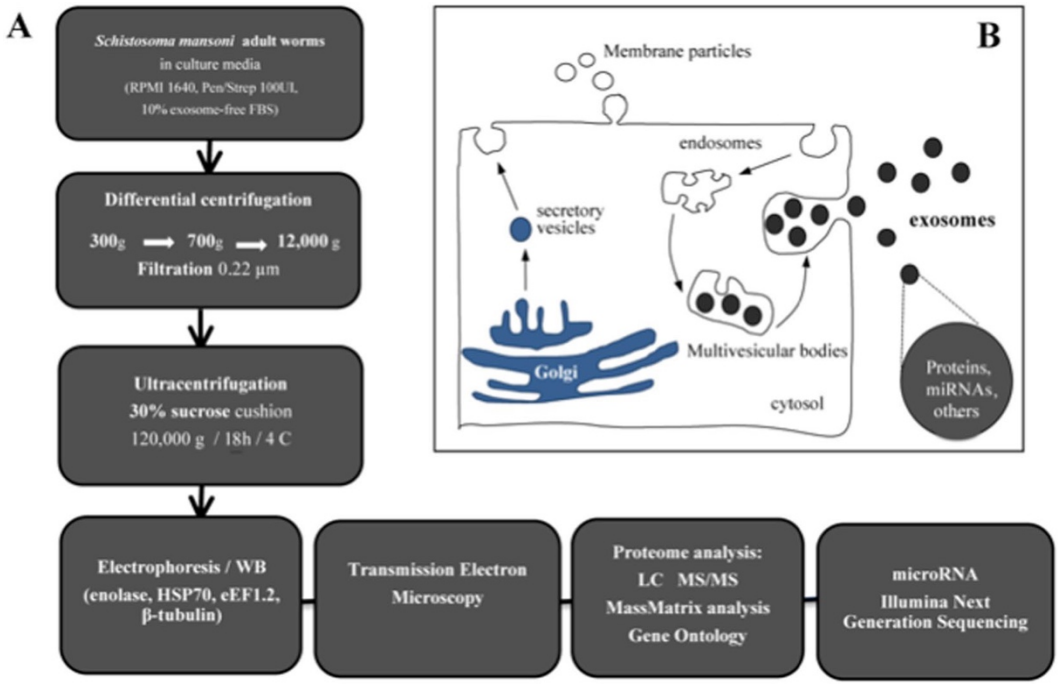

Figure 1. Overview of the procedure used for isolation and characterisation of secreted exosome-like vesicles from *Schistosoma mansoni*. (**A**) Adult male and female worms were cultured 48-72 h in media containing exosome-depleted serum. Vesicles were purified from the culture media by differential centrifugation, followed by filtration through a 0.2 µm membrane and ultracentrifugation on a discontinuous 25, 30, 35% sucrose gradient, as described [34]. The purification was monitored by western blot (WB) analysis, using antibodies against known

exosomal markers (e.g. enolase) and electron microscopy (EM), prior to analyses of protein and miRNA content. (**B**) Schematic of the two major types of secretory extracellular vesicles. Membrane particles (or ectovesicles) are formed by outward budding of the plasma membrane. Exosomes are derived from the endocytic pathway via the formation of large multivesicular body (MVB) intermediates, which fuse with the plasma membrane releasing the vesicular cargo, exosomes, as well as other contents into an extracellular environment. Alternatively, the MVBs can be directed to lysosomes and degraded.

Exosomal proteins were classified by GO annotation according to molecular function, biological process and predicted cellular compartment (Fig. 3). The results show a high prevalence of proteins with catalytic and/ or binding activity ("molecular function") and proteins involved in metabolic and cellular processes ("biological process"). Enzymes are the most common type, representing nearly half of the proteins. The majority of these enzymes are involved in glucose metabolism. Enzymes from other pathways, for example taurine metabolism (taurocyamine kinase), amino acid metabolism (ornithine-oxo-acid transaminase), purine metabolism (hypoxanthine-guanine phosphoribosyltransferase, HGRPT) and detoxification (redox) metabolism (e.g. glutathione-S-transferase (GST), glyoxalase) were detected. The schistosome vesicles are rich in proteases, including metallopeptidases, cysteine peptidase and serine peptidases. The most abundant of these enzymes, based on the number of spectral matches, are thimet oligopeptidase, leucine aminopeptidase (LAP), calpain, hemoglobinase and cathepsin B.

Many proteins were assigned to GO biological processes terms that relate to signal transduction ("signalling", "response to stimulus", "immune processes") and biological regulation (Table 1). Some of these proteins are common exosomal markers that have broad spectrum signaling activities, for example 14-3-3 proteins [44]. We also identified proteins associated with calcium-dependent signaling (e.g., calponin), GTP-mediated signaling (rpgr interacting protein 1), a serine/threonine protein kinase, phosphatase-associated proteins, a grb2-like protein normally involved in receptor tyrosine kinase signaling, and two putative potassium channels, one of which is activated by cAMP. A few proteins listed in this category have activities in immune processes and blood coagulation pathways, which could be important for the host-parasite interaction. One example is annexin, a calcium-dependent phospholipid-binding protein implicated in a wide range of cellular processes. Some types of annexins have powerful anti-inflammatory activity [45]; others function as plasminogen receptors and stimulate fibrinolysis, thus reducing the formation of blood clots [46]. Also noteworthy are schistosome homologues of ATP-diphosphohydrolase 1, N.N-

dimethylarginine dimethylaminohydrolase (DDAH), kynurenine aminotransferase and integrins. In mammals, these proteins have important immune and/or anti-clotting effects. ATP-diphosphohydrolase 1 suppresses inflammation, inhibits platelet aggregation and prevents blood clots by breaking down extracellular pro-inflammatory and prothrombotic ATP and ADP [47]. DDAH and kynurenine aminotransferase control the levels of important regulators of immune function by indirectly controlling nitric oxide production [48] and the levels of kynurenine, a tryptophan metabolite that regulates vasodilation and immune responses [49]. Finally, integrins mediate cell adhesion and are implicated in a wide range of signaling mechanisms associated with immunity [50]. We found alpha and beta integrin subunits, as well as talin, which anchors integrins to the cytoskeleton and mediates subsequent signaling, in schistosome exosomes.

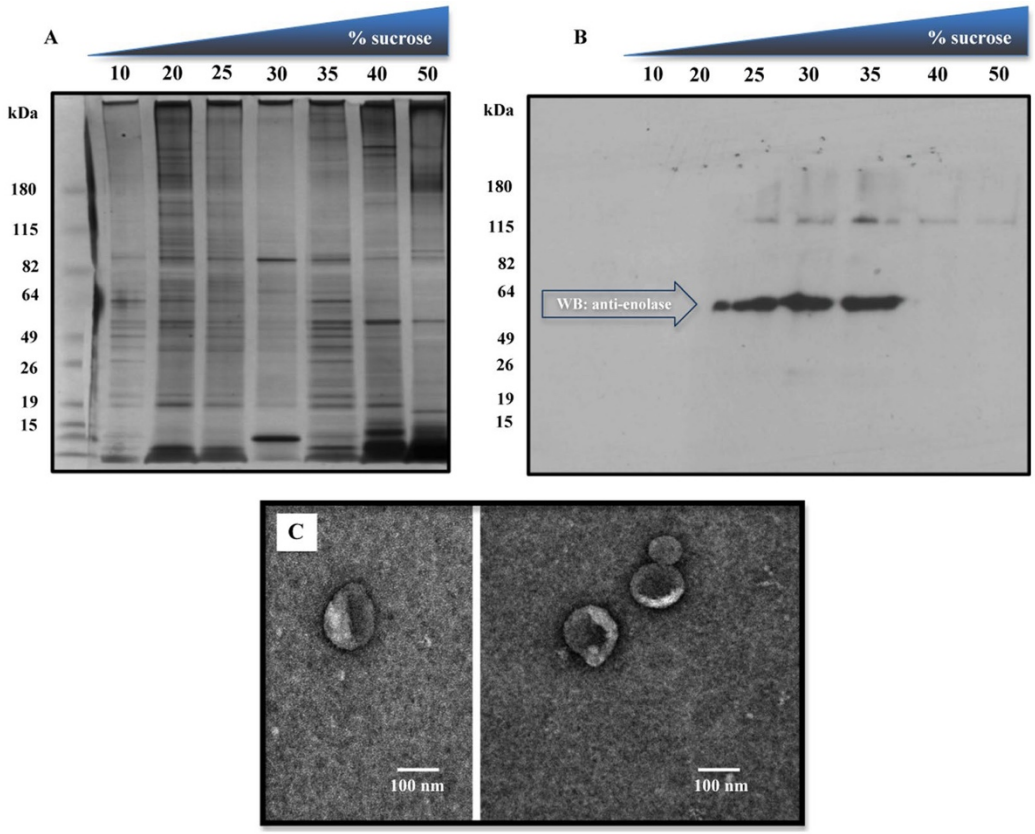

Figure 2. Purification of exosome-like vesicles from *S. mansoni.* Vesicles were collected from worm culture media and partially purified through differential centrifugation as shown in Fig. 1. The resulting crude vesicular pellet was resuspended in PBS, filter sterilised (0.2 µm filter) and subsequently fractionated on a discontinuous 10–50% sucrose gradient. Gradient fractions were tested for total protein content by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (**A**) and then western blotting (WB) with an antibody against enolase, a common exosomal marker (**B**). The results show enolase immunoreactivity between the 25% and 35% sucrose fractions. All

subsequent purifications were performed by applying the filter-sterilised, crude vesicular pellets directly onto a discontinuous 25, 30, 35% sucrose gradient followed by ultracentrifugation, as described [34] (**C**) Transmission electron microscopy analysis of purified exosome-like vesicles from *S. mansoni*. The scale bar indicates 100 nm.

"Cellular compartment" analysis shows a high proportion of proteins associated with membrane-bound compartments (vesicles, organelles) as well as the plasma membrane. Membrane proteins constitute roughly 30% of identified proteins (Fig. 3). Some of the most abundant proteins in the dataset are membrane proteins, notably fer-1, a homologue of vertebrate dysferlin, which has been implicated in membrane structure, fusion, and repair associated with wound healing [51]. Other membrane proteins include transport proteins (e.g., aquaporin, glucose transporter), structural proteins (e.g., prominin), signaling proteins (e.g., tetraspanin, integrins, ion channels), membrane-associated enzymes (e.g., calpain) and a variety of schistosome tegumental antigens (e.g., 200 kDa GPI-anchored glycoprotein) (Table 1).

MS spectra were also searched against the *Mus musculus* database to test if the schistosome vesicles contained host proteins, which are known to be present in parasite-derived exosomes [19,26]. Only 7 mouse proteins were reliably identified (Table 1), of which 4 were keratins, a common contaminant in proteomics studies. Three additional mouse proteins (enolase, actin and plasma membrane calcium ATPase) were disregarded because the peptides matched the schistosome homologues with higher scores. Aside from keratins, the most significant hit was an isoform of importin, a protein typically involved in nuclear transport.

3.3. Analysis of miRNA content

The majority of miRNA reads mapped to non-miRNA sequences (mRNAs, other noncoding RNAs), repetitive sequences, or identified as "no hits" (Table S3). These sequences were removed from the datasets and not analysed further. Of the remaining reads, we focused on those that aligned to *S. mansoni* or Platyhelminth (flatworm) miRNAs in miRbase. Reads that did not match known miRNAs were considered only if they mapped to the genome of *S. mansoni* and the extended sequences at the mapped genome positions were predicted to form hairpins.

Table 1. Protein content of *S. mansoni* exosome-like vesicles.

Accession#	Annotation[a]	peptides[b]	ES protein[c] references
	Metabolic Enzymes		
CCD82906.1	Glycogen phosphorylase [Sm]	50	[8]
P16641.3	Taurocyamine kinase [Sm]	30	[8,13]
ABU49845.1	Creatine kinase [Sm]	28	[9]
Q27877.1	**Enolase [Sm][d]**	**25**	[8-13]
CCD76480.1	**Pyruvate kinase [Sm]**	**22**	[8,9]
CCD81281.1	Glucose-6-phosphate isomerase [Sm]	22	[9]
CCD82636.1	**Lactate dehydrogenase [Sm]**	**18**	[8,9,11]
CCD75004.1	Malate dehydrogenase [Sm]	17	[8,9,11,12]
CCD75874.1	Phosphoenolpyruvate carboxykinase [Sm]	17	[8,9,11,12]
XP_002571535.1	Ornithine-oxo-acid transaminase [Sm]	9	[9]
P41759.1	Phosphoglycerate kinase [Sm]	8	[8,9,11-13]
CCD76263.1	Transketolase [Sm]	8	[9,12]
XP_002581246.1	Phosphoglucomutase [Sm]	7	[8,9]
CCD79691.1	**Fructose 1,6-bisphosphate aldolase [Sm]**	**7**	[8,9,11-13]
CCD59265.1	Aldehyde dehydrogenase, putative [Sm]	4	[9,11]
CCD59437.1	Adenosylhomocysteinase, putative [Sm]	4	
CCD75628.1	**Glyceraldehyde-3-phosphate dehydrogenase [Sm]**	**3**	[8,9,11-13]
P09383.1	Hypoxanthine-guanine phosphoribosyltransferase [Sm]	3	
AFH56663.1	Methylthioadenosine phosphorylase [Sm]	3	[9]
XP_002577577.1	Aconitate hydratase [Sm]	3	[11]
Q27778.1	6-phosphofructokinase [Sm]	3	[8,9]
CCD81640.1	Long-chain-fatty-acid-CoA ligase [Sm]	2	
CCD75611.1	Glycogenin-related [Sm]	2	
CCD58348.1	6-phosphogluconate dehydrogenase,putative [Sm]	2	
	Xenobiotic / Redox Metabolism		
P09792.1	Glutathione S-transferase 28 kDa isozyme [Sm]	14	[8,9,11-13]
XP_002582203.1	Glutathione S-transferase 26 kDa [Sm]	10	[9,11-13]
CCD59704.1	Aldo-keto reductase, putative [Sm]	6	[11]
AAA29889.1	Glutathione S-transferase, partial [Sm]	5	[9,11]
CCD77979.1	Glyoxalase I [Sm]	3	
	Proteases		
CCD59179.1	Thimet oligopeptidase (M03 family) [Sm]	19	

CCD78710.1	Leucine aminopeptidase (M17 family) [Sm]	14	[8,9]
CCD76462.1	Calpain (C02 family) [Sm]	11	[9]
P09841.3	Hemoglobinase (Antigen SM32) [Sm]	7	
CCD74613.1	Cathepsin B-like peptidase (C01 family) [Sm]	6	[9,10]
XP_002572619.1	Prolyl oligopeptidase (S09 family) [Sm]	5	
CCD77256.1	SpAN g.p. (M12 family) [Sm]	5	
AHB79081.1	Serine protease 2 precursor [Sm]	3	[10]
XP_002578277.1	Subfamily M12B unassigned peptidase (M12 family)[Sm]	3	
CCD80658.1	Family S9 non-peptidase homologue (S09 family) [Sm]	3	
CCD79473.1	Xaa-Pro dipeptidase (M24 family) [Sm]	2	
	Fatty acid binding		
1VYG	Fatty Acid Binding Protein [Sm]	3	[8-11,13]
	Transporters / Channels		
CCD60986.1	Plasma membrane calcium-transporting atpase [Sm]	14	
CCD78964.1	Sodium potassium transport ATPase alpha subunit [Sm]	8	
CCD77470.1	Glucose transport protein [Sm]	7	
CCD80392.1	Cation-transporting ATPase [Sm]	5	
XP_002578298	Choline transporter-like protein 2 (Ctl2) [Sm]	4	
CCD75891.1	Aquaporin-3 [Sm]	3	
XP_002578385.1	Chloride channel protein [Sm]	3	
CCD77770.1	Anion exchange protein [Sm]	2	
	Signal Transduction and Biological Regulation		
Q26540.1	**14-3-3 protein homolog 1 [Sm]**	**10**	[8,9,12,13]
CCD75054.1	**14-3-3 epsilon [Sm]**	**9**	[9,12,13]
XP_002578585.1	**Annexin [Sm]**	**8**	[9,10]
CCD74824.1	sh3 domain grb2-like protein B1 (endophilin B1) [Sm]	6	
CCD74840.1	Kynurenine aminotransferase [Sm]	6	
CCD77507.1	Talin [Sm]	6	
XP_002577660.1	Integrin alpha-ps [Sm]	6	
CCD74661.1	Integrin beta subunit [Sm]	6	
CCD79674.1	Atp-diphosphohydrolase 1 [Sm]	5	[8]
XP_002578604.1	rpgr-interacting protein 1 related [Sm]	4	

CCD58613.1	Hyperpolarization activated cyclic nucleotide-gated potassium channel, putative [Sm]	4	
XP_002576557.1	N, N-dimethylarginine dimethylaminohydrolase [Sm]	3	
XP_002569666.1	ral guanine nucleotide dissociation stimulator ralgds[Sm]	3	
CCD75701.1	Proline-serine-threonine phosphatase interact protein [Sm]	3	
CCD78505.1	Lip-related protein (liprin) alpha [Sm]	3	
XP_002581393.1	**Tetraspanin [Sm]**	**3**	
XP_002580804.1	Serine/threonine protein kinase [Sm]	3	
CCD78813.1	rap1 [Sm]	2	
CCD58662.1	Calponin homolog, putative [Sm]	2	[8,13]
CCD78057.1	Voltage-gated potassium channel [Sm]	2	
CCD80562.1	**Syntenin [Sm]**	**2**	
	Cytoskeletal /Structural		
CCD58796.1	Fer-1-related [Sm]	40	
P53471.1	**Actin-2 [Sm]**	**19**	[9,12]
CCD79944.1	Alpha tubulin [Sm]	13	[9,12]
CCD79871.1	Tubulin beta chain [Sm]	12	[8,9]
XP_002574516.1	Tubulin subunit beta [Sm]	12	[8,9,12]
CCD76120.1	Collagen alpha-1(V) chain [Sm]	11	
CCD60034.1	Prominin (prom) protein, putative [Sm]	10	
CCD77450.1	Rab GDP-dissociation inhibitor [Sm]	7	
CCD82967.1	**Actin [Sm]**	**6**	[8,9,12,13]
CCD60380.1	Synaptotagmin, putative [Sm]	6	
AAA29882.1	Fimbrin [Sm]	5	[8,9]
CCD82452.1	Signal recognition particle 68 kD protein [Sm]	5	
XP_002572341.1	Gelsolin [Sm]	4	
CCD77020.1	Intermediate filament proteins [Sm]	4	
CCD76586.1	Cytoplasmic dynein light chain [Sm]	3	[8,13]
XP_002572850.1	Collagen alpha chain type IV [Sm]	2	
CCD79854.1	Microtubule-associated protein 9 [Sm]	2	
CCD82782.1	Rab-2,4,14 [Sm]	2	
CCD60258.1	Rab11, putative [Sm]	2	
CCD74879.1	Ran [Sm]	2	
	Tegumental antigens		
CCD81232.1	200-kDa GPI-anchored surface glycoprotein [Sm]	24	

CCD76403.1	Tegumental protein Sm 20.8 [Sm]	9	[8,13]
P14202.1	Tegument antigen SmA 22.6 [Sm]	7	[10]
CCE94318.1	Tegumental antigen [Sm]	3	
CCD59158.1	Sm23, putative [Sm]	2	
CCD76286.1	Sm29 [Sm]	2	
	Histones		
CCD77737.1	Histone H3 [Sm]	4	[8]
AAG25601.1	Histone H4 [Sm]	4	[8,12]
CCD75757.1	Histone H2B [Sm]	3	[8,12]
	Chaperones		
CAZ34365.1	**Heat shock protein 70 (hsp70)-4, putative [Sm]**	**7**	[8,9,12,13]
Q26565.1	Peptidyl-prolyl cis-trans isomerase [Sm]	5	[8,11]
CCD76203.1	Heat shock protein-HSP20/alpha crystallin family [Sm]	5	
XP_002577613.1	Chaperonin containing t-complex protein 1 epsilon subunit tcpe [Sm]	3	
	Translation		
CCD76432.1	**Elongation factor 1-alpha (ef-1-alpha) [Sm]**	**7**	[8,9,12,13]
CCD79146.1	**Eukaryotic translation elongation factor [Sm]**	**5**	[9,12]
	Others		
XP_002576729.1	SPRY domain containing protein [Sm]	10	
CCD60716.1	Cell division control protein 48 aaa family protein (transitional Endoplasmic reticulum atpase), putative [Sm]	7	
CCD82557.1	Band 4.1-like protein [Sm]	6	
CCD58670.1	Centrosomal protein of 135 kDa (Cep135 protein) [Sm]	6	
CCD82376.1	Excision repair helicase ercc-6-related [Sm]	5	
XP_002575991.1	Ubiquitin (ribosomal protein L40) [Sm]	4	[8,9,11,13]
CCD76953.1	Ubiquitin-protein ligase BRE1 [Sm]	4	
XP_002578337.1	Mixed-lineage leukemia 5 mll5 [Sm]	4	
CCD58616.1	Basic helix-loop-helix transcription factor, putative [Sm]	3	
CCD77867.1	Late embryogenesis abundant protein [Sm]	3	
CCD80225.1	Zinc finger protein [Sm]	2	

Hypothetical / unnamed		
CCD75352.1	hypothetical protein Smp_140590 [Sm] (similar to Galectin family)	9
XP_002570696.1	hypothetical protein [Sm] (enolase, fragment)	7
CCD81381.1	hypothetical protein Smp_007640 [Sm] (1,6-glucosidase)	7
CCD80386.1	unnamed protein product [Sm] (actin-like protein)	6
CCD78834.1	hypothetical protein Smp_134750 [Sm]	5
CCD82007.1	hypothetical protein Smp_080920.3 [Sm]	3
CCD77946.1	hypothetical protein Smp_133590 [Sm] membrane, Ca binding vesicle fusion, neurotransmitter exocytosis	3
CCD82509.1	hypothetical protein Smp_024220 [Sm]	2
CCD58986.1	hypothetical protein Smp_155620 [Sm]	2
CCD79363.1	hypothetical protein Smp_145450 [Sm]	2
XP_002575612.1	hypothetical protein [Sm]	2
CCD75804.1	hypothetical protein Smp_159020 [Sm]	2
CCD76102.1	hypothetical protein Smp_006830.1 [Sm]	2
***Host proteins* (*Mus musculus*)**		
XP_006536151	importin-8 isoform X3 [Mm]	4
XP_006521203.1	keratin, type II cytoskeletal 1b isoform X1 [Mm]	3
XP_006520948.1	keratin, type II cytoskeletal 79 isoform X1 [Mm]	3
XP_006521177.1	keratin Kb40 isoform X2 [Mm]	2
XP_006520575.1	keratin, type I cytoskeletal 18 isoform X1 [Mm]	2
XP_006523578.1	axin-1 isoform X1 [Mm]	2
XP_006531840.1	39S ribosomal protein L21 [Mm]	2

[a]MS data were used to search the *S. mansoni* and mouse (*Mus musculus*) genome datasets available at NCBI; Sm, *S. mansoni* protein; Mm, mouse protein. [b]Number of unique (non-overlapping) peptides matching the designated protein across three independent experiments. [c]Proteins previously identified in proteomics analyses of schistosome excretory/secretory (ES) products. Relevant references are provided (refer to reference list for full citations). [d]Proteins in boldface print are featured in ExoCarta's "top 25" list of the most common exosomal markers from all different species and tissues.

The analysis identified 158 known and predicted miRNAs in whole worm extracts, 143 were also detected in exosomes but their relative abundances differed. This includes only sequences represented by >10 in one of the samples; miRNAs present at <10 reads in both samples were omitted from the analysis. The most abundant exosomal miRNAs (>100 reads) are described in Table 2 and a complete list is provided in Table S4. Among the top hits, roughly 70% are available in miRBase, including known *S. mansoni* (sma) sequences and conserved homologues from *S. japonicum* (sja) and the planarian *Schmidtea mediterranea* (sme). The remaining are novel, putative miRNAs that mapped to the *S. mansoni* genome within predicted hairpins. Most miRNAs are underrepresented in vesicles, in some instances by more than 100-fold (Fig. 4). Some, however, are present at the same or even higher levels; two of the more abundant miRNAs, sma-miR-71a and sma-miR-125b, were both present at about the same level in the two samples, and other miRNAs, such as sma-bantam and sma-miR-36-3P, were moderately enriched in vesicles. Bantam is an invertebrate-specific miRNA previously detected in serum of helminth-infected hosts and was reported to be secreted by the parasite [16,17,52]. Our results support these earlier studies and further suggest that sma-bantam is released, at least in part, in secretory vesicles.

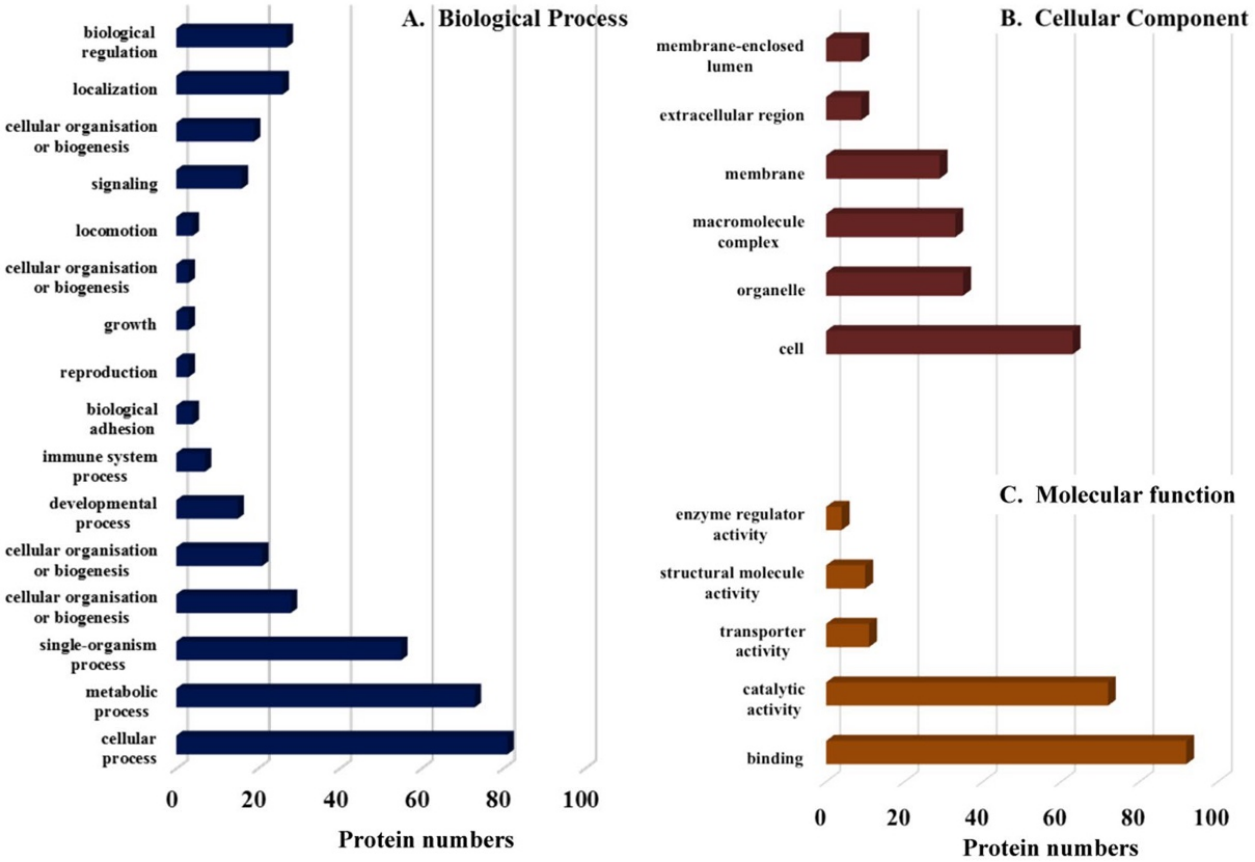

Figure 3. Gene Ontology (GO) analysis of proteins recovered from *S. mansoni* exosome-like vesicles. The identified proteins analysed with Blast2GO [39] and were classified according to Biological Process (**A**), Cellular Component (**B**) and Molecular Function (**D**), as defined by the GO consortium.

3.4. Detection of exosomal miRNAs in infected mice

To test if *S. mansoni* releases exosome-like vesicles *in vivo*, we purified exosomes from serum of infected mice and screened for schistosome-derived miRNAs by qRT-PCR. The average worm burden was 30–40 worm pairs per mouse. As controls, we used uninfected mice maintained for the same length of time. Although most vesicles isolated using ExoQuick kit are likely of host origin, we hypothesised that even a small proportion of parasite-derived exosomes would be sufficient to detect specific *Schistosoma* miRNAs by qPCR. To facilitate detection, we focused on the abundant *S. mansoni* exosomal miRNAs from the RNA seq analysis and used a stem-loop RT method [41] combined with TaqMan probe-based qPCR to improve specificity. Attempts to amplify miRNA using a polyA tailing RT method [53] and SYBR green for qPCR produced non-specific results (not shown). In contrast, the stem-loop TaqMan probe method was able to

specifically detect sma-miR-125a, sma-miR-125b, sma-bantam, sma- miR-71a (Fig. 5). The uninfected sample provides a measure of background amplification, presumably due to non-specific amplification of homologous sequences present in mouse-derived exosomes. The four miRNAs tested showed significant ($p < 0.05$) amplification compared to background levels and their median signal-to-noise ratios ranged from ~300- to 12,000-fold (Fig. 5). It was surprising that ELVs isolated from infected mice showed notable difference in the various miRNA level. This was particularly notable for sma-miRNA-125b (Fig. 5).

4. Discussion

Exosomes are important agents of intercellular communication in mammals, invertebrates and even prokaryotes [23,24]. For pathogens, exosomes constitute a powerful mechanism by which virulence factors and other bioactive molecules can be delivered to a host cell to promote infection. This has been well documented for microbial pathogens, in particular fungi, *Leishmania* and *T. cruzi* [25,54]. Evidence for exosome-mediated secretion in helminth infections is more limited [19,26,27] and little is known about their functional roles. We further show that *S. mansoni* releases ELVs and characterise adult schistosome exosomal proteins and miRNAs.

Table 2. List of most abundant miRNAs in purified *S. mansoni* exosomes.

miR name[a]	miR sequence	Norm reads[b] exosome	Norm reads whole worm
sma-miR-125b_R-1[c]	TCCCTGAGACTGATAATTGCT	43,394	49,431
sma-bantam[d]	TGAGATCGCGATTAAAGCTGGT	**13,148**	**7,495**
sma-miR-71a_R+1	TGAAAGACGATGGTAGTGAGAT	**12,322**	**12,408**
sma-miR-125a	TCCCTGAGACCCTTTGATTGCC	12,075	33,572
sma-miR-36-3p	CCACCGGGTAGACATTCATTCGC	**8,977**	**4,146**
sma-miR-10-5p	AACCCTGTAGACCCGAGTTTGG	3,281	19,497
sma-miR-61_R+1	TGACTAGAAAGTGCACTCACTTC	3,145	7,471
sma-miR-2a-3p_R-1	TCACAGCCAGTATTGATGAAC	2,198	6,603
sme-lin-4-5p_	TCCCTGAGACCTTAGAGTTGT	1,893	5,902
sja-miR-2162-3p	TATTATGCAACGTTTCACTCT	1,794	3,246
sja-miR-277_R+2	TAAATGCATTTTCTGGCCCGTT	653	1,354
PC-5p-12974_124[e]	GAGAGATTAAGACTGAACGCC	**580**	**298**
sja-miR-277_R+1	TAAATGCATTTTCTGGCCCGT	532	1,717
PC-3p-8606_176	ACGGGCTTGGCAGAATTAGCGGGG	**304**	**167**
PC-5p-1634_720	TCCCTGAGACCTTAGAGTTGTCT	275	595
sme-mir-749-p5	GTCCGGGGTGCAGGCTTC	**275**	**188**
sma-let-7	GGAGGTAGTTCGTTGTGTGGT	254	3,535
sma-miR-71b-5p	TGAAAGACTTGAGTAGTGAGACT	**243**	**254**
PC-5p-15294_107	ACACTGCGAGGCATTGAAT	207	563
PC-3p-8590_176	GAGATGGATAGTGGCTAGCATTT	**190**	**39**

PC-5p-10382_150	CCTCCGGAATCCCATAGTACT	**160**	**156**
sma-miR-3479-3p	TATTGCACTAACCTTCGCCTTG	140	1,545
PC-5p-14055_115	TGGCGCTTAGTAGAATGTCACCG	121	200
PC-5p-1776_672	TGATGGATGTAGTATAGG	117	436
sja-miR-3492	ATCCGTGCTGAGATTTCGTCT	**111**	**42**

[a]Only the most abundant exosomal miRNAs (>100 reads) are shown. A complete list of all the miRNAs that were detected is provided in Table S2. [b]Normalised ("norm") reads were calculated after adjustment for the size of the library, as described in the Methods; [c]The suffix R followed by a negative number indicates that the read sequence is shorter than the annotated miRNA in miRbase by one (R-1) or two nucleotides (R-2) at the 3′ end. Conversely, R + 1, R + 2 show that the read sequence is longer than the annotate miRNA by one or two nucleotides; [d]miRNAs indicated by bold-face printare present at the same or higher levels in exosomes compared to whole worms. [e]The prefix PC (potential candidate) is used to describe novel miRNA sequences.

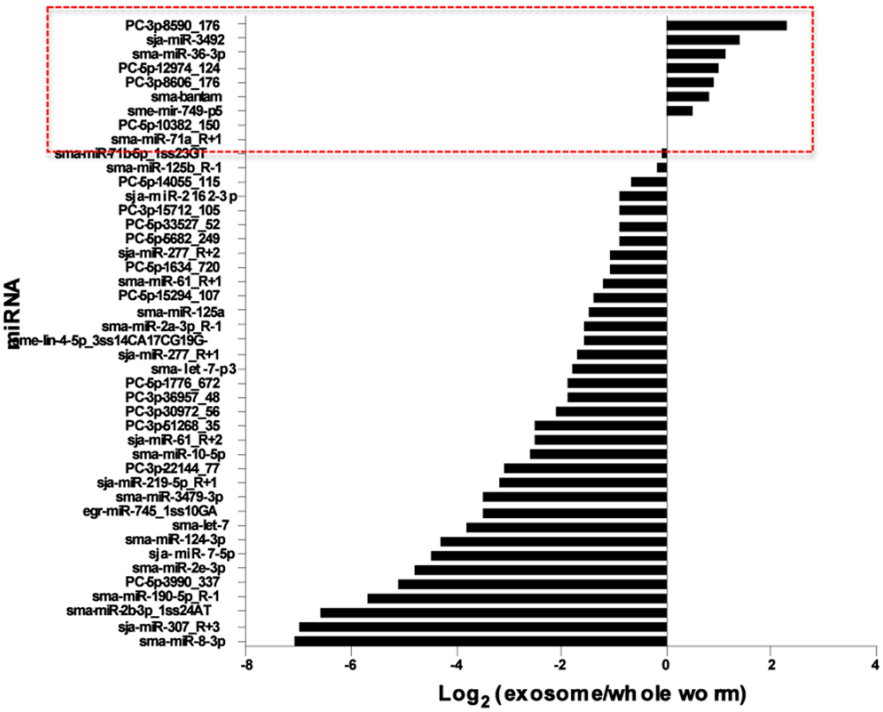

Figure 4. Comparative analysis of *S. mansoni* microRNAs (miRNA) obtained from whole worms and purified exosome-like vesicles. The data are shown as the Log2 ratio of normalised reads in the exosomal sample relative to the whole worm sample. Only the most abundant miRNAs are shown. Those miRNAs that are present at about the same level in the two samples, or are enriched in exosomes (Log2 ≥ 0) are marked.

Purified *S. mansoni*-secreted exosomes and biophysical characterisation demonstrated that these vesicles were bona fide exosomes rather than crude vesicular pellets or large protein aggregates. Our approach selects for exosomes based on their distinctive size and floatation properties on a sucrose gradient [36]. Previous studies have shown that exosomes sediment at density corresponding to ~30% sucrose [36], a finding confirmed here. This protocol minimises contamination by larger membrane vesicles that typically have higher densities [36]. EM analysis confirmed that the ELVs used in the proteomic studies were ~60–150 nm in diameter and contained most of the "signature" (top 25) exosomal markers described in ExoCarta [43]. We recognise there may be other secretory vesicles (e.g., plasma membrane-derived ectovesicles) that have similar size and density. Thus, we refer to the material under study as exosome-like vesicles (ELVs).

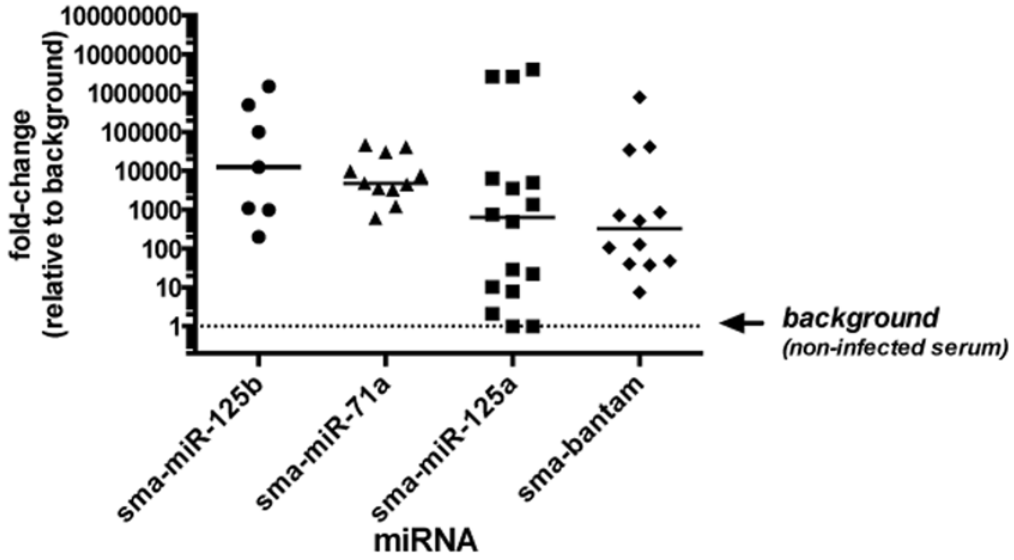

Figure 5. Quantitative qRT-PCR analysis of *S. mansoni* exosomal miRNAs in sera of infected mice. Circulating exosomes were purified from sera of *S. mansoni* –infected mice at 6–7 weeks post-infection or uninfected controls of the same age, using ExoQuick. RNA was extracted from the purified exosomes and then used for amplification of four *S. mansoni* miRNAs (Sma-mir-125a, Sma-mir-125b, Sma-mir-71a, Sma-bantam) by qRT- PCR. Data were median normalised relative to a "spike-in" synthetic miRNA [17,55] and are shown as the fold- change relative to the uninfected control sample (background).

A large proportion of the proteins recovered from *S. mansoni* ELVs are metabolic enzymes, particularly enzymes associated with glycolysis. The prevalence of glycolytic enzymes in

exosomes has been reported in other organisms; many of the signature exosomal proteins in ExoCarta are glycolytic enzymes, such as enolase, aldolase, GAPDH, etc. For schistosomes, the discovery of these enzymes in vesicles helps to explain how they are secreted by the parasite, despite lacking classical secretion signal sequences. Why exosomes contain so many glycolytic enzymes is unclear. It is possible that exosomes increase glycolytic activity in a target cell after docking and fusion. Schistosome ELVs contain abundant glycogen phosphorylase and a glucose transporter, which would make more glucose available for metabolism, either through glycogenolysis or increased transport from the outside. Alternatively, these enzymes could have other activities. It is well known that glycolytic enzymes have "moonlighting" functions unrelated to glycolysis. For example, extracellular forms of enolase, aldolase and GAPDH bind to mammalian plasminogen [56]. If any of these enzymes is present on the surface of the schistosome vesicles, as was recently shown for *Leishmania*-derived exosomes [57], it is tempting to speculate that the plasminogen binding activity could help prevent blood clots and facilitate parasite migration, an important activity for the blood-dwelling schistosome. Other "moonlighting" activities of extracellular glycolytic enzymes include adherence to the extracellular matrix, angiogenesis and the modulation of immune function [56], all of which could be important for schistosomes.

Possible roles of exosomes in modulation of clotting pathways deserve consideration. Like other blood parasites, schistosomes must prevent blood clotting and remove clots once formed. This is of particular importance to *S. mansoni* because of the large size of the worm pair relative to the narrow mesenteric veins in which they reside. The presence of worms is likely to disrupt blood flow and stress the vessel wall, conditions that stimulate coagulation. Schistosomes employ a variety of mechanisms to prevent platelet aggregation and disrupt formation of clots [58]. Interestingly, some of the proteins found in the *S. mansoni* exosomes have anti-clotting activities, including ATP-diphosphohydrolase 1 (ATPDase-1), a membrane-associated protein that hydrolyses extracellular prothrombotic ATP and ADP, inhibiting platelet aggregation and activation [47]. Previous studies of the schistosome homologue of ATPDase-1 revealed that the enzyme is present on the tegument [59] and has ATP and ADP hydrolysing activity [60]. Our results show that this enzyme is also secreted in vesicles. Secretion of ATPDase-1 was not reported *in vitro* [60], but the methods employed may not have detected vesicle-associated enzyme. Exosomal

ATPDase-1 may represent an important mechanism of haemostatic control. Other exosomal proteins with potential anti-clotting effects include the glycolytic enzymes listed above (enolase, GAPDH, aldolase) due to their plasminogen binding activity [56], the schistosomal antigen Sm22.6, which inhibits thrombin [61], annexin [46] and the calcium-dependent protease calpain. In the host, calpain has a variety of functions, one of which is the regulation of platelet aggregation [62]. Vesicles carrying parasite calpain could alter normal calpain activity in the host and perhaps disrupt endogenous control of platelet activation. Likewise, if parasite proteins such as integrins and talin function in the same way as the mammalian homologues, vesicle-mediated delivery of these proteins to host cells could help the parasite to modulate the immune response and, importantly, control the formation of blood clots.

S. mansoni exosomes carry a variety of other proteases, including metalloproteases, cysteine and serine pro- teases that are believed to play important roles in exosome-mediated signaling [63], contribute to invasion, migration, nutrient acquisition, immunomodulation and, haemostatic control [64]. Research on schistosome proteases has focused on secreted enzymes (e.g., cathepsin B) and cercarial elastases that contribute to skin penetration by infective larvae. There is a rich diversity of other proteases in schistosomes, most of which have only recently been identified [65] and their functions are poorly understood. Our results show that some of these enzymes are secreted in vesicles and therefore could have important roles in the host-parasite interaction. Examples include a novel type 2 serine protease (SmSP2) [66], a prolyl-oligopeptidase of the S9 serine protease family (SmPOP), recently shown to target bradykinin and angiotensin I [67] and a homologue of leucine aminopeptidase (LAP). LAP is of considerable interest in flukes due to its involvement in the immune response to schistosome eggs and egg hatching [68]. LAP is present in the adult worm gut, where it contributes to digestion of blood proteins, in the parasite tegument [69], and is secreted [8,9], though it lacks a signal peptide. LAP secretion by *F. hepatica* is vesicle-mediated [26] and our results suggest a similar mechanism for *S. mansoni*.

A proteomic analysis of ELVs derived from adult *S. mansoni* has been recently reported [28]. Although we identified some common proteins, notable discrepancies are evident (Table S5). Proteins found in both studies include enolase, GAPDH, glutathione-S-transferase, calpain, LAP, Sm20.8 and Sm22.6. Notable differences include glycogen phosphorylase, taurocyamine kinase

and cathepsin B, high abundance proteins detected in this study that were also found in schistosome ESP [8-10,13]. Differences could be due to variations in protocols.

Proteomic analysis of schistosomulae-derived EVs revealed shared proteins between schistosomulae- and adult worm-derived EVs (e.g., taurocyamine kinase, enolase, glutathione S-transferase, calpain, 14-3-3 epsilon and Sm20.8; Table S5), but also differences. For example, schistosomulae EVs did not contain glycogen phosphorylase or glucose transporters [29]. LAP, the enzyme responsible for the final stage in catabolism of host hemoglobin [69] was also absent from schistosomulae-derived EVs [29], probably because the gut is not functional early in schistosomulum development [70]. Such variations in ESPs between different parasite life stages have previously been reported in helminths [71,72] and could be used as specific life-stage markers in diagnosis.

Exosomes carry different types of RNA molecules, including miRNAs [21]. miRNAs are small molecules of ~23 nucleotides that regulate post-transcriptional silencing of genes. They bind to complementary sequences, typically in the 3′ untranslated region (3′-UTR) of a target transcript, resulting either in translational repression or sequence-specific RNA degradation [73]. miRNAs can be secreted and much of that secretion is vesicle (exosome)-mediated. Exosomal miRNAs are more stable than extravesicular forms and can be delivered to a target cell when exosomes fuse with the cell membrane [21]. Helminth parasites have a rich diversity of miRNAs [52,73-76], some of which are secreted [15-17,19,77], and helminth-secreted miRNAs modulate expression of specific host genes [19], suggesting an important role in the host-parasite interaction. Here we provide evidence of vesicle-mediated miRNA secretion in adult schistosomes. Over 140 miRNAs were identified in purified vesicles, some at very high levels, including miRNAs found in sera of schistosome-infected animals [16,17]. We confirmed the presence of schistosome miRNAs in circulating exosomes from infected mice by qRT-PCR, indicating that exosomal miRNA secretion occurs in the infected host.

The biological relevance of schistosome exosomal miRNA secretion is unknown. Preliminary searches for potential human targets using computational tools (TargetScan Custom (5.2)) detected conserved seed regions in many of the schistosome miRNAs described here, suggesting these could, in principle, recognise human transcripts. For example, sma-miR125b, an

abundant miRNAs in schistosome vesicles, has >600 potential human targets based on a conserved 8-mer seed region of the mature miRNA. Similar analysis of Sma-bantam, an invertebrate-specific miRNA, identified 39 potential human targets (not shown). These bioinformatics analyses must be refined and combined with gene expression studies in the context of different host cell environments to elucidate miRNA function. As previously observed in *D. immitis* [72], it is likely that ELVs secreted by male and female schistosomes vary in content. It was also noted that the level of some miRNAs, in particular sma-miR125b, showed a broad variation in the level of miRNA found in the ELVs isolated from the serum of individual mice. These differences could be due to; i) genetic difference in the murine host since CD1 are outbreed mice which may have different responses to *S. mansoni* infections, ii) difference in worm burdens, or iii) possible differences in the stability of the miRNA transcript. It is also possible that some of the vesicular traffic is directed towards other schistosomes as a mechanism of animal-to-animal communication, as suggested recently for *C. elegans* [78]. Future studies will need to consider potential miRNA targets in the parasite as well as the host. miRNAs found in schistosomulae EVs [29] were not among the 25 most abundant miRNAs in adult ELVs (Table 2), suggesting that the presence of these miRNA in schistosome ELVs may be stage-specific.

It is easy to envision that vesicles secreted within the confines of the mesenteric veins could directly target the endothelial lining of the blood vessels, platelets or cells of the immune system. Most research on parasite-derived vesicles has focused on the immune response. Protozoal pathogen exosomes have either pro- or anti-inflammatory effects, depending on the parasite and type of vesicle [25], and modulate expression of a variety of host genes associated with immunity [79]. Whether these effects extend to schistosomes is unclear. A recent study of *S. japonicum* reported that treatment of macrophage RAW264.7 cells with parasite-derived vesicles promoted M1-type polarisation and increased production of pro-inflammatory cytokines such as TNF-α and IL-12 [27]. We tested *S. mansoni* exosomes in cultures of bone marrow-derived mouse (C57/BL6) macrophages and detected no change in TNF-α or IL12 production at concentrations of up to 50 µg exosomal protein/ml (data not shown). This discrepancy could be a function of the different target cells (RAW264.7 versus bone-marrow derived primary cells), different purity of vesicle preparations or different species of schistosome.

We have characterised miRNAs present in adult *S. mansoni* ELVs, which constitute the first report of exosomal miRNA secretion from adult schistosomes. We also provide a molecular profiling of exosomal protein content, offering contrast to a previous report [28] (Table S5), which is possibly due to differences between our protocols. Both proteomic and miRNA analysis of schistosomulae-derived EVs [29] revealed variations between schistosomulae- and adult worm-derived EVs (Table S5), which could serve as specific life-stage markers in diagnosis. However, further investigation will be required to exploit their potential in the development of vaccines, therapeutics and diagnostic methods.

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Supplementary Material

Table S1: List of primers, TaqMan probes and synthetic RNAs used in this study

Stemloop Primers for Reverse Transcription	
sma-miR- Bantam	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CCA GC
sma-miR- 71a	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CTC AC
Sma-miR-125a	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GCA AT
Sma-miR-125a	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA GCA AT
Spike-in control	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT ACG TG
Universal Stemloop Reverse Primer for qPCR	
Universal reverse primer	CCA GTG CAG GGT CCG AGG TA
Stemloop Forward Primers for qPCR	
sma-miR- Bantam	CAG GCG TGA GAT CGC GAT TA
sma-miR-71a	CAG GCG TGA AAG ACG ATG G
Sma-miR-125a	CAT GCA TCC CTG AGA CCC T
sma-miR-125b	CAC GCA TCC CTG AGA CTG A
Spike-in control	CAC GCA CGT ATC GAG TGA TG
TaqMan Probes for qPCR[a]	
sma-miR- Bantam	6FAM-TGG ATA CGA CAC CAG CTT-MGBNFQ
sma-miR-71a	6FAM- CTG GAT ACG ACT CTC ACT A-MGBNFQ
Sma-miR-125a	6FAM-GAT ACG ACG GCA ATC AA -MGBNFQ
sma-miR-125b	6FAM- TGG ATA CGA CAG CAA TTA-MGBNFQ
Spike-in control	6FAM-CAC TGG ATA CGA CTA CGT GA -MGBNFQ
Synthetic microRNAs	
sma-miR- Bantam	UGA GAU CGC GAU UAA AGC UGG U
sma-miR-71a	UGA AAG ACG AUG GUA GUG AGA
sma-miR-125a	UCC CUG AGA CCC UUU GAU UGC C
sma-miR-125b	UCC CUG AGA CUG AUA AUU GCU
Spike-in control	CGU AUC GAG UGA UGU CAC GUA

[a] TaqMan probes were purchased with a FAM (6-carboxyfluorescein) dye label on the 5'end and a minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end.

Table S2: Mass spectrometry data: Protein and peptide list

Protein Score	Decoy%	# of Peptides Identified	# of Spectral Matches	Unique peptide	GI (genInfo identifier)	Accession number	
							Metabolic Enzymes
1476	0,00%	50	116	50	360045358	CCD82906.1	Glycogen phosphorylase [Sm]
816	0,00%	30	51	30	391358187	P16641.3	Taurocyamine kinase [Sm]
744	0,00%	29	48	28	156118911	ABU49845.1	Creatine kinase [Sm]
723	0,00%	31	77	25	3023710	Q27877.1	Enolase [Sm]
661	0,00%	25	61	22	353230309	CCD76480.1	Pyruvate kinase [Sm]
442	0,00%	22	28	22	360043735	CCD81281.1	Glucose-6-phosphate isomerase [Sm]
499	0,00%	22	45	18	360045088	CCD82636.1	Lactate dehydrogenase [Sm]
430	0,00%	17	33	17	353228833	CCD75004.1	Malate dehydrogenase [Sm]
326	0,00%	17	21	17	353229703	CCD75874.1	Phosphoenolpyruvate carboxykinase [Sm]
215	0,00%	9	11	9	256070409	XP_002571535.1	Ornithine-oxo-acid transaminase [Sm]
172	0,00%	8	9	8	1172460	P41759.1	Phosphoglycerate kinase [Sm]
111	0,00%	8	10	8	353230092	CCD76263.1	Transketolase [Sm]
138	0,00%	7	10	7	256090542	XP_002581246.1	Phosphoglucomutase [Sm]
78	0,00%	9	10	7	353232336	CCD79691.1	Fructose 1,6-bisphosphate aldolase [Sm]
147	0,00%	9	9	4	350645988	CCD59265.1	Aldehyde dehydrogenase, putative [Sm]
66	0,00%	4	4	4	350645892	CCD59437.1	Adenosylhomocysteinase, putative [Sm]
726	0,00%	23	53	3	353229457	CCD75628.1	Glyceraldehyde-3-phosphate dehydrogenase [Sm]
80	0,00%	3	4	3	123502	P09383.1	Hypoxanthine-guanine phosphoribosyltransferase [Sm]
48	0,00%	3	3	3	383930643	AFH56663.1	Methylthioadenosine phosphorylase [Sm]
44	0,00%	3	4	3	256082671	XP_002577577.1	Aconitate hydratase [Sm]
31	1,82%	3	3	3	3122305	Q27778.1	6-phosphofructokinase [Sm]
46	0,00%	2	3	2	360044093	CCD81640.1	Long-chain-fatty-acid-CoA ligase [Sm]
41	0,00%	2	2	2	353229440	CCD75611.1	Glycogenin-related [Sm]
34	1,82%	2	4	2	350643977	CCD58348.1	6-phosphogluconate dehydrogenase,putative [Sm]
							Xenobiotic / Redox Metabolism
577	0,00%	21	58	16	121700	P09792.1	Glutathione S-transferase 28 kDa isozyme [Sm]
206	0,00%	10	15	10	256093080	XP_002582203.1	Glutathione S-transferase 26 kDa [Sm]

95	0,00%	6	6	6	350645579	CCD59704.1	Aldo-keto reductase, putative [Sm]
94	0,00%	5	7	5	161007	AAA29889.1	Glutathione S-transferase, partial [Sm]
32	1,82%	3	3	3	353231561	CCD77979.1	Glyoxalase I [Sm]
							Proteases
343	0,00%	19	21	19	350646132	CCD59179.1	Thimet oligopeptidase (M03 family) [Sm]
223	0,00%	14	21	14	360043297	CCD78710.1	Leucine aminopeptidase (M17 family) [Sm]
119	0,00%	11	14	11	353230291	CCD76462.1	Calpain (C02 family) [Sm]
182	0,00%	7	17	7	729709	P09841.3	Hemoglobinase (Antigen SM32) [Sm]
221	0,00%	6	13	6	353228442	CCD74613.1	Cathepsin B-like peptidase (C01 family) [Sm]
65	0,00%	5	5	5	256072593	XP_002572619.1	Prolyl oligopeptidase (S09 family) [Sm]
56	0,00%	4	5	5	353230839	CCD77256.1	SpAN g.p. (M12 family) [Sm]
48	0,00%	3	3	3	564131894	AHB79081.1	Serine protease 2 precursor [Sm]
43	0,00%	2	8	3	256084114	XP_002578277.1	Subfamily M12B unassigned peptidase (M12 family) [Sm]
43	0,00%	3	14	3	353233303	CCD80658.1	Family S9 non-peptidase homologue (S09 family) [Sm]
35	1,82%	2	3	2	353232118	CCD79473.1	Xaa-Pro dipeptidase (M24 family) [Sm]
							Fatty acid binding
76	0,00%	5	7	3	55670478	1VYG	Fatty Acid Binding Protein [Sm]
							Transporters/channels
395	0,00%	15	38	14	350644272	CCD60986.1	Plasma membrane calcium-transporting atpase, putative [Sm]
68	0,00%	8	10	8	360043551	CCD78964.1	Sodium potassium transporting ATPase alpha subunit [Sm]
166	0,00%	7	18	7	353231052	CCD77470.1	Glucose transport protein [Sm]
62	0,00%	7	6	5	353233037	CCD80392.1	Cation-transporting ATPase [Sm]
67	0,00%	4	6	4	256084157	XP_002578298	Choline transporter-like protein 2 (Ctl2) [Sm]
124	0,00%	3	9	3	353229720	CCD75891.1	Aquaporin-3 [Sm]
33	1,82%	3	5	3	256084334	XP_002578385.1	Chloride channel protein [Sm]
42	0,00%	2	12	2	353231352	CCD77770.1	Anion exchange protein [Sm]
							Signal Transduction and Biological Regulation
209	0,00%	15	38	10	3023193	Q26540.1	14-3-3 protein homolog 1 [Sm]
166	0,00%	11	14	9	353228883	CCD75054.1	14-3-3 epsilon [Sm]
283	0,00%	11	26	8	256084742	XP_002578585.1	Annexin [Sm]

186	0,00%	9	20	6	353228653	CCD74824.1	sh3 domain grb2-like protein B1 (endophilin B1) [Sm]
35	1,82%	6	6	6	353228669	CCD74840.1	Kynurenine aminotransferase [Sm]
30		6	12	6	353231089	CCD77507.1	Talin [Sm]
148	0,00%	7	14	6	256082840	XP_002577660.1	Integrin alpha-ps [Sm]
96	0,00%	6	9	6	353228490	CCD74661.1	Integrin beta subunit [Sm]
34	1,82%	5	5	5	353232319	CCD79674.1	Atp-diphosphohydrolase 1 [Sm]
55	0,00%	4	9	4	256084780	XP_002578604.1	rpgr-interacting protein 1 related [Sm]
43	0,00%	4	6	4	350646699	CCD58613.1	Hyperpolarization activated cyclic nucleotide-gated potassium channel, putative [Sm]
122	0,00%	4	9	3	256080579	XP_002576557.1	ng, ng-dimethylarginine dimethylaminohydrolase [Sm]
37	1,82%	4	6	3	256052204	XP_002569666.1	ral guanine nucleotide dissociation stimulator ralgds[Sm]
36	1,82%	3	5	3	353229530	CCD75701.1	Proline-serine-threonine phosphatase interacting protein [Sm]
33	1,82%	3	8	3	360043093	CCD78505.1	Lip-related protein (liprin) alpha [Sm]
93	0,00%	3	18	3	256090851	XP_002581393.1	Tetraspanin [Sm]
34	1,82%	3	4	3	256089413	XP_002580804.1	Serine/threonine protein kinase [Sm]
61	0,00%	2	4	2	360043400	CCD78813.1	rap1 [Sm]
37	1,82%	2	3	2	350646635	CCD58662.1	Calponin homolog, putative [Sm]
33	1,82%	2	4	2	360042647	CCD78057.1	Voltage-gated potassium channel [Sm]
30	3,23%	2	2	2	353233207	CCD80562.1	Syntenin [Sm]
							Cytoskeletal /Structural
815	0,00%	40	105	40	350646584	CCD58796.1	Fer-1-related [Sm]
586	0,00%	19	46	19	1703114	P53471.1	Actin-2 [Sm]
233	0,00%	14	23	13	353232589	CCD79944.1	Alpha tubulin [Sm]
313	0,00%	12	21	12	353232516	CCD79871.1	Tubulin beta chain [Sm]
307	0,00%	12	21	12	256076432	XP_002574516.1	Tubulin subunit beta [Sm]
51	0,00%	11	12	11	353229949	CCD76120.1	Collagen alpha-1(V) chain [Sm]
320	0,00%	12	21	10	350645253	CCD60034.1	Prominin (prom) protein, putative [Sm]
81	0,00%	7	9	7	353231032	CCD77450.1	Rab GDP-dissociation inhibitor [Sm]
150	0,00%	10	14	6	360045419	CCD82967.1	Actin [Sm]
169	0,00%	6	13	6	350644919	CCD60380.1	Synaptotagmin, putative [Sm]
106	0,00%	5	7	5	495668	AAA29882.1	Fimbrin [Sm]
39	1,82%	5	5	5	360044904	CCD82452.1	Signal recognition particle 68 kD protein [Sm]

72	0,00%	4	5	4	256072032	XP_002572341.1	Gelsolin [Sm]
37	1,82%	4	7	4	353230603	CCD77020.1	Intermediate filament proteins [Sm]
57	0,00%	4	10	3	353230415	CCD76586.1	Cytoplasmic dynein light chain [Sm]
32	1,82%	2	3	2	256073059	XP_002572850.1	Collagen alpha chain type IV [Sm]
32	1,82%	2	2	2	353232499	CCD79854.1	Microtubule-associated protein 9 [Sm]
64	0,00%	2	3	2	360045234	CCD82782.1	Rab-2,4,14 [Sm]
49	0,00%	2	3	2	350645028	CCD60258.1	Rab11, putative [Sm]
37	1,82%	2	2	2	353228708	CCD74879.1	Ran [Sm]
							Tegumental antigen
282	0,00%	30	42	24	360043686	CCD81232.1	200-kDa GPI-anchored surface glycoprotein [Sm]
211	0,00%	13	23	9	353230232	CCD76403.1	Tegumental protein Sm 20.8 [Sm]
144	0,00%	7	11	7	135578	P14202.1	Tegument antigen SmA 22.6 [Sm]
56	0,00%	3	5	3	390124514	CCE94318.1	Tegumental antigen [Sm]
111	0,00%	2	11	2	350646174	CCD59158.1	Sm23, putative [Sm]
69	0,00%	2	7	2	353230115	CCD76286.1	Sm29 [Sm]
							Histones
58	0,00%	5	6	4	353231319	CCD77737.1	histone H3 [Sm]
55	0,00%	4	6	4	10953803	AAG25601.1	histone H4 [Sm]
70	0,00%	5	8	3	353229586	CCD75757.1	histone H2B [Sm]
							Chaperones
105	0,00%	8	9	7	238663484	CAZ34365.1	Heat shock protein 70 (hsp70)-4, putative [Sm]
100	0,00%	7	12	5	27805450	Q26565.1	Peptidyl-prolyl cis-trans isomerase [Sm]
85	0,00%	5	5	5	353230032	CCD76203.1	Heat shock protein-HSP20/alpha crystallin family [Sm]
46	0,00%	3	3	3	256082744	XP_002577613.1	Chaperonin containing t-complex protein 1 epsilon subunit tcpe [Sm]
							Translation
148	0,00%	7	9	7	353230261	CCD76432.1	Elongation factor 1-alpha (ef-1-alpha) [Sm]
55	0,00%	5	5	5	353231791	CCD79146.1	Eukaryotic translation elongation factor [Sm]
							Others
325	0,00%	10	25	10	256080932	XP_002576729.1	SPRY domain containing protein [Sm]

102	0,00%	7	8	7	350644553	CCD60716.1	Cell division control protein 48 aaa family protein (transitional Endoplasmic reticulum atpase), putative [Sm]
126	0,00%	6	11	6	360045009	CCD82557.1	Band 4.1-like protein [Sm]
50	0,00%	6	6	6	350646643	CCD58670.1	Centrosomal protein of 135 kDa (Cep135 protein) [Sm]
48	0,00%	5	11	5	360044828	CCD82376.1	Excision repair helicase ercc-6-related [Sm]
105	0,00%	10	22	4	256079432	XP_002575991.1	Ubiquitin (ribosomal protein L40) **[Sm]**
52	0,00%	4	4	4	353230536	CCD76953.1	Ubiquitin-protein ligase BRE1 [Sm]
30	3,23%	5	6	4	256084236	XP_002578337.1	Mixed-lineage leukemia 5 mll5 [Sm]
32	1,82%	3	5	3	350646702	CCD58616.1	Basic helix-loop-helix transcription factor, putative [Sm]
31	1,82%	3	3	3	353231449	CCD77867.1	Late embryogenesis abundant protein [Sm]
32	1,82%	2	4	2	353232869	CCD80225.1	Zinc finger protein [Sm]
30		1	1	1	1084298248	XP_018652220.1	Gata zinc finger domain-containing protein [Sm]
							Hypothetical/Unnamed
183	0,00%	9	18	9	353229181	CCD75352.1	hypothetical protein Smp_140590 [Sm]
227	0,00%	7	22	7	256068121	XP_002570696.1	hypothetical protein [Sm]
103	0,00%	7	7	7	360043835	CCD81381.1	hypothetical protein Smp_007640 [Sm]
436	0,00%	15	32	6	353233031	CCD80386.1	unnamed protein product [Sm]
45	0,00%	5	7	5	360043421	CCD78834.1	hypothetical protein Smp_134750 [Sm]
45	0,00%	3	3	3	360044459	CCD82007.1	hypothetical protein Smp_080920.3 [Sm]
37	1,82%	3	4	3	353231528	CCD77946.1	hypothetical protein Smp_133590 [Sm]
61	0,00%	2	4	2	360044961	CCD82509.1	hypothetical protein Smp_024220 [Sm]
52	0,00%	2	4	2	350646356	CCD58986.1	hypothetical protein Smp_155620 [Sm]
37	1,82%	2	5	2	353232008	CCD79363.1	hypothetical protein Smp_145450 [Sm]
35	1,82%	2	4	2	256078659	XP_002575612.1	hypothetical protein [Sm]
33	1,82%	3	10	2	353229633	CCD75804.1	hypothetical protein Smp_159020 [Sm]
33	1,82%	2	4	2	353229931	CCD76102.1	hypothetical protein Smp_006830.1 [Sm]
							Host proteins (*Mus muscuslus*)
264	0,00%	4	11	4	569017314	XP_006536151	importin-8 isoform X3 [Mm]
141	0,00%	6	3	3	568992803	XP_006521203.1	keratin, type II cytoskeletal 1b isoform X1 [Mm]

115	0,00%	4	3	3	568992275	XP_006520948.1	keratin, type II cytoskeletal 79 isoform X1 [Mm]
97	0,00%	2	4	2	568992745	XP_006521177.1	keratin Kb40 isoform X2 [Mm]
62	0,00%	2	3	2	568991509	XP_006520575.1	keratin, type I cytoskeletal 18 isoform X1 [Mm]
51	0,00%	2	3	2	568998710	XP_006523578.1	axin-1 isoform X1 [Mm]
48	0,00%	2	2	2	569005850	XP_006531840.1	39S ribosomal protein L21 [Mm]

Table S3: Summary of RNA sequencing results

# Reads	exosomes	whole worms
Total ("raw") reads	10,953,926	12,462,586
Mappable reads[a]	8,639,784	10,232,965
Reads mapped to known miRNAs[b]	74,223	236,809
Reads mapped to putative miRNAs[c]	4,418	13,186
Reads mapped to other RNAs (mRNA, Rfam or Repbase)[d]	5,450,691	7,979,750
Unmapped reads ("no hits")	2,824,993	1,600,243
Others[e]	285,459	402,977

[a] Reads remaining after removal of impurities, low-quality sequences and sequences < 15 nucleotides; [b]reads aligned with known *S. mansoni* or platyhelminth miRNAs available in miRBase vs. 20;
[c] reads did not map to known miRNAs but aligned to the *S. mansoni* genome between predicted hairpins;
[d] reads mapped to *S. mansoni* mRNAs, repetitive sequences in Repbase (http://www.girinst.org/repbase) or other (non-miRNA) small RNA species available in the Rfam database (http://rfam.janelia.org);
[e] reads mapped to the *S. mansoni* genome but are not predicted to form hairpins (disregarded).

Table S4: miRNA sequences detected in exosomes and whole worms

miR_seq	miR_name	norm_EXO_SM	norm_W_SM
TCCCTGAGACTGATAATTGCT	sma-miR-125b_R-1	43,394	49,431
TGAGATCGCGATTAAAGCTGGT	sma-bantam	13,148	7,495
TGAAAGACGATGGTAGTGAGAT	sma-miR-71a_R+1	12,322	12,408
TCCCTGAGACCCTTTGATTGCC	sma-miR-125a	12,075	33,572
CCACCGGGTAGACATTCATTCGC	sma-miR-36-3p	8,977	4,146
AACCCTGTAGACCCGAGTTTGG	sma-miR-10-5p	3,281	19,497
TGACTAGAAAGTGCACTCACTTC	sma-miR-61_R+1	3,145	7,471

TCACAGCCAGTATTGATGAAC	sma-miR-2a-3p_R-1	2,198	6,603
TCCCTGAGACCTTAGAGTTGT	sme-lin-4-5p_ 3ss14CA17CG19G-	1,893	5,902
TATTATGCAACGTTTCACTCT	sja-miR-2162-3p	1,794	3,246
TAAATGCATTTTCTGGCCCGTT	sja-miR-277_R+2	653	1,354
GAGAGATTAAGACTGAACGCC	PC-5p-12974_124	580	298
TAAATGCATTTTCTGGCCCGT	sja-miR-277_R+1	532	1,717
ACGGGCTTGGCAGAATTAGCGGGG	PC-3p-8606_176	304	167
TCCCTGAGACCTTAGAGTTGTCT	PC-5p-1634_720	275	595
GTCCGGGGTGCAGGCTTC	sme-mir-749-p5	275	188
GGAGGTAGTTCGTTGTGTGGT	sma-let-7	254	3,535
TGAAAGACTTGAGTAGTGAGACT	sma-miR-71b-5p_ 1ss23GT	243	254
ACACTGCGAGGCATTGAAT	PC-5p-15294_107	207	563
GAGATGGATAGTGGCTAGCATTT	PC-3p-8590_176	190	39
CCTCCGGAATCCCATAGTACT	PC-5p-10382_150	160	156
TATTGCACTAACCTTCGCCTTG	sma-miR-3479-3p	140	1,545
TGGCGCTTAGTAGAATGTCACCG	PC-5p-14055_115	121	200
TGATGGATGTAGTATAGG	PC-5p-1776_672	117	436
ATCCGTGCTGAGATTTCGTCT	sja-miR-3492	111	42
GTCCCTATCTACTATCTAGCGAAACCACAGCC	PC-5p-33527_52	90	172
TCCTGGACGCTGGCAAATGCT	PC-3p-15712_105	77	147
ATCTGTTAGTACTTTGGTCCT	PC-3p-21727_78	65	26
GAGAGATTAAGACTGAACGCCT	PC-3p-19439_87	65	16
ATGATGGATGTAGTATAGG	PC-5p-5682_249	62	119
ACCCGTACCCAATCGTCGAC	PC-3p-46742_38	60	66
TACCGCTGTCCATAACTATGT	PC-3p-70423_25	57	43
TGGCGAAGAGTTCTGATTAGT	PC-3p-82189_22	54	23
TGGAAGACTGGTGATATGTTGTT	sja-miR-7-5p	50	1,093
TGACTAGAAAGTGCACTCACTTCT	sja-miR-61_R+2	50	283
TCGACGATTGGGTACGGGTCT	PC-3p-49221_36	50	23
TGATGGATGTAGTATAGGTGGGAGCTGGGTG	PC-3p-36957_48	49	186
CAGGTCGGGCGTCTAGGCACTT	PC-5p-38154_46	48	14
CGTACTATGGGATTCCGGAGT	PC-3p-55957_32	48	4
AGGATACTGGAGCCCATGTGTTT	PC-3p-36544_48	45	11
ATATCGATGTGTATATGCTCT	PC-3p-45469_39	45	12
TTCTTAGATTGTAAGCACTCT	PC-5p-58341_30	44	32
CGTCTCAAGGGACTGTGAAACT	sma-miR-2b-5p_ 1ss22AT	43	38
CTCTTAGCAGGATTGGTGTCT	PC-3p-72151_25	43	6
TATTGGCAAACAATCTGACT	PC-3p-14824_110	40	54
CATTTCAGATATACTCTGCT	PC-3p-26877_64	40	25
AACCGATGTTAGGACGTTTGT	PC-3p-25083_69	39	9
CATACAACCGACTGGCTTTCC	sma-let-7-p3	38	132
TGGACGGGGCAATATCTTAAT	PC-3p-126893_14	38	19
TTCCGATTCGGCATTTCTGATT	PC-5p-61230_29	37	9
ACTGAGACGGGTGTGGATACT	PC-5p-54721_32	37	4
AGGCCAATTGAACTACCCTTT	PC-5p-37607_47	35	20
TGAAAGACGATGGTAGTGAGATAT	egr-miR-71_R+3	34	16
TGTCCGCGGAACAGTGTACC	PC-5p-54980_32	33	51

TTTGGTCCCCTTCAACCAGCTGT	sme-miR-133a-3p_L+1_1ss11GT	33	37
TGATATGTATGGGTTACTTGGT	sma-miR-190-5p_R-1	31	1,659
TGAAAGACGATGGTAGTGAGATA	egr-miR-71_R+2	31	64
TCCGCGGACATACGCCGACT	PC-5p-89649_20	30	21
AGAGGTAGTGATTCAAAAAGTT	sme-let-7d	30	7
TCGAGACGGTTACTAGACACT	PC-5p-109992_16	28	4
TCCCTTGTTCGACTGTGATGTTT	sma-miR-2c-5p_R+1_1ss22GT	27	21
GATTTGTCTGACGTTATGTGT	PC-5p-66976_27	27	6
ATAATCAGACTGAATATGTGT	PC-5p-108368_16	26	26
TTAGGATCCAAGTATAGCGTTT	PC-3p-84103_21	26	10
TCCGTGCTGAGATTTCGTCA	sma-miR-3492_L-1	24	26
TCGACAGATGTTAGTGACTGT	PC-3p-73847_24	24	8
CCGATGTTAGGACGTTTGTCT	PC-3p-148935_12	23	8
TCACAGGAATCGAAATACTCT	PC-3p-124334_14	23	11
TTTCGGCAGTTCAGTGGTACT	PC-3p-68130_26	23	4
CACTCGTCGCACTGCATGCCT	PC-3p-30972_56	21	92
GAGAAGCCTGATGGTCGGAGT	PC-3p-87239_20	21	4
TGTCATGGAGTTGCTCTCTA	PC-3p-22144_77	20	168
TCGGGCAGTACATTTCTGATT	PC-5p-140928_12	20	11
CAGAGAATTCATGCAGACTAT	PC-5p-122898_14	20	5
TCCGGGCATTGGTATAACAGT	PC-3p-124567_14	20	4
GAGGGCTAGGTCATCTGCGATT	PC-5p-100544_18	20	3
ATAAACGATTGTCTGAACATT	PC-5p-83501_21	18	6
CAGACAGAAGGTGTAGCTATT	PC-5p-128535_14	18	1
TAATACTGTTAGGTAAAGATGCC	sma-miR-8-3p	17	2,404
TGCTGCCTGATAAGAGCTGTGA	egr-miR-745_1ss10GA	17	187
TGATTGTCCATTCGCATTTCTT	sja-miR-219-5p_R+1	17	161
ACGCCGACATAAACCGTGAAT	PC-3p-114367_15	17	18
TTAACAATCTGGTAGATGTCT	PC-3p-87348_20	17	17
CAGACCGTGGACGCCAGTTCT	PC-5p-93611_19	17	16
CCAATGGTGCACATGGGCTCTT	PC-5p-59222_30	17	13
GACCCGATTGAGATACGCGCT	PC-5p-108611_16	17	11
CAACTCGGAGAGGCCAGGTTTTT	PC-5p-130301_13	17	6
CACGTGTTCCGAGTCTTCTCT	PC-5p-131361_13	17	1
AAGAAGACCCTGTTGAGCTTGACTCTAGTCCGACGTTGTG	PC-3p-28386_61	17	11
CTGTTAACCGAGGCCATCTCT	PC-3p-53629_33	16	21
TTGGATCCCCAACTTTCGACT	PC-5p-151851_11	16	10
ACCACAAGGGCTGATGAGACT	sma-mir-3492-p3_1ss18AG	16	10
TTCCGGAAGTTTTGAGTGATT	PC-3p-167297_10	16	1
TGATGGATGTAGTATAGGTGGGAGCTGGGT	PC-3p-51268_35	15	86
TGGATGCATATGGCTATGTCT	PC-3p-57721_31	14	15
AGAGATCCCGCTAGAAGCTTT	PC-3p-54871_32	14	8
AGGAAGCCCACTGTCGACATT	PC-3p-101048_17	14	8
TATACCGAGGCTTGAGATGG	PC-5p-149581_12	14	6
AATAGTCTGCATGAATTCTCT	PC-5p-191011_9	14	5
ACCCCAAGGAGTCTAGTGAATT	PC-3p-165038_10	14	4
AGATCCAGAGAACATTGAAGT	PC-5p-123862_14	14	3
CTCCGGAGATGTTTTGGGGCT	PC-5p-201291_8	14	2

AGCTCAGTGGTCTATCGGTTATT	PC-5p-26623_65	14	11
AAAAAGTCCCTGTTCGAGCT	PC-3p-27975_62	13	51
ACTTCGGCTGGTGATTTCCC	PC-3p-129633_13	13	10
TAAGGCACGCGGTGAATGTCA	sma-miR-124-3p	13	260
ACGACGTATTCCGAGGTCTCT	PC-5p-47560_37	13	30
TGAAGAATGTTGGAAAATCT	PC-5p-47385_37	13	20
CCGAGCTATCGCACTGTTGAT	PC-5p-272816_6	13	13
TGGAAGACTTGTGATTTAGTTGTT	PC-5p-3990_337	11	399
GTTTGAGTCTTGATGTG	PC-3p-79807_22	11	33
ATCGGTATTAATGTTGGTGCTT	PC-3p-146279_12	11	11
TACCGTCACTTTGGCTGTACT	PC-3p-46989_38	10	22
CAGCCAAAGTGACTCAGGTATGC	sma-mir-125a-p3	10	18
AGTGCTCTGAATGTC	egr-mir-219-p5 _1ss11TA	10	16
TGAGTAGAGTGAATTATGACT	PC-5p-108973_16	10	12
ACTGGTAGGTCCTGGGTTCTTT	PC-3p-47447_37	10	33
TCCCTGAGACCCTTTGATTGT	sja-miR-125a_R-1	9	23
ACCAGGGCATGACCAGACTCT	PC-5p-95204_19	9	16
TATCACAGCCCTGCTTGGGACACT	sma-miR-2b-3p _1ss24AT	7	699
TGTGGGTCTCTTTCTTGTCCATT	sma-mir-61-p5 _1ss23GT	7	10
AAAACATTGTCGATTTCTCC	PC-3p-91555_19	7	10
TATCACAGTCCAAGCTTTGGT	sma-miR-2e-3p	6	159
TGCTGCCTGATAAGAGCTGTGAT	PC-3p-49410_36	6	69
TTGCACGAATCTAACGCCTGT	PC-3p-52431_34	6	12
GAAGATTCAGACAAACAATAGC	PC-3p-88291_20	6	11
TTTTGCTGGCCTGGTATGAATT	PC-3p-97828_18	6	10
GTAGACATGTGACCT	sme-mir-2150-p3 _1ss5TA	4	61
GAACCCAGTGGTCTATCGGTTAAGTGCT	PC-5p-64128_28	4	18
TTTGAGTCCCGAATCTTGCGT	PC-5p-112939_16	4	17
TTGCTGAGATCGCCTTTTTGT	PC-3p-99572_18	4	11
GATTCGGCTCAGTGGTCTATCGGTTAAGTGCT	PC-5p-67365_26	4	24
AAGTCCCTGTTCGAGCTGATT	PC-3p-115660_15	4	12
TCACAACCTACTTGATTGAGGGG	sja-miR-307_R+3	3	371
AAATTCGAGTCTATAAGGA	sma-miR-10-3p_R-4	3	55
CCAGTGACCAGACATATCCCT	sma-miR-190-3p_L+1	3	44
TTCAACCATGCCATTAGCTGT	PC-5p-95371_19	3	18
TCACAGCCAATATTGATACCC	PC-3p-118142_15	1	30
TCTCGCTTCCCCGCCTTTCCCG	sma-mir-71a-p3	1	15
TACCAACTTTGACTGAGTTATACTGCTTTTGT	sma-miR-2e-5p_R+11	1	13
TAACTCAGTGGTCTATCGGTTAAGTGCT	PC-5p-86492_21	1	13
GCTCCGGTAGCTTAGTTGGT	sja-miR-3488	1	18
GAGTTCAGTGGTCTATCGGTTAAGTGCT	PC-5p-106009_17	1	10
TAGCCGTGGAGCTGTTCATCGCATTT	PC-5p-4596_298	0	31
GATTGAGCTGTACTGCTTGGGCT	PC-5p-152299_11	0	24
TTGGTCCCTATCAACCAGCTAT	sja-miR-133_1ss21GA	0	20
CAACGTCATCCTCATAGTGATT	PC-5p-82254_22	0	20
TATCACAGCCCTGCTTGGGACACTT	egr-miR-2b_R+3	0	18
NGGAATGTGGCGAAGTATGGTC	sja-miR-1_1ss1TN	0	17

ATAACTCAGTGGTCTATCGGTTAAGTGCT	PC-5p-78919_23	0	16
GAAGAGTGCCGACCCGGTTAGCC	sma-mir-36-p5	0	16
TTGACGTAATGATTTGAG	PC-3p-110194_16	0	15
GTCATCCTTGGATTGTGATTTT	sma-mir-2d-p5	0	14
TATCACAGTCCTGCTTAGGTGACG	sma-miR-2d-3p_R+2	0	13
GTGAGCAAAGTTTCAGGTGT	egr-miR-87-3p	0	13
TTGGTCCCTATCAACCAGCT	sja-mir-133-p5	0	12
AGGGTTATCCGTTTGCTCCGTT	PC-5p-191498_9	0	11
TGTAGGGCGGGATTGAGC	PC-5p-127121_14	0	10

Table S5: Comparison of protein content with published proteomics analysis of extracellular vesicles from adult and schistosomule *S. mansoni*.

Metabolic Enzymes						
			Adults		**Somules**	
GI (genInfo identifier)	**Accession number**	**Name**	**Samoil *et al.***	**Sotillo *et al.***	**Nowacki *et al.***	**ES protein***
360045358	CCD82906.1	Glycogen phosphorylase [Sm]	Yes	No	No	Yes
391358187	P16641.3	Taurocyamine kinase [Sm]	Yes	No	Yes	Yes
156118911	ABU49845.1	Creatine kinase [Sm]	Yes	No	Yes	Yes
3023710	Q27877.1	Enolase [Sm]	Yes	Yes	Yes	Yes
353230309	CCD76480.1	Pyruvate kinase [Sm]	Yes	No	No	Yes
360043735	CCD81281.1	Glucose-6-phosphate isomerase [Sm]	Yes	Yes	No	Yes
360045088	CCD82636.1	Lactate dehydrogenase [Sm]	Yes	Yes	No	Yes
353228833	CCD75004.1	Malate dehydrogenase [Sm]	Yes	No	No	Yes
353229703	CCD75874.1	Phosphoenolpyruvate carboxykinase [Sm]	Yes	No	No	Yes
256070409	XP_002571535.1	Ornithine-oxo-acid transaminase [Sm]	Yes	No	No	Yes
1172460	P41759.1	Phosphoglycerate kinase [Sm]	Yes	No	Yes	Yes
353230092	CCD76263.1	Transketolase [Sm]	Yes	Yes	No	Yes
256090542	XP_002581246.1	Phosphoglucomutase [Sm]	Yes	No	No	Yes
353232336	CCD79691.1	Fructose 1,6-bisphosphate aldolase [Sm]	Yes	Yes	Yes	Yes
350645988	CCD59265.1	Aldehyde dehydrogenase, putative [Sm]	Yes	No	No	Yes
350645892	CCD59437.1	Adenosylhomocysteinase, putative [Sm]	Yes	No	No	No
353229457	CCD75628.1	Glyceraldehyde-3-phosphate dehydrogenase [Sm]	Yes	Yes	Yes	Yes
123502	P09383.1	Hypoxanthine-guanine phosphoribosyltransferase [Sm]	Yes	No	No	No
383930643	AFH56663.1	Methylthioadenosine phosphorylase [Sm]	Yes	No	No	Yes
256082671	XP_002577577.1	Aconitate hydratase [Sm]	Yes	No	No	Yes
3122305	Q27778.1	6-phosphofructokinase [Sm]	Yes	No	No	Yes
360044093	CCD81640.1	Long-chain-fatty-acid-CoA ligase [Sm]	Yes	No	No	No
353229440	CCD75611.1	Glycogenin-related [Sm]	Yes	No	Yes	No
350643977	CCD58348.1	6-phosphogluconate dehydrogenase,putative [Sm]	Yes	No	No	No
Xenobiotic / Redox Metabolism						

121700	P09792.1	Glutathione S-transferase 28 kDa isozyme [Sm]	Yes	No	Yes	Yes
256093080	XP_002582203.1	Glutathione S-transferase 26 kDa [Sm]	Yes	Yes	Yes	Yes
350645579	CCD59704.1	Aldo-keto reductase, putative [Sm]	Yes	No	No	Yes
161007	AAA29889.1	Glutathione S-transferase, partial [Sm]	Yes	No	No	Yes
353231561	CCD77979.1	Glyoxalase I [Sm]	Yes	No	No	No
Proteases						
350646132	CCD59179.1	Thimet oligopeptidase (M03 family) [Sm]	Yes	No	No	No
360043297	CCD78710.1	Leucine aminopeptidase (M17 family) [Sm]	Yes	Yes	No	Yes
353230291	CCD76462.1	Calpain (C02 family) [Sm]	Yes	Yes	Yes	Yes
729709	P09841.3	Hemoglobinase (Antigen SM32) [Sm]	Yes	No	No	No
353228442	CCD74613.1	Cathepsin B-like peptidase (C01 family) [Sm]	Yes	No	No	Yes
256072593	XP_002572619.1	Prolyl oligopeptidase (S09 family) [Sm]	Yes	No	No	No
353230839	CCD77256.1	SpAN g.p. (M12 family) [Sm]	Yes	No	No	No
564131894	AHB79081.1	Serine protease 2 precursor [Sm]	Yes	Yes	No	Yes
256084114	XP_002578277.1	Subfamily M12B unassigned peptidase (M12 family) [Sm]	Yes	No	No	No
353233303	CCD80658.1	Family S9 non-peptidase homologue (S09 family) [Sm]	Yes	No	No	No
353232118	CCD79473.1	Xaa-Pro dipeptidase (M24 family) [Sm]	Yes	No	No	No
Fatty acid binding						
55670478	1VYG	Fatty Acid Binding Protein [Sm]	Yes	No	Yes	Yes
Transporters/channels						
350644272	CCD60986.1	Plasma membrane calcium-transporting atpase, putative [Sm]	Yes	No	No	No
360043551	CCD78964.1	Sodium potassium transporting ATPase alpha subunit [Sm]	Yes	No	No	No
353231052	CCD77470.1	Glucose transport protein [Sm]	Yes	No	No	No
353233037	CCD80392.1	Cation-transporting ATPase [Sm]	Yes	No	No	No
256084157	XP_002578298	Choline transporter-like protein 2 (Ctl2) [Sm]	Yes	No	No	No
353229720	CCD75891.1	Aquaporin-3 [Sm]	Yes	No	No	No
256084334	XP_002578385.1	Chloride channel protein [Sm]	Yes	No	No	No
353231352	CCD77770.1	Anion exchange protein [Sm]	Yes	No	No	No
Signal Transduction and Biological Regulation						
3023193	Q26540.1	14-3-3 protein homolog 1 [Sm]	Yes	Yes	Yes	Yes
353228883	CCD75054.1	14-3-3 epsilon [Sm]	Yes	No	Yes	Yes
256084742	XP_002578585.1	Annexin [Sm]	Yes	Yes	Yes	Yes
353228653	CCD74824.1	sh3 domain grb2-like protein B1 (endophilin B1) [Sm]	Yes	No	No	No
353228669	CCD74840.1	Kynurenine aminotransferase [Sm]	Yes	No	No	No
353231089	CCD77507.1	Talin [Sm]	Yes	No	No	No
256082840	XP_002577660.1	Integrin alpha-ps [Sm]	Yes	No	No	No
353228490	CCD74661.1	Integrin beta subunit [Sm]	Yes	No	No	No
353232319	CCD79674.1	Atp-diphosphohydrolase 1 [Sm]	Yes	No	No	Yes
256084780	XP_002578604.1	rpgr-interacting protein 1 related [Sm]	Yes	No	No	No
350646699	CCD58613.1	Hyperpolarization activated cyclic nucleotide-gated potassium channel, putative [Sm]	Yes	No	No	No
256080579	XP_002576557.1	ng, ng-dimethylarginine dimethylaminohydrolase [Sm]	Yes	Yes	No	No

256052204	XP_002569666.1	ral guanine nucleotide dissociation stimulator ralgds[Sm]	Yes	No	No	No
353229530	CCD75701.1	Proline-serine-threonine phosphatase interacting protein [Sm]	Yes	No	No	No
360043093	CCD78505.1	Lip-related protein (liprin) alpha [Sm]	Yes	No	No	No
256090851	XP_002581393.1	Tetraspanin [Sm]	Yes	No	Yes	No
256089413	XP_002580804.1	Serine/threonine protein kinase [Sm]	Yes	No	No	No
360043400	CCD78813.1	rap1 [Sm]	Yes	Yes	Yes	No
350646635	CCD58662.1	Calponin homolog, putative [Sm]	Yes	No	No	Yes
360042647	CCD78057.1	Voltage-gated potassium channel [Sm]	Yes	No	No	No
353233207	CCD80562.1	Syntenin [Sm]	Yes	Yes	No	No
		Cytoskeletal /Structural				
350646584	CCD58796.1	Fer-1-related [Sm]	Yes	No	No	No
1703114	P53471.1	Actin-2 [Sm]	Yes	Yes	Yes	Yes
353232589	CCD79944.1	Alpha tubulin [Sm]	Yes	No	No	Yes
353232516	CCD79871.1	Tubulin beta chain [Sm]	Yes	No	No	Yes
256076432	XP_002574516.1	Tubulin subunit beta [Sm]	Yes	No	No	Yes
353229949	CCD76120.1	Collagen alpha-1(V) chain [Sm]	Yes	No	No	No
350645253	CCD60034.1	Prominin (prom) protein, putative [Sm]	Yes	No	No	No
353231032	CCD77450.1	Rab GDP-dissociation inhibitor [Sm]	Yes	No	Yes	No
360045419	CCD82967.1	Actin [Sm]	Yes	Yes	Yes	Yes
350644919	CCD60380.1	Synaptotagmin, putative [Sm]	Yes	No	No	No
495668	AAA29882.1	Fimbrin [Sm]	Yes	No	Yes	Yes
360044904	CCD82452.1	Signal recognition particle 68 kD protein [Sm]	Yes	No	No	No
256072032	XP_002572341.1	Gelsolin [Sm]	Yes	No	No	No
353230603	CCD77020.1	Intermediate filament proteins [Sm]	Yes	No	No	No
353230415	CCD76586.1	Cytoplasmic dynein light chain [Sm]	Yes	Yes	No	Yes
256073059	XP_002572850.1	Collagen alpha chain type IV [Sm]	Yes	No	No	No
353232499	CCD79854.1	Microtubule-associated protein 9 [Sm]	Yes	No	No	No
360045234	CCD82782.1	Rab-2,4,14 [Sm]	Yes	No	No	No
350645028	CCD60258.1	Rab11, putative [Sm]	Yes	No	Yes	No
353228708	CCD74879.1	Ran [Sm]	Yes	No	No	No
		Tegumental antigen				
360043686	CCD81232.1	200-kDa GPI-anchored surface glycoprotein [Sm]	Yes	No	No	No
353230232	CCD76403.1	Tegumental protein Sm 20.8 [Sm]	Yes	Yes	Yes	Yes
135578	P14202.1	Tegument antigen SmA 22.6 [Sm]	Yes	Yes	No	Yes
390124514	CCE94318.1	Tegumental antigen [Sm]	Yes	No	No	No
350646174	CCD59158.1	Sm23, putative [Sm]	Yes	No	No	No
353230115	CCD76286.1	Sm29 [Sm]	Yes	Yes	Yes	No
		Histones				
353231319	CCD77737.1	histone H3 [Sm]	Yes	No	No	Yes
10953803	AAG25601.1	histone H4 [Sm]	Yes	Yes	No	Yes
353229586	CCD75757.1	histone H2B [Sm]	Yes	No	No	Yes
		Chaperones				
238663484	CAZ34365.1	Heat shock protein 70 (hsp70)-4, putative [Sm]	Yes	No	No	Yes
27805450	Q26565.1	Peptidyl-prolyl cis-trans isomerase [Sm]	Yes	No	No	Yes

353230032	CCD76203.1	Heat shock protein-HSP20/alpha crystallin family [Sm]	Yes	No	No	No
256082744	XP_002577613.1	Chaperonin containing t-complex protein 1 epsilon subunit tcpe [Sm]	Yes	No	No	No
Translation						
353230261	CCD76432.1	Elongation factor 1-alpha (ef-1-alpha) [Sm]	Yes	Yes	Yes	Yes
353231791	CCD79146.1	Eukaryotic translation elongation factor [Sm]	Yes	No	No	Yes
Others						
256080932	XP_002576729.1	SPRY domain containing protein [Sm]	Yes	No	No	No
350644553	CCD60716.1	Cell division control protein 48 aaa family protein (transitional Endoplasmic reticulum atpase), putative [Sm]	Yes	No	No	No
360045009	CCD82557.1	Band 4.1-like protein [Sm]	Yes	No	No	No
350646643	CCD58670.1	Centrosomal protein of 135 kDa (Cep135 protein) [Sm]	Yes	No	No	No
360044828	CCD82376.1	Excision repair helicase ercc-6-related [Sm]	Yes	No	No	No
256079432	XP_002575991.1	Ubiquitin (ribosomal protein L40) [Sm]	Yes	No	Yes	Yes
353230536	CCD76953.1	Ubiquitin-protein ligase BRE1 [Sm]	Yes	No	No	No
256084236	XP_002578337.1	Mixed-lineage leukemia 5 mll5 [Sm]	Yes	No	No	No
350646702	CCD58616.1	Basic helix-loop-helix transcription factor, putative [Sm]	Yes	No	No	No
353231449	CCD77867.1	Late embryogenesis abundant protein [Sm]	Yes	No	No	No
353232869	CCD80225.1	Zinc finger protein [Sm]	Yes	No	No	No
Hypothetical/Unnamed						
353229181	CCD75352.1	hypothetical protein Smp_140590 [Sm]	Yes	No	No	No
256068121	XP_002570696.1	hypothetical protein [Sm]	Yes	No	No	No
360043835	CCD81381.1	hypothetical protein Smp_007640 [Sm]	Yes	No	No	No
353233031	CCD80386.1	unnamed protein product [Sm]	Yes	No	No	No
360043421	CCD78834.1	hypothetical protein Smp_134750 [Sm]	Yes	No	No	No
360044459	CCD82007.1	hypothetical protein Smp_080920.3 [Sm]	Yes	No	No	No
353231528	CCD77946.1	hypothetical protein Smp_133590 [Sm]	Yes	No	No	No
360044961	CCD82509.1	hypothetical protein Smp_024220 [Sm]	Yes	No	No	No
350646356	CCD58986.1	hypothetical protein Smp_155620 [Sm]	Yes	No	No	No
353232008	CCD79363.1	hypothetical protein Smp_145450 [Sm]	Yes	No	No	No
256078659	XP_002575612.1	hypothetical protein [Sm]	Yes	No	No	No
353229633	CCD75804.1	hypothetical protein Smp_159020 [Sm]	Yes	No	No	No
353229931	CCD76102.1	hypothetical protein Smp_006830.1 [Sm]	Yes	No	No	No