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Characterisation of secreted exosomes from the intestinal nematode *Heligmosomoides polygyrus*

Gillian Coakley

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Declaration

I, the undersigned, hereby declare that this thesis is my own composition, the work presented in the thesis has been conducted by myself, unless acknowledged otherwise, and it has not been submitted for any other degree or professional qualification except as specified.

Sections of the Introduction have been previously published in two review articles published in Reviews in Parasitology, and Molecular and Biochemical Parasitology. Data from Chapter 3 and 5 have been previously published in a research article in Nature Communications, with author contributions stated in the manuscript. The mass spectrometry analysis in Table 3.2.6 was compiled by Thierry Le Bihan. All published papers are attached in the appendix; text and data reproduced here are my own work

Gillian Coakley Date 01/02/17

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Abstract

The parasite secretome has been shown to play a key role in both pathogenicity and the regulation of host defence, allowing pathogens, such as helminths, to establish a chronic infection within the host. The recently discovered presence of extracellular vesicles within parasite-derived excretory-secretory products introduces a new mechanism of potential cross-species communication. Extracellular vesicles (EVs), such as exosomes, facilitate cellular communication through the transfer of small RNAs, lipids and proteins between cells and organisms across all three kingdoms of life. In addition to their roles in normal physiology, EVs also transport molecules from pathogens to hosts, presenting parasite antigens and transferring infectious agents.

Here, I examine secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus,* and their potential role in the host-helminth interactions. Transmission electron microscopy reveals vesicle-like structures of 50-100 nM in the ultracentrifuged secretory product, and potential evidence of multi-vesicular bodies in the worm intestine. This, coupled with information from the exoproteome, helped support the hypothesis that exosomes originate from the parasite intestinal tract.

I have completed a series of studies looking at the fundamental properties of exosome-cell interactions, providing comparative studies between mammalian and *H. polygyrus*-derived exosomes. I have determined some of the key factors influencing exosome uptake, including time of incubation, cell type and exosome origin. Through microarray analysis of *H. polygyrus* exosome-treated small intestinal epithelial cells, we see significant gene expression changes, including those involved in the regulation of signalling and the immune response, such as DUSP1 (dual-specificity phosphatase) and IL1RL1 (the receptor for IL-33). The modest reduction of inflammatory cytokine responses by exosomes in small intestinal cell lines was amplified in immune cells, such as macrophages. Exosomes can significantly reduce expression of classical activation markers, as well as inflammatory cytokine production in the macrophage cell line RAW 264.7, and this is further supported by similar responses in bone marrow-derived macrophages.

Owing to their suppressive nature, I demonstrate that immunization of mice with an exosome/alum conjugate generates significant protection from a subsequent *H. polygyrus* larval challenge, as seen through a reduction in egg counts and worm burden.

I have investigated the role of the IL33 receptor (IL-33R); a key molecule associated with parasitic resistance that is suppressed by exosomes in type-2 associated immune responses. Uptake of *H. polygyrus*-derived exosomes by alternatively activated macrophages caused the suppression of type 2 cytokine/protein release and the reduction of key genes associated with this phenotype. In addition, there was also significant repression of both transcript and surface T1/ST2, a subunit of the IL-33R). Using a model of lung inflammation, *in vivo* studies demonstrate that, in both prophylactic and co-administration experiments, exosomes modulate the innate cellular response. This is represented by changes in the number of innate lymphoid cells (ILCs), bronchoalveolar lavage eosinophils and type-2 cytokine output. In this system, the expression of T1/ST2 on type 2 ILCs was also significantly reduced.

I have extended the investigation on exosome-IL-33R responses by using T1/ST2 knockout mice. Despite generating strong antibody responses, vaccination against exosomes could not protect T1/ST2 knockout mice against a subsequent infection.

This work suggests that exosomes secreted by nematodes could mediate the transfer and uptake of parasite products into host cells, establishing cross-species communication to suppress the host ‘danger’ or inflammatory response.

Lay summary

Almost a quarter of the world's population suffer from soil-transmitted helminth infection, with the majority prevailing in impoverished regions such as eastern Asia, sub-Saharan Africa and the Americas. The increasing incidence of anti-helminthic drug resistance in livestock poses a huge socio-economic problem, in addition to the potential human threat via infection. Therefore, it is essential that we harness a greater understanding of the interactions that occur between parasites and their hosts, as this can lead to the development of more effective treatments for helminthic disease. Correspondingly, helminth parasites generally establish long-term infections, reflecting their ability to drive a new physiological and immunological status within their host. Identification of the immunosuppressive molecules that helminths use to evade host defence could potentially provide a new approach for the treatment of inflammatory and allergic-based conditions such as colitis and asthma.

Unfortunately, human helminthiases are difficult to study in a laboratory setting, and research can be further hindered due to poor financial investment and public awareness. Therefore, animal models of helminth infection are used due to similarities in the pathogenesis and lifecycle stages, which can then be extrapolated for human study.

It has been shown that parasites' secretory products, including glycans, extracellular vesicles and metabolites, can play a key role in both pathogenicity and host immunoregulation. Exosomes are small cell-derived vesicles (50-100nm), which facilitate communication through the transfer of cellular components (such as antigens and small nucleic acids), and are released from the majority of cells.

We have demonstrated that secreted vesicles from the murine gastrointestinal nematode *Heligmosomoides polygyrus* (a model of human hookworm infection) exhibit a range of immunosuppressive and regulatory properties on murine cell lines and *in vivo*. However, fundamental questions still remain about host-parasite communication.

Comparative studies between mammalian and *H. polygyrus*-derived exosomes highlight some of the key factors responsible for exosome uptake. This allowed us to then focus on blocking parasite-derived exosome entry into mammalian cells *in vitro*, inhibiting any parasite-mediated effects on the host cell.

Additionally, I showed that immunization of mice using an exosome/alum conjugate contributes to significant protection from a subsequent *H. polygyrus* infection. This vaccination generates sterile immunity against larval challenge, seen through the initiation of antibody responses against exosomes, resulting in a reduction of parasitic egg counts and worm burden.

Conversely, we looked to exploit the immunomodulatory features of *H. polygyrus* exosomes. Using a model of lung inflammation, we found that nasal administration of exosomes suppressed the innate cellular immune response in the lung tissue (one which would normally drive the symptoms of asthma). This demonstrated the ability of parasite-derived exosomes to suppress an inflammatory-mediated disease, and highlights a potential starting point to test therapeutic strategies using exosomes, especially for airway allergy.

This project provides a greater understanding of the properties of helminthic exosomes and host-parasite communication, and offers new strategies on how we can interfere with or mimic these processes to treat infectious or inflammatory disease.

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Abbreviations

AAM - Alternatively Activated Macrophages
Ab – Antibody
Ag – Antigen
AIDS – Acquired Immunodeficiency Syndrome
APC – Antigen Presenting Cells
BAL – Bronchoalveolar Lavage
BMDM – Bone Marrow-derived Macrophages
BMDC – Bone Marrow-derived Dendritic Cells
CD - Cluster of Differentiation
DALY – Disability-adjusted Life Year
DAMP – Danger Associated Molecular Patterns
DC – Dendritic Cell
DNA – Deoxyribonucleic Acid
DUSP1- Dual Specificity Phosphatase-1
ELISA - Enzyme Linked Immunosorbent Assay
ES – Excretory/Secretory products
ESCRT - Endosomal Sorting Complexes Required for Transport
EV – Extracellular Vesicle
FACS – Fluorescence Activated Cell Sorting
FCS – Foetal Calf Serum
FSC – Forward Scatter
h - hour
HES – *Heligmosomoides polygyrus* excretion-secretions
HIV - Human Immunodeficiency Virus
HRP – Horse Radish Peroxidase
HSP – Heat Shock Protein
i.p. injection - Intraperitoneal Injection
i.v. injection - Intravenous Injection
IFNγ – Interferon Gamma
Ig - Immunoglobulin
IL – Interleukin
ILC2 – Type-2 Innate Lymphoid Cells
ILVs - Intra-luminal Vesicles
iNOS - inducible nitric oxide synthase
kDa – Kilodalton
KO – Knockout
LPS - Lipopolysaccharide
LN - Lymph Nodes
mAb - Monoclonal Antibody
MAPK - Mitogen-Activated Protein Kinase
MFI - Mean Fluorescence Intensity
MHC - Major Histocompatibility Complex
Min – Minute
miRNA – micro RNA
MLN - Mesenteric lymph node
mRNA - Messenger RNA
MVB – Multi-vesicular Body
nm - Nanometre
NTD – Neglected Tropical Disease
OVA – Ovalbumin
Ova - haploid female reproductive cell
PAMPs – Pathogen-associated Molecular Patterns
PBS - Phosphate Buffered Saline
PRRs – Pattern Recognition Receptors
q-RTPCR – Quantitative Polymerase Chain Reaction
RELMα/β - Resistin-like Molecules Alpha/Beta
RNA - Ribonucleic Acid
R&D – Research and Development
S.D. - Standard Deviation
S.E.M – Standard Error of Mean
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM – Scanning Electron Microscopy
siRNA - Short Interfering RNA
SSC – Side Scatter
STAT - Signal Transducer and Activator of Transcription
STH – Soil-transmitted Helminth
Sup – HES depleted of exosomes
TEM – Transmission Electron Microscopy
TGF-β - Transforming Growth Factor Beta
TLR – Toll-Like Receptor
TNF - Tumour Necrosis Factor
TSLP - Thymic Stromal Lymphopoietin
TSPAN -Tetraspanin
WB - Western Blot
WHO – World Health Organisation
WT - Wild Type
Ym1 - chitinase-3-like-3 (Chi3l3)

Chapter 1: Introduction

1.1 Neglected tropical disease

1.1.1 Introduction – epidemiology and current statistics

More than one billion people worldwide are affected by parasitic disease, including malaria [1] and neglected tropical diseases (NTDs) such as leishmaniasis, Chagas disease and helminthiases [2] with the majority prevailing in developing regions such as eastern Asia, sub-Saharan Africa and the Americas. One way to assess overall disease impact is to score the disability-adjusted life year (DALY). This method is used by the World Health Organisation (WHO) to evaluate the number of years of 'healthy' life lost due to premature mortality combined with years lost to disabilities induced by a particular disease. Data from the Global Burden of Disease Study in 2013 states that malaria and NTDs account for approximately ~5% (more than 26 million DALYs) of the total number of years lost due to ill-health, disability or early death worldwide [3].

Susceptibility to disease correlates highly with the geographical location and economic status of an individual. Currently, just under half of the world's population live in low-to-middle-income countries. Approximately 65 -80% of people requiring treatment for NTDs live within these regions (generally from lower-to-middle income countries [2]). Social determinants in impoverished areas; including poor education, diet, financial instability, agriculture, struggling healthcare systems and a lack of

government intervention are major factors for the persistence of NTDs today [4]. Furthermore, the lack of sanitation (both in terms of availability and education), is a major player for NTD transmission and longevity, especially in soil-transmitted helminth infections [5]. The consequences of some NTDs, such as long-term illness, decreased productivity and absenteeism from work, contribute to an endless cycle of poverty, leading to larger socioeconomic consequences worldwide.

The London Declaration for eradication or control of ten NTDs (lymphatic filariasis, onchocerciasis, soil-transmitted helminths, schistosomiasis, trachoma, leprosy, human African trypanosomiasis, Chagas disease, visceral leishmaniasis and dracunculiasis) in 2012, called for the participation of large pharmaceutical companies, governments of affected impoverished nations and charitable organizations or foundations and private financial donors in order to achieve this goal [6]. This was generated with the aim of providing easy accession to drugs and chemotherapeutics, as well as support for other preventative methods such as distribution of mosquito netting and the installation of clean water systems. Indeed, around 70% of R&D invested in infectious disease in 2014 has been focused on 'the big three'; malaria, HIV/AIDS or tuberculosis (TB), and less than 20% spent on the other major NTDs identified by WHO [7]. Furthermore, investment on research/therapeutics of major helminth diseases (lymphatic filariasis, hookworm infection, trichuriasis, schistosomiasis, and ascariasis) was only ~17% of the funds distributed in 2011, despite being responsible for 87% of DALYs and behind three-quarters of all mortalities caused by NTDs [8].

1.1.2 Health status and co-morbidities

Most DALY statistics associated with NTDs could be attributed to the chronic nature of some of these diseases. These can lead to lifelong disabilities such as mental and physical retardation, malnutrition, anaemia and lymphoedema [9], contributing to productivity loss in the global workforce. Most NTDs, including soil-transmitted helminthiasis, are not often thought of as fatal diseases when compared to mortality rates of HIV/AIDs or TB patients. However, they actually do contribute to the significant burden of "co-morbidities" with either the top three infectious diseases, as well as many other health conditions [10]. This is especially concerning, when the major group affected by helminthiasis are children of school-age [11]. The main species of helminths that infect people are the roundworm (*Ascaris lumbricoides*), the whipworm (*Trichuris trichiura*) and the hookworms (*Necator americanus* and *Ancylostoma duodenale*). The geographical overlap is not the only factor that contributes to co-infections. For example, the ability of helminths to establish an 'immunosuppressed' or type-2 environment within the host during chronic infection [12], can help support the establishment and transmission of other infections, such as HIV, malaria and tuberculosis [10]. There are also data suggesting that helminthic infection can limit vaccine efficacy, such as associations with enteric-stage *Schistosoma japonicum* infection and the hepatitis B vaccine [13]. However, there are some conflicting reports of this phenomenon in vaccination against other viruses [14]. This may be due to the potency or construct of the vaccine, which may have to be altered following the consideration of a helminth co-infection and its potential consequences [15]. Correspondingly, most of the immunomodulatory effects induced

by helminth infection can be reversed following drug-mediated parasite clearance [16-18].

1.1.3 Current therapeutics and limitations

One of the current priorities for WHO looks at tackling the 17 major NTDs by 2020 [19]. WHO estimates that just 0.1% of domestic expenditure on health projected to be spent in low to middle income countries for the next ~15 years would be sufficient to fund their contribution to universal NTD treatment [2]. Achieving internationally agreed targets of NTD control and elimination [6] will result in significant improvements in health and reductions in morbidity, with a projected >80% of the global reduction in low to lower-middle income countries (the worst-affected by NTDs). This would also reduce the prevailing between-country health inequalities normally associated with poverty. Targets include vector transmission control (mosquito nets, topical treatments, etc.) and ensuring availability of both preventative chemotherapeutics and anti-parasite drugs. Drug donations to United States Agency for International Development, have exceeded $11 billion in value, and are to be distributed in mass treatment campaigns within a number of developing countries [20].

Whilst there has been significant progress in drug-donation programs, there are a number of limiting factors that could prevent total success, considering the large number of people (more than 1 billion) targeted in more than 100 countries worldwide. However, the prospect of drug resistance is alarming, with an increasing incidence both in livestock (with the potential threat of zoonotic transmission [21]),

and in humans [22], as well as having strong economic and social implications [23]. Furthermore, most of the targets for elimination focus on inhibition of transmission and infection cycles, with little recognition given to treatment of NTD-induced morbidity. A comprehensive programme to treat NTDs must also include management of morbidity. For example, reducing the prevalence of disease-associated stigma, generating support for mental health care, funding surgical alleviations and providing livelihood initiatives for those unable to work due to morbidity [24]. A longer-term solution, especially for NTDs such as soil-transmitted helminth (STH) infections, would be to promote the large-scale improvement in sanitation, access to clean drinking water and to educate on the merits of good hygiene practice [5].

1.2 The immune response to helminth infection

As previously stated, almost one third of the global population is infected with helminths, whereby chronic infection has strong implications for co-morbidity, permanent disabilities, and the global economy [19]. Unfortunately, human helminthiases are difficult to study in a laboratory setting, and research can be further hindered due to low study numbers, the variability of subjects, the development of potential co-morbidities and poor financial investment. Therefore, veterinary models of helminth infection, such as those found in rodents, livestock and canines are used [25], due to some similarities in the pathogenesis and lifecycle stages, helping to elucidate the host immune response to helminths. One such model is the gastrointestinal nematode, *Heligmosomoides polygyrus*, a natural parasite of wood

mice. Although the immune response to *H. polygyrus* is still being characterized, the parasite induces a mixed Th2/T regulatory environment, maintaining longevity and establishing a chronic infection within the host [311]. As a murine parasite, H. polygyrus offers a tractable model to study chronic helminth infection in a laboratory setting (given the length of time it can reside in the murine host), and resides in the same Clade (Clade V) as the human hookworms *Necator americanus* and *Ancylostoma duodenale*. Although rodent models of hookworm infection, such as *H. polygyrus* and *Nippostrongylus brasiliensis*, offer an alternative model of study to human-infective species, they fail to fully recapitulate the characteristic responses to human hookworm infection, such as hyporesponsiveness to low level infection, and most importantly, anaemia (caused by blood feeding by the parasite) [434]. Despite this, we can extrapolate some of the information taken from animal studies, such as the detailed characterisation of the type-2 immune response to helminth infection, and use this as a framework for future studies in humans. An overall summary from what is known of the immune response to helminth infection is shown in Figure 1.1.

1.2.1 Innate Immune Responses

The primary encounter between parasite and host will often occur at a barrier surface (such as skin or intestinal epithelium), which can mediate pathogen recognition via pattern recognition receptors (PRRs) or release various ‘alarmins’ [26]. Whilst the role of toll-like receptors (TLR) in type-1 pathogen pattern recognition is now well understood [27], there is no defined system recognition of Th2-inducing organisms such as helminths or fungi. Despite this, there is some literature describing the presence of TLR ligands from helminths, including the lyso-phosphatidylserine

glycolipid from *Schistosoma mansoni* [28] and double-stranded RNA in SEA (soluble extract from *S. mansoni* eggs), which is shown to activate TLR3 [29]. There are also other receptor systems, such as the C-type lectin receptors (CLRs), which recognise nematode glycans to facilitate host immunity [30].

Some alarmins are released to promote mucosal barrier repair, such as protease-resistant Trefoil factor family peptide-2, which initiates IL-33-driven hookworm expulsion and tissue repair [31]. The release of alarmin cytokines, including IL-25, IL-33 and Thymic Stromal Lymphopoietin (TSLP), is closely associated with helminth-mediated tissue damage [32-34]. These cytokines promote a pro-allergic and anti-helminth immune response by promoting the maturation of monocytes, function of innate cells and type-2 immune responses. More recently, tuft cells (an intestinal epithelial crypt population) were shown to be a major source of IL-25 during helminth infection, promoting a strong ILC2 response and goblet cell hyperplasia [35]. Additionally, these cells expand during both IL-4Rα activation or *Nippostrongylus brasiliensis* infection [36]. These cells have chemosensory properties, potentially used in the detection of parasite-derived molecules, with both studies demonstrating that tuft cells are central to IL-25-driven intestinal immunity against helminths. Consequently, blocking the IL-25, IL-33 or TSLP response (or a combination thereof) can sustain a chronic helminth infection [37, 38]. Importantly, these cytokines are crucial for the activation of type-2 innate lymphoid cells (ILC2) [39]. ILC2s are a prominent early source of type-2 cytokines, such as IL-5 and IL-13 [40], and promote both innate eosinophil responses [41] and adaptive Th2 responses [42]. A recent paper also demonstrates that ILC2s may be a source of IL-4, required

for Th2 differentiation during *Heligmosomoides polygyrus* infection *in vivo* [43]. Interestingly, IL-9 was shown to be critical for ILC2-mediated cytokine responses and barrier repair during the lung stages of *N. brasiliensis* infection [44].

Other innate effector cells also play a role in helminth-mediated immunity. Basophils are an early source of IL-4 during helminth infection [45] that release vasoactive substances like histamine upon degranulation, and are also important for mediating IgE-dependent secondary immunity to helminth infection [46]. A strong indicator of both helminth infection and type-2 allergic responses is often observed by the induction of eosinophilia [47, 48]. Driven by both IL-5 and eotaxin, eosinophils are suggested to be another early source of IL-4 during nematode infection [49]. Furthermore, eosinphillic granule proteins were shown to mediate protection against a primary infection with the filarial parasite *Brugia malayi,* regulating IgE antibody responses and lung inflammation [50]. In addition to direct parasite killing, eosinophils also limit tissue larval stage survival and migration in secondary infections in other nematodes [48].

There is also literature demonstrating the importance of the type-2 immune driven mucus response, also known as the 'weep' response. The mucus layer provides a physical barrier, and is maintained by specialised epithelial cells known as goblet cells. Goblet cells are shown to provide luminal antigens to antigen presenting cells (APCs) [51], as well as releasing effector molecules to drive the anti-parasite response, such as mucins and resistin-like molecules (RELM) [52, 53]. Innate sources of IL-4 and IL-13 promote the secretion of mucin-5ac and RELM-β from goblet cells,

and were shown to be essential for *N. brasiliensis* expulsion [54]. Notably, either mucin-2 or mucin-5ac deficiency led to limited worm expulsion in different gastrointestinal nematode infections [55, 56], demonstrating the importance of mucosal responses in parasitic immunity. Furthermore, goblet cell-derived RELM-β is also implicated in immunity to *H. polygyrus*, by reducing both adult worm fitness and fecundity [52].

In addition to the mucus response, there is evidence that pulmonary epithelial cells can release surfactant-protein-D following the lung stages of *N. brasiliensis* infection. This is shown to enhance both the ILC2 and alveolar macrophage responses, driving immunity and subsequent killing of the parasite [57].

1.2.2 M2 macrophages

There has long been an appreciation for the role of macrophages in helminth infection, often providing a link between innate and acquired immune responses [58]. These cells, which can be initially driven under the influence of IL-4 and IL-13, polarise to an anti-parasite or wound-healing phenotype, where they become known as alternative activated macrophages (AAM), where they are also classified as M2 macrophages [59]. The sources of IL-4 and IL-13 can derive from innate cells, such as ILC2s [60], basophils [45], and possibly eosinophils [49], with adaptive T cell-derived IL-4/IL-13 required for a sustained AAM response that promotes wound healing [61]. Furthermore, AAM polarisation can be significantly enhanced by IL-33 (with AAMs expressing the cognate receptor, IL-33R or T1/ST2) during airway

allergy and inflammation [62]. Knockout studies have also demonstrated the importance of global [63], and macrophage-specific, IL-4 receptor-alpha (IL-4Rα) [64] in facilitating responses to gastrointestinal nematodes, as well as expression of downstream TFs, such as Signal Transducer and Activator of Transcription-6 (STAT-6) [63]. IL-4-driven macrophage recruitment and subsequent effector mechanisms, such as intestinal smooth muscle contractility, are required to mediate parasite expulsion [65]. IL-4Rα signalling in AAMs was shown to be important for activation of these cells, helping to ameliorate acute inflammatory responses during *S. mansoni* infection [66]. IL-4Rα signalling is also important for wound healing during helminth infection e.g. caused by *N. brasiliensis* larvae migrating through the lung. This was shown to drive AAM-mediated release of molecules such as insulin-growth factor-1 (IGF-1), which is known to facilitate tissue repair [67]. In addition, AAM expression of programmed death ligand 2 (PD-L2) is dependent on the IL-4/STAT6 pathway. This inhibitory ligand binds to a cognate T cell receptor PD-1 (programmed-death 1), regulating Th2 cell proliferation and function during *N. brasiliensis* infection in the lung [68]. Furthermore, there is data demonstrating the capacity of AAMs to undergo site-specific proliferation, driven by IL-4, during infection with the filarial nematode *Litomosoides sigmodontis* [69]. This overcomes the potentially counter-active inflammatory response that could be induced by blood monocyte recruitment. Taken together, these data show varied roles for IL-4Rα in AAMs during type-2 immune responses, required at different stages of infection, from clearance to repair.

However, it should be mentioned that recent evidence suggests that an IL-4Rα independent pathway could also drive AAM polarisation; such is seen in *Trichinella*

spiralis infection [70]. Additionally, there is data suggesting that IL-4Rα$^{-/-}$ macrophages can display some hallmarks of alternative activation, and can still immobilize the larval stage of *H. polygyrus* when supported by specific antibodies generated against the parasite [71]. Furthermore, M2 polarization is becoming increasingly more complex, with the identification of an epigenetic regulator Jmjd3 and its target, the TF IRF4, shown to induce M2 polarization to both *N. brasiliensis* infection and following the administration of chitins (sugars found in fungi and parasites) *in vivo* [72]. Finally, the role of cellular metabolism and it's influence on macrophages polarisation has been described. It has been shown that aerobic glycolysis is required for the rapid generation of an M1-centric innate anti-bacterial response, driving the production of reactive-oxygen species and nitric oxide whilst blocking other methods of metabolism, such as oxidative phosphorylation. Consequently, it is oxidative metabolism that drives the differentiation of macrophages to a M2 state, and this can also be enhanced by IL-4 stimulation, subsequently leading to mitochondiral biogenesis and respiration [73].

Macrophages, like epithelial cells, have a suite of PRRs on their surface. Despite the wealth of literature surrounding M1 macrophage PRRs, there is limited data available for M2 macrophages, with the focus largely directed to helminth-derived PAMPs on DC innate receptors [33]. Some studies now show that parasite secreted molecules which activate the MAPK pathway via PRRs can induce IL-10 production in macrophages [74]. IL-10 is an anti-inflammatory cytokine strongly associated with AAMs, and suppression of T cell-mediated pathology during infection [75]. The critical balance of TLR signalling is evident from studies with *S.*

mansoni, where elevated levels of CD14 (an inflammatory TLR4 accessory protein), correlated with reduced STAT6 activation and corresponding M2 responses, potentially to limit any type-2 mediated pathology [76].

AAMs secrete a number of factors involved in the type-2 immune response to helminths. The macrophage-derived protease inhibitor, serpinB2, has been shown to promote both Th2 and mucosal immunity, as well as inducing positive feedback for macrophage polarization and infiltration [77]. Conversely, parasite-derived serpins have been associated with dampening the alarmin cascade or degrading components of the cellular immune response [78]. There is also interesting data on the macrophage migration inhibitory factor (MIF), a pleiotropic mediator, which is normally considered to promote macrophage-driven inflammation [79]. Recently, it was shown that MIF deficient mice had stronger Th2-driven anti-parasite responses, mediating greater expulsion during *N. brasiliensis* infection [80]. The paper also showed that MIF deficiency resulted in reduced numbers of IL-6-producing $CD4^+$ T cells, suggesting a further role of IL-6 deficiency in promoting Th2-mediated expulsion of *H. polygyrus* [81]. Interestingly, MIF deficient mice are more susceptible to *Taenia crassiceps* [82], but this may be due to the mixed Th1/Th2 response induced by this parasite, where early Th1 responses are required for protection against the larval stages [83].

Chitinases such as YM1 [chitinase-3-like-3 (Chi3l3)] and AMCase (acidic mammalian chitinase) are strongly upregulated in AAM during both allergic responses and nematode infection, and are produced to break down chitins from

parasites or other allergens [84, 85]. The function of Ym1 is still relatively unknown, although it has been shown to initiate IL-17A-driven neutrophilia in the lung following infection by *N. brasiliensis*. However, whilst the host Ym1/IL-17A response limits parasite survival in the small intestine, it results in an increased likelihood to develop acute lung pathology [86]. The association of AMCase with allergic airway responses are well defined, where it serves as a biomarker in human asthma [87]. AMCase is also upregulated during nematode infection [84] although their role, until recently, was less understood. AMCase-deficient mice failed to clear infection by *N. brasiliensis*, with reductions in Ym1 and IL-13 expression in the lung and intestine respectively [88]. Furthermore, these mice, although capable of expelling a primary *H. polygyrus* infection, failed to clear a secondary infection, and this was associated with reductions in IL-13, Ym1 and RELMβ and a decrease in mucus production. Another key protein associated with AAM is the resistin-like molecule, RELMα [85], with expression correlating to both nematode infection and IL-4Rα signalling. Previous studies have highlighted a counter regulatory role of RELMα (from both macrophages [89] and eosinophils [90]) in parasite infection. During *S. mansoni* infection, both pulmonary inflammation and parasite expulsion were enhanced in Retnla$^{-/-}$ mice, demonstrating the potential role of RELMα in balancing type-2 immunity. Lastly, the enzyme arginase-1 is another important marker for AAM. Arginase competes with the classically activated macrophage molecule, inducible nitric oxide synthase (iNOS), for a shared arginine substrate, shifting the immune response to prevent inflammation. Arginase-1 has also been proposed to modulate intestinal inflammation during a *S. mansoni* infection by promoting T regulatory cell responses [91]. In the context of *H. polygyrus*, higher

transcript levels of *arg1* were found in more resistant strains of mice during infection [92]. Recent evidence suggests that arginase can facilitate trapping of *H. polygyrus* larvae by antibodies [71], demonstrating a direct inhibitory function of this protein, in addition to balancing type-1/type-2 inflammation.

These studies collectively suggest that macrophage-secreted molecules contribute to type-2 immunity in a context-dependent manner, and this should be noted during future studies into allergies, atopies and parasitic infections. AAMs play a crucial role during helminth infection; enabling parasite expulsion through IL-4Rα-driven 'weep and sweep' responses [59], as well as promoting both innate and Th2 cell responses. AAMs also contribute to host protection, both through the release of molecules that enable tissue repair, as well as facilitating immune regulation through the release of immunomodulatory cytokines and proteins.

1.2.3 Dendritic cells

DCs are professional APCs that can polarize a Th2 response against helminth infection [93], and like macrophages, can often represent a link between innate and adaptive immunity. DCs, in a similar fashion to macrophages and ILC2s, can initiate the response to parasites by sensing epithelial alarmins including IL-25, TSLP and IL-33 [32, 33]. DCs can also recognize a host of PAMPs, such as double-stranded RNAs in SEA via TLR3 [29] and calreticulin from the larval stages of *H. polygyrus* via the scavenger receptor [94]. Additionally, the role of CLRs in in parasite product recognition/uptake by dendritic cells is becoming more established [95]. Specifically,

there is data demonstrating that the SEA antigen, omega-1 [96], and glycans derived from *Fasciola hepatica* ES [97], can bind to mannose-type receptors, leading to their internalisation. Interestingly, the uptake of these glycans may favour the parasite rather than the host, by inducing type-2 polarisation of DCs to limit TLR-driven immunity and inflammatory responses. In addition to innate recognition, dendritic cells also play a crucial role in priming adaptive immunity. Specific depletion of $CD11c^+$ dendritic cells resulted in diminished Th2 effector responses during chronic infection in two helminth mouse models; *S. mansoni* and *H. polygyrus* [98, 99]. Particular transcription factors (TFs) have been highlighted when identifying dendritic cells that promote this type of immunity. For example, IRF4 (interferon-regulatory family member-4) expressing dendritic cells were important for driving Th2 polarization and immunity to *N. brasiliensis* [100, 101]. Furthermore, mice deficient for the TF BATF3 had enhanced type-2 immune responses in helminth infection, with accelerated clearance of *H. polygyrus* infection [102]. Although their function during helminth infection is still to be fully characterized [103], these data give insight into the important role that DCs play in inducing and promoting Th2 immune cell responses.

1.2.4 Acquired immune responses

Acquired immunity to helminths involves a concerted effort of different immune cells. Following their activation by cells in the innate immune compartment, CD4+ Th2 cells play a central function in the adaptive immune response to helminths. During a helminth infection, naïve T cells interact with ILC2s or APCs to recognize

antigen and receive co-stimulation [42, 101]. These cells subsequently receive polarizing IL-4 signals from innate cell sources [45, 49, 60], to differentiate into Th2 cells. Generally, CD4+ T cell responses are considered to rely on parasite-derived antigens to initiate protective adaptive responses [104]. However, there is some data suggesting they can take on a more 'innate' role following helminth infection [105]. This study demonstrated that a secondary challenge, with either a different helminth or an airway allergen, resulted in Th2 effector responses independent of the T cell receptor. Activated Th2 cells release a host of cytokines that have been previously shown to facilitate type-2 immunity, including IL-4, IL-5, IL-9, IL-10 and IL-13 [106]. T cell-derived IL-4 and IL-13 have been shown to mediate B cell maturation and IgE/IgG1 class-switching [107]. This response is of particular relevance to vaccination studies during a model *H. polygyrus* infection, whereby protection against larval challenge is mediated by IgG1 antibodies [108]. T cell-derived IL-4/IL-13 is also shown promote AAM generation and innate immune cell recruitment during nematode infection [54, 109].

T cells can also release the immunosuppressive cytokine, IL-10, in response to repeated skin challenge with *S. mansoni* cercariae, regulating local inflammation and T cell proliferation [110]. Moreover, helminths have been shown to drive the activation of T regulatory cells (T regs) [111]. Specifically, *H. polygyrus* larvae have been shown to drive local T reg expansion in peyer's patches and MLN [112]. These cells limit Th2-mediated immunity and pathology [113], and are shown to correlate with chronicity of parasite infection [111, 114]. Interestingly, dampening the effector immune response will benefit both host and parasite, controlling excessive

inflammation in the host (caused by barrier damage or granuloma formation), but at a cost of suppressing active immunity or killing of the parasite. Incidentally, the immunosuppressive response induced during some STH infections can render the host more susceptible to co-infection [10], or limit the efficacy of particular vaccines [13].

Despite a prominent regulatory environment, the induction of Th2 memory responses was shown to be critical for parasite expulsion during secondary infection [115]. There is also data suggesting that T follicular helper cells act as an alternative source of IL-4 [116], driving the secondary humoral response within lymphoid follicles during nematode infection [117].

B cells can also support Th2-mediated immunity during helminth infection [107]. During *H. polygyrus* infection, B cells promote T cell expansion, maintenance of antibody responses (discussed below) and worm expulsion [107, 118]. B cells have also been shown to support antigen-dependent CD4+ T cell immunity to *N. brasiliensis* infection [119]. A large body of literature supports the capacity of antibody responses to mediate anti-helminth immunity, with elevated levels of IgE or IgG associated with protection in animals [120] and humans [25, 121]. Indeed, it has been shown that polyclonal IgG1 responses, but not IgE or IgA, were essential for parasite expulsion during *H. polygyrus* infection [108]. Functionally, IgE was shown to inhibit migration of *N. brasiliensis* larvae by binding to basophils via the Fcε-receptor [122]. IgE was also shown to promote mast cell recruitment to eliminate *T. spiralis* larvae [123]. The capacity for helminths or helminth-derived antigens to drive strong antibody responses offers an interesting perspective for the development of

future anti-parasite treatments. However, given the variability of protection mediated by B cells during different infections [118], consideration must be given to other effector cells working in tandem with the humoral response to establish anti-helminth immunity.

1.3 Modulation of the host immune system by parasites

The co-evolution of helminth parasites with their hosts has occurred over millions of years [124]. This has required these pathogens to develop a host of molecular adaptations to manipulate, suppress or activate different host cells in order to maximise parasite survival in their chosen niche [125]. Helminths suppress host detection systems that would otherwise initiate a 'danger' response, and then effectively tolerise the immune system to parasite antigens (Figure 1.2). Consequently, helminth-induced immunoregulation can dampen responses to bystander antigens in allergy or autoimmunity [126]. The on-going modulation of the host immune response requires a constant dialogue between the parasite and host, something that can be reversed using anti-helminthic treatments [16-18]. Ivermectin has been shown to inhibit the secretion of immunosuppressive proteins by *B. malayi* [127], whereas other anti-helminthics have also been shown to suppress the parasite-induced T reg response [128]. These therapeutic interventions can thereby improve responses to co-infections like TB and malaria [129, 130], as well as promoting better vaccine efficacy in helminth-endemic regions [131]. However, as repeated anti-

helminthic treatment can lead to drug resistance [22] it is essential that future interventions (such as vaccines) are developed to enable longer-term immunity.

1.3.1 The hygiene hypothesis

The ability of helminths to suppress the host immune system has been widely associated with the 'hygiene hypothesis' (HH) [132]. This was first recognized back in 1968, whereby 'immunological disturbances' generated by multiple parasite infections could lead to the low level prevalence of autoimmunity in tropical Africa [133]. The official proposal of the HH occurred in 1989, following observations that increased microbial exposure in early life could protect children from developing immune hypersensitivities later in life [134]. In the case of helminth infection, the HH refers to the inverse correlation between rates of helminthic burden with the incidence of autoimmune or hypersensitivity disorders, such as Inflammatory Bowel Disease (IBD), allergy, multiple sclerosis and rheumatoid arthritis [132]. Additionally, there is added complexity of the influence of helminth infection on the establishment of the intestinal microbiota, which has subsequent consequences on development of the immune system [135]. A recent study found that helminth infection controlled colonization of particular bacterial species associated with protecting or promoting IBD [136]. This is supported by an earlier study, which demonstrated use of helminth infection as a therapeutic against Idiopathic Chronic Diarrhoea by restoring populations of protective intestinal flora [137]. The relationship between helminths and local microbiota is immensely complex, having effects on host immunity and nutritional status, parasite viability and bacterial colonization. This emerging field of

research will require further study to determine whether this represents a viable alternative for anti-helminthic treatment.

These data have led to the use of helminths, their ova (egg cells) or excretory-secretory products (ES) in the treatment against atopic and autoimmune diseases [138, 139]. Allergic disease has been more difficult to control using helminth-based therapies [140], as overall, studies show that helminth infection could not control asthma in humans. This is surprising given the potency of some helminth-ES products in suppressing airway allergy in mice [141, 142], which suggest that these interventions may improve inflammation-induced pathology. However, human trials may not consider outside factors like time of trial (considering the seasonality of some atopies), or may be due to limitations in dosage of the parasites or their ova. It may also be due to inadequate study design, as recent restructure of a hookworm-based trial for coeliac disease improved clinical outcomes [143]. The immunosuppression that is caused by helminth infection is both incredibly complex and context dependent. As such, isolating helminth products with similar immunomodulatory properties, such as those found in ES, represent a more focused option for treatment.

1.3.2 Evasion strategies

Immune evasion is imperative for parasites to maintain a chronic infection within their host. Some intracellular parasites, such as Leishmania, escape complement-mediated lysis and immune cell recognition through alterations of surface antigens [144]. In parallel, some parasitic nematodes can undergo rapid turnover of their

cuticle, resulting in shedding of surface antigens preventing an antigen-specific immune response [145]. Indeed, the large diversity of surface antigens in parasites could hinder the development of an effective vaccine. Larval life stages of helminths, such as *T. spiralis* and *Echinococcus spp*., occupy physical niches, such as nurse cells and hyatid cysts, to evade immune attack [146]. Interestingly, immune evasion may be stage-specific, requiring adult-stage ES to induce modulation to host responses [147].

1.3.3 Parasite excretory-secretory products

Parasites are able to modulate the host immune response to a remarkable degree [148, 149]. Parasite excretory/secretory (ES) products may play a significant role in immunomodulation, as, for example *S. mansoni* secretes an omega-1 glycoprotein demonstrated in a number of studies to promote Th2-skewing of dendritic cells and T cells during infection [96, 150]. Indeed , a lot of literature points to the immunomodulatory effect of ES on both macrophages and DCs through PRRs [149]. The interactions between innate receptors and parasite products offer a potential target for future intervention, potentially to limit uptake of parasite products or inhibit skewing of host immunity. *H. polygyrus* secretes a functional transforming growth factor-β (TGF-β) homologue to initiate immune regulation [151]. Indeed, extensive data attest to the ability of singular parasite molecules to reduce immune activation [149]. The ES-62 molecule, secreted by *Acanthocheilonema viteae,* is shown to have potent anti-inflammatory properties on mast cells [152] and affects downstream signalling of TLR4 in macrophages, promoting bias towards a M2 phenotype [153]. Another example is the immunomodulatory lipoprotein, Antigen B,

secreted by *Echinococcus granulosus*, which facilitates Th2 polarisation and limits migration of neutrophils to the site of infection [154]. Helminth cystatins are also known to have a range of immunomodulatory functions, limiting both antigen presentation and DC differentiation [155] to induction of IL-10 release [74]. Protozoan parasites similarly secrete a range of immunomodulatory molecules. For example *Trypanosoma cruzi* mucins have been associated with suppression of active T cell immune responses by inducing arrest in the cell cycle [156]. Secreted parasite proteins have also been proposed to be involved in metabolic adaptation to the host environment [157] and tissue invasion where proteases are shown to play a major role [158]. Owing to their importance, we have to consider how a large variety of different parasite-derived molecules are packaged and delivered successfully to the host, evading host immune clearance to carry out their specific functions.

1.4 Extracellular vesicles

1.4.1 Origin and discovery

In mammalian systems, extracellular vesicles (EVs) represent a previously under-appreciated mechanism that allows successful transfer of genetic material, proteins and lipids between cells and tissues. These complex vesicles, which include exosomes, ectosomes and microparticles, communicate by carrying a range of specific molecules and targeting motifs to reach and interact with their cell of interest [159]. Microvesicles can be difficult to distinguish from exosomes, but are generally up to 1 μm in diamteter and bud from the plasma membrane, incorporating certain lipids, surface proteins and other molecules prior to fission [160]. Exosomes are

classified as endocytic vesicles of around 50-100 nm size, which are released from most cell types [159]. In recent years, the literature surrounding EV function has exploded as their ubiquity in many biological and disease contexts has become realized [161]. Historically, these were first identified in reticulocytes as a mechanism to release transferrin receptors during maturation [162, 163]. However, early on it was generally considered that exosomes function as disposal units of cellular waste, transporting obsolete proteins to the lysosomal compartment for degradation [159]. Almost two decades later, exosomes then became of interest to immunologists as EVs released by B cells and DCs contain major histocompatibility complexes and can present antigens [164, 165]. However, following the report that functional messenger RNA (mRNAs) and microRNAs (miRNAs) are transferred between mast cells via exosomes [166], there was further momentum in studying EVs as a mechanism of cell-cell communication.

1.4.2 Biogenesis and cargo selection

Exosome biogenesis is initiated by inward budding of multi-vesicular endosomes or multi-vesicular bodies (MVBs) (Figure 1.3), forming intra-luminal vesicles (ILVs). Upon fusion of the MVB with the plasma membrane and their subsequent release, these vesicles are then known as exosomes [167]). The molecular sorting of the cytosolic contents of the parent cell, and subsequent release, is regulated by intercellular Rab GTPases [168], which enable encapsulation of proteins, lipids and RNAs into ILVs. The specificity of cargo loading has been demonstrated for miRNAs, signalling molecules, tetraspanins, lipids, and others [167]. Consequently, exosomes express markers of their parent cells, and their presence in bodily fluids

make them useful biomarkers revealing the condition of host cells and tissue injury [169] including the diagnosis of some cancers [170]. The presence of many key proteins, as well as RNA species, in secreted vesicles highlights both the complexity and diversity of cargo within exosomes, with a correspondingly wide range of potential interactions within recipient cells [171]. Exosomes are also specifically enriched in other molecules associated with their biogenesis by the endosomal sorting complexes required for transport (ESCRT) pathway, such as Alix, which is used to initiate vesicle formation [172], or tetraspanins such as CD9 or CD63, which support vesicle formation and targeting to recipient cells [173]. The ESCRT pathway, composed of approximately 30 proteins that group into 4 complexes, are recruited into the multi-vesicular endosome upon recognition of ubiquitinated proteins [174], inducing invagination of membrane into vesicle 'buds', and drive vesicle scission (reviewed in [175]). The release of EVs into the extracellular environments, mediated by MVB fusion to the host cell plasma membrane, is poorly understood, although has been associated with fusogenic machinery such as SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) [176], in addition to Rab-GTPases [168].

Exosomes are retained within late endosomes (MVBs) until they are released into the extracellular environment. Cargo selection, MVB maturation and subsequent plasma membrane fusion is an active process, requiring a host of cell factors that are still relatively unknown. Once these limitations are overcome, we can develop new methods to manipulate exosome biogenesis and release, important for the generation of new exosome or EV-based therapeutics and interventions. Adding to this complexity, is establishing the route whereby exosomes are internalised by host

cells. It is routinely suggested that exosomes, and other EVs, are internalised by cellular endocytosis. Of note, a recent study demonstrated the method by which exosomes are said to "surf" on filopodial extensions from the cell prior to internalisation, a method which the authors show is dependent on actin polymerisation [177]. However, other work has shown that EVs may also enter by direct fusion [178], macropinocytosis or indeed phagocytosis (all reviewed in [179]). This is discussed in more detail in Chapter 4.

1.4.3 Functions and therapeutic potential

Extracellular vesicles have been shown to play a variety of functions in immune cell activation and suppression [180] and are also proposed to play a role in disease development and tissue homeostasis [181]. An ever-expanding literature has also demonstrated various roles of EVs in cancer, since tumours also secrete these vesicles with oncogenes [170], including those seen in gastrointestinal stromal tumour cell lines [182]. Exosomes and other EVs are now part of larger clinical initiatives to test their properties in drug delivery, for example, transporting small RNAs and chemotherapeutic agents [183]. As previously mentioned, they have also been hailed for their potential as diagnostic biomarkers [169], and function as modulators in disease, for example, by cell regeneration [184]. Whilst the majority of this work has focused on oncology [185], these vesicles also have exciting implications across a range of infectious diseases. The development of a directed anti-pathogen response by host exosomes has also been explored. Several studies have shown that host exosomes collected from parasite antigen-primed dendritic

cells induce protection from different protozoan infections, including *Toxoplasma gondii* [186] and *Leishmania major* [187].

1.5 Parasite-derived extracellular vesicles

The functional importance of EVs during parasitic infection is becoming greatly appreciated [188], distinguishing their roles in immunomodulation, and transfer of material between parasites or with their host (see Table 1). In this broader setting, exosomes and other microvesicles may not only serve as a functional mechanism used by parasites to evade host responses, but equally host exosomes may be part of an effective anti-parasite strategy (Figure 1.4).

1.5.1 Intracellular protozoan parasites - host manipulation by EVs

Several protozoan parasites have been shown to release exosomes and/or microvesicles including *Leishmania* species [189] and *Trypanosoma cruzi* [190-192], the parasites which cause human leishmaniasis and Chagas disease respectively. Seminal reports showed that promastigote and amastigote forms of *Leishmania donovani* and *Leishmania majo*r can release exosomes that are detected in host cells and selectively induce IL-8 secretion from macrophages [193, 194]. The subsequent chemokinetic recruitment of neutrophils has been proposed as a 'Trojan horse' effect, whereby *Leishmania* can invade these cells and gain access to macrophages upon phagocytosis of the infected neutrophils [193, 195]. *Leishmania* exosomes have

also been shown to induce the release of the immunosuppressive cytokine IL-10, and inhibit the inflammatory cytokine tumour necrosis factor (TNF) in human monocyte-derived dendritic cells in response to interferon γ (IFNγ). Pre-treatment of mice with exosomes derived from *L. major* and *L. donovani* resulted in exacerbated infection and pathogenesis *in vivo,* associated with enhanced IL-10 production and a skewed Th2 response preventing parasite expulsion, as a type-1 response is normally required for clearance [193]. Specific components of *Leishmania* exosome cargo have also been identified and shown to be involved in immunomodulation, including elongation factor 1 alpha (EF-1α) and the membrane-bound metalloprotease GP63 [196]. These have both been associated with a suppression in signalling events during a pro-inflammatory IFNγ response by monocytes (and potentially, subsequent Th1 polarisation) [194, 197]. GP63 is also associated with a number of downstream modulatory effects during *Leishmania* infection, including the modulation of inflammation by activating macrophage protein tyrosine phosphatase (PTP) signalling. This metalloprotease has also been shown to impact protein sorting into the exosomes and to inhibit miRNA processing in host cells by targeting the endoribonuclease DICER [196] [198, 199].

At least two types of EVs have been identified from the infective (metacyclic trypomastigotes) and non-infective (epimastiogotes) forms of *T. cruzi* parasites: both forms release microvesicles from the plasma membrane as well as exosomes presumed to derive from the endocytic pathway [190]. Following initial identification of these EVs [200], they were shown to contain a cohort of proteins associated with immune modulation and virulence and include the homolog to the

multifunctional metalloprotease GP63 [190] described above. Notably, following inoculation of parasite microvesicles and subsequent infection with *T. cruzi*, mice develop heightened cardiac parasitism and increased inflammatory infiltrates associated with higher levels of IL-4 and IL-10 [192]. These cytokines induce the polarization of a Th2 response, as well as lower levels of iNOS in the tissue, suggesting that these microvesicles may serve to promote parasite dissemination and enhance survival. Acid phosphatases involved in adherence and infection of different trypanosome strains have also been shown to be present in the microvesicles [201].

1.5.2 Intracellular protozoan parasites - host manipulation to release EVs

In addition to the direct secretion of exosomes and microvesicles by these parasites, both *Leishmania spp.* and *T. cruzi* induce the release of exosomes from the cells that they infect. A study of *L. mexicana*-treated macrophages *in vitro* showed that exosomes released from infected cells are capable of inducing phosphorylation of signalling proteins and significantly upregulating immune-related genes, including adenosine receptor 2a (Adora2a) on macrophages [197]. Interestingly, Adora2a receptor activation on these cells by *Escherichia coli*, another pathogen which normally drives type 1 immune responses, has been associated with increased IL-10 and down-modulated TNF [202]. Conversely, a recent study suggests that exosomes from *L. amazonensis*-infected macrophages can prime other naïve macrophages to initiate anti-parasitic Th1 responses through the release of inflammatory cytokines IL-

12, IL-1β and TNF [203]. *T. cruzi* also induces the release of microvesicles from infected host cells, including lymphocytes and monocytes *in vitro* and erythrocytes *in vivo*. These microvesicles express surface TGF-β, which has been shown to facilitate eukaryotic cell invasion by the parasite and leads to maturation and continuation of the lifecycle [204]. The microvesicles also protect extracellular life cycle stages of *T. cruzi* (including epimastigotes from the vector and trypomatigotes from ruptured cells) from complement-mediated attack, thus facilitating parasite invasion of host cells [205]. More specifically, monocyte-derived microvesicles develop a complex with the complement C3 convertase C4b2a on the parasite surface, limiting the interaction with its substrate C3. The inhibition of this crucial step prevents complement-mediated lysis, opsonisation and the release of anaphylatoxins, subsequently leading to increased parasite survival [204]. In an analogous manner, erythrocytes infected with the malaria parasite, *Plasmodium falciparum,* produce microvesicles that enhance dose-dependent secretion of proinflammatory cytokines such as IL-1β, IL-6, and IL-12 from monocytes following phagocytosis [206]. In the context of malaria infection, it has been hypothesized that these cytokines may aid endothelial cell activation and erythrocyte sequestration, thereby prolonging infection.

1.5.3 Extracellular parasites – communication within their environment

An obvious function of EVs in extracellular pathogens is their ability to protect secreted cargo and move this into host cells. However mechanistic aspects of

this are not understood, including whether there is specificity in the uptake by certain cell types, whether the parasite cargo is recognized by the host immune system, and how communication is carried out between two phylogenetically distant species. Among extracellular protozoan parasites, comparative analysis of the secretome of *Trypanosoma brucei* subspecies, the causative agent of African sleeping sickness, identified a number of exosome-associated proteins such as enolase, heat-shock protein 70, and the clathrin heavy chain. Different members of the metallopeptidase family are also found in the secreted microvesicles and may serve as potential drug targets or even diagnostic biomarkers during stages of African trypanosomiasis [207]. Complimentary studies on the *T. brucei* secretome also demonstrate the presence of 50-100 nm vesicles budding from the plasma membrane of the infective parasite [208]. The parasitic protozoan *Trichomonas vaginalis,* which can cause infertility through sexual transmission, has been shown to release functional exosomes that can play a role in both parasite-to-parasite, as well as parasite-to-host communication [209]. Virulence proteins, such as metalloproteases, are present within the exosomes that are able to specifically downregulate IL-8 secretion by ectocervical cells (potentially limiting neutrophil migration in order to prevent pathogen clearance).

1.5.4 EVs from microorganisms and ectoparasites - more players at the extracellular surface

Other eukaryotes, such as the pathogenic fungus, *Paracoccidioides brasiliensis,* release highly immunogenic EVs that are detectable in the sera of paracoccidioidomycosis patients [210]. One such immunogenic epitope is the

cellular membrane carbohydrate, galactose-α-1,3-galactose (α-Gal), which is not found in human cells. Although α-Gal enriched EVs may generate a robust immune response in the host, they are suggested to be beneficial to the pathogen, both by binding to host lectins, and potentially stimulating a suppressive type 2 response. This is in accordance with previous literature showing that α-Gal enriched *T. cruzi* exosomes are able to stimulate IL-4/IL-10 expression in cardiac tissue and splenocytes [192]. Indeed, many types of opportunistic fungi including *Cryptococcus neoformans, Candida albicans* and *Histoplasma capsulatum,* release EVs [211, 212], which have been suggested to contain virulence-associated factors including polysaccharides and lipids, reviewed further in [213]. The EVs released by *C. neoformans,* for example, are enriched in virulent capsular components such as glucosylceramide and glucuronoxylomannan (GXM) [214]. Interestingly, a recent study has shown the importance of phospholipid translocases (flippases) in *C. neoformans* exosome packaging and transport, whereby mutant Apt1 flippase-knockout fungi have diminished levels of GXM, and are consequently unable to successfully colonize the lung and brain of infected mice [215]. Furthermore, the yeast *Malassezia sympodialis,* a component of natural human flora, is able to release extracellular vesicles capable of inducing IL-4 and TNF secretion from peripheral blood mononuclear cells, enhancing an inflammatory response in patients afflicted with atoptic dermatitis [216]. Fungal-released EVs may also induce antimicrobial activity by host cells: EVs released by *C. neoformans* are taken up by macrophages *in vitro* and stimulate TNF, IL-10, TGF-β, and nitric oxide production [217].

A recent study in the argasid tick, *Ornithodoros moubata,* suggests that some immunomodulatory proteins may be secreted in arthropod saliva and it is tempting to

speculate that EVs would also be found in this environment. Proteomics of the tick saliva reveal a number of exosome associated proteins e.g. aldolase and enolase, as well as anti-inflammatory lipocalins, which serve as scavengers of leukotrienes, and adenosine nucleotides at the location of the bite [218]. It is clear that we are only at the beginning of many new discoveries concerning extracellular parasites and the functionally diverse EVs they might secrete. There are a growing number of reports containing proteomic matches to exosome proteins in parasite secretomes, and this should cement the idea that these are probably used by most, if not all pathogens at some stage in their life cycle. The effect that parasite-derived EVs may exert at this interface will be of particular importance in the context of anti-parasite treatment. Addtionally, due to the ability of some parasite-derived EVs to suppress an innate immune response [219] these may also be useful tools to ameliorate inflammatory-associated disease [149].

1.5.5 Extracellular pathogens – helminth-derived EVs

Helminth worms are ubiquitous pathogens of plants and animals that have co-evolved with their hosts for hundreds of millions of years, using sophisticated mechanisms for manipulating them [149]. It has only recently been demonstrated that these complex parasites also secrete exosomes, and potentially other classes of EVs, into the environment where they can be internalized by host cells. Marcilla *et al* [220], showed that EVs derived from the trematodes, *Fasciola hepatica* and *Echinostoma caproni*, are detectable on tegumental surface of the parasites. Furthermore, these EVs are internalised by rat intestinal epithelial cells *in vitro*, and

contain homologues of proteins found in mammalian exosomes. Notably, earlier work examining the glycocalyx of *S. mansoni* cercariae demonstrated the potential presence of structures similar to multi-vesicular bodies adjacent to the schistosomula tegument [221]. Furthermore, a recent study has discussed the presence of exosome-like vesicles secreted by *S. japonicum* adults, which were shown to induce macrophage polarisation to a M1 phenotype, thereby highlighting the potential immunomodulatory properties of schistosoma-derived exosomes and their role during infection [222].

A previous report demonstrated that the model free-living nematode *Caenorhabditis elegans* releases peptide-containing exosomes using a defined apical secretion pathway [223] and it is expected that EVs may be used by all nematodes, either as a mechanism of cell-to-cell communication within the organism or, when exported outside of the organism, as a mode of communication with other species. In addition to the above reports, analyses of secretion products from other helminths suggest the presence of exosome-associated proteins, including CD63-like tetraspanins from the cestode *E. granulosus* [224]. As previously mentioned, tetraspanins are a set of membrane-bound proteins which have been implicated in the formation and targeting of exosomes to recipient cells [173]. As these proteins appear to be conserved amongst mammalian and pathogenic EVs, their role in EV function must be investiagted further. Interestingly, tetraspanins have independently been suggested as promising targets for vaccination against another parasite, *Echinococcus multilocularis,* the causative agent of alveolar echinococcosis [173, 225]. *Sotillo et. al.* further reported that adult *S. mansoni* worms release 50-130nm-

sized exosome vesicles, containing over 80 identifiable proteins, 5 of which are tetraspanins and an abundant saposin-like protein [226]. Finally, a recent report showed that the liver fluke *Opisthorchis viverrini*, a trematode prevalent in parts of South-East Asia, releases secretory material contained exosomes with spectrum of associated proteins, including tetraspanins [227]. Most significantly, *O. viverrini* exosomes were found to stimulate cell proliferation in a human cholangiocyte cell line, and also induce the production of the pro-inflammatory cytokine IL-6 in a manner that was partly inhibited by an anti-tetraspanin antibody. This suggests targeting exosomes via their surface proteins may provide an important anti-parasite vaccination strategy.

1.6 EVs in other communication

1.6.1 Host to parasite communication –defence or support?

As parasites have evolved to secrete vesicles that are able to effectively interact with the host, it is only logical that the host would also use this pathway as a defence mechanism. Indeed, during infection with a rodent malaria parasite, *Plasmodium berghei*, plasma cell-derived microvesicles induce CD40 on antigen presenting cells, generating a potent inflammatory response through T cell priming and effector initiation [228]. Subsequently, macrophage activation may be responsible for clearance of the parasite. This is further supported by studies in *Plasmodium vivax* infection in humans, whereby immune cell-derived microvesicles are associated with higher acute inflammation in the pursuit of parasite eradication [229]. These mechanisms can be exploited in a therapeutic context; for example, murine

reticulocytes infected with the non-lethal *Plasmodium yoelii* X strain can significantly attenuate pathogenesis when transferred into mice which are then infected with lethal strain, *P. yoelii XL* [230]. On a separate note, intestinal epithelial cells were shown to increase the release of antimicrobial peptide-containing exosomes in response to *Cryptosporidium* infection, which is driven by enhanced toll-like receptor 4 signalling following recognition of the protozoan parasite [231]. The facultative intracellular bacterium *Mycobacterium tuberculosis* induces exosome release by infected macrophages, which consequently promotes recruitment of lymphocytes through heightened inflammatory chemokine secretion (such as RANTES and MIP-1α) [232]. In addition, exosomes derived from *Mycobacterium bovis*-infected macrophages are able to promote dendritic cell activation, as well as generating an antibacterial T cell response *in vivo* [233].

Host-derived exosomes also play important functions in antigen presentation. Dendritic cells pulsed with *T. gondii* antigens are able to induce both a systemic and local humoral response against the parasite *in vivo*, thereby serving as an efficient vaccine against toxoplasmosis [186, 234]. Similar results are seen in a vaccine trial with *L. major*-challenged DC exosomes, showing that DC-derived exosomes are able to mediate protective Th1 immunity against cutaneous leishmaniasis in a cell-independent manner [187]. Importantly, several studies have emerged using DC-derived exosomes for protection against common livestock parasites. Chickens vaccinated with *Eimeria* parasite antigen-loaded DC exosomes were able to successfully ameliorate symptoms of avian coccidiosis caused by several species,

Eimeria tenella, Eimeria maxima and *Eimeria acervulina,* as well reducing mortality rates [235].

1.6.2 Parasite to parasite communication – crosstalk

In addition to manipulation of the host immune response, EVs can also mediate intercellular communication between parasites. It has been reported that microvesicles traffic between *P. falciparum*-infected erythrocytes enhances the commitment of asexual parasites to the sexual stages (gametocytes) to promote transmission [206, 236]. Furthermore, it is suggested that EVs (described by Regev *et al*. as "exosome-like" [236]) secreted by erytrocytes following infection with transgenic *P. falciparum* parasites can rescue parasitic growth by transferring DNA encoding a drug resistance marker to other *P. falciparum* in infected cells. Thus, genetic material can be transferred between the infected erythrocytes via microvesicles, and this may also contribute to the sexual development mentioned above. This pathway has been shown to be dependent on trafficking mechanisms which transport parasite-encoded proteins to the host-erythrocyte membrane through membranous structures called Maurer's clefts which are found in infected erythrocytes [236].

A further example is provided in the case of the extracellular parasite *Trichomonas vaginalis.* Pre-incubation with exosomes released from a more adherent strain of the parasite, B7RC2, can induce better adherence of weaker strains, such the lab strain G3, in a dose-dependent fashion, which is not seen in the converse scenario [209]. These are some of the few examples to date of vesicle involvement in parasite-

to-parasite communication. This likely represents a bias in the literature, which focuses largely on the immunomodulatory properties of parasite secretion products. In the microbial context, it is well established that secreted vesicles play a role in microbe-microbe communication and genetic exchange, reviewed in [237]. There are still many gaps in our understanding of how different eukaryotic parasites communicate with one another to regulate aspects of their life cycles, including reproduction or commitment to transmission stages. It will be interesting to see if this is a functional niche occupied by EVs that extends beyond protozoan parasites.

1.7 Aims of the thesis

Broadly, this work aimed to characterise extracellular vesicles found in the excretory-secretory products released by adult *H. polygyrus* worms. The field of exosome research is still very much in its infancy, requiring standardization in vesicle detection, identity, and origin. This has been an ongoing process, given the breadth of the field, whereby extracellular vesicles have been shown to be released by virtually all mammalian cell types, and are associated with a number of host diseases. The release of vesicles by an extracellular parasite adds further novelty to this field and in the context of the host-parasite relationship. It was therefore important to isolate *H. polygyrus*-derived exosomes for further study, establishing new methods for their detection, and determining their role in cross-species communication. The mains questions of this study are;

Where do exosomes originate from in H. polygyrus, and how can they be characterised?

There is limited evidence available about helminth-derived exosomes, and nothing is currently known about their biogenesis and origins in nematodes. In this novel biological system, it is necessary to understand the localization and mechanisms of how exosomes are formed, as this may be key to their function. I plan to address this by:

- Analysing the proteomic profile of exosomes – determining enrichment of surface markers to hypothesise origin and unique characteristics compared to total HES.

- Using common methods of extracellular vesicle analysis and optimising their use for parasite-derived exosomes e.g. silver stain, western blot, flow cytometry and qNANO particle analysis.
- Developing tools, e.g. a *H. polygyrus* exosome-specific antibody, to detect exosomes.
- Identifying the vaccine candidates present in exosomes for future therapeutic interventions.

What are the fundamental interactions between H. polygyrus exosomes and host cells? How does this affect host cell immunity?

The dynamics of exosome-cell interactions are poorly understood. As exosomes contain a suite of bioactive molecules, such as small RNAs, lipids and proteins, we must determine how these contents could be delivered into host cells, and whether they mediate any effect. This will be especially interesting given that these exosomes are isolated from *H. polygyrus* ES (HES), which is known to have a broad immunoregulatory functions in the host. It is therefore prudent to define the potential mechanisms of exosome uptake, and how exosomes may function in host cells. This was addressed by:

- Visualising exosome uptake over the course of time and under different stimulatory conditions.
- Attempting to interfere with exosome uptake, and establishing how this may affect the function of exosomes.
- Determining any effects on the host by miRNAs that are enriched in parasite-

derived exosomes.

- Determining the modulatory effects of exosomes on different cells *in vitro*; e.g. epithelial cells and macrophages, during both type 1 and type 2 immune responses (as both would occur during a gastrointestinal helminth infection).
- Examining exosome uptake in the small intestine using a model organoid culture.

Can exosomes be used in a therapeutic capacity during allergic/inflammatory responses in vivo?

Both *H. polygyrus*, and its excretory/secretory product (HES) can create a down-modulated immune environment *in vivo*, suppressing responses to bystander antigens during allergic or autoimmune disease. As exosomes derive from HES, their potential contribution to these responses must be explored. This was addressed by:

- Determining the modulatory effects of exosomes in a model of airway allergy, testing their ability to modulate immunity in a prophylactic manner or following recall responses.
- Analysing the different cell populations and cytokines associated with the alarmin/type-2 response in this model following exosome treatment.

Do exosomes contribute to the establishment of a H. polygyrus infection?

It has been previously demonstrated that vaccination against the ES products of *H. polygyrus* can protect from a subsequent larval challenge, thus demonstrating the

importance of HES in establishing infection. It would be useful to elucidate the role of exosomes during natural infection, and determine whether they are immunogenic. The importance of this can be realised when designing anti-helminthic drugs and future vaccinations. This was addressed by:

- Employing the alum-adjuvant vaccination model, vaccinating mice with exosomes, HES or HES depleted of exosomes before a challenge infection.
- Determining the antibody responses elicited by exosomes, and whether this is specific to these vesicles or shared amongst other components of HES.

Why does H. polygyrus-derived exosomes suppress the IL-33 receptor in mammalian cells? Does this contribute to their functional properties in host cells or during infection?

A secondary unit of work in this thesis focused on the associations between exosomes and the IL-33 receptor (commonly referred to as T1/ST2), which was shown to be suppressed in a number of the models investigated during my PhD. Given the strong association of this receptor with anti-parasitic responses (whereby T1/ST2 knockout mice have differing susceptibilities to helminth infection), the suppression of IL-33R by exosomes was studied in greater detail. This was addressed by:

- Analysing the effects of exosomes on alternative activation of macrophages in the absence of IL-33R.
- Determining the susceptibility of T1/ST2 knockout mice to *H. polygyrus* infection.

- Determining the effects of exosome vaccination in T1/ST2 knockout mice following a larval challenge.
- Establishing whether exosomes can still induce antibody responses in T1/ST2 knockout mice following vaccination.

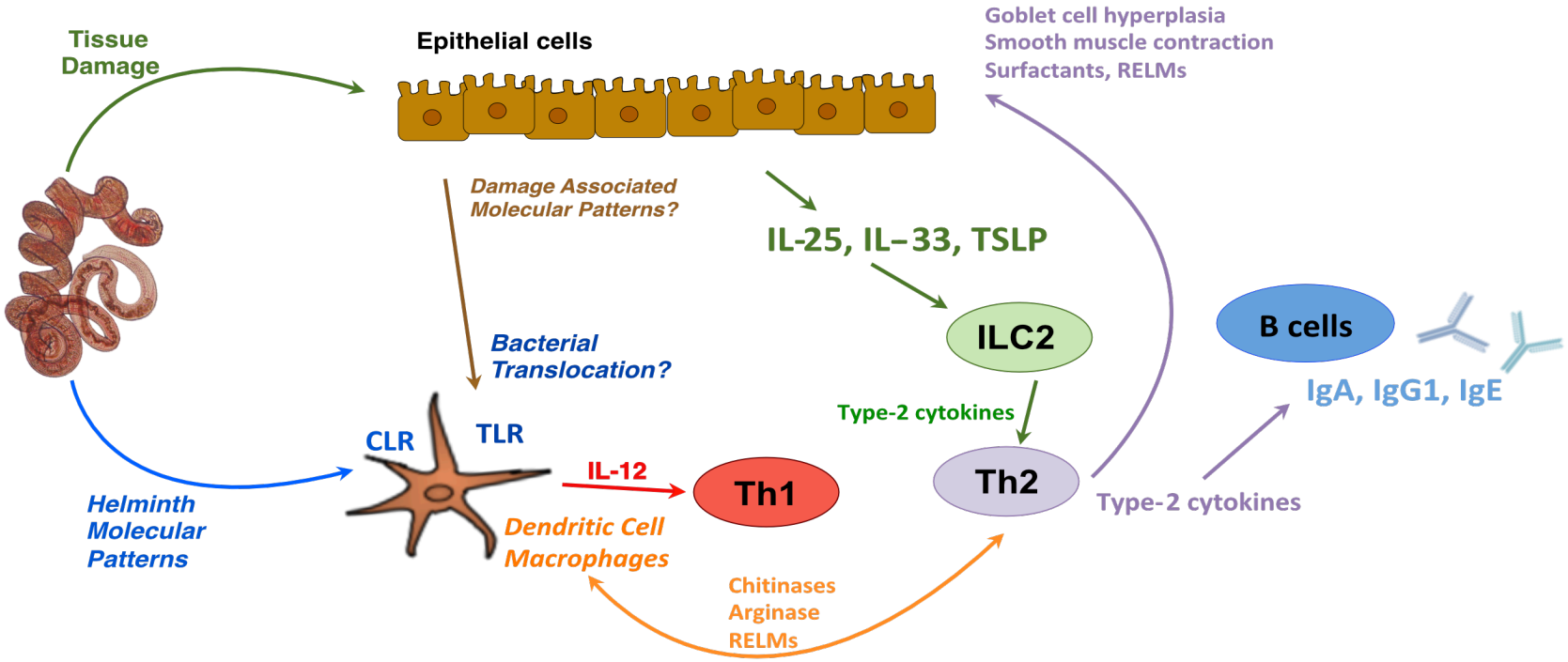


Figure 1.1 Pathogen recognition systems and immunity in helminth infection
Innate mechanisms respond to tissue injury with release of alarmins (e.g. IL-25, IL-33 and TSLP) which can initiate a type 2 response by driving type-2 innate lymphoid cells (ILC2s). Pathogen associated molecular patterns may also be recognised e.g. by Toll Like Receptors (TLRs) or C-type lectin receptors (CLRs), and these molecular patterns may be directly presented by helminths, or indirectly through bacteria translocating through injured epithelium. Antigen Presenting Cells (APCs) release mediators, such as chitnases, RELMs, arginase and cytokines to promote skewing of Th2 cells, and also aim to limit type-2 induced inflammation. ILC2s (and other cells) provide a source of IL-4, in conjunction with antigen presentation by APCs, driving Th2 cell differentiation. The release of type-2 cytokines, such as IL-4, IL-5, IL-9 and IL-13, from ILC2s and Th2 cells, promote an anti-parasite response in the intestine, driving the 'weep and sweep' response through increased mucus production and smooth muscle contractility. Type-2 cytokines also affect B cell responses, driving antibody production and establishing humoral immunity.

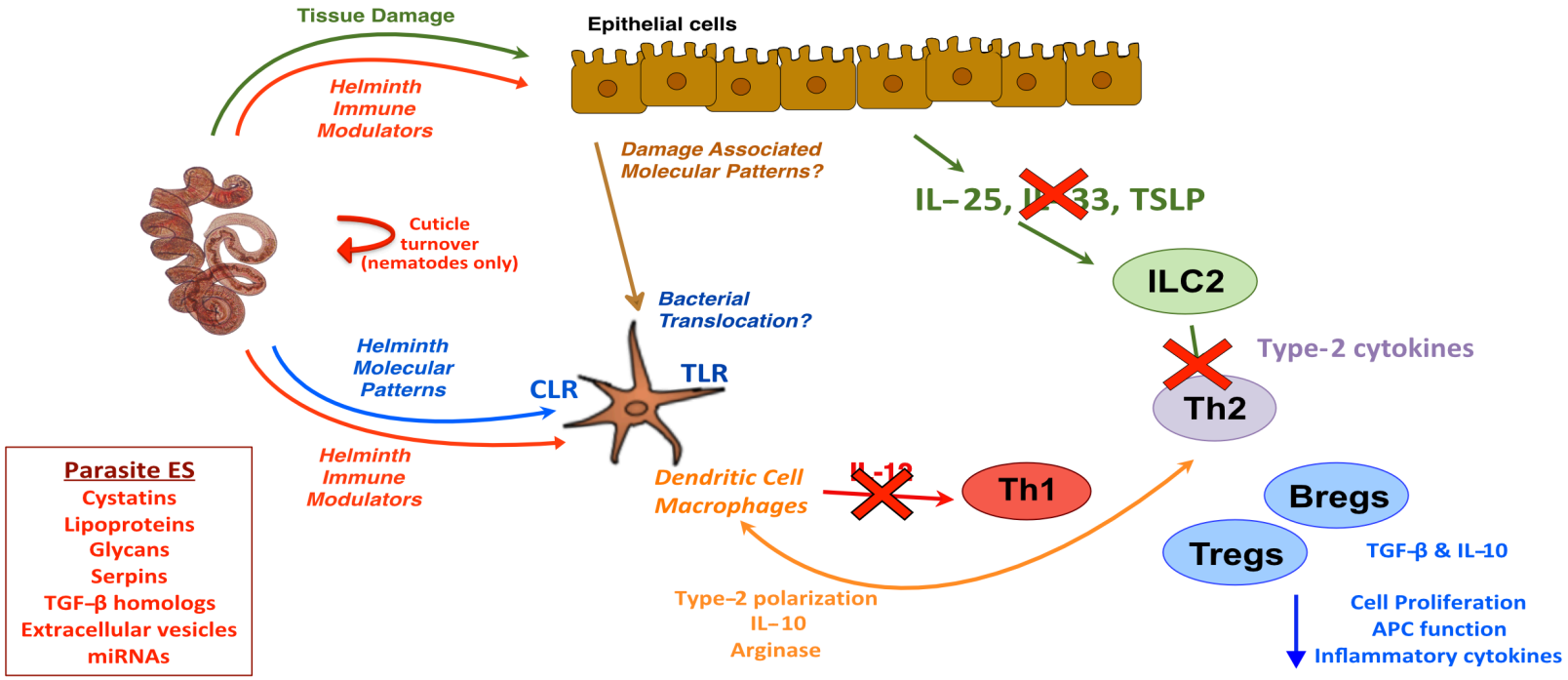


Figure 1.2 Nematode immunomodulation

Helminths, including nematodes, release excretory-secretory products (ES) consists of a large cohort of immunomodulatory molecules which are released in order to evade host immunity. In some cases, nematodes can also undergo cuticle turnover (unlike trematodes or cestodes, which possess a functional membrane known as the tegument), to evade antigen-specific humoral immunity. Innate mechanisms respond to tissue injury with release of alarmins (eg IL-33, TSLP) which can initiate a type 2 response; helminths can block alarmin release or receptors for alarmins such as ST2 (the IL-33R) on ILC2s and epithelium. Pathogen associated molecular patterns may also be recognised by TLRs or CLRs, and these molecular patterns may be directly presented by helminths, or indirectly through bacteria translocating through injured epithelium. In the latter case, the Th1 response driven by IL-12 is blocked by helminth secreted immune modulators, and some components of the immune response, such as TLRs, may be degraded by parasitic serpins. The alternative activation of macrophages induces secretion of tissue healing factors and release of IL-10, aiding barrier repair. Helminth factors in ES also promote APC modulation, which in turn supports the activation of T regulatory cells and B regulatory cells. The release of TGF-β and IL-10 induce immunosuppression in recipient cells, inhibit Th1/Th17 immune responses and limit APC function.

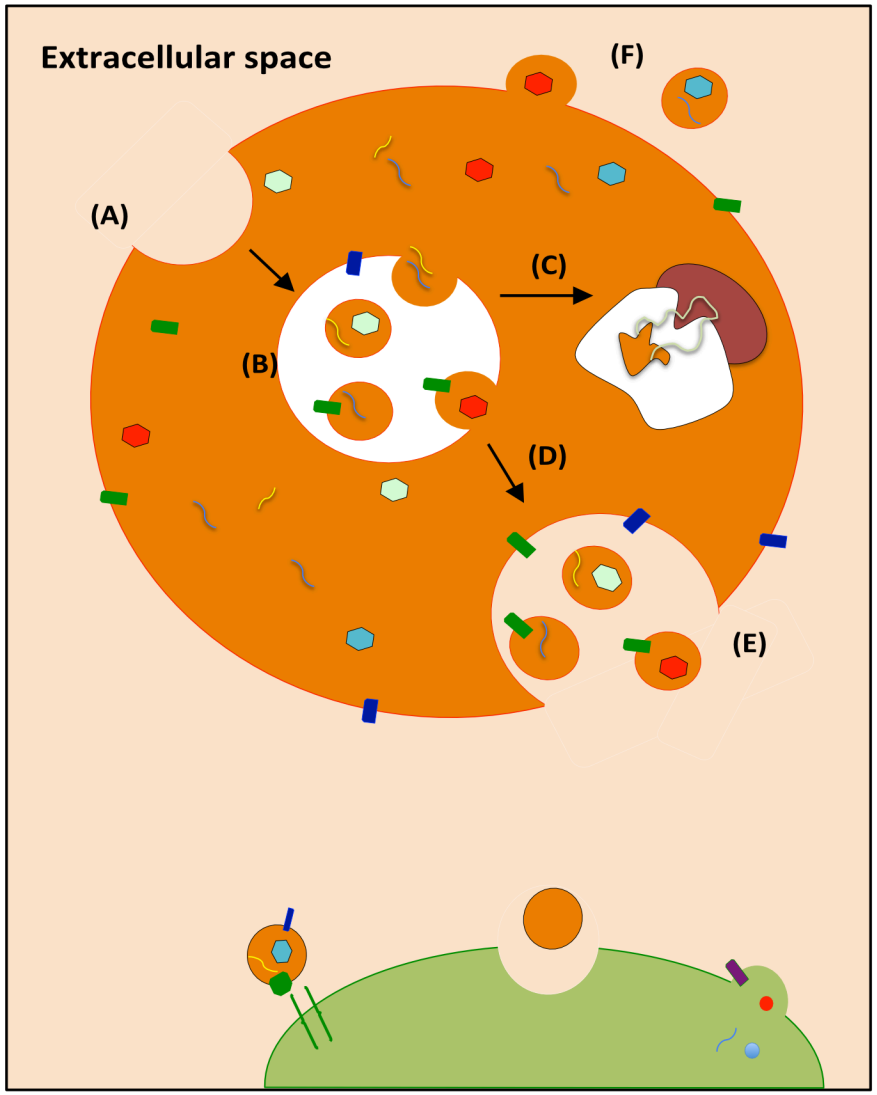


Figure 1.3. The biogenesis and transfer of different extracellular vesicles (EVs)

(A) Endocytosis of the host cell plasma membranes promotes uptake of different bioactive molecules e.g. nucleic acids, proteins, and membrane lipids, from both the extracellular space and the plasma membrane itself. Once inside the cell, the compartment is know as an early endosome. **(B)** Upon differentiation into a late endosome, inward budding allows capture of the host cell cystolic contents, including miRNAs and peptides in intraluminal vesicles (ILVs). The late endosome is also referred to as a multi-vesicular body (MVB). **(C)** Upon signalling, some mature MVBs may fuse with the hydrolytic lysosome, where the vesicle cargo is subsequently degraded. **(D)** MVBs can also fuse directly with the plasma membrane of the host cell, allowing release of their ILVs, now known as exosomes, into the extracellular space **(E)** where they can function upon uptake in recipient cells through receptor-mediated endocytosis, direct fusion or via signalling cascades. **(F)** Other vesicles such as ectosomes are released into the extracellular space following direct budding from the host cell plasma membrane.

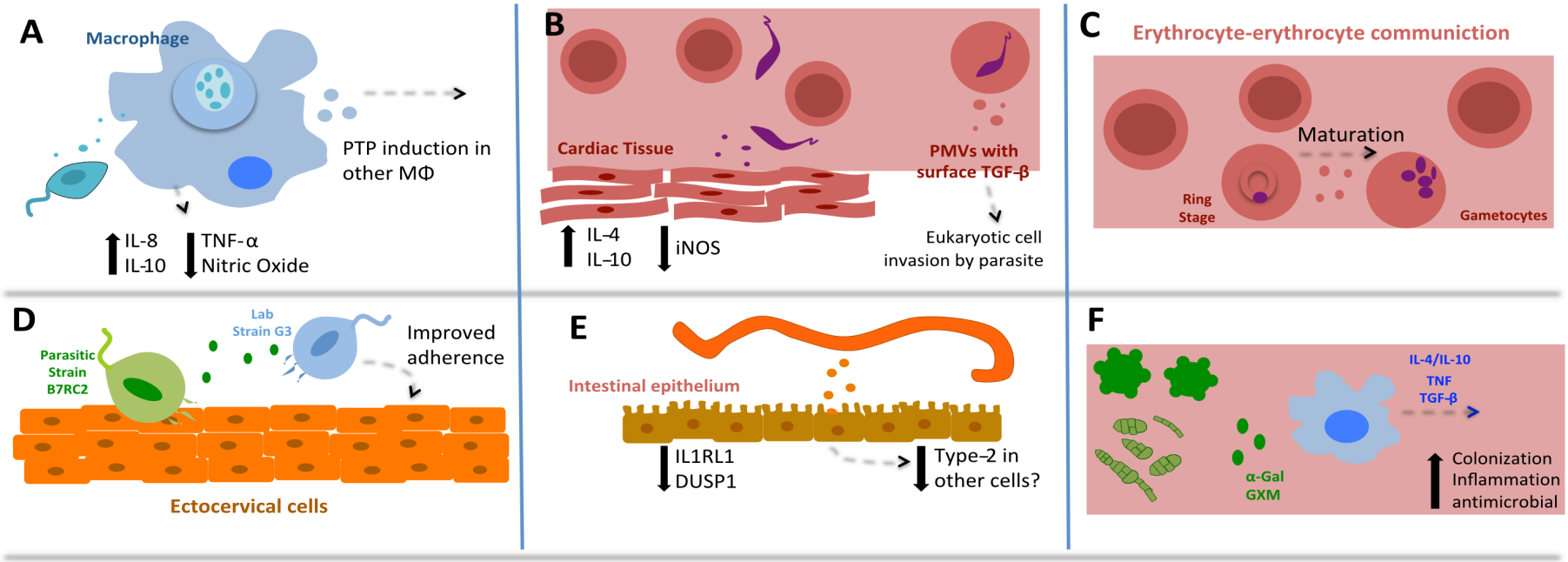


Figure 1.4 Schematic Representation of the Different Functions of Parasite-derived Extracellular Vesicles (EVs)

(A) Leishmania spp. promastigotes release exosomes, which can modulate immune properties of monocytes, shown by an increase in the production of IL-8 and IL-10 and a decrease in tumor necrosis factor (TNF) and nitric oxide . Infected monocytes also release exosomes that have immunomodulatory properties in recipient cells (indicated by broken line), such as the induction of protein tyrosine phosphatases (PTPs) and changes in gene expression. **(B)** Trypanosoma cruzi trypomastigote-shed microvesicles can induce type 2 helper (Th2) polarization [seen by an increase in IL-4 and IL-10 and a decrease in inducible nitric oxide synthase (iNOS)] and invasion of cardiac tissue (indicated by broken line). Infected erythrocytes and lymphocytes release microvesicles containing surface transforming growth factor beta (TGF-b). **(C)** The malaria parasite P. falciparum, induce release of exosome-like vesicles between erythrocytes promoting sexual maturation from blood ring stage to gametocytes (shown by broken line), demonstrated through the transfer of genetic material via EVs. **(D)** The extracellular protozoan Trichomonas vaginalis secretes exosomes, which can promote better adherence of weaker strains to ectocervical cells. **(E)** Adult Heligmosomoides polygyrus worms secrete exosomes as part of their excretory– secretory product in the lumen of the small intestine. These are potentially taken up by intestinal epithelial cells, where they modulate gene expression of the mitogen-activated protein (MAP) kinase regulatory phosphatase gene dusp1 and the IL-33 receptor (ILRL1) and can have downstream suppressive effects on anti-parasite type 2 responses. **(F)** Extracellular fungal micro-organisms, including Paracoccidioides brasiliensis, Candida albicans and Cryptococcus neoformans, secrete immunogenic EVs contains virulence factors like galactose-α-1,3-galactose (α-Gal) and glucuronoxylo- mannan (GXM), promoting colonization and host immunity. Fungal EVs can also stimulate monocyte-derived cells such as macrophages and DCs, to secrete cytokines and promote anti-microbial responses.

Chapter 2: Materials and Methods

2.1 Buffers and Solutions

2.1.1 Protein lysis buffer

1% Nonidet P-40 (Sigma-Aldrich)

50 mM Tris-HCl pH 7.4 (Thermo Fisher Scientific)

150 mM Sodium Chloride (Sigma-Aldrich)

2 mM EDTA (Sigma-Aldrich)

1/100 Protease and Phosphatase inhibitors (Sigma-Aldrich)

2.1.2 Western Blot Buffers

Western Blot 4X Reducing Sample Buffer

NuPAGE® LDS sample buffer (4X) (Invitrogen)

8% β-2-mercapthoethanol (Sigma-Aldrich)

Western Blot Running Buffer

NuPAGE® MOPS SDS Running Buffer 20X for BisTris gels (Invitrogen)

Western Blot Transfer Buffer

NuPAGE® Transfer Buffer 20X for BisTris gels (Invitrogen)

10% Methanol (Thermo Fisher Scientific)

Western Blot Wash Buffer

1X Phosphate Buffered Saline pH 7.2 (Gibco)

0.1% Tween-20 (Scientific Laboratories Supplies)

2.1.3 Flow Cytometry (FACS) Buffer

1X PBS pH 7.2 (Gibco)

0.5% Bovine Serum Albumin (Thermo Fisher Scientific)

2.1.4 ELISA buffers

Carbonate buffer

45 ml of 1M $NaHCO_3$ (Sigma-Aldrich)

18 ml of $NaCO_3$ (Sigma-Aldrich) → made up to 1 L with ddH_20, pH 9.6.

Wash Buffer

1X Phosphate Buffered Saline pH 7.2 (Gibco)

0.05% Tween-20 (Scientific Laboratories Supplies)

2.1.5 Silver stain reagents

Fix – 100 ml ethanol and 25 ml acetic acid → make up to 250 ml with ddH_2O

Sensitisation - 0.5 g Sodium thiosulphate, 17 g sodium acetate and 75 ml ethanol → make up to 250 ml with ddH_2O

Silver stain - 0.1 g silver nitrate in 40 ml ddH_2O

Develop - 2.5 g Sodium carbonate, 100 ml ddH_20 and 20 µl formaldehyde

Stop - EDTA-3.65 g Ns2H20 → make up to 250 ml with ddH_2O

2.1.6 Organoid Immunofluorescent reagents

Glycine/PBS rinse buffer

130 mM NaCl (Sigma-Aldrich)

7 mM Na_2HPO_4 (Sigma-Aldrich)

3.5 mM NaH_2PO_4 (Sigma-Aldrich)

100 mM Glycine (Thermo Fisher Scientific)

Immunofluorescence buffer

130 mM NaCl (Sigma-Aldrich)

7 mM Na_2HPO_4 (Sigma-Aldrich)

3.5 mM NaH_2PO_4 (Sigma-Aldrich)

7.7 mM NaN_3 (Sigma-Aldrich)

0.1% Bovine Serum Albumin (Thermo Fisher Scientific)

0.2% Triton X-100 (Sigma-Aldrich)

0.05% Tween-20 (Scientific Laboratories Supplies)

2.2 Cell isolation and culture

2.2.1 Culture Media

Parasite Media

RPMI 1640 medium (Gibco)

100 U/ml penicillin/100 μg/ml streptomycin (Lonza)

2 mM L- glutamine (Lonza)

5% sterile D-glucose (Sigma-Aldrich)

1 μg/ml of gentamycin (Sigma-Aldrich)

Complete RPMI (used for RAW 264.7 cell line culture [238])

RPMI 1640 medium (Gibco)

10% FCS (Invitrogen)

100 U/ml penicillin/100 μg/ml Streptomycin (Lonza)

2 mM L-glutamine (Lonza)

Complete DMEM

Dulbecco's Modified Eagle Medium (Sigma-Aldrich)

10% FCS (Invitrogen)

100 U/ml penicillin/100 μg/ml Streptomycin (Lonza)

2 mM L-glutamine (Lonza)

(For bone marrow macrophages – add 20% L929 medium – contains M-CSF)

(For bone marrow dendritic cells – add 10% GM-CSF)

(For MODE-K cells [239] – add 1% non-essential amino acids and sodium pyruvate (Gibco))

Complete Media (for lungs)

Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich)

100 U/ml penicillin (Lonza)

1.8 mM $CaCl_2$ (Sigma-Aldrich) and 1 mM $MgCl_2$ (Sigma-Aldrich)

(Digestion media – add 4 U/ml Liberase TL (Roche) and 160 U/ml DNase I (Sigma))

Organoid media

Advanced DMEM/F12 media (Invitrogen)

2 mM L-glutamine (Lonza)

10mM HEPES (Sigma-Aldrich)

100 U/ml penicillin/100 µg/ml Streptomycin (Lonza)

1% N2 supplement (Invitrogen) and 2% B27 supplement (Invitrogen)

50 ng/ml EGF (Invitrogen)

100ng/ml Noggin (Peprotech)

500ng/ml R-spondin (R&D systems)

2.2.2 Isolation of bone marrow for macrophage/dendritic cell culture

Donor mice were sacrificed and the femur and tibia were isolated. Bone marrow cells were extracted in PBS, and red blood cells were lysed by incubating cell suspensions in red blood cell lysis buffer (Sigma-Aldrich) at room temperature for 5 minutes. For macrophage culture bone marrow cells were kept in specific culture conditions (see above), and media changed on day 2, 4 and 6. Macrophages were mature on day 7. For dendritic cell (DC) culture, bone marrow cells were kept in specific culture conditions (see above), and media changed on day 2, 4 and 6 and 8. DCs were mature on day 10. For macrophage or dendritic cell polarization, see table 2.1

2.2.3 Cell stimuli/reagents and restimulation

For antigen-specific restimulation, 2.5 x 10^6 cells were plated in duplicate in a 24-well flat bottom plate (Costar) in complete RPMI medium with or without 1 µg/ml exosomes, or 2 µg/ml anti-CD3/CD28 (eBiosciences) at 37℃ with 5% CO_2 for 72 h. Supernatants were then collected and frozen at -20° C prior to analysis. For

restimulation prior to intracellular cytokine staining, 1-2 x 10^6 cells were plated in a 96-well round bottom plate (Costar) and stimulated with 0.5 μg/ml PMA (Sigma-Aldrich), 1 μg/ml Ionomycin (Sigma-Aldrich) and 10 μg/ml Brefeldin A (Sigma-Aldrich) overnight at 37℃ with 5% CO_2.

Table 2.1 Specific cell culture reagents for *in vitro* assays

Reagent	Supplier	Concentration used at	Function in cell culture assay
LPS	Sigma	100-500 ng/ml	Stimulate classical activation
Recombinant IL-4/IL-13	Peprotech	20 ng/ml	Stimulate alternative activation
Recombinant IL-33	Peprotech	20 ng/ml	Activate ST2 alarmin responses & enhance alternative activation
Cytochalasin D	SLS	4 μg/ml	Inhibit actin polymerisation
Naïve rat IgG	Sigma	1 μg/ml	Fc Receptor block
Polyclonal exosome antisera	In House	1:2000	Binding to exosomal proteins
Polyclonal anti-TSPAN11	Eurogentec	1:2000	Binding to TSPAN11

2.3 Mice, parasites and products

C57BL/6, BALB/c, CF1 (C57BL/6 & CBA background) and IL-33R/$ST2^{-/-}$ (BALB/c background) mice were bred in-house and housed in individually ventilated cages (IVCs) according to UK Home Office guidelines.

2.3.1 *H. polygyrus* infections and lifecycle

Parasite lifecycles were maintained in CF1 mice using 500 *H. polygyrus* L3 stage larvae for infection. Mice were infected with 200 *H. polygyrus* L3 stage larvae in 200 μl ddH_20 by oral gavage as described in [240] for experimental infections. To determine infection status, 3-5 faecal pellets were collected from mice on days 14, 21 and 28. Pellets were weighed and dissolved in 2 ml ddH_20. 2 ml of saturated salt solution (400 g NaCl in 1L $ddH20$) was added and eggs were calculated using a

McMaster egg counting chamber. Egg counts are represented as eggs/g faecal material. Adult worms were harvested from the small intestine on day 28, and recorded as total worms in the intestine. Granuloma counts were conducted following isolation of the small intestine.

2.3.2 HES production and exosome isolation

For collection of HES (H. polygyrus excretory secretory product), adult worms were isolated from the small intestine on day 14, and were kept in serum-free media in vitro and the secretion product is collected every 3 days for 18 days (as per previous publications for HES collection, see [236]). This was amended to 14 days of collection, given the reduced exosome protein yield (as measured by Quibit) in the latter days of collection. Eggs are removed from the collected product by spinning at 400 g before filtering through a 0.2 μm filter (Millipore). Purified media is then spun at 100,000 g for 2 h in polypropylene tubes at 4 °C in a SW40 rotor (Beckman Coulter). Ultracentrifuged material is washed twice in filtered PBS at 100,000 g for 2 h. In some instances, (and this is also routinely done for total HES [236]), the level of LPS contamination in a given exosome batch was quantified using a Chromogenic LAL assay (Limulus Amebocyte Lysate, Lonza) according to the manufacturer's instructions. If LPS levels are <1 U LPS per 1 μg exosomal protein, samples were used for further experimentation (note; all batches tested were signficantly below this limit ~0.15U/μg). The resulting supernatant (and total HES) was concentrated using a Vivaspin 6 5000 MWCO tubes (Fisher) at 5,000 g and washed twice with PBS.

2.4 Exosome quality control

2.4.1 Transmission electron microscopy

For visualization of the vesicles, the purified ultracentrifuged pellet from *H. polygyrus* ES (100 μg/ml protein concentration) was fixed in 2% paraformaldehyde

(PFA), deposited on Formvar-carbon-coated EM grids and treated with glutaraldehyde before treatment with uranyl oxalate and methyl-cellulose

In parasite morphology experiments, adult *H. polygyrus* parasites were washed with PBS before fixation in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer overnight. Parasites were rinsed three times with 0.1 M Na cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1 h. After rinsing in 0.1 M Na cacodylate buffer, they were sequentially dehydrated in a graded acetone series. Finally, samples were sequentially incubated for 30 min in a araldite:acetone solution and filtered in resin and polymerized at 60°C for 24 h. Ultrathin slides (50 nm) were stained with 2% uranil acetate prior to viewing.

2.4.2 Qubit for protein quantitation and qNano analysis

Exosome protein concentration was quantified using the Qubit® Protein Assay Kit (Thermo Fisher Scientific), using the Qubit® 2.0 Fluorometer, according to the manufacturer's instructions. Samples are diluted in supplied buffer with protein reagent, and concentration is measured against BSA standards. Exosomes are quantified by qNano (Izon Science). For exosome samples, a polyurethane nanopore rated for particles <100 nm (NP100-, Izon Science) and was stretched to 47 mm, as measured from adjacent teeth on the qNano unit. 40 µL of sample diluted to an appropriate protein concentration in filtered PBS and vortexed prior to acquisition. The sample was passed through a nanopore by single-molecule electrophoresis, by establishing a voltage (between 0.3V and 0.5V) and suitable pressure (between 6-12 mbar). Optimally, measurement durations were greater than two minutes. All measurements were calibrated with 115 nm (NP100) beads. Data analysis were carried out on the Izon Control Suite software v2.2 (Izon Science).

2.4.3 Silver stain

1. 3 µg exosomes are subjected to SDS-PAGE → Add 150 ml Fixation buffer to gel for 2 h on rocker.

2. Remove Fixation buffer and add 150 ml Sensitisation buffer for 30 min.
3. Wash 3x 5 min with ultrapure water on rocker.
4. Add 150 ml Silver Stain for 20 min → Cover as this is light sensitive.
5. Wash 2x 1 min with ultrapure water on rocker
6. Add 150 ml Developer solution → Monitor visualization of bands
7. Add 150 ml Stop solution when satisfied with band development for 10 min

2.5 Enzyme-linked Immunosorbent Assays

2.5.1 Supernatant quantitation ELISAs (table 2.2)

Supernatants from cell culture assays were measured for cytokine levels by ELISA using monoclonal capture and biotinylated detection antibody pairs (see table 2.2), according to the manufacturer's instructions. Plates were developed with streptavidin-alkaline phosphatase and p-nitrophenyl phosphate substrate (both Sigma-Aldrich). Cytokine concentrations were determined by reference to a standard curve of doubling dilutions of a recombinant cytokine standard. Levels of IL-1α, IL-1β, IL-4, IL-5, IL-10, IL-13, IL-17A and IFNγ were detected in BAL fluid using BD cytometric bead array kits (BD Biosciences), and acquired on a BD FACSArray.

2.5.2 Antibody ELISAs (table 2.2)

In serum antibody ELISAs, whole blood from naïve mice or mice used in vaccination experiments, was collected via cardiac puncture. Blood was clotted for 1 h at room temperature and then spun for 20 min at 12,000 *g* to remove red blood cells. After blocking at 37°C with 10% BSA in carbonate buffer, serum was subsequently added in serial dilutions to ELISA plates coated with either 1 μg/ml HES, exosomes or HES Sup, goat anti-mouse Ig (Southern Biotech) at 1 μg/ml or anti-IgE (clone R35- 72, BD Biosciences) at 1.5 μg/ml, in carbonate buffer.

Antibody binding was detected using HRP-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgA or IgE (Southern Biotech) and ABTS Peroxidase Substrate (KPL), and read at 405 nm.

Table 2.2 Enzyme-linked Immunosorbent Assay reagent list

Cytokine	Capture Antibody	Used at	Top standard	Detection Antibody	Used at	Source
IL-6	MP5-20F3	2 µg/ml	25 ng/ml	MP532C11	0.5 µg/ml	BD Biosciences
IL-10	JES5-16E3	0.5 µg/ml	10 ng/ml	SXC-1	0.5 µg/ml	eBioscience
IL-12p40	C15.6	2 µg/ml	50 ng/ml	554476	0.5 µg/ml	BD Biosciences
IL-13	eBio13A	4 µg/ml	10 ng/ml	eBio1316H	0.5 µg/ml	eBioscience
CCL17	DY529	2 µg/ml	2 ng/ml	DY529	0.2 µg/ml	R&D Duoset
IFNγ	R46A2	0.5 µg/ml	50 ng/ml	XMG1.2	2 µg/ml	eBioscience
RELMα	500-P214	0.25 µg/ml	100 ng/ml	P214Bt	0.25 µg/ml	Peprotech
TNF	TN3-19	2 µg/ml	20 ng/ml	13-7341-85	2 µg/ml	eBioscience
Ym1	DY2446-05	2.88 µg/ml	10 ng/ml	DY2446-05	0.14 µg/ml	R&D Duoset
Detection Substrate		Extravidin-AP			**Source**	Sigma

Cytokine	Capture Antibody	Used at	Top standard	Detection Antibody	Used at	Source
IgA	C10-3	1:300	0.5 µg/ml	1040-05	1:4000	BD/ Southern Biotech
IgE	R35-72	1:300	0.5 µg/ml	1110-05	1:4000	BD / Southern Biotech
IgG1	Polyclonal - 101004	1:1000	0.5 µg/ml	1070-05	1:6000	Bio-Rad/ Southern Biotech
IgG2a	Polyclonal - 101004	1:1000	0.5 µg/ml	1080-50	1:4000	Bio-Rad/ Southern Biotech
IgM	Polyclonal - 101004	1:1000	0.5 µg/ml	1020-05	1:2000	Bio-Rad/ Southern Biotech
Detection Substrate		ABTS-Peroxidase			**Source**	KPL

2.6 Fluorescence Activated Cell Sorting

2.6.1 Surface staining

Single-cell suspensions were made from bone marrow myeloid populations,

mesenteric lymph nodes, peritoneal washes or the spleen, and were subsequently washed in phosphate-buffered saline (PBS). To exclude dead cells, cells were stained with live/dead Fixable Yellow or Aqua dyes (Invitrogen) at a 1/10000 dilution in 100 μl PBS for 15 m at 4°C. Subsequently, Fc receptors were blocked 50 μl of FACS buffer containing 100 μg/ml of naïve rat IgG (Sigma Aldrich) for 15 min at 4°C. Samples were then washed several times in FACS buffer and surface stained in 20 μl of FACS buffer containing different cocktails of antibodies (See Table 2.3), or with appropriate isotype controls (as recommended by the manufacturer for each individual antibody). Samples were acquired on a Becton-Dickinson LSRII flow cytometer (BD Biosciences).

2.6.2 Intracellular staining

To measure intracellular cytokines, cell suspensions were stimulated for 4 h at 37°C in the presence of PMA (50 ng/ml), Ionomycin (1 μg/ml), and Brefeldin A (10 μg/ml) (Sigma). Following any cell surface staining, cells were permeabilised for 30 min at 4°C in Cytofix/Cytoperm solution (BD), and then washed twice in 200 μl of Perm/Wash (BD). Cells were stained for intracellular cytokine expression (For antibodies, see Table 2.3), or with appropriate isotype controls in 20 μl perm/wash. For intracellular cytokines; Foxp3, IL-5 and IL-13, corresponding isotype controls were used (Rat IgG2a-κ Control or IgG1-κ Control respectively). For macrophage-derived RELMα and Ym1, corresponding isotype antibodies were used (Normal Goat IgG control (R&D) and Normal Goat IgG-biotin (R&D), respectively). After staining, cells were washed twice in 200 μl of FACS buffer before acquisition on the Becton-Dickinson LSR II flow cytometer (BD Biosciences).

Table 2.3 List of antibodies for flow cytometry

Antibody target	Clone	Fluorophore	Conc. used	Source
Lineage markers				
CD3	17A2	FITC	1/100	BioLegend
CD4	RM4-5	FITC	1/100	BioLegend
CD5	53-7.3	FITC	1/100	BioLegend
CD8α	53-6.7	FITC	1/100	BioLegend
CD11b	M1/70	FITC	1/100	BioLegend
CD11c	N418	FITC	1/100	BioLegend
CD19	6D5	FITC	1/100	BioLegend
Gr1	RB6-8C5	FITC	1/100	BioLegend
CD49b	DX5	FITC	1/100	eBioscience
Foxp3	NRRF-30	PE	1/50	eBioscience
Innate lymphoid cells				
Lineage negative	See above			
ICOS	15F9	PerCP-710	1/100	eBioscience
ST2	DJ8	biotin	1/100	MD bioproducts
IL-5	TRFK5	PE	1/50	eBioscience
IL-13	eBio13A	eF660	1/50	eBioscience
Myeloid cells				
F4/80	BM8	PeCy7	1/300	BioLegend
CD11b	M1/70	Pacific Blue	1/100	BioLegend
CD11c	N418	APC	1/200	BioLegend
ST2	DJ8	FITC	1/100	MD bioproducts
MHC II (I-A/I-E)	M5/114.15.2	AlexaFluor 700	1/400	eBioscience
CD86 (B7-2)	GL1	PE	1/300	eBioscience
Relm-α	226033 (R&D) and rabbit IgG-AF647 labelling reagent kit (Invitrogen)			
Ym1	Biotinylated goat α-mouse Chitinase 3-like 3 (R&D) and Streptavidin-PeCy7 (BioLegend)			
Other lymphocytes/immune cells* Excluding lineage markers or previous				
CD4	RM4-5	AF700	1/100	BioLegend
CD3	17A2	FITC	1/100	BioLegend
CD25	PC61.5	PE	1/100	eBioscience
SiglecF	E50-2440	PE	1/100	BD Biosciences
B220	RA3-6B2	PCP	1/100	BioLegend
CD45.2	104	Pacific Blue	1/100	BioLegend
Streptavidin		APC/PerCP	1/200	BD Biosciences

Table 2.4 List of antibodies for exosome flow cytometry and western blot

Specificity	Clone	Host	Dilution (WB)	Dilution (FACS)	Source
Whole *H. polygyrus* exosomes	N/A	Rat	1:2000	1:100	In House
Whole *H. polygyrus* exosomes	N/A	Mouse	1:2000	1:100	In House
TSPAN 11 - variant 1	SY6093	Rabbit	1:1000	1:100	Eurogentec
ALIX	3A9	Mouse	1:1000	1:200	Cell Signalling
DUSP1	C-19	Rabbit	1:1000	N/A	Santa Cruz
ST2	P14719	Goat	1:1000	N/A	R&D Systems
β-Actin	3-A9	Rabbit	1:3000	N/A	Cell Signalling
HRP-conjugated antibodies					
Rabbit IgG		Goat	1:3000	N/A	Cell Signalling
Mouse IgG		Goat	1:3000	N/A	Cell Signalling
Rat IgG		Goat	1:3000	N/A	DAKO
Fluorescent antibodies					
Rabbit IgG-DyLight 800		Goat	1:10,000	N/A	Life Technologies
Mouse IgG-AlexaFluor 680		Goat	1:10,000	N/A	Life Technologies
Rat IgG - AlexaFluor 680		Goat	1:10,000	N/A	Life Technologies
Rabbit IgG-Alexa Fluor 594		Goat	N/A	1:3000	Thermo Fisher Scientific
Mouse IgG-AlexaFluor 488		Goat	N/A	1:3000	Thermo Fisher Scientific
Rat IgG-AlexaFluor 647		Goat	N/A	1:3000	Thermo Fisher Scientific

2.6.3 Exosome-Latex bead detection

5 μg of exosomes were conjugated overnight to aldehyde-sulphate latex beads in PBS (4 μm, 4.2 g/100 ml, Invitrogen), washed in PBS with 0.5% BSA, and incubated with primary antibodies (see Table 2.4 for list) or naïve sera for 30 min. After several washes, a secondary antibody conjugated to a fluorophore (Table 2.4), is added prior to acquisition on the MacsQUANT (BD Biosciences).

NB: all flow cytometry data was analysed using FlowJo software (TreeStar).

2.7 Western blot

2.7.1 Protein lysis, quantitation and sample preparation

BM-derived macrophages or MODE-k cells (1×10^6) were stimulated as indicated and washed with PBS on ice. For whole-cell lysates, cells were lysed in lysis buffer (described in 2.1.1), for 30 min on ice, spun at 12,000 *g* for 5 min at 4°C. The post-nuclear supernatants were transferred to 1.5 ml eppendorf tubes and, if not used immediately, were stored at -80°C.

Total protein was determined by the BSA quantification method (Pierce). In other experiments testing the affinity of exosome antisera, 5 µg of whole exosomes, HES or HES depleted of exosomes (HES Supernatant) were used (protein concentration assessed by Qubit quantification – described in 2.4.2).

5 µl of 4X reducing sample buffer (2.1.2) were added to samples (made up to 20 µl with ddH_20) and mixture was boiled for 5 min at 95°C on a heat block. The samples were briefly centrifuged prior to gel loading.

2.7.2 Running and transfer protocol

For 1-dimensional gels, the protein ladder (PageRuler Pre-stained Protein Ladder, 10 to 180 kDa, Thermo Fisher Scientific) and samples were loaded and run down a well of a 15-well NuPAGE 4-12% BisTris gel (Life Technologies), in 1x MES buffer, at 200 V for 50 min at room temperature with constant stirring.

For 2-dimensional gels, exosomes (2 µg) were diluted into a final volume of 125 µl with rehydration solution (7M urea, 2M thiourea (both BDH), 65 mM DTE (Fluka), 4% CHAPS (Sigma-Aldrich), 0.8% resolytes (GE Healthcare), trace bromophenol blue (Sigma-Aldrich), and used to rehydrate 7 cm pH 3–10 IPG strips (GE Healthcare) for 14 h at 20 °C. Isoelectric focusing was performed (500 V for 30 min; 1000 V for 30 min; gradient to 8000 V for 5 h; total ~20 kV h) using an IPGphor (Pharmaca Biotech). Strips were reduced and alkylated in 20mg DTE and 80 mg idoacetamide, before second dimensional electrophoresis using NuPAGE 4–12% BisTris gels and NuPAGE MES SDS running buffer for 2 h at 150 V.

Sample proteins on the gel were transferred to 0.45 μm nitrocellulose membranes (BioRad) or PVDF membranes (Millipore), which were first activated by soaking in 100% ice-cold methanol for 30 s, using 1x NuPAGE transfer buffer (Life Technologies) for 90 min at 120 V in a wet transfer apparatus (BioRad).

Blots were blocked in 2% BSA–TBS with 0.05% Tween 20 (or 5% milk in TBSt) for 2 h at RT, before primary antibody probe overnight at 4 °C (see 2.4 for antibodies). In some instances, membranes were incubated for 1 h in secondary Ab, (HRP-conjugated, see table 2.4) with 2% BSA-TBSt, in conjunction with Amersham ECL prime Western blotting detection reagent (GE Healthcare) to visualize bands using X-ray film (Kodak). Using the Licor-based fluorescence detection system, membranes were incubated with fluorophore-conjugated antibodies (table 2.4) in 5% milk-TBSt for 45 min and acquired in PBS using the Odyssey Scanner (Li-Cor).

2.8 Molecular biology techniques

2.8.1 Reverse transfection of MODE-k cell line

To prepare the transfection reaction; Lipofectamine 2000 was prepared with opti-MEM (both Thermo Fisher Scientific) to a final reaction concentration of 0.3% and incubated for 5 min at room temperature. To prepare RNA complexes, DUSP1 siRNA (small interfering RNA), a mix of synthetic *H. polygyrus* miRNAs (Let-7, miR71, miR200 and miR425, Thermo Fisher Scientific) or a non-gene targeting control, Select Negative Control No.1 siRNA (NT1) (Ambion), were prepared with opti-MEM media to a final reaction concentration of 25nM. Equal volumes of the two mixtures were added together and incubated for 20 min at room temperature. The mixture was then aliquoted into wells and 1.5 x 10^4 MODE-k epithelial cells were seeded in a 96-well plate in complete DMEM (antibiotic-free). After 24 h, samples were assayed for transcriptional changes in *il1rl1* and *dusp1* by qRT-PCR and/or western blot.

2.8.2 Isolation and quantitation of RNA

(1) A small piece of tissue (for Alternaria experiments – trachea and lung) were placed in 1ml of TRIzol reagent (Invitrogen). Tissue was first homogenized using a steel bead and TissueLyser II machine (both Qiagen) for 2 min at 20Hz. Samples were then centrifuged at 10000 *g* for 5 min at 4^0C to remove debris and the resulting supernatants moved into fresh eppendorf tubes. (2) For cultured cells (commonly at a density of 1 $x10^6$ /ml in a 24-well plate), plates were washed twice in PBS before addition of 0.5ml-1ml TRIzol. To allow for nucleoprotein dissociation, cells were incubated for 5 min at room temperature before lysed RNA were aspirated and placed into eppendorf tubes.

Chloroform was added (200 µl/1 ml TRIzol) and samples were inverted continuously for 1 min. After a further 3 min incubation at room temperature, samples were centrifuged at 12,000 *g* at 4°C for 15 min. The clear aqueous phase (harbouring total RNA) was carefully recovered and transferred to a new 1.5 ml eppendorf tube, avoiding any aspiration of the white interphase (containing precipitated DNA), and the organic phase (containing cellular debris). Isopropanol (500 µl/1 ml TRIzol) and 0.5 µl of 15 mg/ml glycogen blue for visualization of RNA (Ambion) were added and left for 10 min at room temperature before centrifugation at 12,000 *g* for 20 min at 4°C. The resulting supernatant was removed and the RNA pellet was washed twice in 75% pre-chilled ethanol and air-dried before being dissolved in 30-50 µl RNase/DNase free H_2O (Gibco) with 0.1 mM EDTA (Promega) and stored at -20°C. The concentration and degree of contamination of the acquired RNA samples was assessed on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The level of UV absorption was measured at wavelengths of 230nm, 260nm and 280 nm. 1.2 µl of purified total RNA sample was used for spectrophotometry, with 1.2 µl RNase/DNase free H_2O/0.1 mM EDTA used to calibrate background. Optimally, RNA samples had a A_{260}:A_{280} ratio of > 1.7 and a A_{260}:A_{230} ratio of > 1.4.

2.8.3 Generation of cDNA and gene detection by quantitative PCR

A fixed amount of 200 ng of extracted RNA was used for the reverse transcription of

cellular RNA to cDNA. Reverse transcription reactions were performed using miScript System kit II (Qiagen) containing 1 µl of Reverse Transcriptase Mix, 2 µl of 5x HiFlex buffer, 1 µl of 10x Nucleics mix and appropriate amounts of RNase-free water and RNA in a total volume of 10 µl. Samples were incubated for 60 min at 37ºC followed by 5 min at 95ºC and were stored at −20°C.

Table 2.5 List of primer sets used in quantitative PCR

Primer	Target mRNA sequence	5'- 3' sequence
Arg-1 FWD	Arginase	GTC TGT GGG GAA AGC CAA T
Arg-1 REV		GCT TCC AAC TGC CAG ACT GT
Dusp1 FWD	Dual specificity phosphatase-1	CTC CTG GTT CAA CGA GGC TAT T
Dusp1 REV		TGC CGG CCT GGC AAT
FAS v1 FWD	Death receptor (variant 1)	AAA CCA GAC TTC TAC TGC GAT TCT
FAS v1 REV		GGG TTC CAT GTT CAC ACG A
GAPDH FWD	Housekeeping gene	CAT GGC CTT CCG TGT TCC TA
GAPDH REV		GCG GCA CGT CAG ATC CA
IL1R1 v2 FWD	IL1 receptor-like 1 (variant 2)	CCT CAC GGC TCT GAG CTT AT
IL1R1 v2 REV		CTG AGG TAG GGT CCA GAA GAG A
IL6 FWD	IL6 cytokine	TGC CTT CAT TTA TCC CTT TGA A
IL6 REV		TTA CTA CAT TCA GCC AAA AAG CAC
iNOS FWD	Nitric oxide synthase	CAG CTG GGC TGT ACA AAC CTT
iNOS REV		CAT TGG AAG TGA AGC GTT TCG
LTβ FWD	Lymphotoxin-β	CCT GGT GAC CCT GTT GTT G
LTβ REV		TGC TCC TGA GCC AAT GAT CT
RELMα FWD	Resistin-like molecule-α	TAT GAA CAG ATG GGC CTC CT
RELMα REV		GGC AGT TGC AAG TAT CTC CAC
TNF FWD	Tumour necrosis factor-α	GGA AAT AGC TCC CAG AAA AGC AAG
TNF REV		TAG CAA ATC GGC TGA CGG TGT G
Ym1 FWD	Murine chitinase	CAT GAG CAA GAC TTG CGT GAC
Ym1 REV		GGT CCA AAC TTC CAT CCT CCA

Real-time SYBR-green PCR assays for mRNA detection were performed using Light Cycler System in 384-Well Reaction Plates (both Roche). Primers (Invitrogen) were designed using the Universal Probe Library Assay Design Center Roche software (available at http://www.roche-applied-science.com), see table 2.5 for primer information. Reactions were performed using SYBR Green System (Roche) according to the manufacturer's instructions. 0.5 µl of cDNA (1:10 dilution)

were used per sample in a total reaction volume of 5 µl. The cycle conditions used were: 5 min pre-denaturation at 95°C, then 45 cycles of denaturation 10 s at 95°C, annealing 10 s at 60°C, 10 s elongation at 72°C. All samples were tested in technical duplicates and nuclease-free water was used as a non-template control.

2.8.4 Immunoprecipitation with exosome anti-sera

HES exosome proteins were immunoprecipitated with polyclonal anti-exosome sera using a Pierce Cross-link Immunoprecipitation kit (Thermo Fisher Scientific), according to manufacturer's instructions. Briefly, antibodies were coupled to Protein A/G agarose resin and cross-linked using 2.5 mM DSS/DMSO. Exosome antigens were immunoprecipitated to the antibody-crosslinked resin overnight. Bound proteins were eluted with a provided buffer and neutralized by 1 M Tris, pH 9.5. Silver stains were run from several stages of the protocol to assess adequate antibody-agarose binding, ensure antibody cross-linking and analyse eluate for presence of antigen. Following rigorous optimisation, modifications included;

- Used 10 µg of antibodies to 40 µl of Protein A/G agarose resin (changed from 1:2 ratio to 1:4)
- Used 60-80 µg of exosome protein for immunoprecipitation (~7x less than recommended amount due to limited resources)

2.8.5 Microarray and Mass spectrometry

For detailed methodology/author contributions of mass spectometry analysis (compiled by Thierry Le Bihan, University of Edinburgh) and microarray data with subsequent target prediction (analysed by Cei Abreu-Goodger, Laboratorio Nacional de Genómica para la Biodiversidad) as shown in Tables 3.2.1A, 3.2.1B and 4.2.4A respectively, see [216].

In brief, for analysis of broad gene changes in murine cells that could be induced by exosomes, MODE-k cells were seeded for 24 h at a density of 20 x 104 per well (in a

24-well plate). The following day the cells were incubated with 5 μg of exosomes for 20 h, before harvest of RNA for extraction prior to preparation for microarray analysis and parasite miRNA target prediction for the 3' UTRs of murine *dusp1* an *il1rl1*. The data generated allowed further investigations (shown in Chapter 4).

The mass spectometry analysis of whole exosome pellet, as in [216], was carried out using 5 μg of exosome protein. For the mass spectometry analysis of exosome proteins isolated by cross-link immunoprecipitation, approximately 50 μg of exosome protein was used, and analysed similarly to the whole-exosome protein analysis as above. Briefly, MS/MS data were searched against the in-house H. polygyrus transcriptome database using the Mascot program (Matrix Science Ltd). For any given protein, a minimum of 2 unique peptide matches were required. Peptide matches with expect values < 0.05 at a Mowse significance threshold of $p < 0.05$ were considered significant.

2.9 Exosome uptake assays

2.9.1 Sample preparation and PKH67

Exosomes purified from *H. polygyrus* adult culture or MODE-K cells, were labelled with PKH67 (Sigma-Aldrich) for 5 min at room temperature, according to the manufacturer's instructions. Approximately 2 μg of dye was used per 5 μg of measured exosome protein. The staining reaction was stopped by adding an equal amount of 1% purified bovine serum albumin (BSA). Subsequently, exosomes were washed in PBS by ultracentrifugation (1 h at 100,000 *g*). A control probe solution was prepared with the PKH67 label in PBS in the absence of exosomes. Cells (BMDMs, MODE-k and RAW 264.7 cells) were given approximately 1-5 μg exosomes per 2 $x10^5$ cells and left in culture for varying time points at 37°C. Cells were then washed twice in PBS before staining with antibodies for determining cells by flow cytometry or confocal analysis. Control samples were treated similarly

before the addition of 50 µl 0.25% trypsin/EDTA (Gibco) for 5 min in order to remove exosomes which may have remained on the cell surface prior to analysis.

2.9.2 Assessment of exosome uptake by flow cytometry and confocal microscopy

For acquisition by flow cytometry, labelled exosomes were incubated with cells for varying time points (as indicated per figure legend), and single-cell suspensions were made from cell populations for subsequent washes in FACS buffer. Live/dead Fixable Aqua dye (Invitrogen) was used to exclude dead cells. BMDMs, BMDCs (using markers indicated in table 2.3), and live singlet populations of MODE-k and RAW 264.7 cells were assessed as % of PKH67+ cells or by the expression intensity of PKH67 following acquisition on the BD FACS Canto II flow cytometer (BD Biosciences). Approximately 50-100,000 events were collected from each data set and analysed using FlowJo software (Tree Star)

For confocal analyses, 3 x 10^4 BMDMs were allowed to attach on to the coverslips overnight and the following day shifted to complete DMEM medium supplemented with 1% L-glutamine. Cells were incubated with labelled exosomes or controls, as described above, before subsequent staining in 1:300 F4/80-AF647 (Table 2.6) in PBS (to determine macrophage identity). Cells were then fixed with 4% PFA (with residual PFA quenched with 50 mM glycine). The coverslips were washed 4x in PBS, and nuclei were stained with 4',6-diamidino-2-phenylindole-supplemented ProLong Fade Gold (Invitrogen) mounting media. Samples were examined on the Leica SP5 II (Leica Microsystems, ×63 objective) using the LAS AP software (Leica). Image analysis was performed using ImageJ software (NIH). In some experiments, cells were treated with culture stimuli in addition to exosomes (as indicated in appropriate figure legends), including LPS, IL-4/IL-13, polyclonal antibodies and cytochalasin D.

Table 2.6 List of antibodies for confocal microscopy

Specificity	Fluorophore	Clone	Host	Dilution	Source
Primary antibodies					
F4/80 (macrophages)	AlexaFluor-647	T45-2342	Rat	1:300	BD Biosciences
E-cadherin (epithelium)	N/A	DECMA-1	Rat	1:2000	Genetex
MUC2C3 (goblet cells)	N/A	N/A	Rabbit	1:500	G. Hannson [241]
Lyzozyme C (paneth cells)	N/A	C-19	Goat	1:100	Santa Cruz
Secondary antibodies					
Anti-Rat IgG	AlexaFluor-594	N/A	Goat	1:500	Life Technologies
Anti-Goat IgG	AlexaFluor-647	N/A	Rabbit	1:500	Life Technologies
Anti-Rabbit IgG	AlexaFluor-488	N/A	Goat	1:500	Life Technologies

2.10 Organoid studies

2.10.1 Small Intestinal Crypt Organoid Culture

The most proximal 10 cm of small intestine was isolated and flushed with ice cold PBS. Intestine was cut longitudinally in order to expose the lumen and scraped gently with a glass cover slip to remove upper mucosa. The intestine was cut into 1-2 mm pieces and washed 3-4x with ice cold PBS. Tissue was then incubated with 2 mM EDTA in PBS for 30 min with agitation at 4°C. After aspirating the EDTA, 10 ml of ice cold PBS was added and crypts were extracted with vigorous pipetting. The supernatant containing crypts was removed and set aside. This process was repeated 4x, with each fraction checked under the microscope for crypt enrichment. The fractions with highest crypt enrichment were combined through a 70 μM cell strainer and washed with advanced DMEM/F12 media (Invitrogen). Crypts were then centrifuged at low speeds (around 150 *g*) to remove single cells. The tissue was then spun at higher speeds (300 *g*) and the pellet re-suspended with ~500 μl Matrigel matrix (Corning). Approximately 20 μl crypt/Matrigel mix are added to each well (based on a 24-well plate). Organoids were incubated at 37°C, 5% CO_2 and culture media (see 2.2.1) was renewed every 2-

3 days. Where indicated, cultures were pre-stimulated for 24 h with recombinant murine IL-4/IL-13 (20 ng/ml) prior to exosome uptake/microinjection assays.

2.10.2 *in vitro* exosome microinjection and co-localisation assays

1mm OD glass capillaries (World Precision Instruments) were pulled into two needles using a micropipette puller (Sutter), according to manufacturer's instructions. Needles were backloaded with 20 µl of injection material (equating to ~ 2 µg labelled exosomes) using a microloader pipette tip. The needle was inserted into the microinjector and tightly sealed. The needle end point is sheared off (~ <0.1 mm) using forceps to allow approximately 1 nanolitre of material to be expelled per injection. Needle tip is moved over organoid of interest at a 30-40° angle and adjusted using the micromanipulator for fine control to lower the needle into the lumen of the organoid (darker in colour). Using a pressure of ~20 kPa, the sample is microinjected 3-4 times to ensure maximal insertion into the organoid lumen.

As previous uptake experiments (See 2.9), PBS is prepared with the PKH67 dye in the same way as exosomes to eliminate any carry-over from labelling. Exosomes isolated from the enterocyte cell line, $MCICL_2$ are used as a comparative mammalian vesicle control.

Organoids were subsequently washed 2x in PBS before 20 min fixation in 4% PFA at room temperature. Samples were then permeabilised in 0.5% Triton X-100 in PBS before 3x rinses in glycine/PBS rinse buffer and blocked for 1 h in immunofluorescence buffer (for both, see 2.1.6) at room temperature. Wells were incubated overnight with primary antibodies (see Table 2.6). The next day, wells were washed 3x in immunofluorescence buffer (20 min each), before incubation with fluorescent conjugated secondary antibody (see Table 2.6) for 50 min. Wells are then washed 1x in immunofluorescence buffer, and 3x in PBS prior to nuclear staining with 4', 6-diamidino-2-phenylindole-supplemented ProLong Fade Gold (Invitrogen) mounting media. Samples were examined on the Nikon N-STORM & A1+ confocal laser scanning microscope (Nikon, ×60 or x100 objective) using the Nikon Nis-Elements (Version 4) software (Nikon). Image analysis was performed using ImageJ software (NIH).

2.11 Exosome vaccination studies

To generate anti-exosome sera, female rats were immunized intraperitoneally with 75 μg exosomes or 75 μg exosomes pre-treated with 0.1% Triton X, in 9% alum adjuvant (Sigma-Aldrich) i.p., then boosted with 15 μg of exosomes on days 28 and 35, before serum collection via cardiac puncture on day 42. For subsequent analyses (by western blot or otherwise) Naïve rat sera was used as an isotype control.

In immunization studies, female C57BL/6, BALB/c or $ST2^{-/-}$ (6-10 weeks old) mice were immunized with 10 μg of exosomes, HES, or HES depleted of exosomes in 9% alum adjuvant (Sigma Aldrich) i.p., then boosted on days 28 and 35 with 2 μg of HES product-alum i.p. prior to serum collection via cardiac puncture on day 42. To ascertain exosome effects in $ST2^{-/-}$ mice, mesenteric lymph nodes were isolated for T cell restimulation (see 2.6.2) and RPMI washes of the peritoneal cavity were taken to look at levels of variable cytokines. In some experiments, mice were challenged with 200 *H. polygyrus* L3 larvae on day 42, and faecal egg counts determined at days 14, 21 and 28 post-infection, and adult worms counted at day 28.

2.12 Exosomes in an Airway Allergy Model

For all experiments presented in these studies, the sample size was large enough to measure the effect size. In all experiments;

(1) All intranasal challenges were carried out under brief isofluorane sedation.
(2) Bronchoalveolar lavage (BAL) was assessed for presence of RELMα and Ym1 by standard ELISA (see 2.5.1), as well as IL-1α, IL-1β, IL-4, IL-5, IL-10, IL-13, IL-17A and IFNγ using BD cytometric bead array Flex-set kits
(3) Cell suspensions (1×10^6) from BAL and lung homogenate (previously treated with digestion media) were taken for analysis of different innate cells; CD4+ T cells, eosinophils, neutrophils, innate lymphoid cells (ILC), macrophages, epithelial cells and dendritic cells (see table 2.3).
(4) Cell suspensions (2×10^6) from lung homogenate were stimulated, as

described in 2.6.2 and [141, 242], for subsequent analysis of intracellular IL-5 and IL-13 in ILCs.

(5) In some experiments, lung tissue was harvested in 1ml RNA-later (Thermo Fisher Scientific) for subsequent RNA extraction.

2.12.1 Challenge-recall using ovalbumin

Mice were administered intranasally on day 0 with 20 μg ovalbumin (OVA), 50 μg *Alternaria alternatus* antigen and/or 10 μg HES or exosomes in 50 μl PBS On days 14, 15 and 16; mice are given an intranasal recall challenge with 20 μg OVA in 50 μl PBS. On day 17 mice were sacrificed for tissue harvest.

2.12.2 Short-term allergic airway modulation

Mice were co-administered intranasally with 10 μg of exosomes, HES or HES depleted of exosomes (or PBS as a control) with 50 μg of *Alternaria alternatus* antigen (Greer). Mice were sacrificed 48 h later for tissue harvest. Mice were administered intranasally with 10 μg of exosomes, HES or HES depleted of exosomes in 50 μl of PBS or PBS alone for 24 h prior to a second intranasal challenge with 50 μg of *Alternaria alternatus* antigen (Greer) and a further 5 μg of exosomes, Sup of HES in 50 μl PBS. Mice were sacrificed 24 h later for tissue harvest.

2.13 Statistical analysis

All statistical analyses were performed using Prism 6 (Graphpad Software Inc.). Unless otherwise indicated in figure legends, data analysis was done as follows;

For comparisons of two groups, a parametric student's t-test, unpaired, and two-tailed was used. When three or more groups were analysed, in-group variance was assessed by Brown Forsythe test and data were log-transformed and analysed by one-way ANOVA, with a Tukey's multiple comparisons post-test. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, N.S. not significant P>0.05.

Chapter 3

Characterisation of *H. polygyrus* secreted exosomes

3.1 Introduction

The murine gastrointestinal nematode, *H. polygyrus*, has long been used as a model to study immunity to helminth infection and has revealed interesting mechanisms of host immunosuppression [243]. It is well known that helminth excretory-secretory products (ES) can modulate the host immune response, through either direct cellular effects or initiation of regulatory T cells [244, 245]. *H. polygyrus* ES (HES) has been shown to immunomodulate host cells [92], as well as well as driving a regulatory pathway, which includes the secretion of a TGF-β homolog [141, 151]. The protein composition of HES has been found to be a complex mixture of molecules, with 374 proteins identified by LC-MS/MS [246]. Following the discovery of exosomes (a type of extracellular vesicle) in the ES of trematodes, *Fasciola hepatica* and *Echinostoma caproni* [220], it led others to postulate the presence of extracellular vesicles (EVs) in the secretory material of other parasitic worms. Exosomes are nanovesicles around 50-100nm in size that are secreted by virtually all cells to facilitate the transfer of selected cargo, mainly lipids, proteins and RNA species, between cells [167]. Exosomes develop within a cell by inward budding of multi-vesicular endosomes, and thus contain components of the parental cell, such as

RNAs or proteins, that may be trafficked into the same compartment. The discovery of extracellular vesicles from kinetoplastids, fungi and bacteria drove the theory that communication via vesicles could operate on a cross-species platform [188]. Data from the trematode studies further suggests that ES-derived exosomes are capable of reaching the host environment, as they appear to be found intact on the parasites' tegument, and were taken up by host intestinal cells *in vitro* [220]. The formation of exosomes by helminths had originally been established in free-living nematodes, with the demonstration that *Caenorhabditis elegans* use a novel secretion pathway from the apical membrane to co-secrete multivesicular bodies, containing exosome-like vesicles, with peptides that normally promote cuticle development. [223]. Exosomes from helminths and protozoa appear to share many specific markers with those known to be present in mammalian exosomes, such as Heat-shock protein 70 (HSP70), endosomal sorting components e.g. ALIX, and surface tetraspanins including CD9 and CD63 [167].

During my PhD, our group published a study documenting the release of exosomes in *H. polygyrus* ES. These exosomes represent approximately 10% of the total protein secretion of an adult worm [219]. Proteomic comparison of the secreted products represented in the soluble and vesicular fractions separated by ultracentrifugation also demonstrated enrichment of a number of key components within the exosomes. Interestingly, *H. polygyrus* exosomes contained a suite of RNA species, including miRNAs such as let-7, miR200 and bantam [219], which were shown to suppress the mouse phosphatase DUSP1 using a reporter assay. Additional data identifying extensive small RNA repertoires, including miRNAs, in helminth-

derived exosomes are now becoming available, including data from nematodes such as *B. malayi* [247] and trematodes including *D. dendriticum* [248] and *S. mansoni* [249]. Most importantly, truly definitive evidence for helminth-derived miRNA acting on host genes remains to be obtained. However, the circumstantial evidence remains enticing; not only are extensive seed sequences shared between helminth and host miRNAs, but the miRNA-rich exosomes (of *H. polygyrus* at least) also carries a worm Argonaute protein [219], suggesting that a functional package for gene repression is being delivered to the target cells.

There is evidence that some pathogens use exosomes to subvert a host immune response, therefore promoting their survival. For example, the protozoan parasite *Leishmania donovani*, not only induces the production of immunomodulatory exosomes from macrophages [197], but also releases its own exosomes that have an immunosuppressive effect on myeloid-derived cells [193]. In addition, exosomes derived from *T. vaginalis* were shown to promote recruitment of neutrophils, which was suggested to support the spread of infection [209]. Therefore, it could be hypothesized that helminths would release exosomes to aid in the infection process by modulating host immune responses.

There is limited evidence available regarding the cellular origin of EVs from within helminths [188]. To address this, researchers have relied on molecular techniques such as proteomics and transcriptomics to suggest the origin of EVs, based on protein enrichment of potential parental cell markers. For example, recent data suggests different populations of EVs from *F. hepatica* may derive from either the gastrodermal layer (larger vesicles) or the tegument (exosome-like vesicles), based on

enrichment of cell origin markers [250]. In this novel biological system, it is necessary to understand the localization of exosomes within the helminth and mechanisms of how exosomes are formed, as this may be key to their function. Furthermore, identification of the immunogenic proteins on the exosome surface could provide future opportunities to interfere with *H. polygyrus* exosome-host cell communication and modulation.

3.2 Results

3.2.1 Existence of extracellular vesicles in *H. polygyrus* excretory secretory products

At the start of this thesis project, there was pilot data to suggest that adult *H. polygyrus* produces EVs that can be found in the ES products, and the goal here was to characterize the properties of these EVs and their functional effects on host cells. Adult *H. polygyrus* worms produce excretory-secretory products (HES) that are harvested *in vitro* by keeping parasites in culture media [240], and assumed to be similar to that released in the intestinal lumen of the murine host. Following HES ultracentrifugation, the pellet material was analysed using transmission electron microscopy (TEM). Indeed, small, irregularly shaped vesicles of around 50-100nm in size can be detected (Figure 3.2.1A), which are similar to those classified in the literature as exosomes [159, 220]. Total protein content of isolated exosomes, compared to HES depleted of exosomes (Sup), or total HES, highlighted distinct differences in the profile of these samples, and enrichment of particular proteins in the exosome fraction (Figure 3.2.1B) To address whether exosome secretion is gender-specific, male and female adult worms were separated following harvest from the murine intestinal tract. Worms were separated by hand under a light microscope according to their distinct physical differences, and HES was collected separately from flasks of male and female parasites. Upon initial TEM analysis, exosomes only appear to be secreted from female *H. polygyrus* (Figure 3.3.1.C, *left panel*). However, when ultracentrifuged HES fractions (which was shown previously to contain exosomes) from both sexes were analysed, there does not seem

to be any difference in protein content (Figure 3.2.1C *right panel*). This suggests that exosomes are produced by both sexes, and that inconsistencies may have occurred during the processing stages for TEM analysis. Finally, mass spectrometry-based proteomic analysis of the exosome pellet was carried out [219], whereby 139 proteins were expressed at significantly higher levels than those found in HES supernatant ($p < 0.05$ associated with the protein change). As part of this project, these data were analysed to produce a shortlist of the top 25 most highly expressed proteins in exosomes compared to the supernatant fraction (Table 3.2.1A). Of note, common exosome markers were found, including RAL-1 (Ras-related GTPase-1), heat-shock protein-70, and tetraspanin-11. An adapted shortlist from [219] and results from our LC-MS/MS analysis identifies more exosome-associated markers, and their determined functions (Table 3.2.1B). The characterization of proteins associated with exosomes will help inform on how they may be generated, as well as their origins and role by looking for markers of biogenesis, specific cell types and other functional components.

3.2.2 Potential origin of *H. polygyrus*-derived exosomes

Next, we sought to determine where exosomes may originate from inside the *H. polygyrus* adult worm. Analysis of the proteomic profile showed that some of the highly expressed proteins in exosomes are homologous to those found in intestinal proteins of other worm species (Figure 3.2.2A) [219]. It seems reasonable to suggest, given that exosomes tend to express markers of their parent cell [159], that these exosomes may originate from a subset of intestinal cells within the worm. We analysed cross-sections of whole adult worm, in order to find any evidence of

exosomes, or multi-vesicular bodies located near the intestinal tract (Figure 3.3.2B-E). During fixation and processing of worms for TEM analysis, it was immensely difficult to straighten worms to ensure longitudinal cross-sections. Furthermore, it was extremely rare to obtain images of an intact intestinal tract (complete with open lumen, etc). Despite this, there appears to be vesicles of the correct shape and size localised near the intestine (Figure 3.2.2F-I), which are consistent with multi-vesicular bodies or late endosomes.

3.2.3 Quantification of *H. polygyrus* extracellular vesicles

There is a growing requirement for standardization within the field of EV research, both with regards to quantitative and qualitative assessment of EVs [251]. Optical methods such as TEM give a rough indicator of size and morphology of the vesicles (Figure 3.2.1A). Additionally, quantitation techniques such as Qubit or bicinchoninic acid assay (BCA), will only reveal protein concentration of the vesicle sample, and this may differ due to the heterogeneity of vesicle populations. It is becoming increasingly apparent that virtually all mammalian cell EV populations can have a degree of heterogeneity [252], and this is now being shown in parasite-derived ES preparations as well [219, 247, 250]. Collectively, these techniques give no indications of the relative quantities of vesicles within a particular isolation preparation. Thus, techniques such as optical single particle tracking analysis or resistive-pulse sensing were developed, commercially known as Nanoparticle Tracking Analysis (NTA) [253] and the IZON qNano technique [254] respectively. The qNano technique quantifies individual EVs by the transient decrease of an ionic current that is generated by the movement of a particle through nanopores in a

specially designed membrane (See methods and materials). This gives a readout of both size distribution and average concentration of EVs within a sample. *H. polygyrus* exosome preparations consist of a population of vesicles, which on average are around 95-112nm in diameter (Figure 3.2.3A). According to qNano analysis, 1µg of the ultracentrifuged HES pellet (measured previously by qubit), which we know by TEM to contain EVs, equates to ~1 x 10^9 vesicles. There is clear enrichment of vesicles within this preparation, compared the remaining supernatant (Sup) concentrate or total HES (Figure 3.2.3B). With regards to infection, it would be of biological relevance to know the average number of vesicles secreted per worm. Based on average worm burdens, the average concentration of total HES secreted during infection [240], and the proportion of the exosome fraction within total HES [219], this equates to approximately 1 x 10^7 vesicles secreted per worm during a two week culture (Figure 3.2.3C). It is noted however that we do not have a clear assessment of the efficiency of vesicle recovered during purification, and whether the approximation is necessarily reflective of vesicles secreted during infection.

3.2.4 *H. polygyrus* exosomes elicit strong polyclonal antibody responses

In order to detect *H. polygyrus* exosomes by western blot or flow cytometry, we developed anti-exosome polyclonal sera. Rats were immunized using an alum vaccination schedule [255], with intraperitoneal doses of exosomes, or exosomes that had been pre-treated with Triton X-100 detergent, which degrades exosomal membranes (Figure 3.2.4A). As we do not know how *H. polygyrus* exosomes interact

in another species *in vivo*, i.e. processing by other cells, direct recognition, etc. it was essential to compare the sera raised from both types of exosome preparations i.e. with or without an intact lipid membrane. The polyclonal sera obtained from both immunization schedules were analysed for reactivity via western blot, and specificity was determined by comparison with sera from a naïve rat (Figure 3.2.4B). Despite antisera recognition of proteins common to exosomes, total HES or HES supernatant, there also appear to be a few bands unique to exosomes (for example, at approx. 38 and 10 kDa). It would be beneficial to determine the proteins that correspond to these exosome-specific proteins. Therefore, a two-dimensional gel electrophoresis was performed to determine the identity of exosome proteins, and showed a range of protein spots when analysed by silver stain (Figure 3.2.4C *top*). The polyclonal rat serum generated against exosomes recognized several of these spots by western blot (Figure 3.2.4C *bottom)*, as well as many others. It should be noted that this polyclonal serum was used in a number of future experiments (Chapter 3, 4 and 6).

Additionally, we received purified polyclonal rabbit sera that had been generated against two peptide sequences from the C-terminus of *H. polygyrus* Tetraspanin-11. This was expected to be highly enriched on the exosome surface [219], given the presence of tetraspanins on surface of mammalian exosomes, where they are suggested to be implicated in a number of exosomal functions, including cargo sorting, target cell selection and anchorage [173]. There appear to be several exosome-specific bands that the anti-TSPAN11 antisera bind to in comparison to HES Sup and total HES, following visualization by western blot (Figure 3.2.4D). However, none of these match the expected size of ~30kDa (based on LC-MS/MS in [219]). The presence of bands with a higher molecular weight than expected could

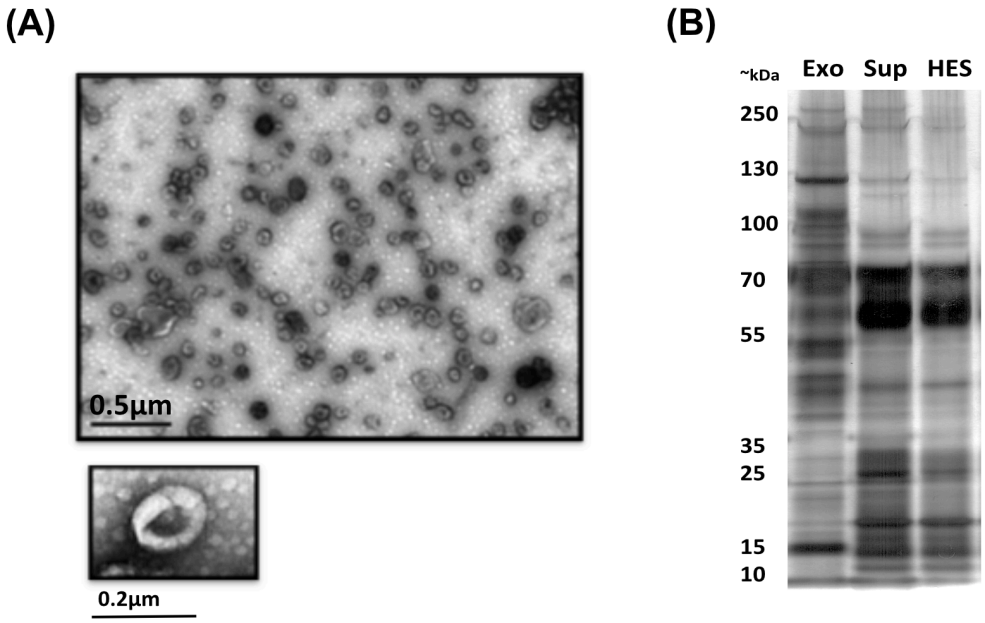
suggest possible post-translational modification of the protein, protein misfolding or non-specific binding to exosome surface proteins. The non-specific nature of this polyclonal serum was kept in mind for any future experiments.

3.2.5 Antibody-based capture of exosomes by flow cytometry

Using a latex bead-based system, we can determine the relative presence of various surface proteins on EVs through detection on a standard flow cytometer [256] (Figure 3.2.5A). We can use this method to determine the binding capacity of polyclonal rat antisera and other antibodies to the proteins on the surface of *H. polygyrus* EVs. Based on the relative fluorescence intensity and histogram (Figure 3.2.5B), rat polyclonal serum that was raised against exosomes recognized a higher repertoire of proteins on the surface of exosomes, compared to either total HES or HES depleted of exosomes. Non-specific binding was assessed with either beads alone, or in comparison to naïve sera. The corresponding histograms show the proportion of binding from naïve serum (green line) or anti-serum to exosomes (red line), and the bead only control (grey). In addition, polyclonal anti-tetraspanin 11 (anti-TSPAN11), antibodies had higher proportion of binding to exosome surface proteins compared to other HES fractions (Figure 3.2.5C), suggesting their potential to interact with exosomes in future studies. Finally, we used a monoclonal antibody against murine ALIX in this system, which has approximately 42% sequence identity to the *H. polygyrus* ALIX protein. Based on the relative binding fluorescence to *H. polygyrus* exosomes, this suggests the presence of cross-reactive ALIX proteins on the nematode exosome surface (Figure 3.2.5D).

3.2.6 Cross-link immunoprecipitation of *H. polygyrus* exosomes

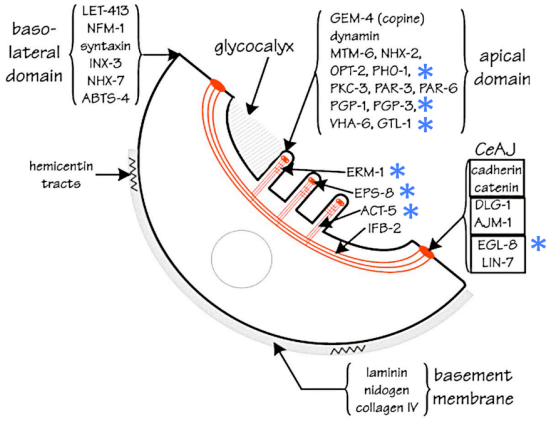
In order to identify the primary exosomal protein candidates as targets for vaccination, we must first isolate exosomal proteins recognized specifically by polyclonal rat serum. I carried out cross-link immunoprecipitation of exosomal proteins by anti-serum, followed by subsequent LC-MS/MS analysis (Workflow in Figure 3.2.6). These data revealed a number of candidate proteins specifically targeted by antibodies (Table 3.2.6), along with proposed functions as exosomal proteins. Some of these proteins are involved in direct vesicle/exosome functions, including zinc metallopeptidase and ubiquitinases, scavenger receptors and vacuolar ATPase subunits [198, 223, 257]. Furthermore, proteins such as Galectin-1, Histone deacetylase-1 and Heat-shock protein 70 are associated with immune modulation [258-260], whereas others such as lysozyme-2 have bacteriolytic activity [246]. The list of proteins recognized by antibodies generated in exosome-immunized animals highlights the immunogenic candidates which require further investigation.

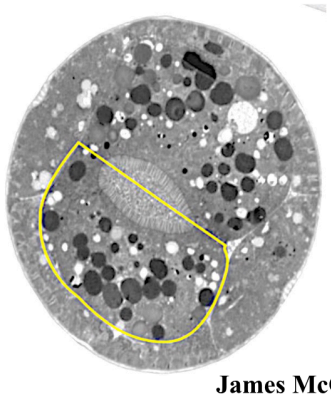


3.2.1 Exosome existence in secretory product of *H. polygyrus*

(A) Electron micrograph of exosomes isolated from HES by ultracentrifugation (scale bar = 0.5 µm and 0.2 µm). Vesicles range from approx. 50-100nm in size. **(B)** 5 µg exosomes and HES depleted of exosomes (Sup) and total HES visualised by silver stained SDS-PAGE. **(C)** Sex differences in exosome abundance and composition. Left, Electron micrograph of isolated potentail exosomes (denoted by arrowhead, Δ) from adult male and female *H.polygyrus*. (scale bar = 0.2 µm top left, 0.5 µm bottom left). Right, Silver staining of the isolated exosomes on SDS-PAGE (M = male, F = female).

(A)



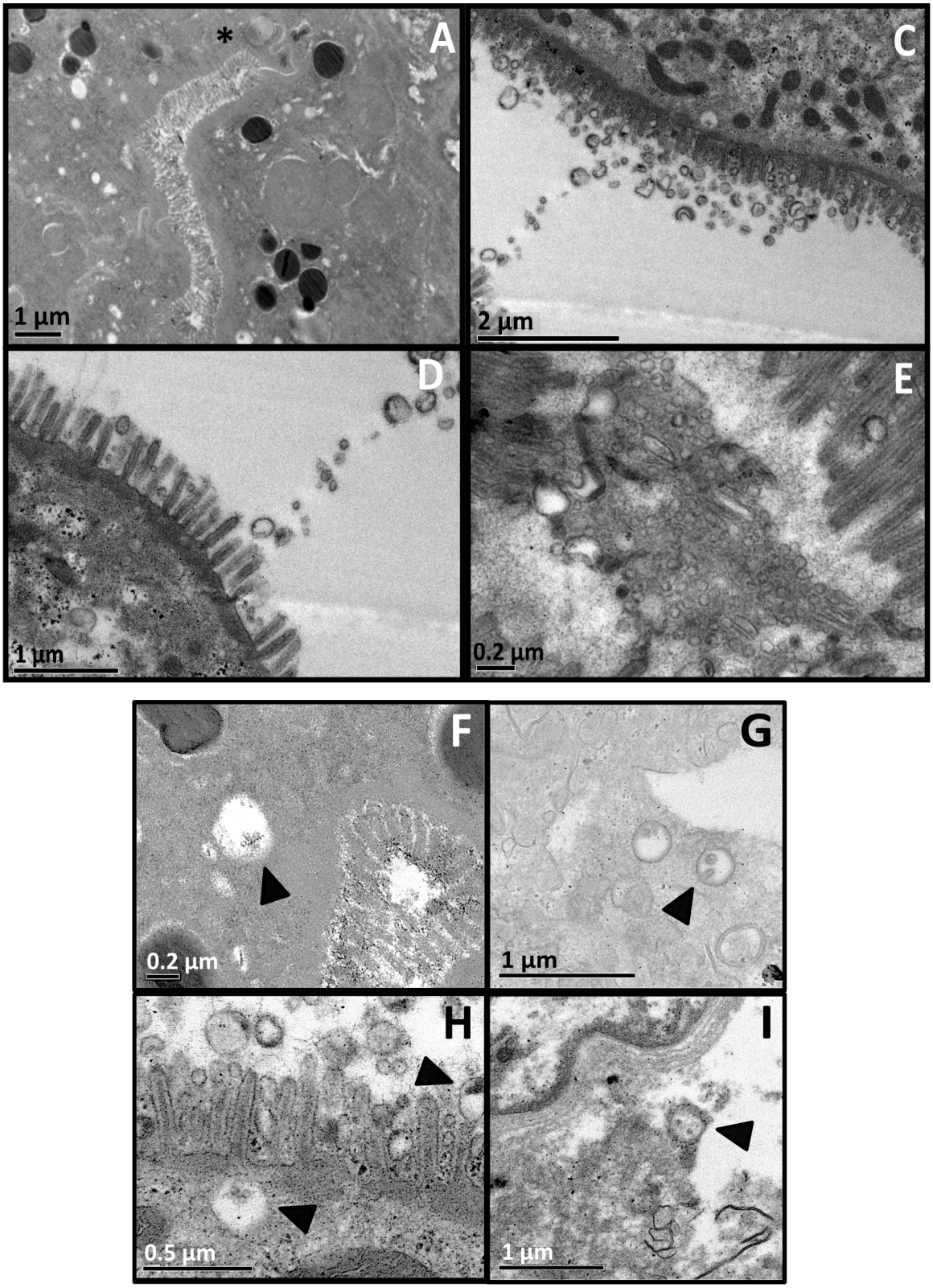


James McGhee, Wormbook (2007)

Rank	Protein Identity	Fold change	Species	Protein similarity
9	CBN-ACT5 protein	57.11	*Caenorhabditis brenneri*	96.71%
12	Intestinal acid PHOsphatase family member (pho-1)	53.45	*Caenorhabditis elegans*	38.89%
14	EPS (human endocytosis) related family member (eps-8)	52.01	*Caenorhabditis elegans*	81.48%
31	P-GlycoProtein related family member (pgp-9)	30.93	*Caenorhabditis elegans*	70.35%
34	CBN-TSP-9 protein	29.78	*Caenorhabditis brenneri*	61.76%
35	Ezrin/Radixin/Moesin family (erm-1)	28.82	*Caenorhabditis elegans*	79.55%
39	CRE-PHO-1 protein	26.26	*Caenorhabditis remanei*	44.48%
45	Glutathione S-transferase	21.75	*Haemonchus contortus*	70.24%
62	Vacuolar H ATPase family (vha-16)	16.26	*Caenorhabditis elegans*	93.26%
77	EGg Laying defective family (egl-4)	12.42	*Caenorhabditis elegans*	87.33%
80	Vacuolar H ATPase family (vha-15)	11.49	*Caenorhabditis elegans*	78.30%
93	CBR-TAG-60 protein	9.42	*Caenorhabditis briggsae*	73.40%
114	CRE-EPS-8 protein	6.01	*Caenorhabditis brenneri*	83.87%

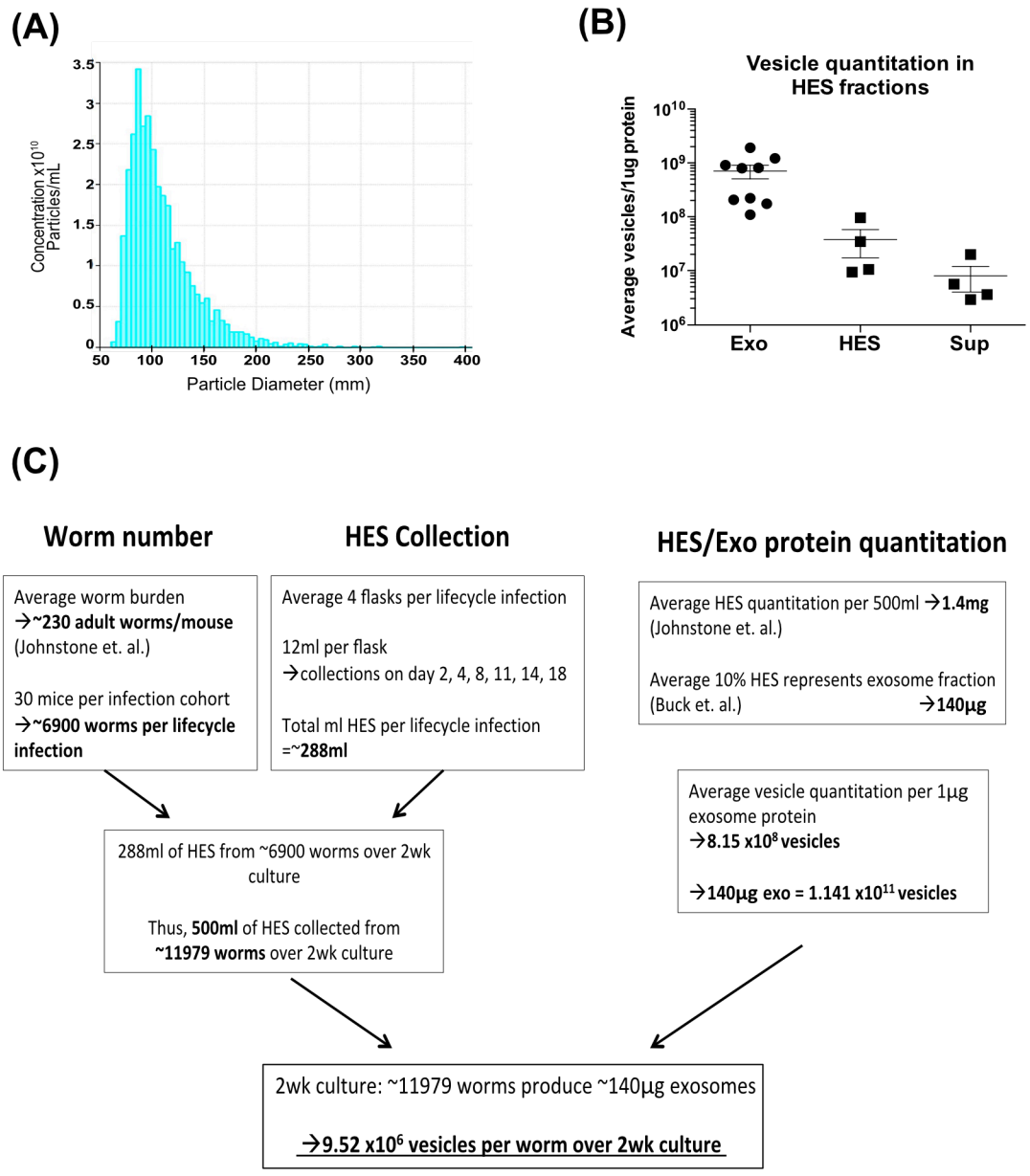
3.2.2 Potential origin of *H. polygyrus*-derived exosomes

(A) Classification of intestinal proteins identified on exosomes secreted by adult *H. polygyrus* worms. Table highlights homology to proteins identified in other nematodes, with additional comparative analysis to the *Caenorhabditis elegans* intestinal cross-section (see ref [267]).



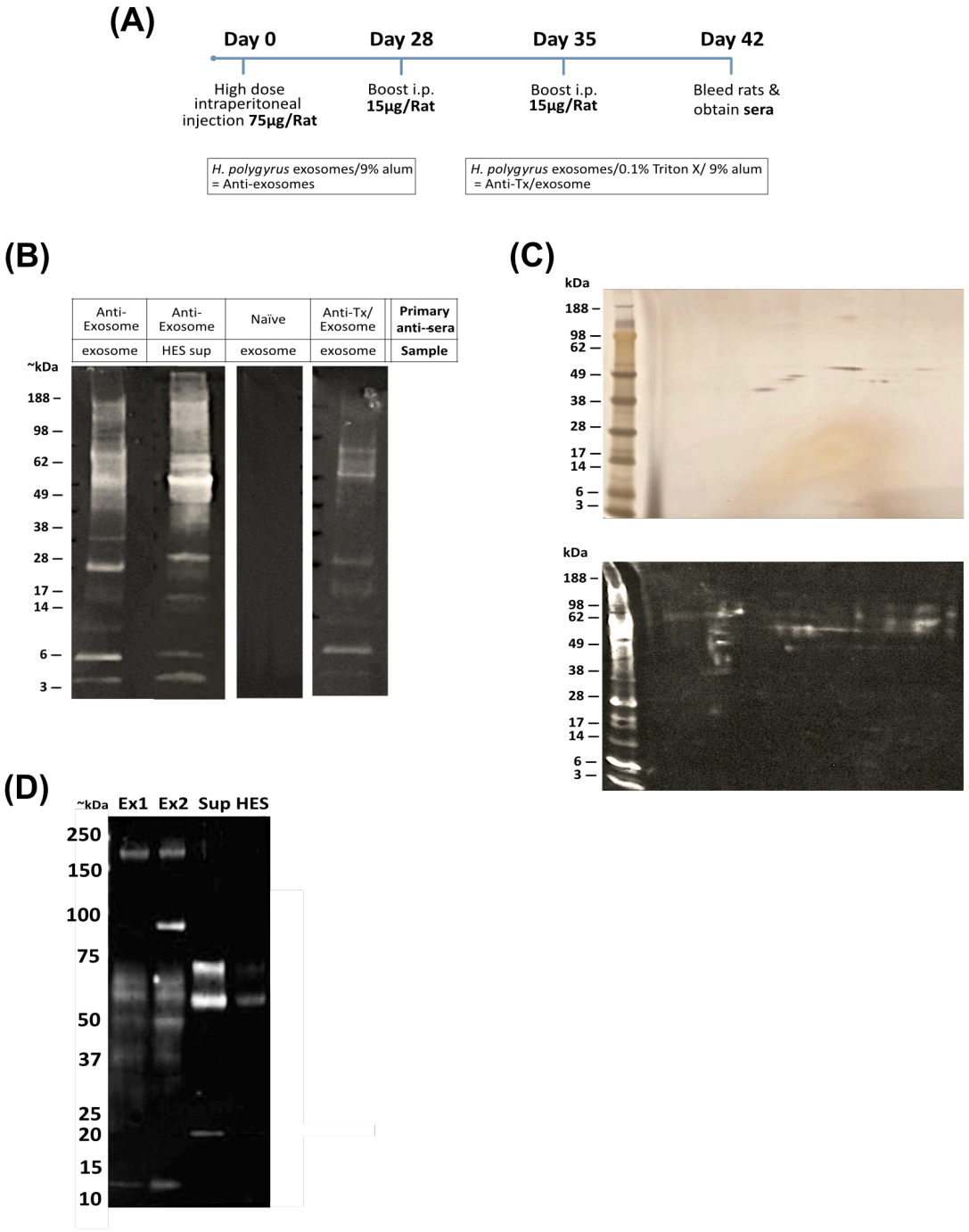
3.2.2 Potential origin of *H. polygyrus*-derived exosomes - *continued*

(B) Low-power micrograph of the intestinal tract of adult *H. polygyrus,* showing brush border epithelium, as well as a ducted secretory gland (marked with *). **(C)** + **(D)** Higher power images of *H.polygyrus* intestinal ultrastructure, with **(E)** zoom of the luminal contents containing a large number of vesicles and macromolecules. **(F-I)** Potential evidence of MVBs near intestine (marked with ▲). All appear to contain vesicles of around 20-100nm in size. For all microscopy, scale bars determine distance as indicated.



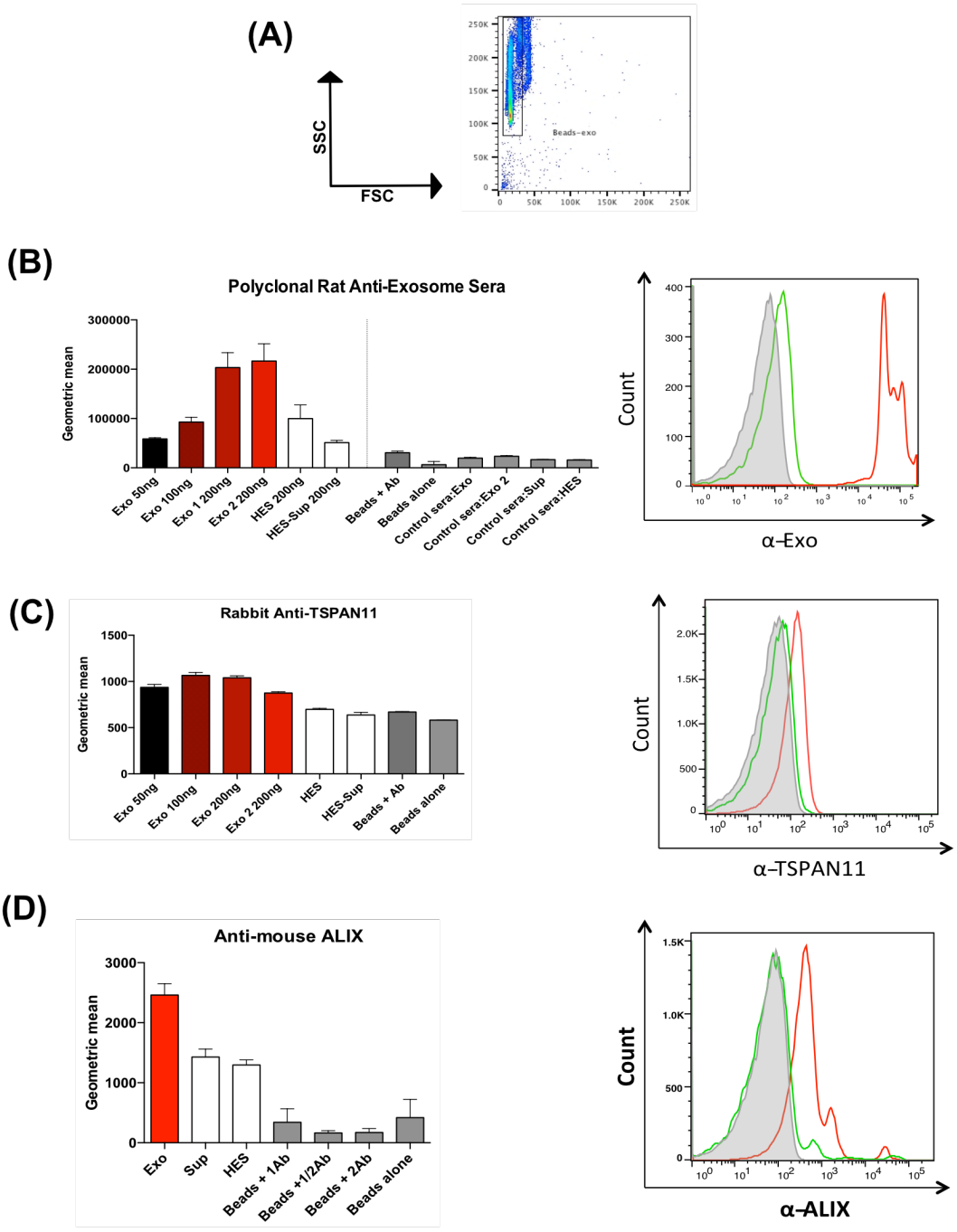
3.2.3 Quantification of *H.polygyrus* extracellular vesicles

(A) Size distribution of *H.polygyrus* exosomes were determined by qNano as particle diameter (x-axis) versus concentration of particles/mL of sample (y-axis). **(B)** Assessment of average particle number per 1 µg of exosomes, HES depleted of exosomes (Sup) or total HES. Data are pooled from 2-3 independent measurements and presented as mean values ± SEM (n = 4-9 samples per group). **(C)** Workflow for determination of approximate number of vesicles secreted by adults worms in culture.



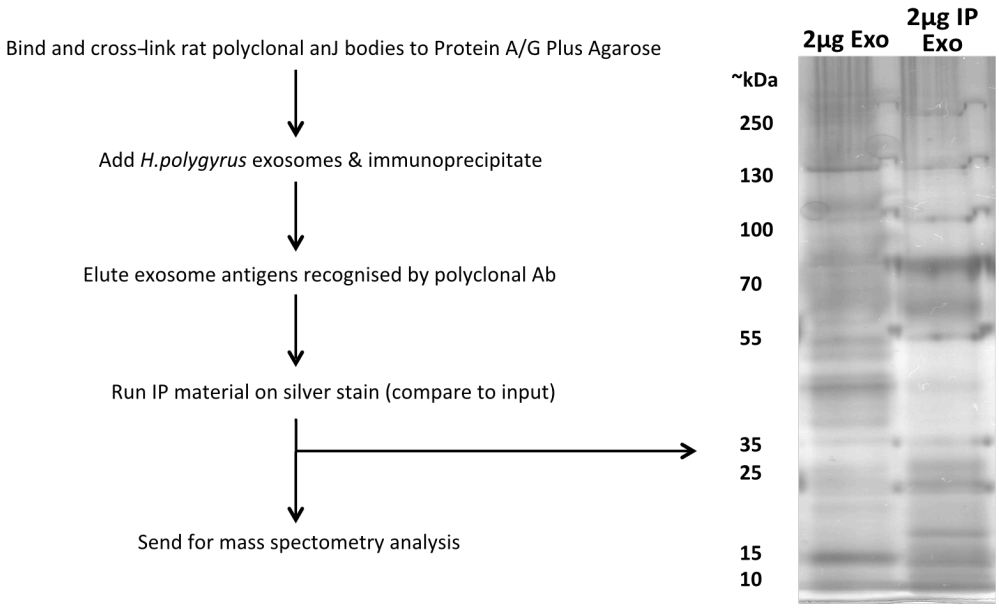
3.2.4 *H. polygyrus* exosomes elicit strong polyclonal antibody responses

(A) Schematic of experimental protocol for production of polyclonal antisera (as described in 2.11). **(B)** Western blot assays for reactivity of rat polyclonal sera against 2 µg exosomes or HES supernatant. Naïve rat sera was used as a negative control. **(C)** Representative 2-Dimensional analysis of *H. polygyrus* exosomes as visualized by silver stain and western blot detected by anti-exosome sera. **(D)** The major protein components of 5 µg exosomes, Sup or HES were determined by western blot using anti-TSPAN11 polyclonal antibodies (1:1000). All blots were detected using anti-rabbit/rat IgG conjugated to HRP (1:5000), and are representative of at least 2 experiments with different exosome isolations.



3.2.5 Antibody-based capture of exosomes by flow cytometry

(A) Representative FACs plot for gating of exosomes conjugated to aldehyde-latex beads. Comparison of the dose-dependent recognition of antibodies generated against **(B)** whole exosomes, **(C)** testraspanin-11 or **(D)** murine ALIX, to bind to exosomes demonstrated by the relative flourescence intensity. Histograms highlight shift in flourescence in exosome-bound beads following secondary recognition by polyclonal sera (red line) compared to beads alone (green bar) or recognition by naive sera (grey). Data are pooled from 2 independent experiments and presented as mean values ± SD (n = 3-4 wells per condition/experiment).



3.2.6 Cross-link immunoprecipitation of H.polygyrus exosomes

Workflow for cross-link immunoprecipitation of *H.polygyrus* exosome proteins by polyclonal anti-exosome antibodies. Representative silver stain of 2 µg original exosome input compared to material isolated following immunoprecipitation.

Table 3.2.1A – Top 25 proteins enriched in exosomes

Rank	Identity	Fold change	No. unique peptides	% peptide coverage	Accession	Species with protein similarities
1	**Hypothetical protein CRE_25189**	274.61	4	9.5	Hp_I15305_IG07249_L1196	*C. remanei*
2	**Protein RAL-1, isoform b**	139.11	3	10.4	Hp_I14286_IG06230_L1419	*C. elegans*
3	**No BLASTX similarities 28267638:28268138 forward**	138.66	2	36.7	Hp_I28260_IG20204_L501	*N/A*
4	**Zinc Metallopeptidase 1**	104.65	19	19.6	Hp_I12518_IG04462_L2600	*A. caninum*
5	**Hypothetical protein CRE_03026**	77.99	3	15.1	Hp_I14432_IG06376_L1375	*C. remanei*
6	**Temporarily Assigned Gene name family member (tag-234)**	73.25	2	16.8	Hp_I11897_IG04033_L376	*C. elegans*
7	**hypothetical protein CAEBREN_05149**	64.35	5	8.9	Hp_I13513_IG05457_L1688	*C. brenneri*
8	**O-Acyltransferase-32**	61.44	2	7.4	Hp_I12671_IG04615_L2329	*A. suum*
9	**ACTIN-5**	57.11	10	28.5	Hp_I15213_IG07157_L1209	*C. brenneri*
10	**Myoglobin**	56.24	3	8.6	Hp_I02590_IG00245_L1152	*H. polygyrus*
11	**Aspartyl protease precursor**	55.2	2	4.9	Hp_I08795_IG02482_L1340	*H. contortus*
12	**Intestinal acid PHOsphatase family member (pho-1)**	53.45	2	6.9	Hp_I16610_IG08554_L997	*C. elegans*
13	**Putative Zinc Metallopeptidase**	52.76	25	25.8	Hp_I12444_IG04388_L2875	*H. contortus*
14	**EPS (human endocytosis) related family member (eps-8)**	52.01	2	16.9	Hp_I30640_IG22584_L466	*C. elegans*
15	**Cell Death abnormality family member (ced-10)**	47.72	3	6.8	Hp_I14321_IG06265_L1411	*C. elegans*
16	**Multidrug resistance protein 1**	46.45	2	8.5	Hp_I18648_IG10592_L810	*A suum*
17	**SCAVenger receptor (CD36 family) related-4**	46.19	5	8.4	Hp_I13316_IG05260_L1787	*C. remanei*
18	**R-RAS related family member (ras-2)**	45.23	2	3.8	Hp_I14240_IG06184_L1427	*C. elegans*
19	**G Protein, Alpha subunit family member (gpa-17)**	43.17	3	6.1	Hp_I14396_IG06340_L1386	*C. elegans*
20	**Tetraspanin-11**	40.02	2	5.9	Hp_I16310_IG08254_L1020	*A suum*
21	**Pepsinogen**	39.99	4	7	Hp_I08139_IG02154_L1715	*H. contortus*
22	**Heat Shock Protein 70**	39.2	16	21.9	Hp_I12624_IG04568_L2400	*D. medinensis*
23	**No BLASTX similarities 12097939:12099055 forward**	38.7	2	7.8	Hp_I09125_IG02647_L1117	*N/A*
24	**Hypothetical protein CBG01308**	38.13	6	23.1	Hp_I07646_IG01907_L1927	*N/A*
25	**Hypothetical protein CBG02206**	35.14	2	3.4	Hp_I06659_IG01391_L1864	*N/A*

Table 3.2.1B - Functions of exosomal proteins identified by LC-MS/MS

Rank	Identity	Fold change	No. unique peptides	% peptide coverage	Accession	Spp. with protein similarities	Function	Ref
2	**Protein RAL-1, isoform b**	139.11	3	10.4	Hp_I14286_IG06230_L1419	*C. elegans*	MVB biogenesis and exosome secretion	[258]
4	**Zinc Metallopeptidase 1**	104.65	19	19.6	Hp_I12518_IG04462_L2600	*A. caninum*	Vesicle cargo selection	[195, 206]
13	**Putative Zinc Metallopeptidase**	52.76	25	25.8	Hp_I12444_IG04388_L2875	*H. contortus*	Vesicle cargo selection	[195, 206]
14	**EPS (human endocytosis) related family member (eps-8)**	52.01	2	16.9	Hp_I30640_IG22584_L466	*C. elegans*	Endosomal trafficking	[259]
20	**Tetraspanin-11**	40.02	2	5.9	Hp_I16310_IG08254_L1020	*A. suum*	Ubiquitous e.g. cell adhesion, motility, membrane fusion, signaling and protein trafficking	[173]
22	**Heat Shock Protein 70**	39.2	16	21.9	Hp_I12624_IG04568_L2400	*D. medinensis*	Ubitquitous e.g. chaperone protein/Ag presentation	[257, 260]
33	**ALIX**	30.16	4	6.7	Hp_I13661_IG05605_L1622	*C. elegans*	Exosome biogenesis and ESCRT-pathway protein	[172]
34	**Tetraspanin-9 protein**	29.78	2	3.7	Hp_I07666_IG01917_L1781	*C. brenneri*	Ubiquitous e.g. cell adhesion, motility, membrane fusion, signalling and protein trafficking	[173]
50	**Rab-11B**	19.7	3	14.5	Hp_I22717_IG14661_L625	*S. salar*	MVB fusion and vesicle release	[261]
63	**Galectin-1**	15.74	8	11.9	Hp_I04721_IG00743_L1466	*H. contortus*	Immune regulation	[255]
94	**Rab-5**	7.2	2	3.4	Hp_I12868_IG04812_L2115	*C. elegans*	Endosomal trafficking	[262]
110	**Heat shock protein 90**	6.34	5	7.8	Hp_I07666_IG01917_L1781	*H. contortus*	Ubitquitous e.g. chaperone protein/immunoregulation/wound healing	[257, 260]
<139	**Enolase**	1.94	9	18.8	Hp_I06749_IG01436_L1580	*H. contortus*	Glycolytic enzyme	[159]

Table 3.2.6 - Vaccine candidates recognized by exosome-specific antibodies

Rank	Identity	No. unique peptides	% coverage	Species with protein identity	Proposed helminth exosome function
1	**Putative Zinc Metallopeptidase**	29	18.5	*H. contortus*	Vesicle cargo selection
2	**Zinc Metallopeptidase 1**	21	12	*A. caninum*	Vesicle cargo selection
5	**VITellogenin structural genes (vit-6)**	8	3.3	*C. elegans*	Yolk protein precursors
7	**Pepsinogen**	9	10.7	*H. contortus*	Pepsin proenzyme
8	**TransThyretin-Related family member (ttr-27)**	6	35.5	*C. elegans*	Mediates phagocytosis/Extracellular fluid transport protein
9	**VITellogenin structural genes (vit-2)**	10	3.8	*C. elegans*	Yolk protein precursors
15	**ACTin family member (act-4)**	2	4.7	*C. elegans*	Cell motility protein
16	**CBN-ACT-5 protein**	2	5.7	*C. brenneri*	Cell motility protein/Intestinal villi structure formation
18	**Antigen H11**	1	3.7	*H. contortus*	Aminopeptidase activity/Vaccine candidate/Intestinal molecule
21	**Ezrin/Radixin/Moesin family (erm-1)**	3	3.1	*C. elegans*	Intestinal structural remodelling
25	**Microsomal aminopeptidase**	1	3	*H. contortus*	Parasite intestinal glycoprotein e.g. antigen H11
28	**Galectin-1**	1	2.7	*H. contortus*	Immune regulation
30	**Nicotinic acetylcholine receptor-α subunit 63a**	2	6.5	*H. contortus*	Muscle neuron receptor - target of anti-helminthic drugs
32	**PI-type proteinase**	1	2.5	*K. pastoris*	Cysteine proteinase. Impairs gram +ve bacterial activity
33	**CLaudin-like Caenorhabditis family member (clc-1)**	1	5.9	*C. elegans*	Regulates channel activity, intercellular signalling, and cell morphology

34	**Heat Shock Protein 70**	1	2.3	*A. suum*	Ubiquitous e.g. Chaperone protein/antigen presentation
35	**CRE-VHA-2 protein**	1	3.5	*C. remanei*	V0 complex of vacuolar ATPase - mediates apical secretion of exosomes
36	**Histone deacetylase 1**	1	2.6	*A. suum*	Epigenetic modification of STAT3/Th2 responses/Induce FOXP3 in T cells
37	**SCAVenger receptor (CD36 family) related-4**	1	2.3	*C. remanei*	Exosome marker, Mediates exosome uptake and phospholipid transfer
41	**Serpin-B6**	1	2.5	*A. suum*	Regulate serine proteinase activity
47	**Lysozyme-2**	1	3.2	*H. polygyrus*	Antimicrobial and bacteriolytic effect
48	**SCAVenger receptor related-1**	1	7.7	*C. brenneri*	Exosome marker, Mediates exosome uptake and phospholipid transfer
50	**glyceraldehyde-3-phosphate dehydrogenase-like 1**	1	13.1	*M. musculus*	C3 inhibitor in *H. contortus.* Vaccine candidate in *S.mansoni* EVs
58	**Keratin-like protein**	1	6.4	*N. brasiliensis*	Structural protein
65	**Ubiquitin family protein**	1	37.8	*T. spiralis*	Vesicle cargo selection

3.3 Discussion

There are now a growing number of reports concerning extracellular parasite-derived EVs, especially from helminths, including liver flukes, [220], Schistosoma species [226, 261], filarial [247] and gastrointestinal nematodes [219, 262]. In this respect, a greater understanding and characterization of these vesicles must be developed in order to understand their function during infection.

The gastrointestinal nematode, *H. polygyrus,* releases exosome-like vesicles within its excretory/secretory product. These vesicles are consistent in shape and size to those described in the literature as exosomes [167]. Proteomic analysis of the *H. polygyrus* ultracentrifuged pellet further confirms the identity of these vesicles, as they are enriched in typical exosome markers such as ALIX, Rab proteins, heat-shock proteins and tetraspanins, proteins which are directly involved in exosome biogenesis and interactions [159]. In some instances of helminth infection, inoculation with either egg or worm stages can induce differential immune responses, as is shown during treatment of airway hyper-reactivity with the nematode *S. mansoni* [263]. However, we do not know currently whether other life stages have the capacity to secrete vesicles. Despite this, we show that exosome release does not appear to be gender specific, supporting most previous reports demonstrating exosome-like vesicles in ES from mixed male and female adult cultures [247, 261]. Although transmission electron microscopy suggested higher exosome abundance in the female worm secretory product, gel analysis showed that the protein composition of the pelleted fractions were identical in secretion product of male and female *H. polygyrus* worms.

Current methods of exosome or EV characterization can reveal their molecular repertoire, concentration per sample, size and morphology [251]. Despite this, there are clear requirements to recapitulate the physiological 'dose' of exosome-like vesicles that are released *in vivo* [264]. This will be an important requirement to take forward for our investigations of *H. polygyrus*-exosome mediated effects on cells *in vitro* and the dosage administered during experimentation *in vivo.* Indeed, there is limited literature available on the quantitative release of vesicles on a per cell basis, or their relative contents e.g. the variable numbers of miRNAs per vesicle [265]. An approximation of the number of vesicles secreted per worm allows us to hypothesise the potential number of vesicles that cells are exposed to during natural infection.

Owing to the novel biology surrounding nematode-derived exosomes [188], it was of interest to determine where exosomes originate from in the adult worms in order to develop a greater understanding of biogenesis and function. Analysis by mass spectrometry of the HES pellet revealed that exosomes were enriched in some intestinal markers homologous to those found in other worm species such as *C. elegans* and *Haemonchus contortus* [266]. These included Pho-1, glutathione S-transferase and actin-5, the latter of which has been shown to be essential for the development of microvilli and other intestinal architecture [267]. Ultrastructural analysis revealed evidence of exosome-like vesicles or multi-vesicular bodies (MVBs) in close proximity to the *H. polygyrus* intestinal tract. Some structures resembled MVBs found in *E. caproni* [220], although these cannot be definitively identified without use of a specific antibody to identify MVB or exosome-specific markers.

Rats were vaccinated with exosomes plus an alum adjuvant, which generated polyclonal antibodies that recognize exosomes, with the aim of it having minimal reactivity to other components in HES. The anti-exosome antisera recognized a large number of bands on exosome protein blots, but also reacted to HES that had been previously depleted of exosomes. This may be due to incomplete exosome purification from HES during ultracentrifugation, or there may be shared proteins in exosomes and HES. Despite this, there are several bands recognized by the antisera, such as those found at approximately 12 and 38- kDa, which appear to be exclusive to exosomes. Further characterisation of the exosome protein profile was performed by two-dimensional gel electrophoresis, which also highlighted the reactivity of exosome antisera by western blot. A comparison with total HES will be important to determine any spots which overlap, as many proteins in HES have already been identified using this method [255]. Despite the low abundance of exosomal proteins detected in the 2D gel (possibly owing to the low protein amount used for this assay), there is some overlap/localization to proteins in total HES that were visualized using the same method [255]. These proteins include enolase, zinc metalloprotease and apyrases, which are further recognized by anti-exosome polyclonal antibodies.

Tetraspanins from parasites have been recently suggested as promising targets for vaccination, as shown in *Echinococcus multilocularis,* the causative agent of alveolar echinococcosis [173, 225]. Parasite tetraspanins have also been recently identified in *S. mansoni* EVs [226], and further implicated in similar vaccination strategies, whereby antibodies blocked tetraspanin-mediated uptake of *O. viverrini* EVs by host cells [227]. This suggests targeting exosomes and their surface proteins

may provide an important anti-parasite vaccination strategy. Consequently, antibodies generated against *H. polygyrus* TSPAN-11 also recognise exosomal proteins by both western blot and flow cytometry. However, given that this antiserum does not recognise proteins of the correct size to TSPAN11, we cannot be confident in their use for future studies. Flow cytometry also allowed us to determine surface protein expression and reactivity by polyclonal anti-exosome antibodies or cross-reactivity by antibodies generated against murine ALIX. However, as stated previously, EV observation by this particular flow cytometry method does not allow EV quantification, or help discriminate between different vesicle subsets [268].

Crosslink immunoprecipitation and subsequent mass spectrometry analysis allowed for the isolation and identification of exosomal proteins that are recognized by polyclonal antibodies. Some of these proteins have been associated with inert exosome functions, including zinc metallopeptidase and ubiquitinases, which have been shown to mediate vesicle cargo selection in both mammalian EVs and pathogenic EVs [198, 209, 269]. Other exosomal proteins, such as scavenger receptors, vacuolar ATPase subunits or transthyretin-like proteins are associated with exosome lipid transfer or in secretion and uptake of EVs [223, 257, 270]. Furthermore, proteins such as Histone deacetylase-1 and HSP70 have been associated with immune modulation through epigenetic inhibition of Th2 responses, or in antigen presentation and immune cell activation [259, 260]. Interestingly, Galectin-1 has been shown to mediate effects through the induction of tolerance, activating T regulatory cell populations [258], and has also been shown to suppress inflammation in experimental colitis [271]. It seems prudent to hypothesise that

helminths may use exosomes to deliver these immunomodulatory molecules to regulate inflammation that is induced by barrier damage and bacterial infiltration. Additionally, exosomes may also mediate bacterial regulation through the secretion of anti-microbial compounds, including lysozyme-2 [246], and PI-type proteinases [272]. Identification of antibody-reactive proteins in this study would allow the development of specific monoclonal antibodies against *H. polygyrus* exosomes. Several proteins on this list, including metallopeptidases, serpins, and the nicotinic acetylcholine receptor were all listed as vaccine candidates against the filarial nematode *Onchocerca volvulus* [273]. Additionally, microsomal aminopeptidases, such as Antigen H11, have long been associated in vaccine development against *H. contortus* [274]. More recently, glyceraldehyde-3-phosphate dehydrogenase-like molecules (GAPDH) have been included in the potential vaccine candidate list from *S. mansoni* EVs [226]. Finally, there are a number of studies showing the effectiveness of vaccination against helminthic HSP70 in protection against parasite challenge, including *S. japonicum* [275], *L. sigmodontis* [276], and *T. spiralis* [277], further cementing the utility of exosome-associated proteins as vaccine targets.

It must be recognized that the proteomic characterization of *H. polygyrus* exosomes only highlights one aspect of their cargo. It has been shown that *H. polygyrus* exosomes contain a suite of small RNAs, including miRNAs [219], and microarray analysis showed that *H. polygyrus* exosomes induced regulation of murine genes, including DUSP1 and the IL-33 receptor. A specific cocktail of these miRNAs suppressed the murine phosphatase DUSP1, a key regulator of the MAP kinase pathway. Interestingly, cystatin molecules secreted by *A. viteae* promoted and sustained transcript expression of IL-10 in macrophages isolated from DUSP1$^{-/-}$ mice

[74]. However, despite having parasitic miRNA binding sites in the 3' untranslated region (UTR) of the gene for the IL-33 receptor, a molecule associated in anti-parasitic responses [278], it was not functionally repressed by several synthetic parasite miRNAs tested. This suggests that whilst some exosomal functions may be exerted or enhanced by miRNAs, this does not account for all suppressive mediators within the exosome cargo. Furthermore, analysis of the lipid repertoire of *H. polygyrus* exosomes highlights the differential enrichment of particular lipid species, such as plasmalogens, compared to mammalian exosomes [279]. This paper demonstrated that *H. polygyrus* exosomes had greater membrane rigidity and enrichment of lyso-phosphatidylserine, a molecule also secreted by *S. mansoni*, which is shown to be important in TLR2-mediated immune modulation [280].

Overall, these data provide an initial characterization of extracellular vesicles found in the ES products of *H. polygyrus,* which is geared towards the development of tools to detect or inhibit these complexes based on their protein markers. These vesicles contain a cohort of proteins enriched in mammalian exosomes, and are morphologically similar to vesicles as described elsewhere [159, 220]. Their origin was hypothesized through imaging and the enrichment of parasite intestinal proteins. The development of polyclonal sera allowed for identification of *H. polygyrus* exosomes by traditional protein detection assays. Proteomic analysis of the exosome pellet also revealed the presence of potential immunomodulatory proteins. To investigate the role of *H. polygyrus* exosomes during infection, I determined the functional properties of these exosomes with both intestinal and immune cells *in vitro*, which is discussed in the next chapter.

Chapter 4

The impact of *H. polygyrus* exosomes on host cell responses *in vitro*

4.1 Introduction

The data discussed in chapter 3 focused on the primary characterization of exosomes isolated from the ES of adult *H. polygyrus*. This revealed the presence of modulatory proteins and suggests a potential mechanism of cross-species regulation used by the parasite. The excretory-secretory products of other parasitic nematodes have been shown to contribute to immunomodulation, facilitating parasite evasion of host immunity, and enabling the establishment of chronic infection [281]. *H. polygyrus* ES (HES) contains a large cohort or bioactive molecules which have potent immunoregulatory functions [243]. For example, *H. polygyrus* secretes a functional TGF-β homologue, which initiates immune regulation through the induction FOXP3+ regulatory T cells [151, 282].

Recently, our group showed that extracellular nanovesicles are present in the ES from adult *H. polygyrus* culture. This study [219], where I contributed to the manuscript as a co-author, highlighted the potential functions of parasite-derived exosomes in the host-helminth relationship. It has long been appreciated that intracellular pathogens can secrete extracellular vesicles, or induce their infected host

cells to do so (See Table 4.1). There is evidence that some intracellular pathogens use EVs to subvert a host immune response, therefore promoting their survival. For example, the Epstein-Barr virus (EBV) has been shown to direct exosome release from infected B cells, which contain viral miRNAs that are used to suppress host anti-viral immunity [283]. Furthermore, the protozoan parasite *L. donovani* is shown to induce the production of immunomodulatory EVs from macrophages [197], and also release their own EVs that have an immunosuppressive effect on myeloid-derived cells [193]. Furthermore, microvesicles secreted by *Plasmodium falciparum*-infected red blood cells are shown to activate both pro-inflammatory and anti-inflammatory cytokine responses [284].

At the beginning of this PhD project, there were only a handful of studies documenting extracellular vesicles in the ES material of extracellular pathogens, including the parasitic protozoan *Trichomonas vaginalis* [209], and the trematodes, *F. hepatica* and *E. caproni* [220]. The discovery of nematode-derived exosomes within adult ES allows us to speculate why a parasite would release such vesicles into harsh host environments, such as the intestine. Indeed, exosomes are shown to protect their contents, including miRNAs [285], from environmental factors such as RNases and can be therefore used as biomarkers based on their presence within a multitude of body fluids. The ability of exosomes to withstand the intestinal environment has been demonstrated through the use of grape-like EVs to treat DSS-colitis [286]. Indeed, our group showed that *H. polygyrus* exosomes protect nematode RNA species, including miRNAs, from degradation [219]. As such, it will be interesting to determine whether other forms within the *H. polygyrus* lifecycle,

such as the the L3 or L4 larval stages, can secrete EVs within their own ES products.

Over the course of this study, more data emerged on extracellular parasite-derived EVs, highlighting their potential as immunomodulators (See Table 4.1). Some EV-derived molecules, such as miRNAs, are hypothesised to have direct immunosuppressive properties, and are found in parasites such as *Brugia malayi* [247] and trematodes including *Dicrocoelium dendriticum* [248] and *S. mansoni* [249]. Thus, parasite-derived EVs have potential as future therapeutics, replicating the immune-regulating properties of the parasites they derive from [287]. Additonally, other molecules, such as cathepsin L1 found on *F. hepatica and Brugia malayi* EVs, have been shown previously to interfere with monocyte pattern recognition and modulate T cell responses [247, 250]. Conversely, some helminthic EVs actually have immunostimulatory properties. *O. viverrini* exosomes were shown to induce production of the pro-inflammatory cytokine IL-6 in a cholangiocyte cells [227], and EVs from *S. japoniucm* were shown to stimulate M1 polarisation [261]. EVs from trematodes also contain a number of proteases [220, 248], which may facilitate tissue migration, expanding the potential roles of parasite EVs during infection.

We must consider the potential interactions occurring at the parasite-host interface during EV-mediated responses. One of the most commonly proposed methods for EV uptake is endocytosis [179, 288], although this may be context dependent, with studies showing exosome uptake by macropinocytosis, direct fusion or phagocytosis (all reviewed in [179]). Interestingly, a recent study has chronicled

the uptake and intracellular trafficking of HEK293 exosomes by human fibroblast cells, and has demonstrated that uptake is initiated through filopodial contact and endocytosis in an actin-dependent manner [177]. Despite recent advances, a majority of this work has focused mammalian exosomes, both in cancer [289, 290] and immunity [178, 291]. However, there are a number of studies demonstrating the uptake of EVs from intracellular pathogens. For example, Leishmania-derived EVs are taken up by macrophages [292], and the uptake of microvesicles from *P. falciparum*-infected RBCs promoted the transfer of genetic material [236]. There is also more detailed data showing that exosomes from EBV-infected cells generate their function in recipient epithelial cells following internalization by caveolin-mediated endocytosis [293]. However, evidence of helminth-EV uptake is scarce, with only two studies demonstrating uptake in intestinal epithelial cells [219, 220]. There was also a recent study showing that *S. japonicum* EVs can be internalized by murine liver cells and transfer their small RNA cargo [294]. Importantly, antibodies generated against EV-tetraspanins from the liver fluke *O. viverrini* can block their uptake by cholangiocytes, thus limiting their function [227]. As detailed in the previous chapter, exosome surface proteins are recognized by specific antibodies, and further analysis may identify proteins that mediate uptake. A greater understanding of helminth EV uptake will be crucial when designing future therapeutic interventions.

This chapter examines the interactions between *H. polygyrus* exosomes and host cells. In particular, the effect on uptake by time and stimulatory conditions is examined in two different cell types. Furthermore, I demonstrate exosome uptake

can be manipulated, either by the use of chemical inhibitors or specific antibodies. The immunomodulatory impact of *H. polygyrus* exosomes on host cells is examined, with particular focus on cells present within the intestinal environment, including macrophages and epithelial cells. This study further investigated how these exosomes can suppress aspects of type-1 and type-2 immune responses, as both would be induced during a natural *H. polygyrus* infection. In order to add more complexity in our investigation of exosome-host cell interactions, and overcome the difficulties of isolating exosome-specific roles in intestinal environment during natural infection we employed the use of an *ex vivo* small intestinal organoid culture. The data presented from the organoid work highlighted some key technical challenges, as well as some interesting preliminary data with regards to uptake in a multicellular system.

Table 4.1 Pathogen-derived EVs and their proposed functions

Pathogen	Exosome origin	Functional response	Effector Mechanism(s)	Ref
Protozoa				
Leishmania amazonensis	Infected macrophages	Promotion of Th1 responses	Naïve macrophages are primed to release IL-12, IL-1β and TNF	[295]
Leishmania donovani	Promastigotes	Invasion/persistance within host cells and delivery of virulence factors	*Leishmania* elongation factor 1a and GP63 activate host protein-tyrosine phosphatases in monocytes. GP63-induced exosome cargo selection and inhibit host miRNA processing	[193, 194, 197, 199, 292]
		Induction of *Leishmania*-peptide carrying exosomes from monocytes	Increase in IL-8 secretion by macrophages. Induces release of IL-10 in human monocytes, whilst suppressing release of TNF	[193, 292]
Leishmania major	Promastigotes	Invasion/persistance within host cells and delivery of virulence factors	*Leishmania* elongation factor 1a and GP63 activate host protein-tyrosine phosphatases in monocytes	[196, 198]
		Increased disease exacerbation and Th2 polarisation *in vivo*	Increase in the number of IL-4 producing CD4 T cells/decrease in the number of IFNϒ-producing CD4+ T cells	[193]
	Stationary phase	Enhanced parasite replication/Lesion exacerbation	Increased levels or IL-4 and IFN-γ, with over-induction of IL-17A and IL-10 in the lymph node	[296]
Leishmania mexicana	Infected macrophages	Immunomodulation of host signalling events promoting parasite survival	Upregulation of adenosine receptor 2A by parasite-derived GP63 contained within host exosomes and activate host protein-tyrosine phosphatase e.g. SHP-1	[197, 297]
	Stationary phase	Inhibition of alarmin response allowing survival	GP63-mediated cleavage of NLRP3 inflammasome, abrogation of ROS production and IL-1β maturation	[298]
Plasmodium berghei	Infected erythrocytes	Activate systemic inflammation and T cell priming	Via MyD88/TLR4 pathway and CD40/CD40L interactions	[299]
Plasmodium falciparum	Infected erythrocytes	Transfer of parasite material and parasite dissemination	Innate cell activation. Cytokine induction in macrophages (IL-6, IL-12, IL-1β & IL-10) in dose-dependent manner	[284]
		Commitment of asexual parasites to gametocytes	Transfer of genetic information between parasites and budding of EVs via PfPTP2	[236, 284]
Plasmodium vivax	Erythrocytes, leukocytes	Higher acute fever and length of malaria symptoms in humans	Unknown mechanism	[300]
Trypanosoma cruzi	Trypomastigotes	Th2 polarisation. Parasite dissemination, adhesion and enhanced parasite survival	Increase in IL-4 and IL-10 secretion, and reduction in iNOS expression in CD4 T cells and macrophages.	[201, 301]
	Trypomastigotes	Modulation of innate inflammation and chronic phase immunopathology	gp85/trans-sialidase (TS) superfamily and α-Gal glycoprotein enriched EVs - TLR2/innate activation and production of IL-10 by splenic T/B cells	[302]
	Infected lymphocytes and erythrocytes	Parasite invasion and inhibition of complement-induced parasite elimination	Plasma membrane-derived vesicles containing surface TGF-β, which promotes entry into host cells	[303, 304]

Table 4.1 - Pathogen-derived EVs and their proposed functions *continued* [a]

Pathogen	Exosome origin	Functional response	Effector Mechanism(s)	Ref
Protozoa				
Trichomonas vaginalis	Mature parasites	Limit neutrophil migration to site of infection	Metalloproteases downregulate IL-8 secretion in ectocervical cells. Promote adherence of weakly adherent non virulent strains.	[209]
Trypanosoma brucei	Procyclic forms of the parasite	Improved entry into host cells, enhanced parasite survival	Abundance of parasite-derived proteases e.g. oligopeptidase B – favours parasite invasion	[207, 305]
	Flagellular nanotubules	Erythrocyte invasion, increased virulence and host anaemia	Transfer of SRA (serum-resistance associated protein) to other parasites to inhibit trypanosome lytic factors	[306]
Fungi				
Cryptococcus neoformans	EVs from fungal cell phase	Promote colonisation of infected tissues	Release virulence factors - glucosylceramide and glucuronoxylomannan	[214]
		Stimulate fungal killing	Enhanced IL-10 and TGF-β secretion nitric oxide production by macrophages	[217]
Malassezia sympodialis	Yeast - skin living flora component	Exacerbation of atopic dermatitis	Promote IL-4 and TNF secretion from PBMCs	[216]
Paracoccidioides brasiliensis	Yeast phase exosomes	Potential to skew to a suppressive Th2 response	Immunogenic EVs enriched in galactose-α-1,3-galactose which may bind host lectins potentially improve infectivity by fungi	[210]
Helminths				
Heligmosomoides polygyrus	Intestinal tract of adult nematode	Suppress classical inflammation and danger responses, promoting parasite survival	Suppression of host targets including IL-33R and DUSP1	[219]
Fasciola hepatica	Tegument or gastrodermis	Host immune-modulation and cell degradation	Immunomodulatory EV cargo e.g. cathepsin L and B peptidases	[220, 250]
Opisthorchis viverrini	Adult worms	Host cell inflammation, tumorigenesis and wound healing	Increase IL-6 production from cholangiocytes and stimulate protein dysregulation	[227]
Schistosoma japonicum	Adult worms	Polarization of host macrophages to M1 phenotype	Unknown mechanism	[261]
		Host gene regulation and modulation	Functional transfer of miRNAs e.g. Bantam, to host cells	[294]
Schistosoma mansoni	Adult worms	Nutrient acquisition and potential host modulation	Secretion of Saposin-like proteins & ferritin isoforms. Transfer of small non-coding RNAs	[226, 249]

[a]Details in each column (from left to right) describe: the parasite species, the life stage and/or cellular origin of the EV, the proposed functional outcome, the mechanistic data in support of this function, and the primary literature reference.

4.2 Results

4.2.1 Tracking differential uptake of *H. polygyrus* exosomes

Previously, it has been established that *H. polygyrus* exosomes can be visualized inside mouse epithelial cells after 24 hours [219]. Here I focused on understanding the timing and requirements for uptake, comparing epithelial cells and macrophages, two cell types highly represented in small intestinal tissue, the natural environment for *H. polygyrus* adult worms [240]. Exosomes purified from adult HES by ultracentrifugation were labelled with a PKH67 fluorescent dye, which incorporates into the lipid-rich membrane of the exosomes, and is detectable by both flow cytometry and confocal microscopy [219]. As an uptake control, the dye was also prepared without exosomes to control for any aggregates that carry over during ultracentrifugation that would label cells (See gating strategy Figure 4.2.1A). Any exosomes present on the surface of cells were removed by treating cells with trypsin 5 min prior to acquisition, and uptake of the dye alone was detectable in <1% of cells, supporting the use of PHK67 positive cells as a marker of exosome uptake. By flow cytometry, initial observations show *H. polygyrus* exosome uptake steadily increasing in $F4/80^+$ bone-marrow derived macrophages (BMDMs), the MODE-K small intestinal epithelial cell line, and the RAW 246.7 cell line (a mixed monocyte/macrophage cell line [238]), over the course of 24 h (Figure 4.2.1B), represented by the % of exosome-positive cells. Notably, this rate of uptake is not exosome specific, as similar uptake rates were observed using mammalian exosomes. By confocal analysis, *H. polygyrus* exosome uptake over time was also verified in $F4/80^+CD11b^+$ BMDMs (Figure 4.2.1C).

4.2.2 *H. polygyrus* exosome internalisation is modulated by both Cytochalasin D treatment and macrophage polarisation

To test whether *H. polygyrus* exosome uptake occurs by an active process, BMDMs were co-treated with exosomes and cytochalasin D, a potent inhibitor of actin polymerization. Exosome uptake was effectively abolished after 1 h, and was still inhibited after 24 h in BMDMs in the presence of cytochalasin D co-treatment, evaluated by both flow cytometry (Figure 4.2.2A) and confocal microscopy (Figure 4.2.2B). It was then established whether the polarization of macrophages to either an M1 or M2 phenotype affects parasite-derived exosome uptake. Both cell types are present in the small intestine during an immune response to both infective larval stages and adult *H. polygyrus,* as well as the bacterial infiltrate resulting from barrier damage [307, 308]. BMDMs were pre-treated with 20 ng/ml IL-4/IL-13 or 500 ng/ml LPS, or media alone for 24 h prior to the addition of *H. polygyrus* exosomes for 1 h, and cells were then assessed for exosome uptake by flow cytometry (Figure 4.2.2C) or confocal microscopy (Figure 4.2.2E). It was found that LPS pre-treatment significantly represses the ability of BMDMs to take up exosomes early on, compared to either stationary BMDMs or those polarised by IL-4/IL-13. A similar pattern of uptake was shown in LPS or IL-4 prestimulated bone marrow-derived dendritic cells (BMDCs) (Figure 4.2.2D). This data is consistent with previous reports showing that nanoparticle uptake is superior in M2-polarised compared to M1-polarised macrophages after 1 h [309]. Additionally, LPS repression of exosome uptake may suggest that some early internalisation occurs by phagocytosis, as this is inhibited in macrophages following LPS pre-stimulation [310].

4.2.3 Antibodies enhance internalisation of *H. polygyrus* exosomes in bone-marrow derived macrophages

Recently, it has been demonstrated that epithelial cell uptake of EVs from *O. viverrini* can be inhibited using specific tetraspanin antibodies (a key exosome protein) [227]. In chapter 3, I demonstrated the capacity of exosomes to generate polyclonal antibody responses following an alum vaccination schedule. I therefore aimed to investigate whether antibodies generated against *H. polygyrus* exosomes would interfere with uptake. Exosomes were incubated with BMDMs stimulated with either LPS, IL-4/13 or media in the presence of rat polyclonal antisera for 1 h, and exosome uptake was assessed by confocal microscopy (Figure 4.2.3A) or flow cytometry (Figure 4.2.3B). Antisera treatment significantly enhanced the early uptake of exosomes, regardless of stimulation, even in LPS pre-treated macrophages, which take up few exosomes in the absence of antibody. We also noted a more dispersed pattern of exosomes within antiserum-treated cells after 1 h, suggesting that their function properties might also be altered by antibody-mediated uptake (tested in further studies below). After 24 h, the enhancement of uptake by polyclonal anti-exosome sera was still evident in either IL-4/IL-13 or LPS-stimulated cells. However, this was not reflected when BMDMs were co-treated with exosomes and TSPAN11 antibodies (Figure 4.2.3C), which may be due to the non-specificity of this antiserum (Figure 3.2.4D). Furthermore, attempts to interfere with exosome-specific antibody-mediated EV uptake were not significantly diminished by using naïve rat IgG to outcompete the Fc receptor.

4.2.4 Microarray of exosome-stimulated MODE-k epithelial cells.

During natural infection, *H. polygyrus* adult worms coil around the villi in the small intestine, and would therefore be in direct contact with the uppermost layer of cells; the intestinal epithelium [307]. Thus, MODE-K cells, an immortalized small intestinal epithelial cell line [239], were used to address the effect of *H. polygyrus* exosomes on global gene expression of murine cells. A microarray analysis determined that approximately 128 genes were differentially expressed upon exosome treatment (using a false discovery rate (FDR) cut-off of $P<0.05$) [219], of which the top ten genes showing highest fold change are shown in Table 4.2.4A. To gain further insight into the potential mechanisms of the exosome gene modulation, the identified 128 genes were mapped to networks available on the Ingenuity database (Ingenuity Systems, www.ingenuity.com). The major networks within canonical and disease pathways, as well as biological effects, were identified and were ranked by the score in the *p*-value calculation of the IPA assay (Table 4.2.4B). The input array data revealed a significant link to the p38 MAPkinase pathway by IPA. Two genes associated with this signalling cascade, *il1rl1* and *dusp1* (a member of the IL-1 receptor family and a key MAPK regulator respectively), are significantly downregulated in MODE-k cells upon exosome treatment (see Figure 4.2.4A for schematic), further confirmed by qRT-PCR (Figure 4.2.4B). Notably, exosome-mediated suppression of IL-33R in MODE-k cells was also evident at the protein level, and was suppressed by co-treatment with cytochalasin D (Figure 4.2.4C).

4.2.5 Transfection of MODE-k epithelial cells

There are a number of potential mechanisms that could mediate depression of DUSP1 and IL-33R, as shown in the microarray analysis of MODE-k cells [219]. The 3′ untranslated region (UTR) of both *dusp1 and il1rl1* messenger RNAs (mRNAs) contain conserved 7-9 nucleotide 'seed sequences', which provide available binding sites for *H. polygyrus* miRNAs (Figure 4.2.5A). I also tested any non-specific or potential downstream effects of either parasite miRNA on both target genes. Of these, transfection of individual parasitic miRNAs yielded no significant differences in the levels of the two target genes (Figure 4.2.5B and C), whereas a direct DUSP1 siRNA could suppress both genes. To control for any non-specific effects of miRNA/siRNA delivery via transfection, I used a non-gene targeting control siRNA, Select Negative Control No.1 (NT1). Additionally, as a positive control for transfection, I used a synthetic siRNA which specifically targets *dusp1,* helping to establish a baseline for transfection efficiency and allows for comparison to *H. polygyrus* miRNA mimics which target this particular gene. It is currently unknown whether the target genes discussed above could be linked in a signaling cascade, and is an interesting avenue for future investigation. However, transfection of MODE-k epithelial cells with a cocktail of parasitic miRNAs suppressed *dusp1* gene expression, but not *il1rl1*, as shown using a luciferase reporter assay [219], suggesting that secreted parasite miRNAs could work in tandem to exert maximal effects on host *dusp1*, whereas *il1rl1* suppression may be mediated by other exosome cargo.

4.2.6 Exosome modulation of type-2 immune responses in small intestinal epithelial cells and RAW 264.7 cells

During intestinal helminth infection, parasite egress into the intestinal lumen results in epithelial cell barrier damage [311]. In response to the resulting bacterial infiltrate, intestinal helminths have been shown to use their ES products to limit host pathology and danger responses, and induce tolerance pathways to facilitate barrier repair [312]. Initial investigations determined whether exosomes could modulate small intestinal epithelial cell line responses to a large range of toll-like receptor ligands and stress molecules, including LPS, PAM-CSK3, Poly I:C, CpG, IFNγ and, HMGB1. Unfortunately, this cell line showed very little response to most types of TLR stimuli (data not shown). However, exosomes, total HES or HES-depleted of exosomes were able to induce of suppression of both LPS or PAM-CSK3-mediated IL-6 production (Figure 4.2.6A and B). Next, it was investigated whether exosomes could modulate TLR-driven inflammation in more immune-responsive cells, as helminth ES has been shown to impair the ability of myeloid cells to respond to TLR stimulation [313, 314], and these cells are also shown to internalize *H. polygyrus* exosomes (Figure 4.2.1 and 4.2.2). Exosomes were shown to suppress both the transcriptional (Figure 4.2.6C) and cytokine (Figure 4.2.6D) hallmarks of LPS-activated RAW 264.7 cells, a macrophage cell line [238], including IL-6, IL-12p40 and TNF, and expression of inducible nitric oxide synthase (iNOS). Accordingly, exosome modulated expression of TLR4, the receptor for LPS, and MyD88, a downstream signalling molecule (Figure 4.2.6E). It should also be noted that exosomes fail to induce toxicity in these cells, as cell-death markers such as FAS and

Lymphotoxin-Beta receptor (LTBR), were also suppressed upon exosome treatment, even in the presence of LPS (Figure 4.2.6F).

4.2.7 Exosomes modulate classical activation in primary macrophages

Whilst the RAW 264.7 macrophage cell line provides a convenient alternative to primary cells, it must be recognized that both phenotype and function of any immortalized cells may change with continuous passage and culture. Mature BMDMs can also be polarized to the so-called 'M1' phenotype upon LPS stimulation. These cells are able to produce pro-inflammatory cytokines and effector molecules to mediate anti-bacterial immunity [315]. Although LPS pre-stimulation inhibited initial uptake of exosomes in BMDMs, we observe around ~50% of cells were exosome-positive after 24 h, which is 2-fold less than cells co-treated with LPS and exosomes (Figure 4.2.7A). Correspondingly, exosomes were still able to significantly suppress TNF and IL-6 release in BMDMs that had undergone LPS pre-treatment (Figure 4.2.7B). Thus, exosomes retain their suppressive properties even in cells previously primed with activating TLR4 ligands.

In a similar pattern to what was shown in RAW 264.7 macrophages (Figure 4.2.6C and D), *H. polygyrus* exosomes suppress the same classical activation markers, IL-12p40, TNF and iNOS, induced by LPS stimulation. This was further shown to be both time (Figure 4.2.7C-E) and dose dependent (Figure 4.2.7F-G). Moreover, like MODE-k epithelial cells, exosomes could also modulate expression of *dusp1* and *il1rl1* in BMDMs (Figure 4.2.7H-I).

4.2.8 Exosomes modulate alternative activation in primary macrophages

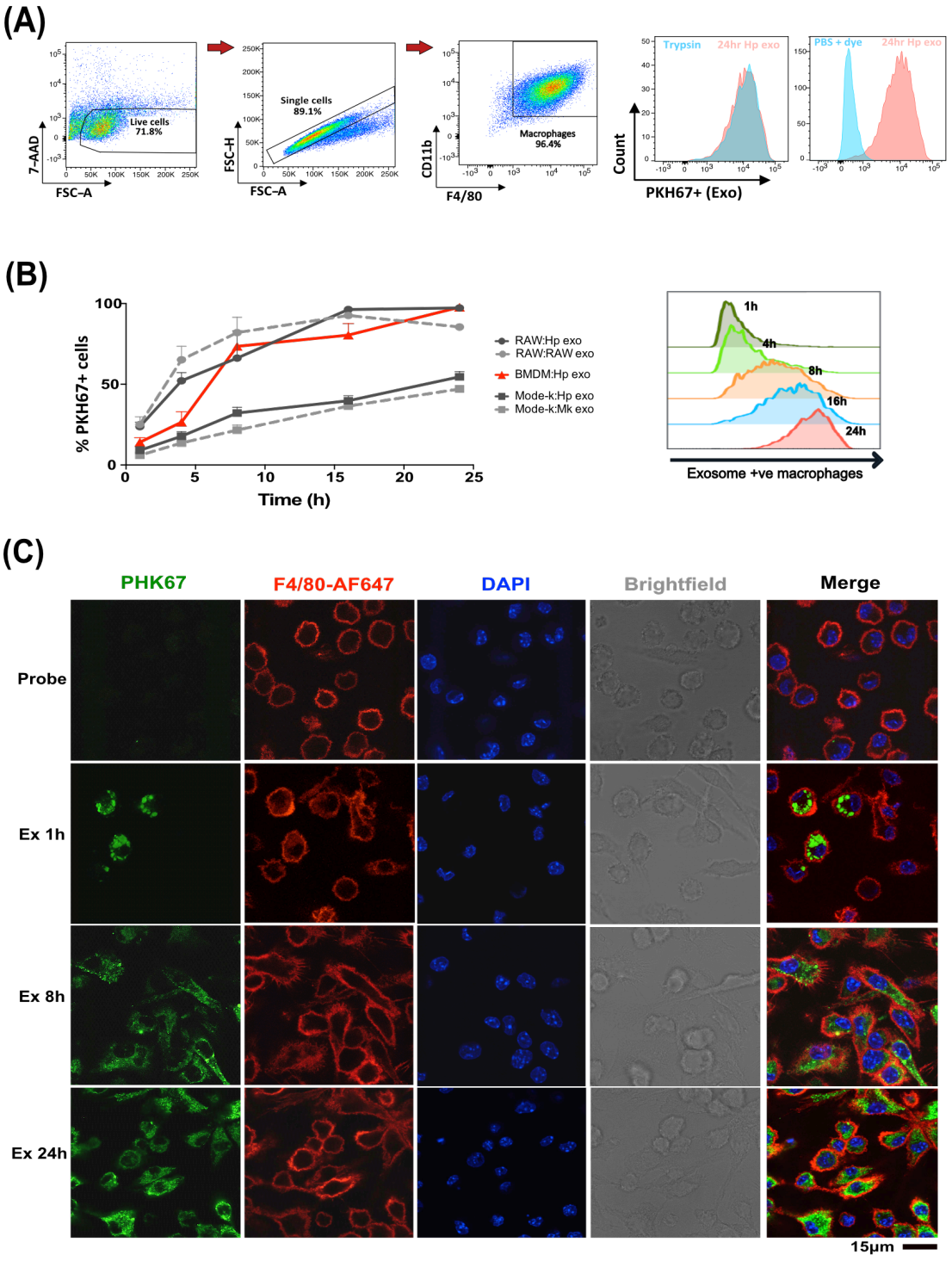
In order to prevent Th2-driven immune pathology and anti-parasitic responses, helminths can induce an immunosuppressive environment within their host. *H. polygyrus* ES products have been shown to activate FOX P3+ T regulatory cells [151], and can also suppress early ILC2 responses and downstream Th2-mediated inflammation in a model of airway allergy [141]. In addition to the studies on classical activation, a key component of this thesis was to examine the potential role of *H. polygyrus* exosomes in type-2 immune responses. As alternatively activated macrophages are shown to play a key role in mediating anti-helminth immunity [316], I therefore directly tested the suppressive effects of exosomes on the alternative activation of BMDMs following a 24 h co-culture with exosomes and IL-4 and IL-13, and included for comparison both total HES or HES that had been depleted of exosomes by ultracentrifugation ("Sup"). There was a marked ablation in transcriptional hallmarks of alternative activation in BMDMs [317], namely, RELMα, Ym1 and Arginase 1 (Figure 4.2.8A). This suppression is also reflected in levels of RELMα, Ym1, and the M2-associated chemokine CCL17 [318] released into culture supernatant (Figure 4.2.8B). To ascertain whether exosomes can also suppress the function and signature of cells that are already alternatively activated, the exosomes were also added to BMDMs at 24 h following IL-4/-13 pre-treatment. Subsequent production of RELMα and Ym1 were markedly reduced, as were release of IL-10 (Figure 4.2.8C) and surface expression of CD206 (the mannose receptor) (Figure 4.2.8D), further corollaries of alternative activation. Blocking exosome uptake by cytochalasin D (Figure 4.2.8D), in alternatively activated BMDMs

demonstrates a similar pattern to observations recorded in naive macrophages (Figures 4.2.2). Although the addition of polyclonal sera enhances uptake in IL-4/IL-13 treated macrophages after 1 h (Figure 4.2.3), similar levels of uptake were observed between polyclonal sera treatment and exosomes alone in IL-4/IL-13 treated macrophages after 24 h (Figure 4.2.8E). Importantly, both of these treatments abrogate the functions of exosomes, as reflected by the ability of AAMs to produce RELMα, Ym1 and CCL17 following co-treatment either with cytochalasin D, whereby exosome entry is blocked, or with polyclonal anti-sera (Figure 4.2.8F). These data suggest that the release of *H. polygyrus*-derived exosomes during infection could be a factor in preventing anti-parasitic host responses by macrophages.

4.2.9 Small intestinal organoids – interactions with exosomes

In order to generate a better understanding of how exosomes may interact with a variety of host cells during natural infection, I employed the use of *ex vivo* small intestinal cultures. Small intestinal organoids were derived from crypt stem cell isolation (see materials and methods), and either parasitic or mammalian exosomes (derived from the enterocyte cell line, MCICL2, [319]) were given directly into the culture media (Figure 4.2.9A and B), whereby uptake was measured after 1 h. To control for dye carry-over, the PKH67 dye was prepared in the absence of exosomes (Figure 4.2.9C). However, during natural infection the adult worms are situated in the lumen of the small intestine, and I hypothesise that the release of HES (and exosomes) by adult worms would also occur here. Therefore, preliminary studies were carried out by microinjecting exosomes into the lumen of these organoids in an

attempt to resemble the conditions that occur during infection. Initial optimization of this technique was determined using an artificial dye, which is demonstrated in Figure 4.2.9D. Organoids were also treated with IL-4 and IL-13 to recapitulate the intestinal conditions observed during a type-2 response to helminth infection [36]. Consistent with the previous study, recombinant IL-4/IL-13 treatment resulted in goblet cell hyperplasia (Figure 4.2.9E). Following exosome microinjection (approximating to 3 ng/organoid), I observed clustering of *H. polygyrus* exosomes within large subcellular spaces of the organoids (Figure 4.2.9F). I also observed a similar pattern of dispersal following microinjection of MCICL2 mammalian exosomes (Figure 4.2.9G). Although I hypothesise these large spaces to be goblet cells, I am currently unable to confirm this by co-localisation staining. However, I did observe the occasional exosome cluster within Paneth cells (Figure 4.2.9H), although this was much rarer. The ability to use multicellular tissues will provide a clearer picture of the fundamental interactions between exosomes and host cells, and represents an exciting body of work to be investigated in the future.



4.2.1 Tracking differential uptake of *H. polygyrus* exosomes

(A) Gating strategy for exosome uptake, denoting: live cells, doublet exclusion and macrophages. The histograms show expression of PKH67 (exosome-positive cells) after 24 h incubation (pink), compared to BMDMs pre-incubated with trypsin (*left*) or incubated with PKH67 dye alone (*right*). **(B)** Percentage uptake of PHK67-exosomes (derived from *H. polygyrus* ES, MODE-k or RAW 264.7 cells) incubated with BMDMs, RAW 264.7 or MODE-k cells from 1 h to 24 h (*left*) or shift in PHK67 expression in BMDMs (*right*). **(C)** Timecoure microscopy of PHK67-exosomes incubated with BMDMs. DAPI stain identifies nuclei and F4/80-AlexaFlour 647 labelleing shows macrophages. Images were taken using a Leica confocal laser microscope SP5 (x63 objective); scale bars indicate 15µm, with representative images shown from 2 independent experiments.

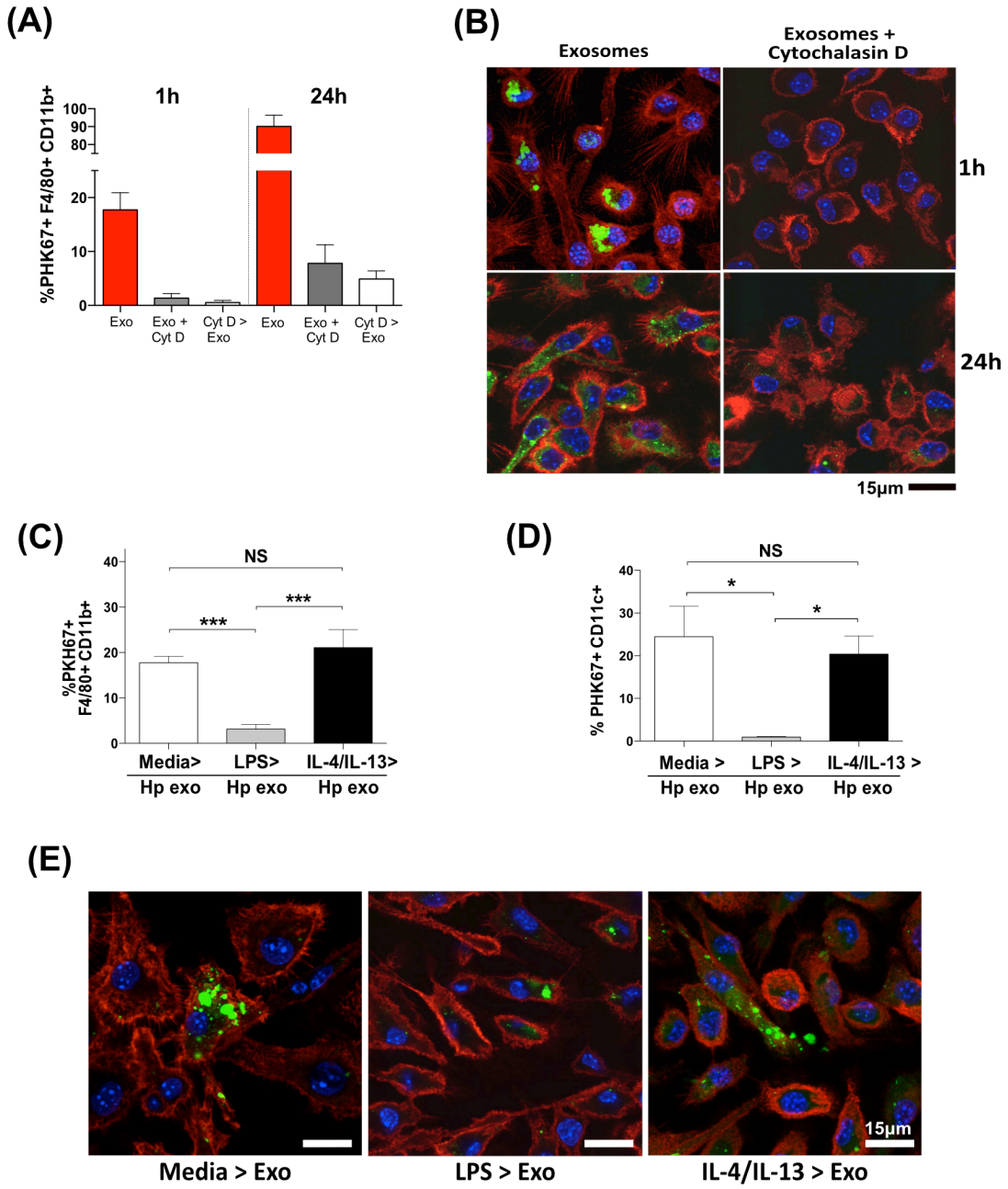


Figure 4.2.2 *H. polygyrus* exosome internalization is modulated by both Cytochalasin D treatment and macrophage polarization

Exosome uptake after 1h or 24h following pre-treatment (indicated by >) or co-treatment of BMDMs with 2ug/ml cytochalasin D, illustrated by proportion of PKH67^{+} macrophages analysed by **(A)** flow cytometry or **(B)** confocal microscopy. Myeloid cells were pre-treated (>) for 24 h with 20ng/ml IL-4/13, 500ng/ml LPS or media prior to addition of PHK67-exosomes for 1 h. Uptake was assesed in **(C)** BMDMs or **(D)** BM-derived dendritic cells by flow cytometry, and **(E)** BMDMs by confocal microscopy. BMDM/BMDC data were pooled from 2-3 independent experiments (in biological triplicate), presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result * = $p<0.05$, *** = $p<0.001$. For all microscopy, the same flourescent markers as stated in 4.2.1; scale bars indicate 15µm, with representative images from 2 independent experiments.

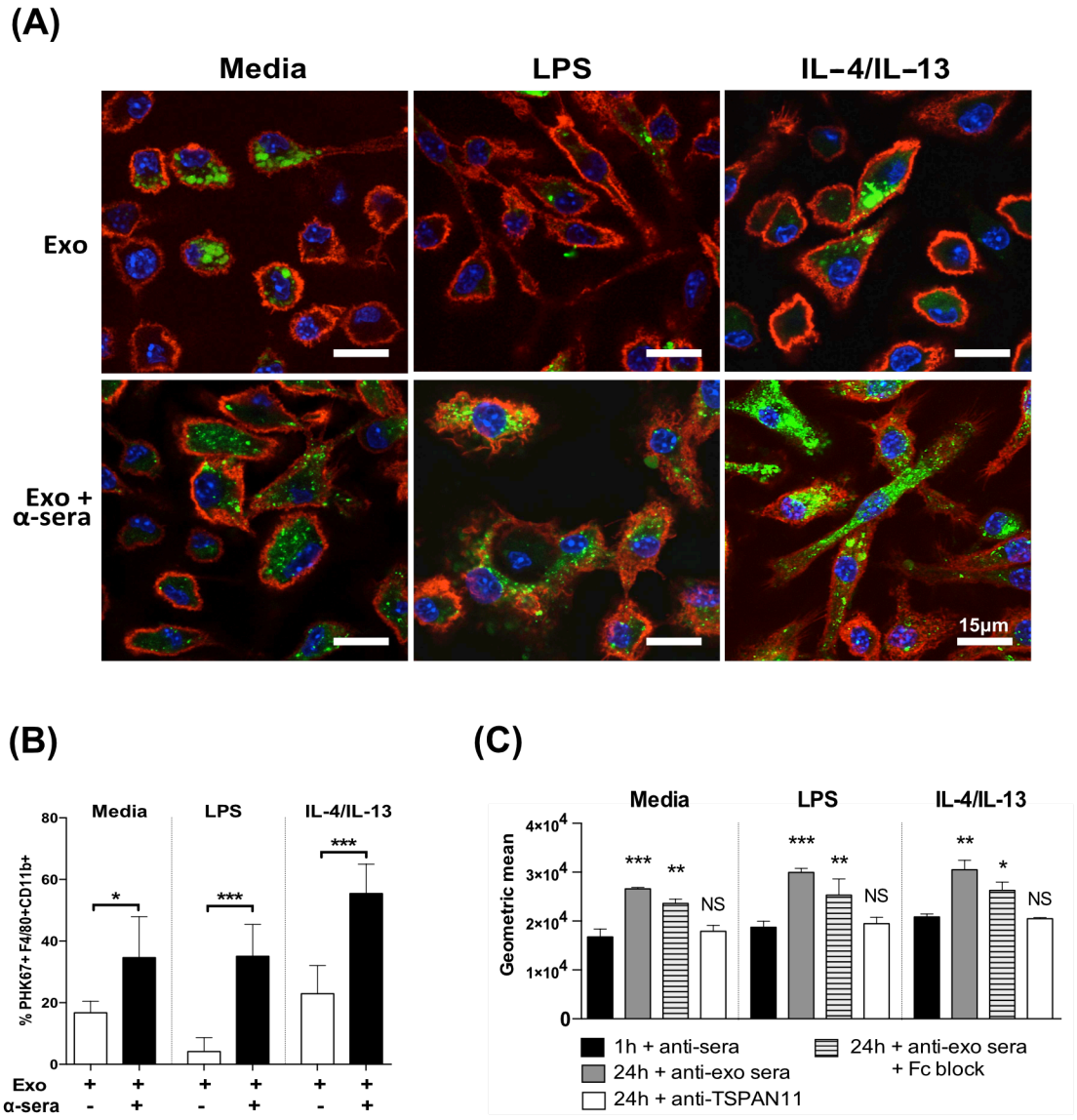
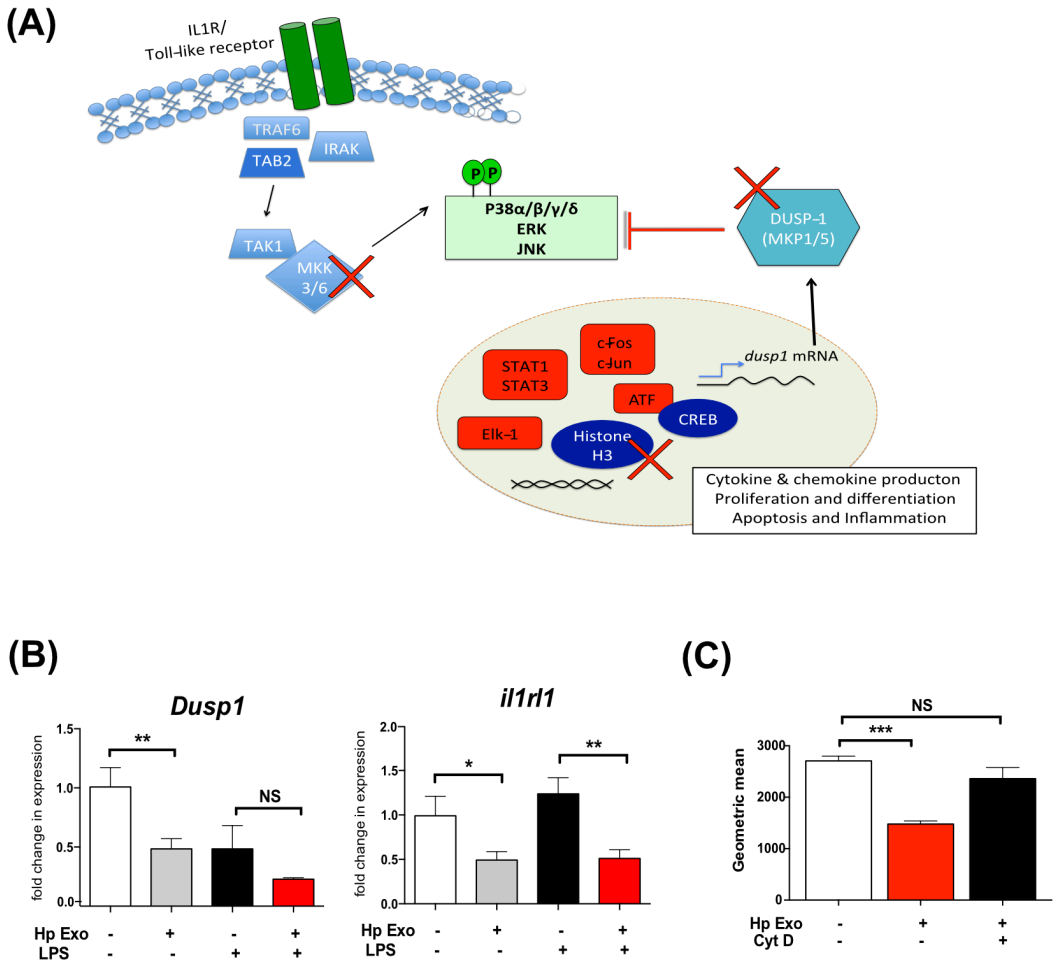


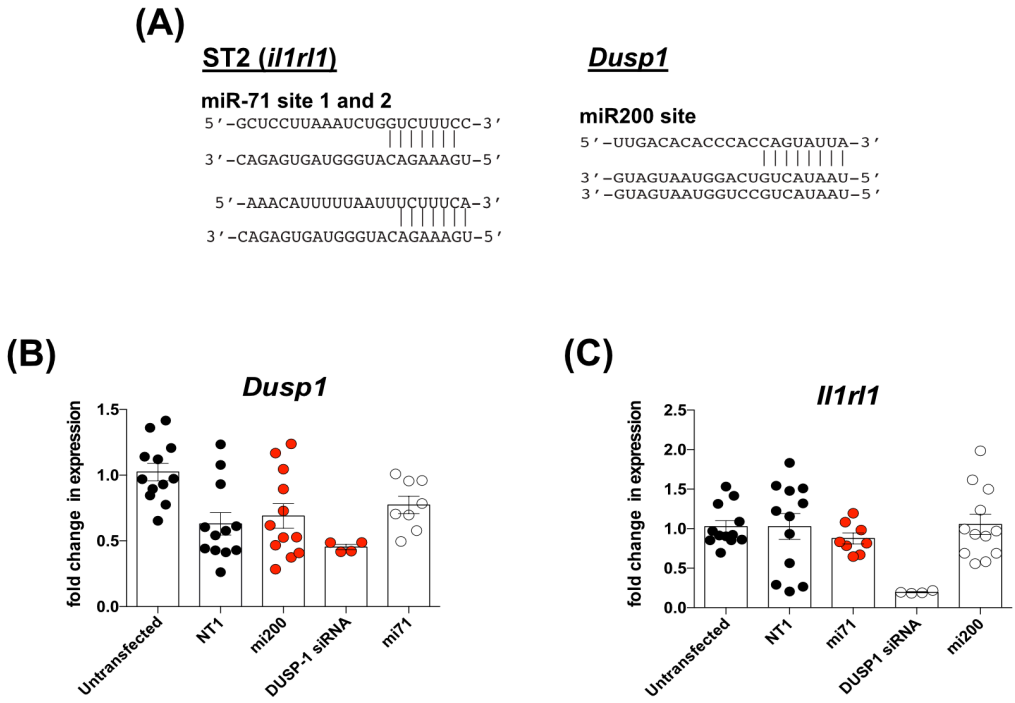
Figure 4.2.3 Specific antibodies enhance internalisation of *H. polygyrus* exosomes in bone-marrow derived macrophages

BMDMs were pre-treated for 24 h with 20ng/ml IL4/13, 500ng/ml LPS or media prior to addition of rat polyclonal exosome anti-sera (1:2000) and PKH67-exosomes for 1h. **(A)** Differential patterns of uptake were observed by confocal microscopy, **(B)** or determined by flow cytometry (% PKH67+ cells), or **(C)** the expression intensity of PHK67 in BMDMs after 1h compared to 24h co-treatment with anti-exosome sera, anti-TSPAN11 antibodies and 1µg/ml naive rat IgG (Fc block). For microscopy, the same flourescent markers were used as stated in 4.2.1; scale bars indicate 15µm, with representative images from 2 independent experiments. Flow cytometry data were pooled from 3 independent experiments (in biological triplicate), presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.



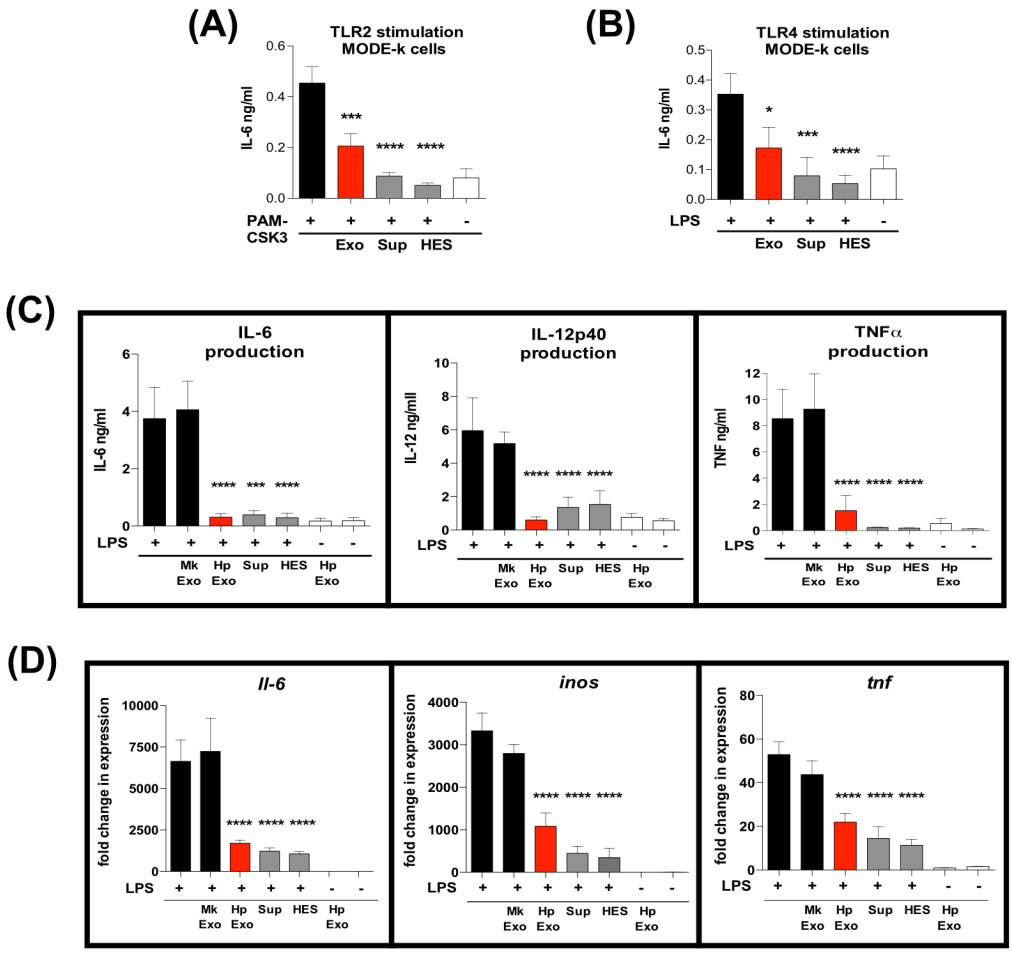
4.2.4 Microarray of exosome-stimulated MODE-k epithelial cells

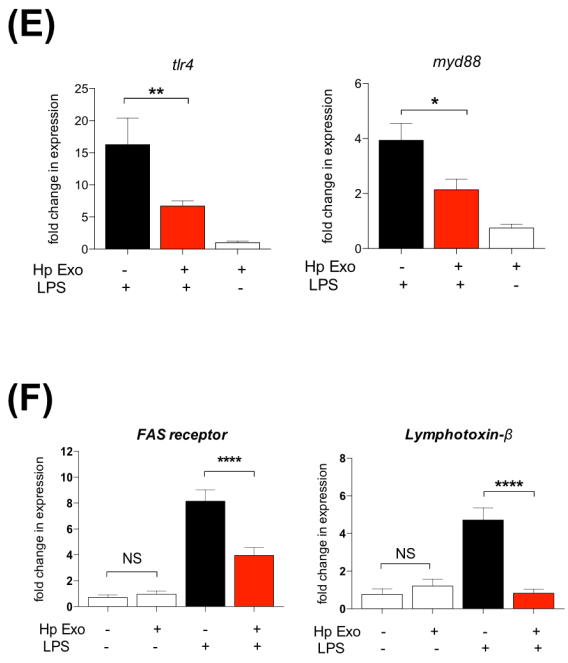
(A) Schematic of genes modified by *H.polygyrus* exosomes (shown by a red X) in the p38-MAP kinase pathway adapted from Ingenuity Pathway Analysis (IPA) of the top 128 genes changed in MODE-k cells from the microarray. DUSP1 (Dual specificity protein phosphatase 1) is transcriptionally induced by MAPK signalling components, e.g. ATF2, c-Jun and CREB, and negatively regulates MAPK by targeting phosphotyrosine and phosphothreonine residues. **(B)** qRT-PCR validation of microarray from MODE-k cells treated with 10ug/ml exosomes +/- 500ng/ml LPS, showing expression of the *Dusp1* or *il1rl1* transcript normalized to GAPDH. **(C)** Expression intensity of ST2 on MODE-k cells following 24 h treatment with 5ug/ml exosomes, +/- 2ug/ml Cytochalasin D. Data are representative of 2 independent experiments, presented as mean values ± SD (n = 8 wells/condition; one-way ANOVA). NS indicates a non-significant result. * = p<0.05. ** = p<0.01, *** = p<0.001.



4.2.5 Transfection of MODE-k epithelial cells with *H. polygyrus* miRNAs

(A) Potential binding sites for *H. polygyrus* microRNAs (miR-71, miR-365, Let-7 and miR-200) were identified by searching for seed-matching sites in murine 3'UTRs of *Dusp1* and *il1rl1*. **(B) + (C)** Relative expression of *Dusp1* and *il1rl1* in MODE-k cells following 24 h transfection with synthetic *H. polygyrus* miRNAs or a DUSP1 siRNA (50 nM), normalized to untransfected cells. Black dots indicate controls (NT1 = non-targeting siRNA control), red dots indicate synthetic siRNAs contain seed sequence match to gene analysed, white dots indicate siRNA does not contain seed sequence match to gene analysed. Data are pooled from 3 independent experiments, presented as mean values ± SEM.





4.2.6 Exosome modulation of type-1 immune responses in small intestinal epithelial cells and RAW 264.7 cells

(A) + (B) MODE-k cells were stimulated with 1µg/ml PAM-CSK3 or LPS +/- 10µg/ml exosome, HES depleted of exosomes (Sup) or total HES for 24h and levels of IL-6 were assessed by ELISA. **(C-F)** RAW 264.7 cells were stimulated with 500ng/ml LPS or media +/- 5µg/ml exosomes, Sup or HES for 24 h. (5µg/ml MODE-k exosomes used as a vesicle control). **(C)** Supernatants were tested by ELISA for levels of IL-6, TNF or IL-12p40. **(D)** Relative expression of classical markers; *il6, inos* and *tnf,* **(E)** LPS-responsive pathways; *tlr4* and *myD88,* and **(F)** stress markers; *fas* and *lymphotoxin-beta* were measured by qRT-PCR and normalized to GAPDH. Data are pooled from 3 independent experiments, presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001

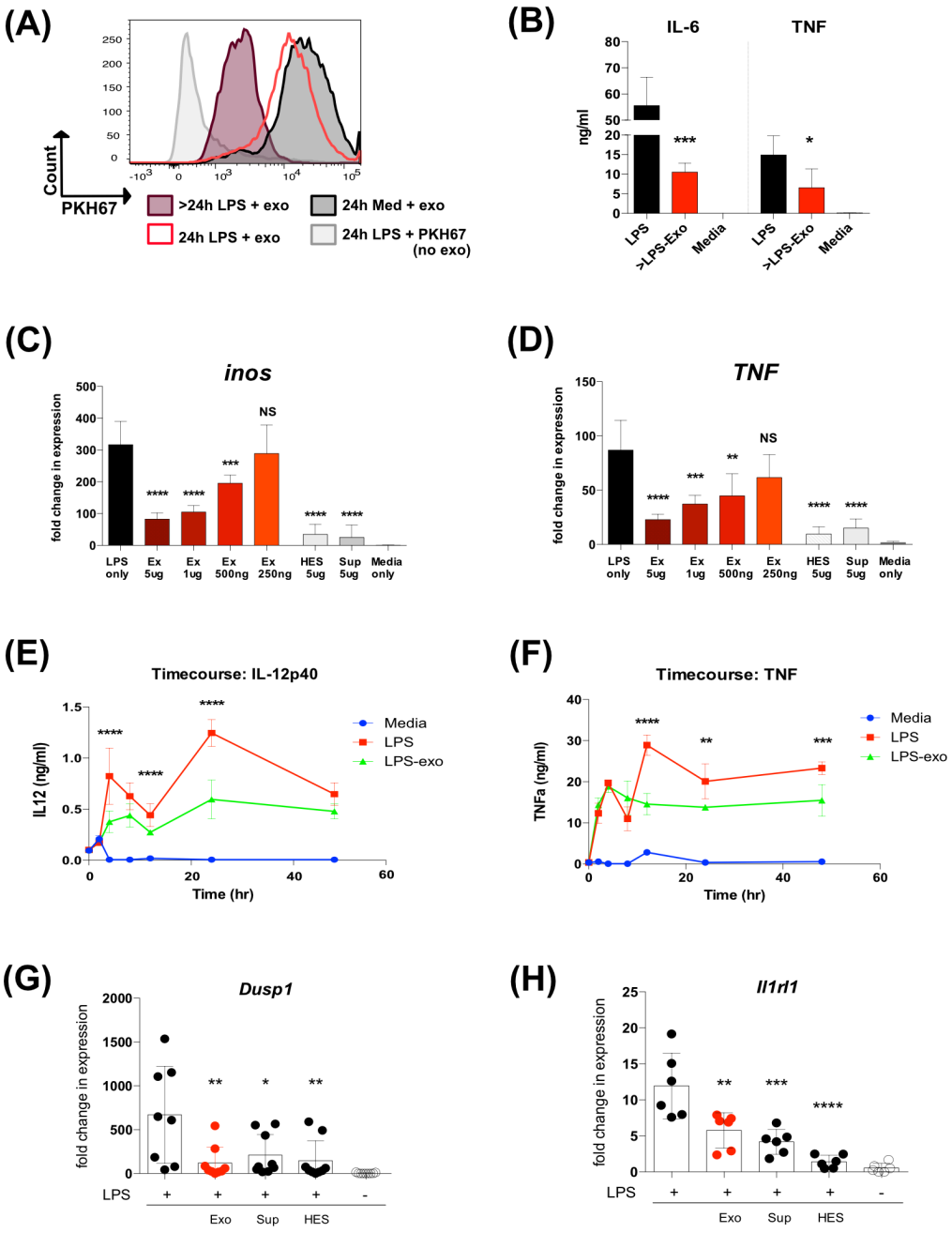
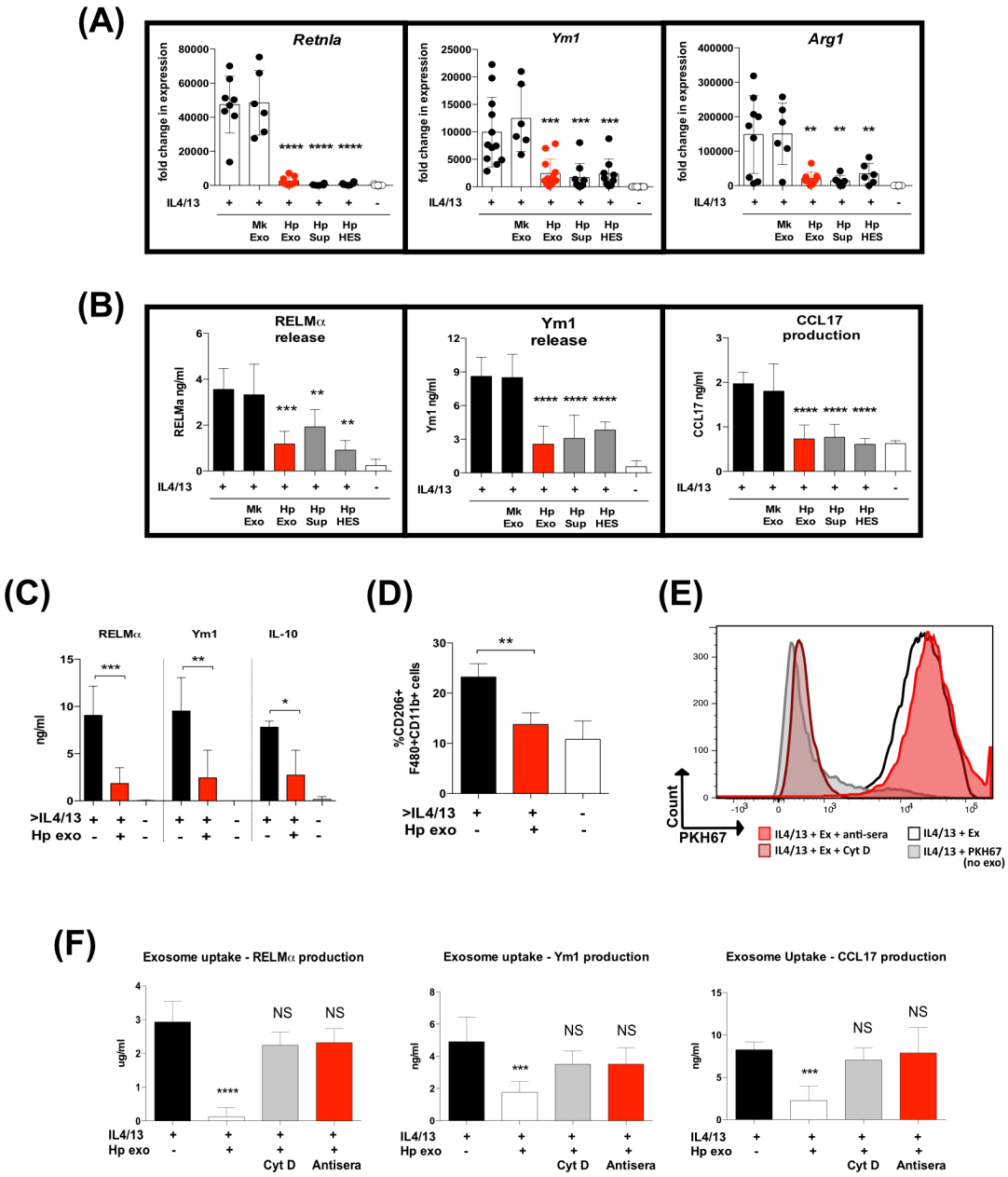


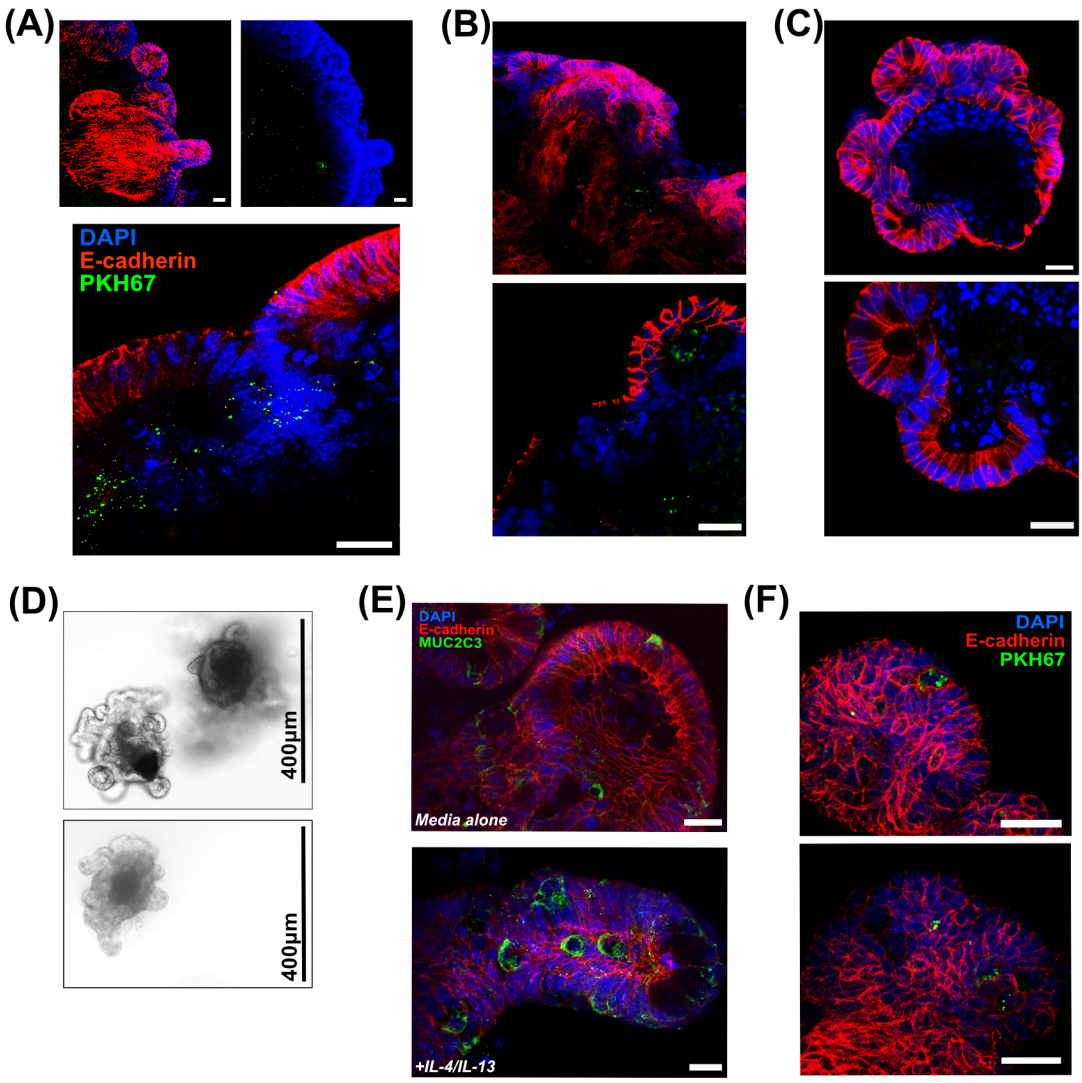
Figure 4.2.7 Exosomes modulate classical activation in primary macrophages

(A) Uptake of PKH67-exosomes determined by shift in flouresence and **(B)** supernatant levels of IL-6 and TNF assessed by ELISA in BMDMs pre-treated (indicated by >) or co-treated with media or 500ng/ml LPS +/- exosomes, Sup and HES. **(C)** + **(D)** Relative expression of *inos* and *tnf* were measured by qRT-PCR and normalized to GAPDH to determine dose dependent effect of exosomes (5-0.25µg/ml) compared to 5µg/ml of Sup or HES on LPS stimulation. **(E)** + **(F)** Supernatant levels of IL-12p40 and TNF released over 48 h in LPS/exosome co-treated BMDMs. **(G)** + **(H)** Relative expression of *dusp1* and *il1rl1* following 24h co-treatment of LPS +/- exosomes, Sup or HES normalised to GAPDH. Data are pooled from 2-3 independent experiments (in biological triplicate), presented as mean values ± SD; one-way ANOVA for **(B-D)** + **(G-H)**; two-way ANOVA for **(E-F)**). * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001



4.2.8 Exosomes modulate alternative activation in primary macrophages

BMDMs were co-treated with 20ng/ml IL-4/13 +/- 5µg/ml exosomes, Sup, HES or media alone for 24 h (5µg/ml MODE-k exosomes used a vesicle control). **(A)** Relative expression of *Retnla, Ym1* and *Arg1* were measured by qRT-PCR and normalized to GAPDH and **(B)** supernatants were assessed by ELISA for levels of RELMα, Ym1 and CCL17. **(C)** Supernatants from IL-4/13 pre-treated BMDMs (indicated by >) were assessed by ELISA for levels of RELMα, Ym1 and IL-10 and **(D)** expression of CD206 was measured by flow cytometry. BMDMs were co-treated with IL-4/13 and exosomes +/- 2µg/ml cytochalasin D or anti-exosome sera for 24 h. **(E)** Uptake of exosomes was determined by fluoresence shift of PKH67 and **(F)** supernatant levels of RELMα, Ym1 and CCL17 were determined by ELISA. Data are pooled from 2-3 independent experiments (in biological triplicate), presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001



4.2.9 Small intestinal organoids - interactions with exosomes

(A) Small intestinal organoids were incubated with 3µg/well of PKH67-labelled *H.polygyrus* exosomes **(B)** 3µg/well mammalian MCICL2 exosomes, or **(C)** dye alone for 1 h and uptake was assessed by confocal microscopy. **(D)** Organoids microinjected with 20 nl of sudan black are visualised by light microscopy. **(E)** Organoids were treated with media or 400 ng/ml IL-4/IL-13 for 24 h to determine goblet cell hyperplasia (green). **(F)** Organoids pre-treated for 24 h with IL-4/IL-13 were microinjected with ~3 ng of PKH67-labelled H. polygyrus exosomes or **(G)** MCICL2 exosomes and uptake was assessed 24 h late -r. **(H)** Confocal microscopy demonstrating co-localisation of exosomes and paneth cells following the same experimental design described in (F).

(A-C) + (F-H) DAPI is used to stain nuclei, PKH67 indicates exosomes, E-cadherin indicates epithelium, Lyzozyme-C indicates paneth cells. **(E)** As above except MUC2C3 indicates goblet cells. Unless otherwise indicated, scale bars indicate 20µm.

Table 4.2.4A – Top genes regulated in MODE-k cells by *H. polygyrus* exosomes

Gene	Full name	FDR p value	Fold change
Dusp1	Dual specificity phosphatase 1	0.001	↓ 1.4
LOC666559	PREDICTED: farnesyl pyrophosphate synthase	0.03	↑ 1.4
Prpf19	PRP19/PSO4 pre-mRNA processing factor 19 homolog	0.03	↑ 1.4
Akr1c18	Aldo-keto reductase family 1, member C18	0.004	↓ 1.4
Nupr1	Nuclear protein 1	0.01	↑ 1.4
Anln	Anillin, actin binding protein	0.03	↓ 1.4
Dgcr6	DiGeorge syndrome critical region gene 6	0.03	↑ 1.3
Il1rl1	Interleukin 1 receptor-like 1, transcript variant 2	0.04	↓ 1.3
Mrps21	Mitochondrial ribosomal protein S21	0.01	↑ 1.3
LOC100048530	PREDICTED: Coiled-coil domain containing 72	0.03	↓ 1.3

Table 4.2.4B – Ingenuity pathway analysis of major networks affected in mammalian cells by *H. polygyrus* exosome treatment

Top canonical pathways	p Value	No. proteins
p38 MAPK	9.25E-06	7
Protein Kinase A	3.16E-05	6
ERK/MAPK	8.66E-05	3
Top disease pathways	**p Value**	**p Value**
Organismal Injury and Abnormalities	8.52E-04 - 1.44E-02	24
Inflammatory disease	8.72E-04 - 1.68E-02	20
Top biological functions	**p Value**	**p Value**
Cellular assembly and re-organisation	1.71E-05 - 1.68E-02	22
DNA replication, recombination and repair	1.71E-05 - 1.68E-02	20
Humoral Immune response	3.67E-05 - 1.44E-02	16
Cell death and survival	5.11E-05 - 1.92E-02	15
Cell-cell signalling and interactions	5.68E-05 - 1.92E-02	12

4.4 Discussion

Various studies have identified functions of pathogen-secreted EVs [188], establishing them as important mediators in cell-cell communication, especially during the immune response. There is still definitive gaps in the literature regarding EV uptake mechanisms [179], and this is especially true for helminth-derived EVs, with only a handful of reports demonstrating uptake in host cells [219, 220, 227, 294]. Here, *H. polygyrus* EVs were used to investigate their uptake capacity into host cells, and to determine functional effects that occur following uptake.

It is clear from these observations that *H. polygyrus* exosome uptake occurs in a time and cell-dependent manner. This finding parallels previous literature, showing that mammalian EV uptake is mediated in a similar way [320, 321]. As there appears to be improved uptake of *H. polygyrus* exosomes in both primary and cell line macrophage/monocyte populations compared to the intestinal epithelial cells, it was hypothesised that uptake in these could be mediated in part by phagocytosis or endocytosis. This is supported by the use of Cytochalasin D, a potent inhibitor of actin polymerization known to block endocytosis and phagocytosis and shown prevent exosome uptake in other systems [289, 322]. Interestingly, the role of cellular filopodia, actin-rich membrane protrusions which facilitate endocytosis, in exosome entry has recently been demonstrated using the small molecule inhibitor SMIFH2. This blocks formin-dependent actin polymerization [177], which has been suggested to be involved in viral endocytosis [323]. After a 1 h incubation of BMDMs with *H. polgyrus* EVs, there appeared to be discrete localization of EVs

within these cells, whereas the EVs appear to be more dispersed after 8 h. It could therefore be hypothesized that, after 1 h, parasite-derived EVs are still within endosomes, similar to previous data described in other systems showing intracellular endosomal aggregates shortly after EV internalisation [324, 325]. This is also further supported by live-cell imaging data, showing that uptake of exosomes led to direct entry into the endosomal sorting compartments or vesicles, with shuttling towards the endoplasmic reticulum [177], or the perinuclear region [288]. However, several questions still remain unanswered with regards to how the exosome/exosome-containing endosomal cargo is released into the cell cytosol. From a drug delivery standpoint, recent work has shown that combinatorial treatment of HeLa-derived exosomes with cationic lipids and a pH-sensitive synthetic peptide (normally used for endosomal escape in other studies) can promote cytosolic release of exosome contents [326]. Other studies have shown a similar responsiveness to low pH, which can enhance EV-membrane fusion in cancer cells [327]. This may also be relevant in terms of fusion with host endosomal membranes, given the data showing late-stage endosomal trafficking of exosomes to the acidic lysosomal compartment following internalization [177, 288, 289]. Interestingly, this is a common occurrence in viral infection, whereby low pH facilitates virus-host endosomal fusion and subsequent cytosolic release [328].

Primary investigations were carried out in intestinal epithelial cells. These cells often represent the first barrier of defence during *H. polygyrus* infection, whereby adults are in direct contact with villus protrusions *in vivo* [307]. Microarray analysis from epithelial cells treated with *H. polygyrus* exosomes demonstrated the subtle modulation of murine host genes, including *dusp1*, a murine phosphatase

which regulates components of the MAPK pathway [329], and notably, the interleukin 1 receptor like-1 (*il1rl1*) gene. This gene encodes the IL-33 receptor (also commonly referred to as T1/ST2), which is strongly associated with initiation of anti-parasite and alarmin responses [34] (Discussed further in Chapter 6). Interestingly, it has been reported that BMDMs isolated from DUSP1 knockout mice ($DUSP1^{-/-}$) display sustained MAPK activation and over-production of inflammatory cytokines such as TNF and IL-6 [330], the latter of which has been shown to enhance susceptibility to *H. polygyrus i*nfection [81]. Furthermore, it has been shown that not only do $DUSP1^{-/-}$ mice have significantly higher systemic levels of the immunosuppressive cytokine IL-10 [330], but $DUSP1^{-/-}$ macrophages secrete increased IL-10 in response to parasitic cystatins isolated from *Acanthocheilonema viteae* [74]. Furthermore, DUSP1/MKP-1 signalling is shown to promote macrophage arginase expression over nitric oxide synthase in response to LPS [330]. Hence, parasite modulation of DUSP1 could block the induction of arginase, known to inhibit *H. polygyrus* larval motility [71]. It may also be a potential mechanism used by other parasites, as arginase is known to suppress *N. brasiliensis*-mediated lung pathology by promoting sequestration of larvae in the skin infection site [122].

The presence of an Argonaute protein and small RNAs within *H. polygyrus* exosomes may also suggest the exosomes enable cross-species RNA interference [219], although the mechanism of this is not yet known. It is becoming increasingly evident that many helminths secrete miRNAs, many of which are associated with parasite-derived EVs [287]. Although *H. polygyrus* exosomes can induce transcriptional suppression of both *dusp1* and *il1rl1*, and contain parasitic miRNAs

with complementarity to the 3'untranslated regions of these murine genes, functional repression by these small RNAs was only shown for DUSP1 [219]. Whilst a number of secreted parasite miRNAs could work simultaneously to repress murine *dusp1* (whereby combinatorial targeting of a single gene is a common feature of miRNAs [331]), *il1rl1* suppression may be mediated by other bioactive molecules within exosomes.

During nematode infection, intestinal epithelial barrier integrity is influenced by migration of mature adults into the intestinal lumen from the submucosa, feeding of adults, and anti-helminth immunity e.g. release of IL-4 [332, 333]. The subsequent infiltration of bacteria can generate type-1 inflammatory or danger responses, which can be initiated through recognition of bacterial or alarmin molecules by PRRs e.g. Toll-Like receptors [334]. In order to regulate this, parasites and their ES products have been shown to initiate wound-healing and regulate immunity during infection [151, 312]. Further investigations of intestinal epithelial cells showed that exosomes could modulate secretion of IL-6, in response to TLR2/4 stimulation. Other than IL-6, neither the MODE-k cells or MCICL2 cells (which have a more crypt-like phenotype [319]) produced any detectable traces of inflammatory cytokines such as IL-12p70, nor alarmin cytokines e.g. IL-25, IL-33 or TSLP with any of the stimuli. However, this may be due to limitations in using immortalized cell lines instead of primary intestinal cells, which lack previous experience and immune priming by the microbiota, as well as being unable to fully recapitulate the physiology of the intestinal epithelial cells e.g. by having a soluble mucus layer, protruding villi and crypts.

In this regard, cells present in the intestinal environment, such as epithelial cells and immune cells like macrophages, may be modulated by environmental cues during infection that could affect potential interactions with parasite-derived exosomes. It was therefore important to establish whether *H. polygyrus* exosome uptake could be affected by stimulation. Macrophages activated by type 1 stimuli (such as bacteria) or type 2 stimuli (fungi or helminths), or through Th1/Th2 cytokines, may differ with respect to phagocytosis or endocytosis [335, 336]. *H. polygyrus* exosome uptake was enhanced in pre-polarized M2 macrophages compared to M1 macrophages after 1 h. These data are consistent with previous reports showing that nanoparticle uptake is superior in M2-polarized macrophages [309, 337]. Whilst LPS pre-treatment inhibited initial exosome uptake in either BMDCs or BMDMs, there was marked improvement of uptake after 24 h incubation. As such, the manner of uptake is not entirely based on polarization and may be context dependent, as whilst IL-4 can limit phagocytosis of bacteria [338], human M2-polarized macrophages display enhanced phagocytosis of apoptotic cells [339]. Therefore, methods of uptake must be regarded on an individual basis. Additionally, it was found pre-treatment of BMDMs with exosome antisera increased the rate of uptake, regardless of the macrophage activation status, potentially through the process of opsonisation. This is supported by previous studies demonstrating that pathogen-immunized sera can actually enhance uptake of pathogen particles by complement-mediated pathways [340]. However, it should be noted that, although FcR-mediated uptake is quantitatively enhanced/accelerated, it may also direct exosomes into a degradative pathway thereby ablating their functional effect.

Macrophages are regarded as a key player in immunity to gastrointestinal helminths [316] and have been commonly associated with, and modulated by, exosomes during parasitic infections (see table 4.1). Since *H. polygyrus* exosomes are efficiently internalized by macrophages, the functional effects were further examined. *H. polygyrus* exosomes were able to suppress secretion and the transcriptional expression of hallmarks of LPS/TLR4 activation in RAW 264.7 macrophages, including inflammatory cytokines, IL-6, IL-12p40 TNF and the enzyme iNOS. Additionally, RAW 264.7 cells and BMDMs were shown to have very similar response patterns to TLR stimulation. The finding that *H. polygyrus* exosomes mediate suppression of classical/type-1 inflammation is unsurprising, given previous data showing that HES can modulate dendritic cell maturation and responses to various TLR stimuli [341, 342]. Interestingly, *H. polygyrus* exosomes modulate expression of both *tlr4* and it downstream adaptor, *myd88*, in response to LPS stimulation. Although MyD88-deficient mice have been shown to be more resistant *H. polygyrus* infection [343], this adaptor can be required for responses to fungal allergens [344], but not adjuvant-induced [242] type-2 airway inflammation. As HES can modulate immune responses in both settings [141, 242], it is possible that the suppressive properties of exosomes could also act in a TLR/MyD88-dependent and independent manner. LTBR and Fas are markers associated with macrophage apoptosis [345, 346], and both are show to be downregulated by *H. polygyrus* exosomes, which may be a potential mechanism used to induce host tolerance. Since *H. polygyrus* exosomes also suppress *il1rl1* expression in macrophages this suggests that they have multi-faceted functions to modulate bacterially-induced classical activation in these cells.

Although helminth ES can directly modulate bacteria/TLR-driven inflammation in immune cells, other data demonstrate the roles of helminth ES products in driving the propagation of $FOXP3^{+}$ regulatory T cells [151], and in promoting induction of alternatively activated M2 macrophages during acute infection and *in vitro* [244]. AAMs are known to play a key role in driving and establishing immunity to helminth infection, secreting anti-inflammatory factors such as IL-10 and RELMα, well as facilitating wound-healing to parasite-mediated tissue damage [316]. Based on the strong associations between M2 macrophages and *H. polygyrus* infection [71, 115], these cells were studied in more detail in this chapter. Exosome treatment caused both mRNA and protein suppression of a number of M2 macrophage hallmarks, including the mannose receptor (CD206), IL-10, arginase, RELMα and Ym1. It is rather interesting that exosomes could suppress markers of M2 activation, given their role in tissue repair and mediation of anti-inflammatory responses [347], which is normally adventitious to parasite survival. However, some of the molecules suppressed e.g. arginase-1 may normally facilitate an anti-helminth response. As discussed previously with regards to exosome-mediated *dusp1* suppression, parasite modulation of DUSP1 could block the induction of arginase, known to inhibit larval motility, limiting migration and infectivity of helminths [71, 122]. Similar to the effects of exosomes shown here *in vitro,* total HES was shown to modulate levels of both Ym1 and RELMα found in the bronchoalveolar lavage during a model of airway inflammation [242]. Importantly, Ym1-induced neutrophilia contributes to worm killing in *N. brasiliensis* [86], suggesting that *H. polygyrus* could use exosomes to suppress Ym1 in an attempt to limit parasite expulsion. Furthermore, expression of CD206 is known to

correlate with nematode resistance, as *S. mansoni* ES products can be internalized following CD206 binding on macrophages [348]. This is a mechanism that may be used by *H. polygyrus* exosomes to suppress of Th2-mediated immunity during infection. It should be noted that exosome-mediated suppression could be generated when macrophages have been pre-primed for 24 h with IL-4/13 and have already begun polarizing to M2-like macrophages. Hence, exosomes do not only suppression the onset of alternative activation, but have the capacity to modulate cells during an on-going type-2 immune response. In addition, treatments that interfere with exosome uptake in alternatively activated macrophages, such as cytochalasin D or anti-exosome sera, prevent any exosome-suppressive effects in these cells, highlighting the potential importance of exosomes in macrophage immunomodulation. The comparative suppression by either total HES or HES depleted of exosomes in both the mRNA transcripts and protein production suggest that there are other factors in HES (out with the vesicle fraction) that can modulate alternative activation of macrophages. Thus, in this study, helminth-derived exosomes are shown to suppress a type-2 immune response in macrophages, and our data suggest that the release of *H. polygyrus*-derived exosomes during infection could be a factor in preventing anti-parasitic host responses by AAMs.

Finally, the use of the *ex vivo* organoid culture system has provided preliminary evidence of potential exosome-host cell interactions within the intestinal environment [36, 349]. These multicellular structures phenotypically and functionally resemble the small intestinal niche that a number of parasitic helminths occupy, including *H. polygyrus* [307]. Both mammalian and *H. polygyrus* exosomes were

detectable in somewhat discrete cellular compartments after 24 h post microinjection into the lumen (although their identity has not been fully clarified). It has still to be determined whether this is by an active or passive process, but this could be clarified by microinjection of beads or artificial vesicles (used in [350]). If parasite-derived exosomes become internalised by specialised intestinal cells, such as paneth cells or goblet cells, this could indicate another target for cross-species regulation. Paneth cells and goblet cells are known to be associated with anti-helminth immunity [351] [307, 308], and also undergo hyperplasia during helminth infection or from the influence of type-2 cytokines [36]. It will be interesting to assess the effects of parasite-derived exosomes on the function of these cells, which could be determined by the presence of effector molecules in the supernatant of organoid culture, or the proportion of these cells within intestinal organoids. Furthermore, given the complexity of cellular responses within these organoids, future studies should aim to determine potential exosome association with other cells in these ultrastructures, such as tuft cells [36]. These cells are shown to be a potent source of IL-25 during both rest and infection, initiating a strong ILC2 response and the differentiation of epithelial cell progenitors which could support parasite expulsion [35, 36].

Collectively these data suggest that exosomes function as modulators of host immunity. A majority of the *in vitro* studies described in this chapter are limited by having a single cell type. These often form monolayers in culture and do not represent the diversity of immune cells present in the intestine during helminth infection, nor recapitulate the environmental cues induced by the presence of a parasite. During helminth infection, a mixed type-1/type-2 response is established

due to both barrier damage and the presence of the parasite. In order to circumvent a strong host response, parasites release ES products to modulate or suppress active immunity, and parasite-derived EVs are fast becoming a key aspect of host regulation [188]. Whilst the organoid studies provide a framework for analysing more complex intestinal responses, the data is still preliminary. Therefore, the next step of this study was to analyse the effects of *H. polygyrus* exosomes *in vivo*, testing their function as immunomodulators and determining their role during *H. polygyrus* infection.

Chapter 5

Interactions and functions of *H. polygyrus* exosomes *in vivo*

5.1 Introduction

The data from chapter 4 demonstrated that *H. polygyrus* exosomes have the capacity to interact with, and suppress host immune cells *in vitro*. I focused on cells that would be present in the intestinal environment during *H. polygyrus* infection, namely epithelial cells and macrophages, that have both previously associated with anti-helminth immunity [307]. However, responses to helminth infection require a range of innate and adaptive effector mechanisms that collaborate to amplify immunity and induce parasite expulsion. The complex nature of these responses must be considered when investigating the immunoregulatory properties of *H. polygyrus* exosomes, and this chapter will focus on their potential functions *in vivo*.

Studying the immunomodulatory properties of helminths during natural infection can be immensely challenging, owing to the complex mechanisms of immune evasion induced by the parasite. These methods include: anatomical sequestration to sites of immune privilege [146], shedding of antigenic surface proteins by cuticle turnover [145], and active suppression of the host immune

response to helminth antigens, producing a state of parasite-specific immune tolerance [281]. During chronic helminth infection, the host requires an additional network of cells to regulate active type-2 immunity and initiate tissue repair. The induction of an immunosuppressive state within the host, coordinated by both regulatory T and B cells [111, 352] as well as alternatively activated macrophages [58], limits immune pathology to the parasite, and can often result in bystander suppression of both autoimmune disease and allergy [353]. The potency of this modulation has been exploited by the helminth, as it creates a host environment permissive for survival.

This effect is becoming of growing interest due to the increasing rates of autoimmune and allergic diseases, such as asthma, in western society [354], which gives basis to the hygiene hypothesis as discussed in Chapter 1 [134]. The potential benefits of parasite immunodulation have led to the use of helminths, their ova or excretory-secretory products (ES) in clinical trials for the treatment of atopy [138] and autoimmunity, such as inflammatory bowel disease (IBD) and multiple sclerosis (MS) [139, 355, 356]. The laboratory model, *H. polygyrus*, has previously been used to investigate the effects of gastrointestinal nematode infection on other co-morbidities such as allergy and autoimmune disease. *H. polygyrus* infection is shown to have strong immunosuppressive effects on type-1 diabetes [357], a murine model of MS [358], experimental colitis [359] and asthma [360]. Although rodent model systems can recapitulate some aspects of human immune disease models, extrapolating these findings to human study has obvious limitations [361]. Experimental infection trials in humans have been carried out on several helminths, including *Trichuris suis* (the pig whipworm) and *N. americanus* (the human

hookworm), and a case report has been published of an individual self-infecting with the human whipworm *T. trichiura* [362]. These trials have given variable results demonstrating the potential of live helminth infection as a therapeutic in asthma, autoimmune disease and allergy (comprehensively reviewed in [363] and [364]).

Although live-helminth based therapy has shown some promise, with limited side effects [365, 366], there are some clear disadvantages to using this as a long term treatment. Ethical considerations limit both the dose size and number of times that patients are given parasites for each respective trial, which may affect their potency as immunomodulators [143]. Furthermore, the potential development of co-infection with other pathogens could be detrimental in immunocompromised individuals, as would the continual remodelling/fibrosis of tissues that would occur to circumvent host damage during chronic infection [361, 367].

A less controversial/restrictive alternative to live-helminth therapy is to use helminth-derived immune-modulatory molecules. A number of *in vitro* studies have pointed to the modulatory capacity of helminth excretory-secretory products, on both human [368] and murine immune cells [155, 369], limiting inflammatory immune responses and cell activation. Furthermore, there are data demonstrating that *H. polygyrus* ES (HES) [242], or ES-62 (a single molecule isolated from *Acanthocheilonema viteae* ES) [370], can modulate airway inflammation *in vivo.* Indeed, the effects of whole HES, or isolated components have been characterized. These include; a TGF-β mimic which facilitates T regulatory responses [282], cystatins which suppress myeloid cell activation [155], and calreticulin, which was demonstrated to induce strong Th2 polarisation *in vivo* [94]. More recently, a study

from our lab showed that exosomes isolated from HES by ultracentrifugation were shown to modulate innate responses in a model of allergic asthma [219], and this will be discussed later in this chapter in more detail.

Conversely, the suppressive nature of helminth ES, whilst prolonging parasite survival within the host, actually represents an attractive vaccine target for prevention of infection. This research has been pursued in the livestock parasite, *Haemonchus contortus*, whereby vaccination against isolated proteins from ES, included antigen H11 [274] and venom allergen-like proteins (VALs) [371], induced immunity in ruminants. Furthermore, combinatorial formulations of ES proteins from *S. mansoni* larvae are shown to drive antibody-induced host protection [372]. Additionally, previous studies have shown that vaccination against total *H. polygyrus* ES induces sterile immunity against a larval challenge [255], which is now appreciated to be directed via humoral IgG1 responses and innate IL-25/IL-4-driven immune cell types [108].

The work in this chapter illustrates the immunomodulatory properties of *H. polygyrus* exosomes in models of airway allergy, examining their capacity to prevent or ameliorate responses to a fungal antigen. In addition, I investigated the role of *H. polygyrus* exosomes during infection, determining whether vaccination against *H. polygyrus* exosomes could induce the same host protection as shown with total HES [108]. Proteins enriched in exosomes from other parasites, such as testraspanins, are now considered in prospective vaccine studies [226, 227] thus highlighting the potential of exosomes as targets for treatment against helminth infection.

5.2 Results

5.2.1 *Alternaria*-induced allergic inflammation to ovalbumin challenge is suppressed by *H. polygyrus* exosomes

Both enteric *H. polygyrus* infection [353] and HES [373] have been previously demonstrated to modulate airway inflammation in sensitized mice challenged with allergens in the respiratory tract. To investigate whether *H. polygyrus* exosomes could recapitulate these protective responses, cellular immunity was assessed in a model of ovalbumin (OVA)-specific airway inflammation induced by co-sensitization with the fungal allergen, *Alternaria alternata* (Figure 5.2.1A), in a similar manner as described in [141].

In these experiments, exosomes, Sup (HES depleted of exosomes) or HES were co-administered at the time of sensitization, and mice were challenged with OVA alone 14-16 days later. Co-administration did not alter the marked increase in total numbers of both bronchoalveolar lavage and lung cells compared to OVA-challenged mice (Figures 5.2.1B and D), but there was significant modulation of airway eosinophilia (shown by absolute counts in the BAL and lung) (Figures 5.2.1C and E), and a modest suppression of total lung neutrophils (Figure 5.2.1E) by each of the interventions. Neither alveolar macrophages, nor CD4+ T cells, were significantly modulated following co-treatment with any of the preparations. Additionally, the proportion of Foxp3+ regulatory T cells were also unaffected by co-treatment with either HES, Sup or exosomes (data not shown), suggesting that exosome modulation of allergic airway responses was potentially confined to the

innate compartments of the lung, and not mediated by OVA-specific T helper/regulatory cell responses.

5.2.2 *H. polygyrus* exosomes suppress type 2 innate lymphoid cells, but not adaptive T helper cell responses to *Alternaria*

During allergic asthma responses, airway eosinophilia is strongly correlated with the presence of type-2 cytokines, such as IL-5 and IL-13, which are known to promote eosinophil recruitment and induce lung pathology, such as fibrosis [374, 375]. Two major cellular sources of these cytokines during this response are type-2 innate lymphoid cells (ILC2s) and T helper cells (Figure 5.2.2A). These populations were isolated from the lung to investigate whether modulations in innate or adaptive sources of these cytokines by exosomes could be responsible for the suppression of airway eosinophilia in this model. Although it was found that the proportion of IL-5 or IL-13-producing CD4+ T cells were unaffected by exosomes, Sup or HES (Figure 5.2.2B), there was significant suppression of both IL-5 and IL-13-producing innate lymphoid cells in mice co-administered with any of the HES fractions compared to *Alternaria*-sensitised mice alone (Figure 5.2.2C). ILC2s are also known to express ST2, the subunit of IL-33R which works in conjunction with the IL-1R accessory protein (IL1RAP) to respond to IL-33 signalling [39]. This is shown in previous chapters to be modulated by exosomes in both MODE-k epithelial cells and primary macrophages in vitro. In the *in vivo* allergy model tested here, exosomes could also suppress both ST2 expression on ILC2s (Figure 5.2.2D), and total lung transcript levels of the gene for IL-33R (*il1rl1*) (Figure 5.2.2E). Additionally, exosomes could

also modulate total transcriptional levels of *dusp1* in the lung, which again, had been shown *in vitro* to be suppressed by exosomes [219] (chapter 4).

5.2.3 *H. polygyrus* exosomes suppress early type-2 responses to *Alternaria*-induced allergic lung inflammation

As exosome modulation of allergic airway inflammation appears to target innate rather than adaptive responses, a short-term model of innate allergic responses to *Alternaria* exposure was tested (Figure 5.2.3A). In this protocol, mice were co-treated intranasally with exosomes, Sup or HES plus *Alternaria* extract in the absence of ovalbumin antigen, and innate immune responses were characterised after 48 h. At this time point, absolute numbers of bronchoalveolar lavage eosinophils, neutrophils and alveolar macrophages were suppressed in exosome/*Alternaria* co-treated mice compared to *Alternaria* challenged mice (Figure 5.2.3B-D). Additionally, lung transcriptional levels of *Retnla* and *Arg1*, hallmarks of type-2 myeloid cell activation present in *Alternaria*-driven inflammation [376], were suppressed in exosome co-treated mice (Figures 5.2.3E and F), although levels of *Ym1* were unaffected (Figure 5.2.3G). Finally, co-treatment with either HES, Sup or exosomes suppressed both the absolute number of ILC2s (Figure 5.2.3H), as well as the expression of ST2 on these cells (Figure 5.2.3I). These data, although preliminary, highlight the modulatory capacity of exosomes (comparable to both Sup and HES), in early responses to allergic airway challenge.

5.2.4 Prophylactic exosome treatment suppresses early innate and type-2 responses to *Alternaria*

In order to assess the prophylactic potential of exosomes compared to total HES or Sup, mice were administered intranasally with a dose of exosomes 24 h prior to *Alternaria* airway challenge (Figure 5.2.4A). In this protocol, innate reactions were assessed 24 h following allergen administration, a time point at which responses are broadly similar to those at 48 h ([141] and personal communication with H McSorley).

In this prophylactic setting; exosomes, Sup and HES all exhibit strong modulation of airway eosinophilia (Figure 5.2.4B), reducing eosinophil numbers in the BAL by 72.3%, 71.9% and 70.2% respectively. Both exosomes and Sup fail to modulate levels of BAL neutrophils induced 24hr post-*Alternaria* challenge, with only a modest suppression induced by HES (Figure 5.2.4C). In addition, exosome co-treatment suppressed both the number of alveolar macrophages present in the BAL (Figure 5.2.4D), as well as the levels of myeloid-derived cytokines, RELMα and Ym1 (Figure 5.2.4E and F). Broad profiling of cytokines present from both the BAL and lung homogenate (data not shown) revealed no significant changes in alarmin/inflammatory cytokine responses (including IL-1α/β, IL-6 and IL-17A) nor modulatory cytokines (such as IL-10). However, levels of type-2 cytokines, IL-5 and IL-13, were significantly lower in mice co-treated with exosomes, Sup or HES (Figures 5.2.4G and H). These data suggest that exosomes can serve as preventative

treatment against airway inflammation, and have the same modulatory capacity as either total HES or HES depleted of exosomes.

5.2.5 Prophylactic exosome treatment suppresses ILC2 responses to *Alternaria*-induced lung inflammation and modulates ST2 expression in a cell-specific manner

During innate responses to airway challenge, ILC2s are a major source of type-2 cytokines, such as IL-5 and IL-13 [377], and were modulated by exosomes (Figures 5.2.2 and 5.2.3) and HES [141] in both long and short-term models of *Alternaria*-induced airway inflammation. The activation and expression of the ILC2 population was therefore measured in the same experiment of prophylactic administration. It was found that 24 h following *Alternaria* administration, there was a profound suppression in the proportion of IL-5 and IL-13-producing ILC2s in recipients of exosome, Sup or HES treatment (Figure 5.2.5A and B). The modulation of ILC2 cytokine expression may contribute to the reduction of IL-5 and IL-13 observed in the BAL following prophylactic exosome treatment (Figure 5.2.4G and H).

Similar to the previous models in this chapter, exosomes can also suppress the expression of ST2 on ILC2s (Figure 5.2.5C), as well as demonstrating modest suppression of *il1rl1* mRNA in total lung (Figure 5.2.5D). However, the suppression of ST2 does appear to be cell specific, as expression of this receptor is unchanged in lung epithelial cells (CD45.2-EpCAM+), regardless of treatment (Figure 5.2.5E). Previous work has shown that HES can modulate levels of the alarmin cytokine IL-33 [141], which is also known to regulate expression of ST2 in ILC2s [378].

Interestingly, although prophylactic treatment by either total HES and Sup can suppress lung homogenate levels of IL-33, exosomes alone failed to do so (Figure 5.2.5F). Finally, consistent with previous data, exosomes can also modulate *dusp1* transcript in total lung, confirming the model-wide suppression of this target gene by exosomes. This data suggest that exosomes, like total HES, can modulate the early events that occur during an allergic airway response, with preferential suppression of the ILC2 compartment, as well as downregulating expression of the alarmin receptor subunit ST2.

5.2.6 Exosomes provide sterile immunity against *H. polygyrus* larval challenge in C57BL/6 mice

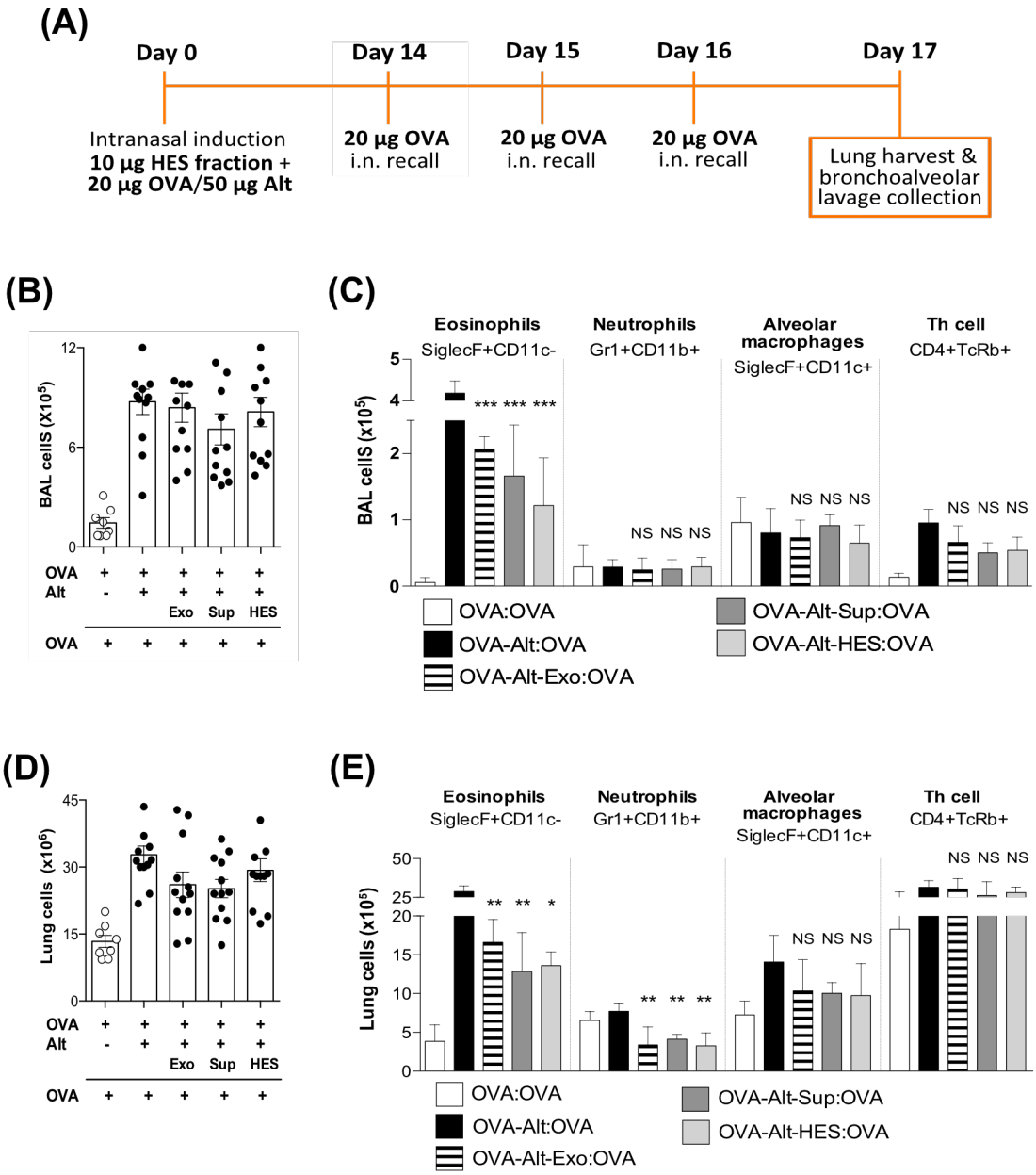
It was important to establish whether vaccination with exosomes could engender protective immunity in mice *in vivo*. This is based on previous data demonstrating that vaccination against total HES could induce sterile immunity against *H. polygyrus* infection [255]. Using an alum-adjuvant model, C57BL/6 mice were vaccinated against either exosomes, HES depleted of exosomes or total HES prior to subsequent larval challenge (Figure 5.2.6A). Any protective immunity induced in this model was assessed by parasitological outcomes.

Faecal egg counts were suppressed by over 90% from days 14 to 28 post-infection in mice vaccinated with either exosomes, Sup or HES compared to PBS/alum vaccinated mice (Figures 5.2.6B-D). Additionally, worm burden was reduced ~82% for exosomes, ~78% for Sup and ~98% for total HES (Figure 5.2.6E). A summary of

egg and worm burdens in vaccinated mice are shown Table 5.2.6. the protection induced showing that exosome vaccination can generate significant protection against parasitic infection, and is comparable to total HES despite only representing approximately 10% of the ES from *H. polygyrus*.

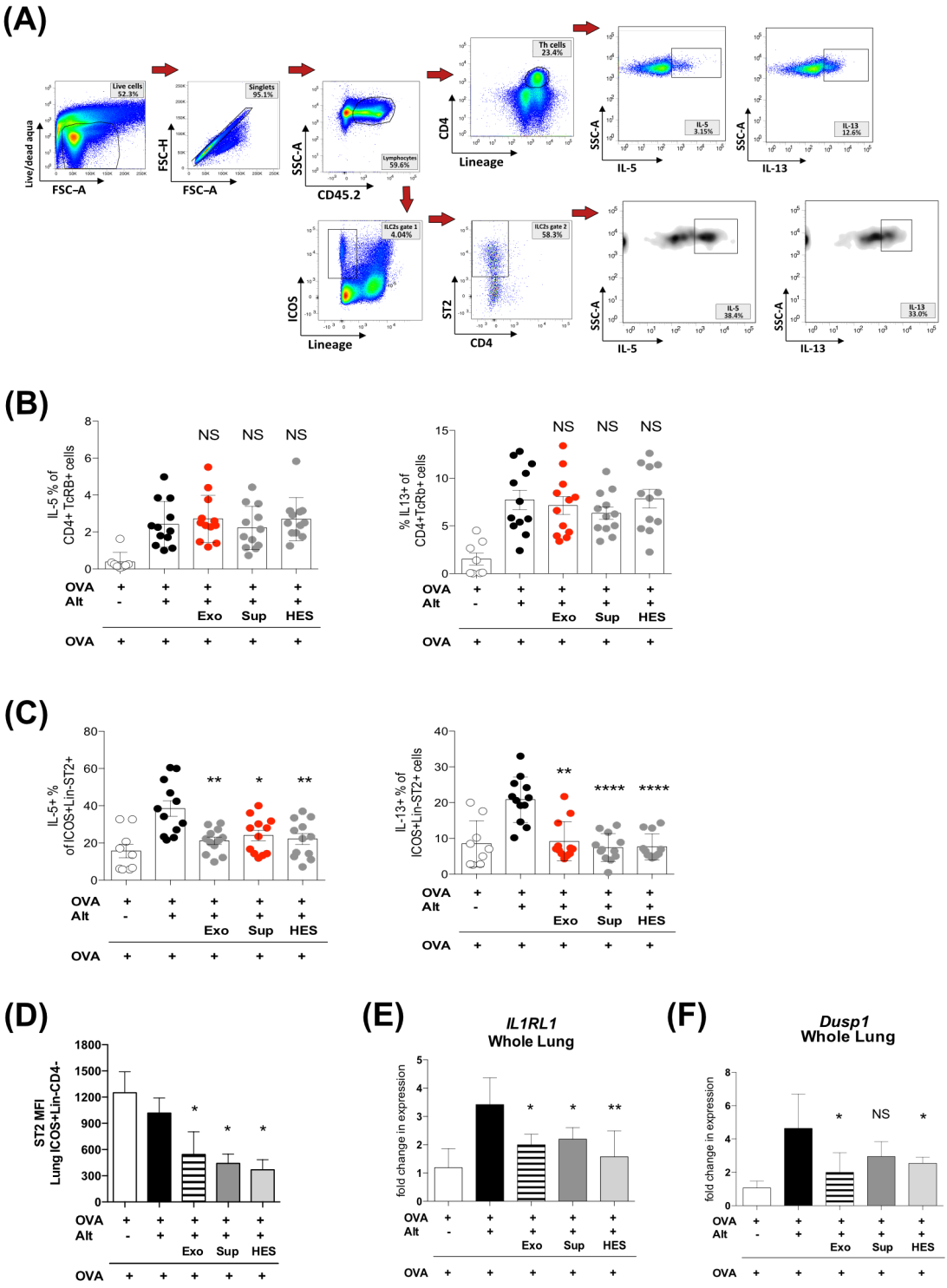
5.2.7 *H. polygyrus* exosomes induce specific antibody responses *in vivo*

Previous work has shown that antibodies play a key role in the secondary immune response to helminth infection [277, 379]. In addition, HES vaccination was shown to elicit a strong antibody response [255]. In chapter 4, I also demonstrated that anti-exosome antibodies block the immunomodulatory effects of exosomes on macrophages *in vitro* (Figure 4.2.8F). Thus, I sought to define the humoral response generated against exosomes *in vivo,* as it may be a factor which contributes to host protection. I examined the repertoire of total serum antibodies elicited by exosome, Sup or HES vaccination alone, prior to challenge infection, in comparison to the PBS-alum mouse control. (Figures 5.2.7A and B). I also determine the serum titres of exosome-specific IgM, IgG1, IgA and IgE, as shown by ELISA (Figure 5.2.7C-F). I also assessed the serum titres of Sup- or HES-specific IgM or IgG1 by ELISA (Figure 5.2.7 G-H). Interestingly, I observed there were significant antibody titres induced between the groups, regardless of whether mice were immunized with exosomes, Sup or HES. This indicates a response to either shared molecular components or to cross-reactive epitopes.



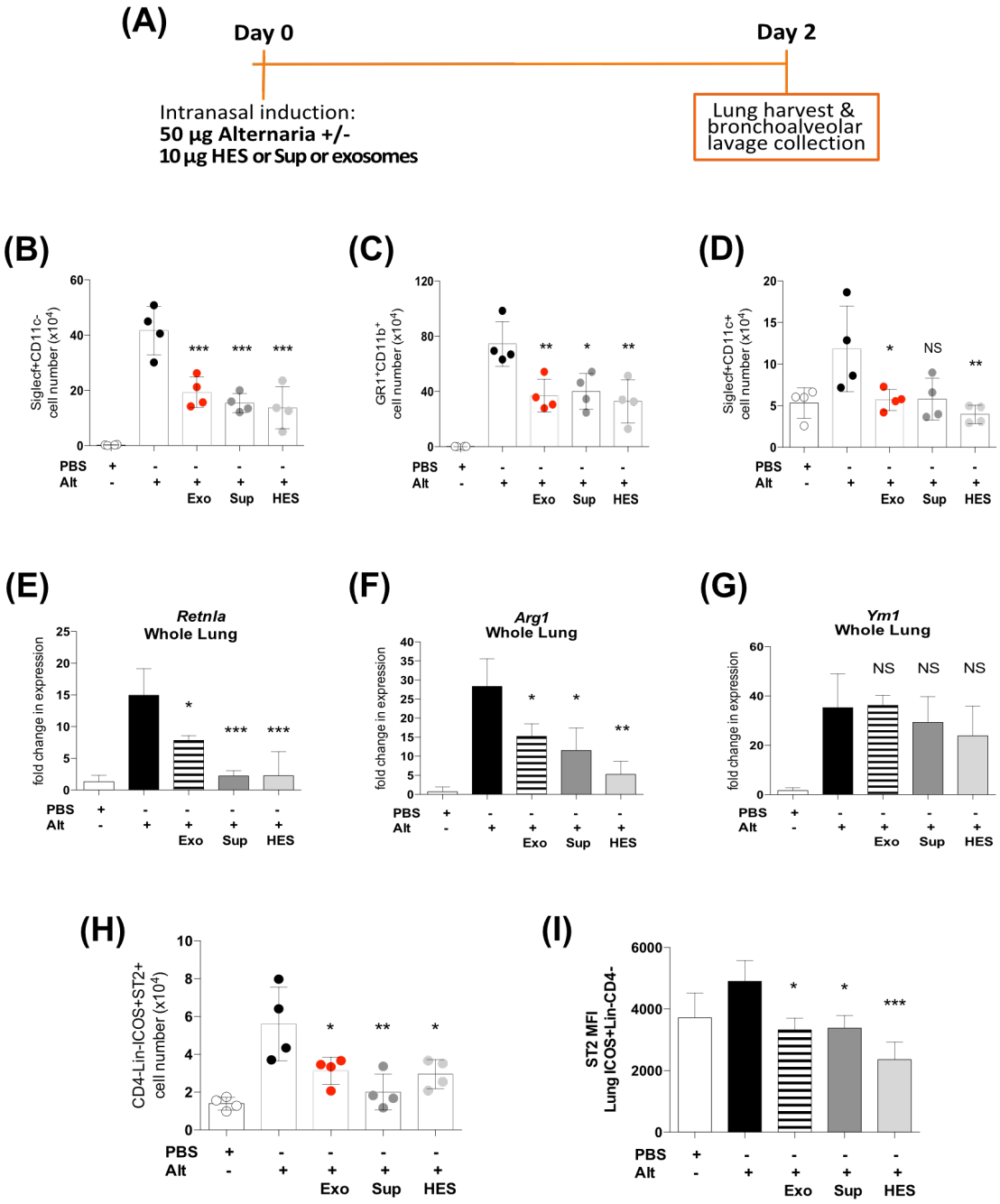
5.2.1 *Alternaria*-induced allergic inflammation to ovalbumin challenge is suppressed by *H. polygyrus* exosomes

(A) Schematic of the *alternaria*-exosome model with ovalbumin (OVA) recall (as described in 2.12.1). **(B)** + **(D)** Graphs represent total cell populations recovered from the bronchoalveolar lavage (BAL) and lung tissue per experimental group. **(C) + (E)** Cell profile of BAL and lung cells: numbers of eosinophils (SiglecF+CD11c-CD11b-), neutrophils (Gr1+CD11b+CD11c-SiglecF-), alveolar macrophages (CD11c+SiglecF+) and T helper cells (CD4+TcRβ+), as assessed by flow cytometry. Data are pooled from 3 independent experiments, presented as mean values ± SD (n = 9-12 mice per group; one-way ANOVA). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001.



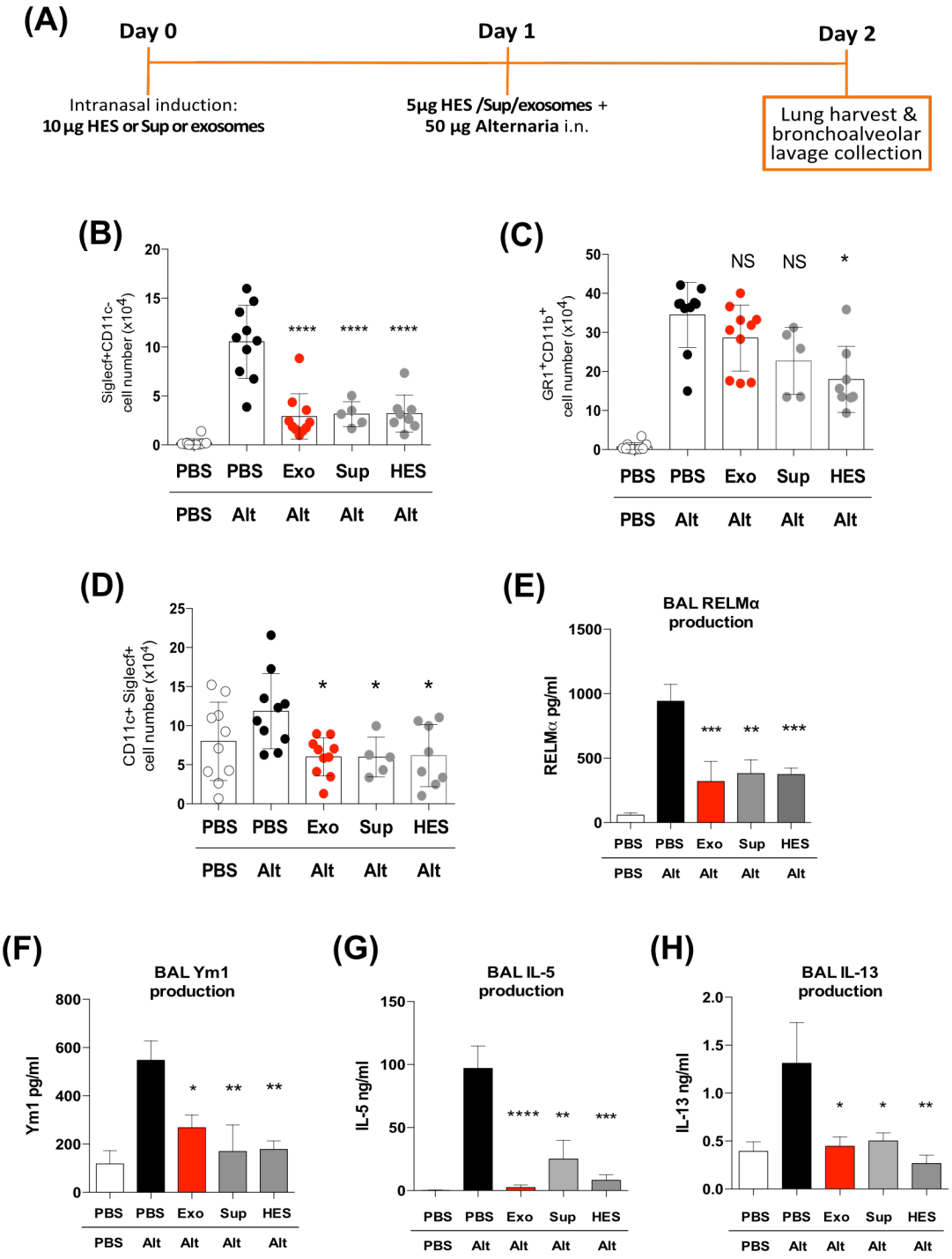
5.2.2 *H. polygyrus* exosomes suppress type 2 innate lymphoid cells, but not adaptive OVA-specific T helper cell responses to *Alternaria*-induced allergic inflammation

(A) Gating of IL-5/IL-13-producing ILC2 and T helper cell compartments of the lung following *alternaria*-OVA challenge. Proportion of IL-5 and IL-13-producing **(B)** T helper cells (CD4+TcRβ+) and **(C)** ILC2s (ICOS+Lineage-ST2+) in the lung. **(D)** Representative data showing expression intensity of ST2 (MFI= mean fluorescence intensity) on lung ILC2s. **(E) + (F)** Relative expression of total lung *il1rl1* and *Dusp1* were measured by qRT-PCR and normalized to GAPDH. Data are pooled from 2 independent experiments, presented as mean values ± SD (n = 9-12 mice per group; one-way ANOVA). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.



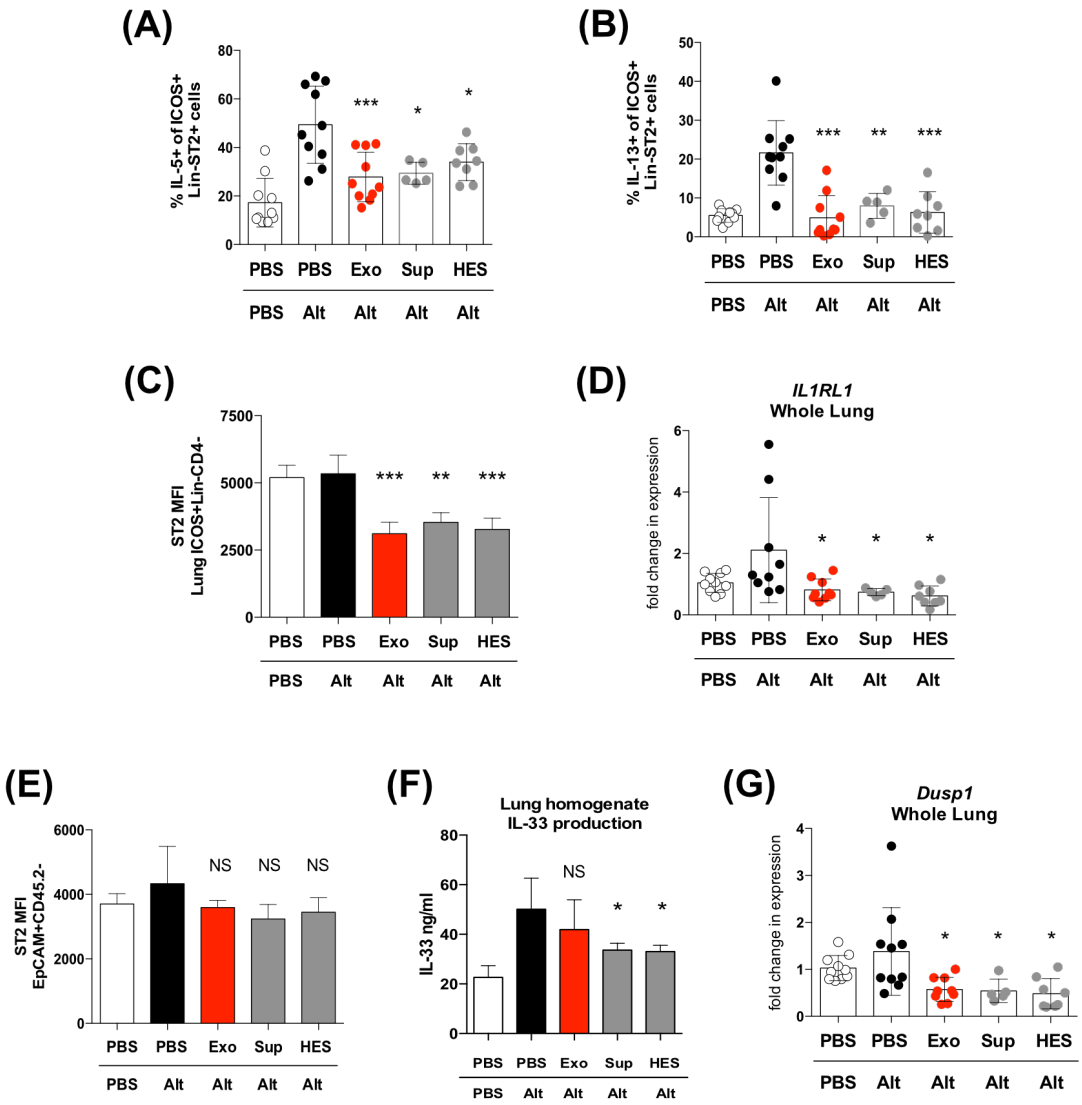
5.2.3 *H. polygyrus* exosomes suppress early innate and type-2 responses to *Alternaria*-induced allergic lung inflammation

(A) Schematic of the 48 h *alternaria*-exosome model to determine exosome effects on innate immunity to fungal allergen (as described in 2.12.2). **(B)** to **(D)** Total cell populations recovered from the BAL per experimental group and gated as per 5.2.1; eosinophils, neutrophils, and alveolar macrophages. **(E)** to **(G)** Relative expression of alternative activation markers in total lung; *Retnla, Arg1* and *Ym1* were measured by qRT-PCR and normalized to GAPDH. **(H)** Absolute numbers of lung type-2 innate lymphoid cells (ICOS+Lineage-CD4-ST2+) and **(I)** expression intensity of ST2 on these cells. Data are from 1 experiment, presented as mean values ± SD (n = 4 mice per group; one-way ANOVA). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001.



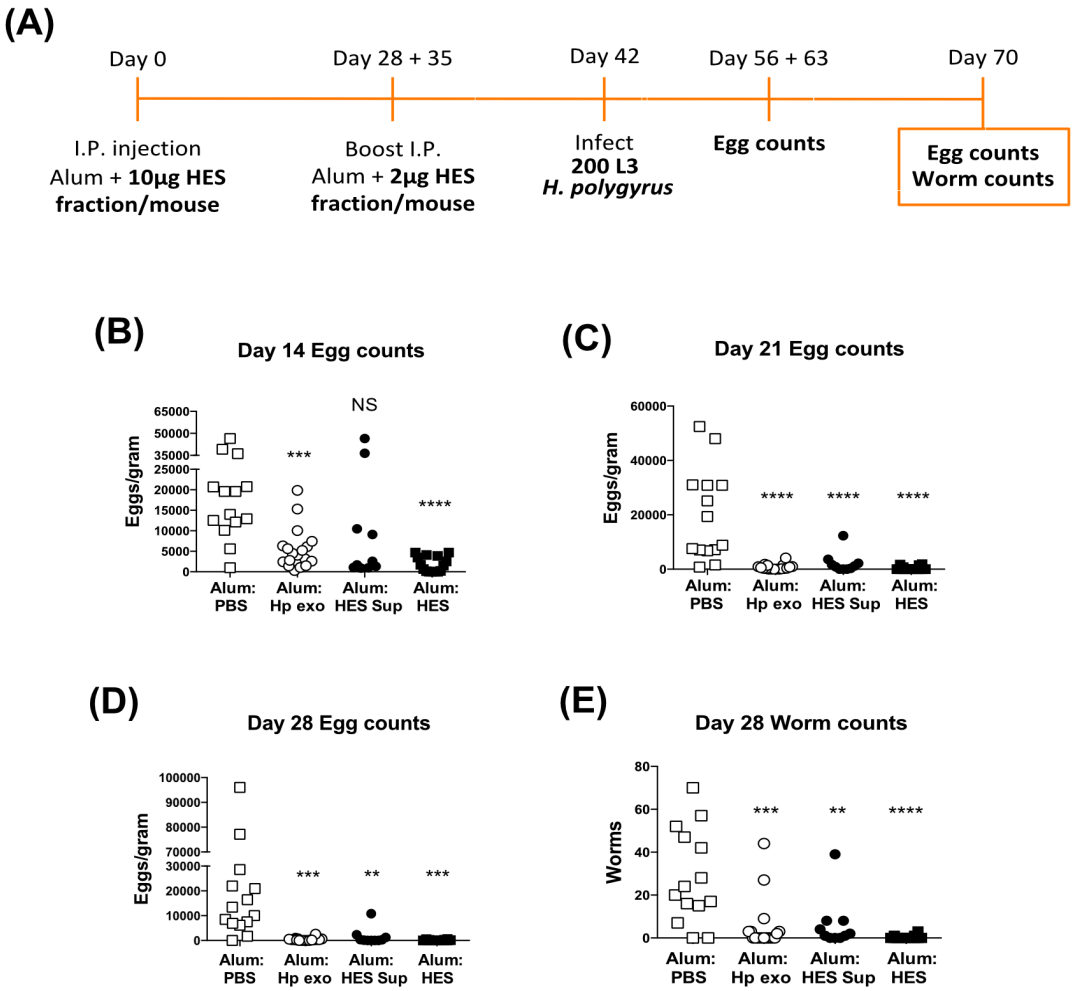
5.2.4 Prophylactic exosome treatment suppresses early innate and type-2 responses to *Alternaria*-induced allergic lung inflammation

(A) Schematic of prophylactic 48 h *alternaria*-exosome model (as described in 2.12.2). **(B)** to **(D)** Total cell populations recovered from the BAL per experimental group; eosinophils, neutrophils, and alveolar macrophages, gated as per 5.2.1. **(E)** to **(H)** BAL fluid was assessed by ELISA for levels of RELMa, Ym1, IL-5 and IL-13 respectively. Data are pooled from 2 experiments, presented as mean values ± SD (n = 5-10 mice per group; one-way ANOVA). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.



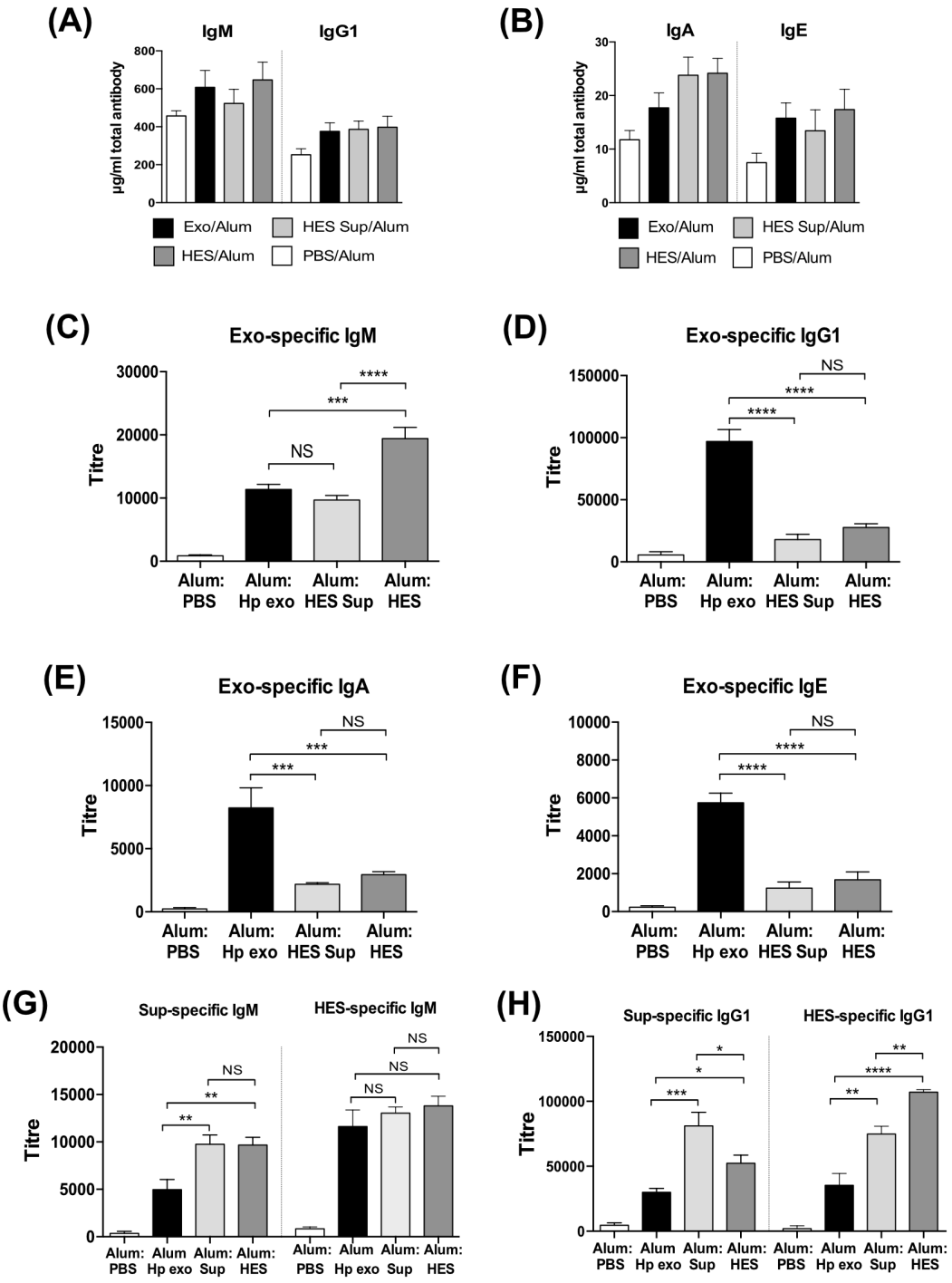
5.2.5 Prophylactic exosome treatment suppresses ILC2 responses to *Alternaria*-induced lung inflammation and modulates ST2 expression in a cell-specific manner

(A + B) Proportions of IL-5 and IL-13-producing ILC2s (ICOS+Lineage-ST2+). **(C)** Representative data of expression intensity of ST2 on lung ILC2s. **(D)** Relative expression of total lung *il1rl1* measured by qRT-PCR and normalized to GAPDH. **(E)** Representative data of expression intensity of ST2 on lung epithelial cells (CD45.2-EpCAM+). **(F)** Lung homogenate was assessed by ELISA for levels of IL-33. **(G)** Relative expression of total lung *Dusp1* were measured by qRT-PCR and normalized to GAPDH. Data are pooled from 2 experiments, presented as mean values ± SD (n = 5-10 mice per group; one-way ANOVA). NS indicates a non-significant result. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$,



5.2.6 Exosomes induce protective immunity against *H. polygyrus* larval challenge in C57BL/6 mice

(A) Aged matched female C57BL/6 were vaccinated with exosomes, Sup, HES or PBS in alum adjuvant prior to challenge with *H.polygyrus* L3 larvae (as described in 2.11). **(B)** to **(D)** Faecal egg counts from *H.polygyrus* challenged mice on days 14, 21 and 28. Symbols denote the differently challenged mouse groups as directly titled below in x-axis. **(E)** Adult worm counts from the small intestine on day 28 in *H.polygyrus* challenged mice. Data are pooled from 3 experiments and calculated by one-way ANOVA (n = 10-18 mice per group).* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.



5.2.7 *H. polygyrus* exosomes induce specific antibody responses *in vivo*

Aged matched female C57BL/6 were vaccinated with PBS, exosomes, Sup, or HES in alum adjuvant (as described in 2.11). **(A, B)** Total serum IgM, IgG1, IgA and IgE levels were determined by ELISA against a standard curve of recombinant mouse IgM, IgG1, IgA or IgE. **(C)** to **(F)** Exosome-specific IgM, IgG1, IgA and IgE serum titres from exosome, Sup, HES or PBS immunized mice were measured by ELISA. **(G)** Sup-specific and HES-specific IgM and **(H)** Sup-specific and HES-specific IgG1 serum titres from exosome, Sup, HES or PBS immunized mice were measured by ELISA. Data are representative from 1 of 2 experiments, presented as mean values ± SEM (n = 5 mice per group). NS indicates a non-significant result, * = p<0.05, ** = p<0.01, ***=<0.001, **** =p<0.0001.

Table 5.2.6 – Degree of protection elicited by exosomes, Sup or HES vaccination

Percentage reductions in egg and worm burden			
	Exo/Alum	**Sup/Alum**	**HES/Alum**
Day 14 Egg counts	72.61%	45.21%	90.90%
Day 21 Egg counts	96.21%	88.80%	97.95%
Day 28 Egg counts	97.99%	93.25%	99.44%
Day 28 Worm burden	**82.08%**	**77.68%**	**98.73%**

Percentage reductions in egg/worm burden in exosome vaccinated mice. Each group is pooled from 3 independent experiments (n= 10-18 mice per group).

5.4 Discussion

The importance of parasite-derived extracellular vesicles during natural infection is becoming increasingly well-established [188]. As such, defining the immune-modulatory mechanisms generated by exosomes, and their role as a helminth excretory-secretory product, could contribute to the generation of new therapeutics against inflammatory disease, replicating the suppressive effects shown during helminth infection [358, 380, 381]. Here *H. polygyrus* exosomes were examined for their potential contribution to the suppression of airway allergy, since this had previously been shown during either infection with *H. polygyrus* [360], or by treatment with total HES [141, 373].

Exosomes, like HES, are able to modulate airway eosinophilia (present in both the bronchoalveolar lavage and lung tissue), induced by ovalbumin recall following allergic antigen sensitization. These effects are not targeted at adaptive immune responses, as the proportion of IL-5 and IL-13-producing Th2 cells were not suppressed by exosomes or total HES. Furthermore, despite previous data showing that helminth infection can promote Foxp3+ T regulatory cells to modulate airway inflammation [353, 360], the levels of these cells were unaffected by exosome co-treatment. Although it has been shown previously that HES can modulate type-2 cytokine producing T helper cells in this model [141], both the previous study and the current one record a more potent modulation of cytokine producing-ILC2s by either exosomes or total HES.

ILC2s are innate cells, which are known to initiate early type-2 immunity to airway allergen challenge following tissue damage/recognition of alarmin cytokines [377, 382-384], and as well as being a potential biomarker in human asthmatic patients, are suggested to sustain airway eosinophilia through the release of type-2 cytokines [385]. The findings of this study suggest that exosomes modulate eosinophilic responses to *Alternaria* antigen by targeting ILC2 cell populations, supporting previous data in similar models with total HES [141], and that this response is independent of adaptive immunity. As such, exosome co-treatment can recapitulate these effects 24-48hr after intranasal allergen challenge and more promisingly, exosomes can also exert their modulatory properties in a prophylactic manner, representing a potential target for development of preventative agents against allergy or inflammation.

Like previous *in vitro* studies [219], *H. polygyrus* exosomes were able to suppress mRNA levels of the MAP-kinase regulator, *dusp1*. Neither IL-6 nor IL-10 levels were modulated significantly by exosome co-treatment, despite the prominent association of these cytokines with DUSP1 (as shown in studies with DUSP1$^{-/-}$ mice [330]). However, this could be due to other factors, including timing, the limited potency of suppression by exosomes in this model, and the nature of the antigen stimulation (given the cited paper refers to LPS-mediated responses in DUSP1$^{-/-}$ mice).

The exosome-mediated suppression of ST2 *in vivo*, either measured at the mRNA level in whole lung tissue, or at the protein level on ILC2s was evident in both long and short-term models of airway inflammation. This also compliments the

in vitro studies in macrophages and intestinal epithelial cells, which are shown in chapter 4. However, the expression of ST2 on EpCAM+ lung epithelial cells was not significantly altered following exosome treatment. As intranasal induction may not induce equal distribution of either the allergen or exosomes in the lung, modulations in epithelial ST2 expression may be lost in a population that account for such a large proportion of the lung microenvironment. Furthermore, the modulation of whole lung il1rl1 mRNA by exosomes is very subtle, and may not reflect changes at the protein level if only a subset of cells received treatment. It may also be, as shown in both cancer and viral studies [293, 386], that exosomes preferentially target/interact with other cells, such as ILC2s or macrophages, which were shown in the previous chapter to demonstrate superior uptake compared to intestinal epithelial cells i*n vitro.* Interestingly, exosome treatment also suppressed the levels of alveolar macrophages in early responses to *Alternaria,* as well as modulating the levels of myeloid-derived cytokines, Ym1 and RELMα (replicating effects shown in alternatively activated macrophages in the previous chapter). The suppression of type-2 cytokine producing-ILC2s could directly affect macrophage polarisation in the lung, and/or be enhanced by a loss of eosinophils, whom are also shown to drive macrophage polarisation in airway responses to allergy [387].

Overall, I hypothesise that exosome-mediated suppression of these responses in the lung could be attributed to a loss of ST2. Although exosomes, unlike total HES, fail to suppress IL-33, it may be reasonable to suggest that different components of HES differentially target aspects of the IL-33/ST2 signalling cascade, given that HES depleted of exosomes can modulate IL-33 levels in the lung. In an experimental model of lung inflammation in T1/ST2$^{-/-}$ mice, both ILC2s were

virtually absent from the lung, and macrophages were unable to polarise nor contribute to pulmonary fibrosis [388]. Additionally, innate IL-5 and IL-13 responses, as well as airway eosinophilia, were drastically reduced in T1/ST2$^{-/-}$ mice in response to *Alternaria* challenge. Importantly, ST2 expression has been shown to correlate with protection against helminth infection [39, 389].

In combination with alum adjuvant, vaccination of mice with exosomes induce high titres of exosome-specific IgM, IgG1, IgA and IgE serum antibodies, compared to the PBS-alum control. The generation of strong exosome-specific IgG1 antibody responses may be responsible for downstream resistance to *H. polygyrus*. Previous work has shown that passive immunization using antibodies raised against VAL proteins or glycans (originating from HES) failed to protect against H. polygyrus infection. However, these components are not highly enriched in exosomes compared to Sup [219], so antibody responses may still be key to the immune protection elicited by exosome vaccination. Despite this, recent data does illustrate the role of IgG1 antibody responses during *H. polygyrus* infection [108]. Generation of these antibodies against the total ES (HES), was essential for the generation of specific type-2 immune responses against a larval challenge and also helped to limit migration of the parasite from the sub-mucosa.

C57BL/6 were vaccinated with exosomes, total HES, Sup or PBS following the same alum-vaccination protocol as stated previously. The resulting egg clearance and successful expulsion of the adult parasite in exosome or HES, and Sup-vaccinated animals demonstrate the potential of *H. polygyrus* secreted products to be a key source of vaccine candidates, as well as playing an important role during

natural infection. Promisingly, the exosome-vaccinated mice, in which the exosome fraction consists of a smaller repertoire of peptides/antigens, could generate similar protection to infection compared to mice vaccinated with total HES or Sup. It is interesting that exosomes and Sup can exert similar effects (in this study and others in this thesis), given that the antibody data suggests that they are distinct in their composition. It will of interest in future studies to ascertain the degree of immune modulation or protective capabilities of either component, and whether there are any responses specific to exosomes.

Understanding the immune-modulatory properties of *H. polygyrus* exosomes serves two purposes. Firstly, they can be exploited for therapeutic treatments in inflammatory or allergic responses. Secondly, targeting exosomes by vaccination can circumvent the immunosuppression that *H. polygyrus* normally induces to prevent expulsion by the host. The data from chapter 4, and the results from the *Alternaria* experiments have illustrated the capacity of exosomes to suppress the ST2 subunit of IL-33 receptor, and the mRNA levels of il1rl1 both *in vitro* and *in vivo*. Therefore, the next chapter will discuss the relationship between exosomes and this receptor in more detail.

Chapter 6

The role of the IL-33 receptor subunit, ST2, during interactions with *H. polygyrus* and exosomes

6.1 Introduction

There is increasing evidence for the role of innate or alarmin responses and their role in anti-helminth responses [390]. The initial interactions between a helminth and its host generally occur at barrier surfaces e.g. the intestinal epithelium, often resulting in host cell damage mediated by parasite migration through the tissue. One consequence of this damage is the concurrent inflammation that occurs at barrier sites where, for example, bacterial translocation could accompany helminth invasion. Host recognition of these invaders is mediated though pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), that drive inflammatory cytokine production. Although generally shown to detect microbial components, some data has described the detection of helminth products by PRRs (as discussed in chapter 1 and [391]). Another major aspect of this response is mediated through the release of alarmins, which are secreted from host cells following a breach of the host epithelium. The key alarmins associated with helminth-mediated barrier damage include; IL-25, IL-33 and TSLP [32, 33, 392], each of which are linked to the

induction of a Type 2 pro-allergic and anti-helminth mode of immune response. Helminths can also partially or entirely circumvent this threat; for example, the response of dendritic cells (DCs) to TLR ligation is effectively negated by products from *Nippostrongylus brasiliensis* and other helminths, with IL-12 production being especially inhibited [393-396], while epithelial cell release of IL-33 is directly blocked by products released by *H. polygyrus* [397].

In previous chapters, I have demonstrated the suppression of ST2, an IL-33R subunit, and the gene encoding ST2, *il1rl1*, in intestinal epithelial cells, macrophages and ILC2s during a model of airway allergy. ST2 is a subunit of the IL-33R, which forms a complex with IL1RAcP to respond to the IL-33 cytokine, both *in vitro* and *in vivo* [398]. IL-33/ST2 interactions are closely associated with the initiation of responses in allergy and infection [34, 399], with the receptor being expressed on a variety of cells, such as innate lymphoid cells, T helper 2 cells, macrophages and epithelial cells. Among these cell types, it is widely accepted that macrophages play a key role in driving and establishing immunity to helminthic infection [57, 71], and are also known to promote type-2 immunity and strongly polarize to M2-like macrophages following activation of T1/ST2 [62]. In addition, the essential requirement for IL-33/ST2 signalling in macrophages is shown during responses to a chronic *H. polygyrus* infection, whereby previous work has demonstrated that the transfer of IL-33-stimulated macrophages into mice can mediate worm expulsion [400].

More broadly, expression of ST2 is associated with host protection from other helminthic diseases, whereby T1/ST2-deficient mice have impaired immune

responses, shown initially during challenge with *S. mansoni* [278] or during infection with *T. spiralis*, *T. muris* or *N. brasiliensis* [39, 389, 401]. Later studies show that T1/ST2 deficiency leads to increased susceptibility to a wide range of infectious pathogens, including filarial infections [402] protozoan infections such as *Toxoplasma gondii* [403], fungal disease such as *Cryptococcus neoformans* [404], and more recently, during the development of cerebral malaria [405].

Owing to their importance in both immunity and infection, both the regulation and induction of the IL-33/ST2 cascade has been investigated. As a member of the TLR/IL-1R (TIR) superfamily, ST2 forms a heterodimer with IL1RAcP following recognition of IL-33. This initiates signaling through components of the MyD88 complex, leading to activation of the MAPK pathway, and transcription factors such as NFκB (reviewed in [406]). Depending on the cell type, activation of this pathway can induce the release of pro-inflammatory cytokines, e.g. in mast cells and basophils, or type-2 cytokines in ILC2s or Th2 cells [407, 408]. The transcription factor, GATA-3, which is highly expressed in ILC2s and Th2 cells, is also associated with the induction and regulation of downstream IL-33 signalling. This transcription factor binds to the promoter of type-2 cytokines, including IL-5 and IL-13, shown to enhance TH2/ILC2 responses to airway allergy [409, 410]. The expression of ST2 on Th2 cells was previously shown to be IL-33/GATA3-dependent [411]. Other work has demonstrated that oxidation of the IL-33 cytokine itself disrupts the ST2 binding site, and may be a form of self-regulation to limit excessive inflammation by limiting the activity of IL-33 in space and time to the site of invasion [412].

Other forms of regulation have also been investigated, such as microRNAs, which are gaining prominence for their involvement in a wide range of immunological processes [413]. For example, mir-155 is expressed in a number of lymphocytes, including T regulatory cells, B cells and myeloid cells, regulating a number of signaling cascades in adaptive immunity [414]. With regards to innate immunity, a recent study has also shown that IL-33-driven induction of miR-155 in ILC2s can regulate ST2 expression, whereby ILC2s isolated from miR155$^{-/-}$ mice had abrogated GATA-3 expression and type-2 immunity to allergen-mediated airway inflammation [415]. Although *H. polygyrus* exosomes contain miRNA with binding sites that recognize the *il1rl1* transcript, these miRNAs alone were unable to modulate the expression of ST2 when transfected into epithelial cells [219]. It is possible that these miRNAs may induce subtle changes, and work in tandem with other bioactive molecules in exosomes to mediate ST2 suppression.

Characterising the molecular mechanisms involved in ST2 suppression by exosomes would provide essential information on how *H. polygyrus* could target this pathway during infection. Using the T1/ST2 knockout mouse model [278], I investigated the potential effects of exosomes in this system during the alternative activation of macrophages. Later studies established the importance of ST2 during a primary *H. polygyrus* infection, before subsequently assessing the effects of exosome vaccination in these mice during infection.

6.2 Results

6.2.1 Exosomes suppress ST2 in alternatively activated macrophages and in *ex vivo* cultured ILC2s

It has been previously shown during a model of airway allergy that HES could suppress IL-33 release [141], mitigating the alarmin response resulting from epithelial damage caused by the fungus *Alternaria alternata*. Both exosomes [219], and HES could also suppress ST2 in this model (Chapter 5), but the role of this receptor subunit in alternatively activated macrophage-exosome interactions had yet to be investigated. The effect of exosomes on *in vitro* generated bone marrow derived macrophages (BMDMs) was therefore tested.

When BMDMs were co-cultured with IL-4/IL-13 and either exosomes, HES or HES depleted of exosomes, there was marked suppression of ST2 surface expression (6.2.1A), whereas mammalian exosomes induced no discernible effect. A similar pattern was observed in the transcriptional levels of *il1rl1* in which the 60-fold increase induced by IL-4/-13 was almost completely ablated by HES products (Figure 6.2.1B).

The addition of polyclonal anti-exosome sera together with exosomes preserved ST2 expression in AAMφ (Figure 6.2.1C). This effect was lost when using rat IgG, which blocks the Fc Receptor (FcR) of cells, out-competeing the antibodies present in anti-exosome sera. Thus, the modulatory effects of exosomes were restored. Interestingly, abrogation of exosome uptake using cytochalasin D demonstrated a potential correlation to ST2 expression, (Figure 6.2.1C-D). The response of macrophages lacking ST2 expression was then tested, and it was found

that exosome uptake was only modestly suppressed in T1/ST2$^{-/-}$ macrophages after a 24 h incubation (Figure 6.2.1E), suggesting that uptake does not necessarily require this receptor.

6.2.2 ST2 deficiency does not affect exosome modulation of IL-4/IL-13-mediated immune responses by macrophages

In order elucidate whether exosome blockade of alternative activation is a consequence of ST2 inhibition, primary macrophages were isolated from T1/ST2$^{-/-}$ mice. Exosomes significantly suppressed the expression of intracellular RELMα (Figure 6.2.2A) and surface CD206 (Figure 6.2.2B) in both ST2-deficient and wild-type BALB/c macrophages following co-stimulation with IL-4/-13. Additionally, exosomes could still suppress these markers in wild-type macrophages when polarisation was enhanced by addition of IL-33. Finally, a similar pattern was observed in the release of Ym1, RELMα and CCL17, whereby exosome co-treatment suppressed the release of these proteins in IL-4/IL-13 activated wild-type and ST2-deficient macrophages alike (Figure 6.2.2C).

6.2.3 T1/ST2 $^{-/-}$ mice are highly susceptible to primary *H. polygyrus* infection

It is well established that ST2-deficient mice have compromised resistance to helminth infection [278]. However, surprisingly little data have been published on the course of *H. polygyrus* infection in this knock out strain, with only one report describing adult worm burden in these mice at day 45 post-infection [37]. The

susceptibility of this genotype in the earlier stages of infection were confirmed following *H. polygyrus* larval challenge, with higher egg counts observed throughout 28 days of infection (Figure 6.2.3A) and higher worm burdens at the final time point (Figure 6.2.3B) than the partially-resistant wild-type BALB/c genotype. Consistent with previous data from T1/ST2$^{-/-}$ mice during *S. mansoni* infection [278], there was a significant difference in the number of granulomas found in the small intestine, with lower abundance in T1/ST2$^{-/-}$ mice (Figure 6.2.3C).

6.2.4 T1/ST2 $^{-/-}$ mice exhibit reduced innate and adaptive cell responses during primary *H. polygyrus* infection

In order to identify factors correlating with susceptibility of T1/ST2$^{-/-}$ mice, both mesenteric lymph nodes and peritoneal cell populations were isolated 28 days post *H .polygyrus* infection. Significantly fewer numbers of key cell populations were recovered from the mesenteric lymph nodes (MLN) isolated from ST2-deficient mice, including macrophages, ILC2s and CD4+ T cells (Figure 6.2.4A-C), whereas the T regulatory cell population were unaffected (Figure 6.2.4D). The paucity of ILC2s from T1/ST2$^{-/-}$ mice is not unexpected, as lower levels of ILC2s were reported in these mice in the lung during both allergic airway inflammation [404] and *N. brasiliensis* infection [416].

As we encountered a marked reduction in T cell MLN numbers, these cells were harvested 28 d post infection and re-stimulated *ex vivo* with either media, 1µg/ml anti-CD3/CD28 or 1µg/ml exosomes. There was a significant increase in the release of IL-13 (Figure 6.2.4E) or IFNγ (Figure 6.2.4F) following exosome

stimulation, and this response was significantly higher overall in wild type cells compared to T1/ST2$^{-/-}$ cells, but no other T cell cytokines; e.g. IL-4 or IL-17 could be detected (data not shown). Thus, not only are there fewer CD4+ Th cells in the draining lymph nodes of ST2-deficient mice, but those cells display weaker responses to *H. polygyrus* exosome antigens. In the peritoneum, as in the MLN, there were significantly lower numbers of macrophages isolated by lavage in T1/ST2$^{-/-}$ mice (Figure 6.2.4G), and correspondingly, lower levels of proteins normally secreted by alternatively activated macrophages (AAMΦ), Ym1, RELMα and CCL17 (Figure 6.2.4H).

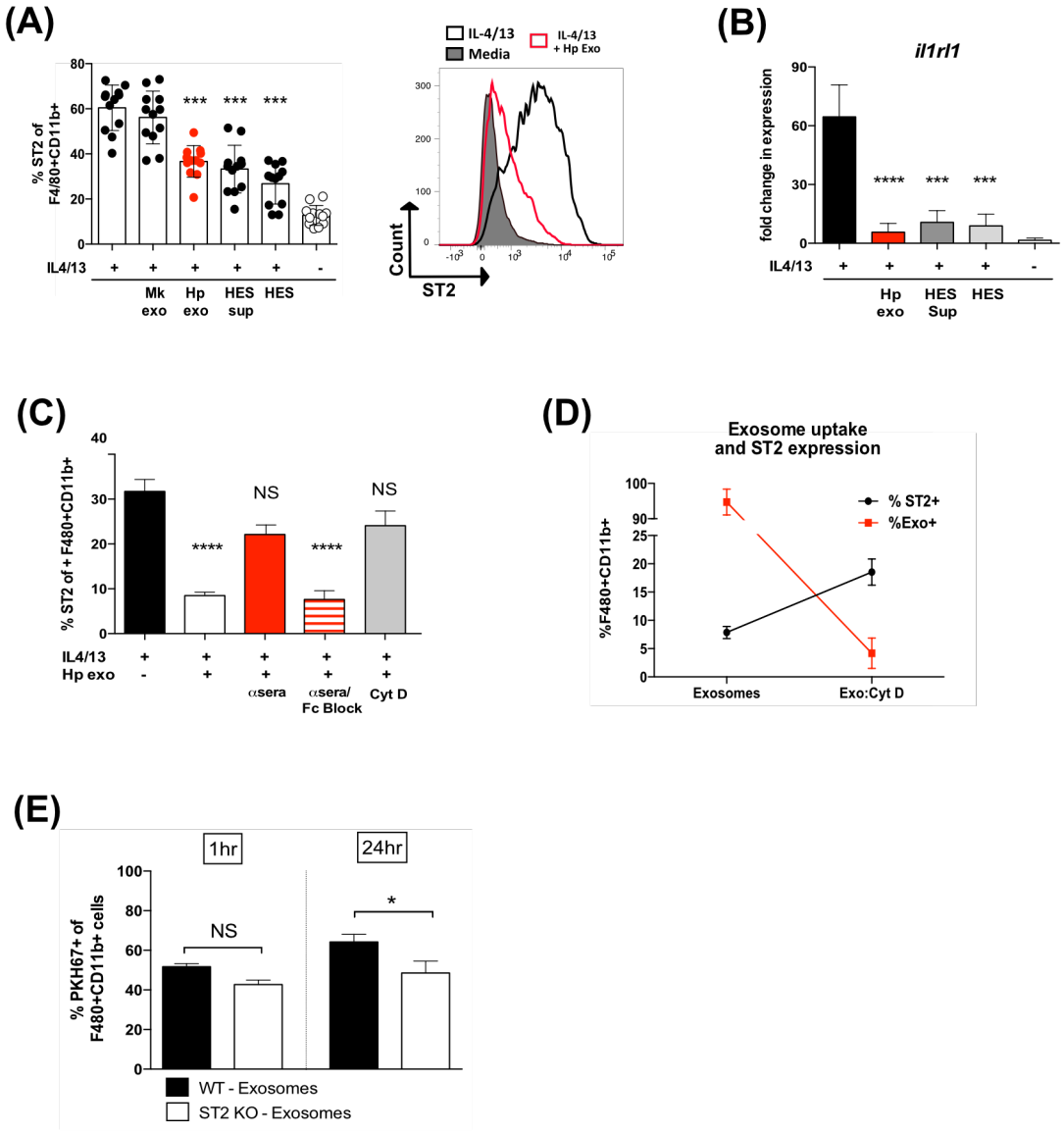
6.2.5 Vaccination against exosomes in T1/ST2$^{-/-}$ mice does not protect against a subsequent *H. polygyrus* larval challenge

As it was previously shown that immunisation of wildtype mice with exosomes was sufficient to generate a high degree of immunity to *H. polygyrus* infection (Chapter 5), the ability of T1/ST2-/- mice to mount an anti-exosome response was investigated. Using the same alum-adjuvant model of vaccination as previously, either wild type BALB/c mice or T1/ST2$^{-/-}$ mice were vaccinated against *H. polygyrus* exosomes, or PBS as a control, before subsequent challenge with infective third-stage larvae and monitoring the course of infection for 28 d. Wild-type mice vaccinated with alum-exosomes had potent immunity to helminth infection, as shown by faecal egg counts (Figure 6.2.5A) and intestinal worm burden (Figure 6.2.5B), summarized in Table 6.2.5. In contrast, T1/ST2$^{-/-}$ mice harbored higher worm numbers with no significant effects induced by exosome vaccination, with

only gradual loss of parasites over time. However, there is a discernable trend, as adult worm burden is reduced by 52% in T1/ST2$^{-/-}$ mice on day 28 (compared to 79% reduction in wild-type mice). Additionally, given the variance, and low animal numbers in this study, these analyses require a larger cohort of mice to confidently assess the data.). Consistent with previous data from T1/ST2$^{-/-}$ mice during *S. mansoni* infection [278], there was a significant difference in the number of granulomas found in the small intestine, with lower abundance in T1/ST2$^{-/-}$ mice (Figure 6.2.5C).

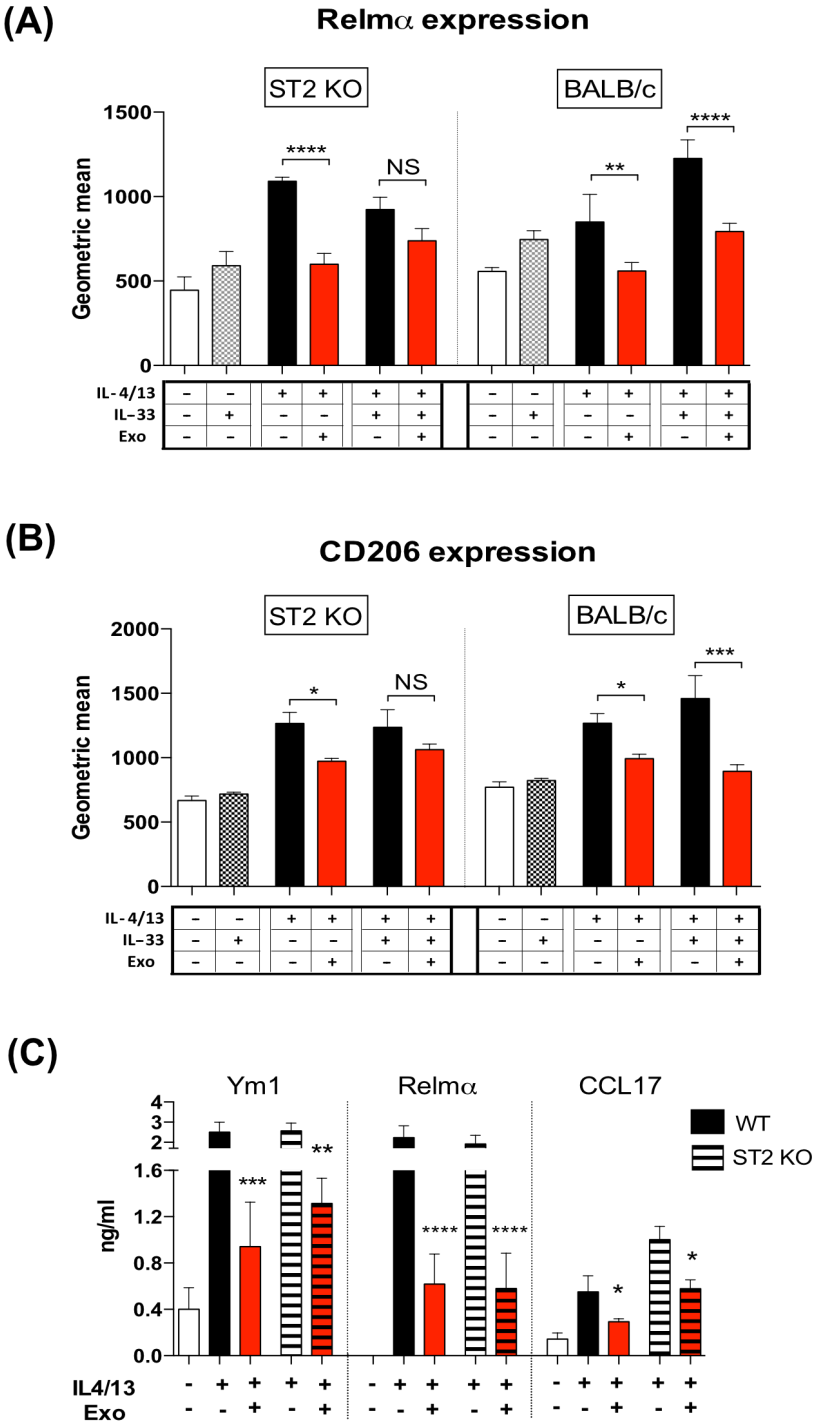
6.2.6 Exosome vaccination induces antibody specific responses in *H .polygyrus* infected T1/ST2 $^{-/-}$ mice

Finally, the profile of total (Figure 6.2.6A), and exosome-specific serum antibodies (Figure 6.2.6B-E), generated in mice during the vaccination/challenge model were determined by ELISA. As antibody responses were shown to facilitate host protection to *H. polygyrus* [108, 255], and other helminths [372], it was important to assess their induction in parasite-susceptible T1/ST2$^{-/-}$ mice following exosome vaccination. Notably, both ST2$^{-/-}$ and wild-type mice generated comparable titres of IgM, IgG1, IgG2a and IgA specific to exosomes, arguing that susceptibility in the absence of IL-33 signalling is due to a deficiency in a cellular, rather than a humoral component, of the immune response.



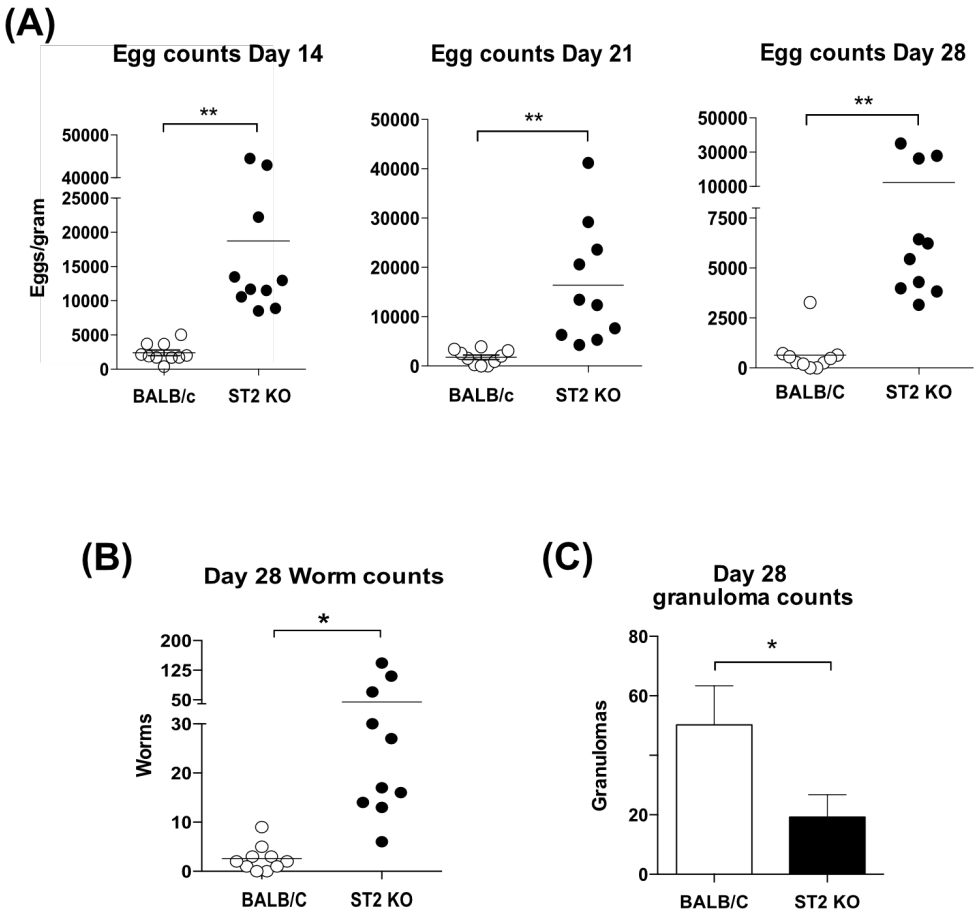
6.2.1 Exosome suppress ST2 in alternatively activated macrophages and *ex vivo* cultured ILC2s

BMDMs were co-cultured with IL-4/13 +/- 5µg/ml exosomes, Sup, HES for 24 h (MODE-k exosomes used a vesicle control). **(A)** The proportion of ST2-expressing F4/80+CD11b+ cells and shift in expression (histogram, *right*) were assessed by flow cytometry, and **(B)** transcriptional levels of *il1rl1* were determined by qPCR and normalised to GAPDH. **(C)** BMDMs were co-treated with either anti-exosome sera, naive rat IgG (Fc block) or cytochalasin D to determine effects on ST2 expression. **(D)** Proportion of PKH67+ cells were correlated to ST2 expression. **(E)** Uptake of exosomes determined in wild-type and ST2$^{-/-}$ BMDMs. **(A-D)** Data are pooled from 3 independent experiments (in biological triplicate), presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. **(E)** Data are from one experiment.



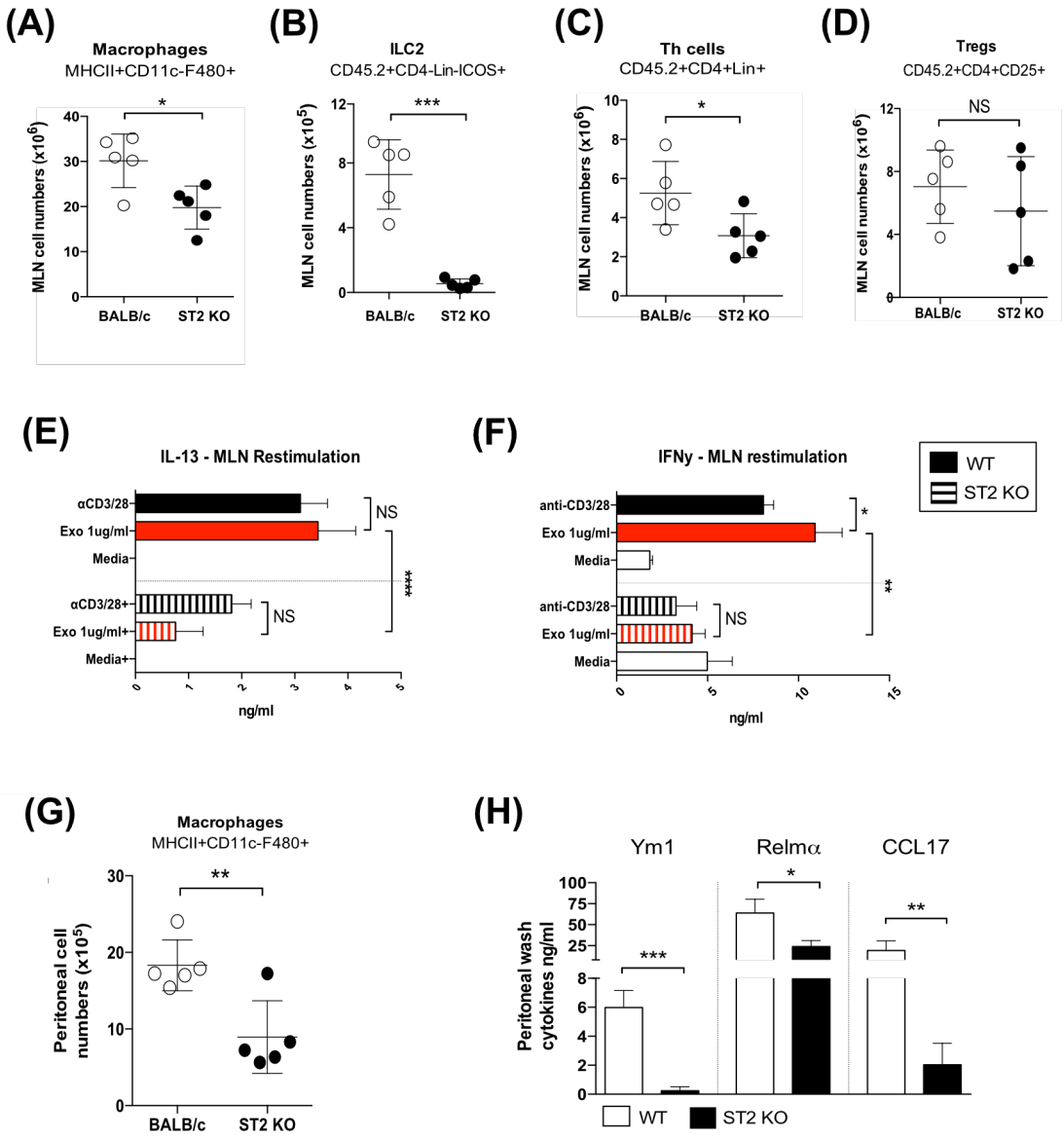
6.2.2 ST2 deficiency does not affect exosome modulation of IL-4/IL-13-mediated immune responses by macrophages

Wild type and ST2$^{-/-}$ BMDMs were treated with 20ng/ml IL-33 and/or IL-4/IL-13 +/- 5µg/ml exosomes. Expression of **(A)** intracellular RELMα or **(B)** surface CD206 were assessed by flow cytometry. **(C)** Levels of Ym1, RELMα and CCL17 found in supernatant were measured by ELISA 24 h post-stimulation. Data are pooled from 2 independent experiments (in biological triplicates) and presented as mean values ± SD; one-way ANOVA. NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.



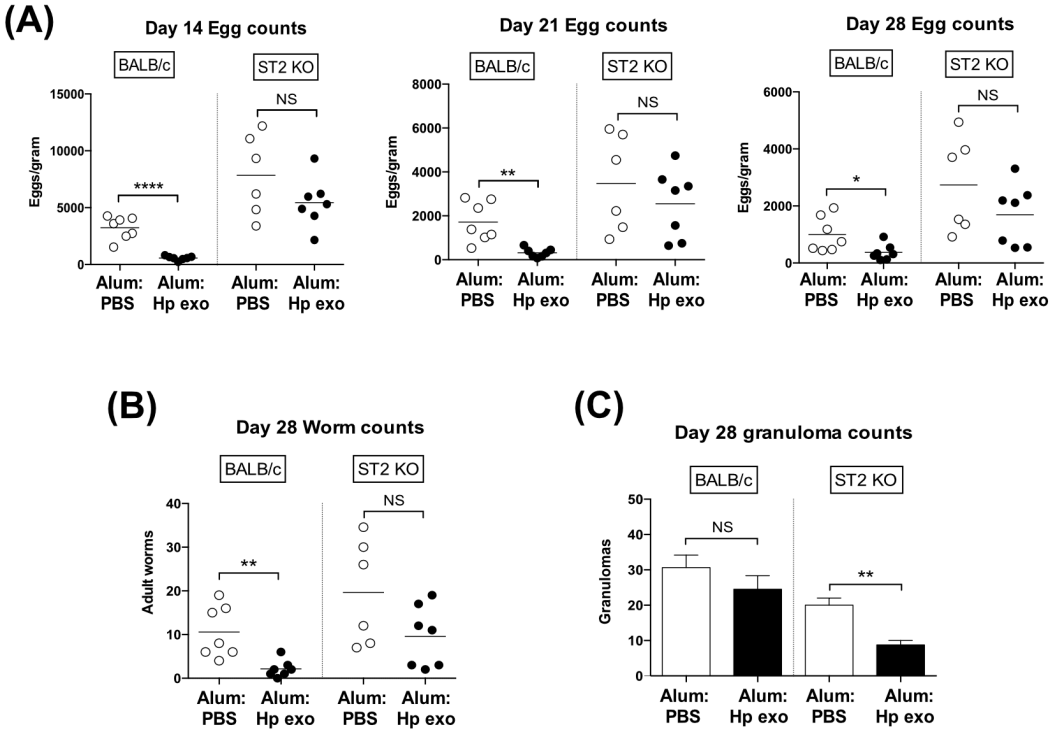
6.2.3 T1/ST2 $^{-/-}$ mice are highly susceptible to primary *H. polygyrus* infection

Female T1/ST2 $^{-/-}$ and wild-type BALB/c mice were infected with 200 *H. polygyrus* L3 larvae. **(A)** Eggs counts/gram faecal matter were calculated on days 14, 21 and 28-post infection. **(B)** Intestinal adult worm burden and **(C)** granulomas were enumerated on day 28. Data are pooled from 2 independent experiments, presented as mean values (n = 10 mice per group; Student's t test). * = $p<0.05$, ** = $p<0.01$



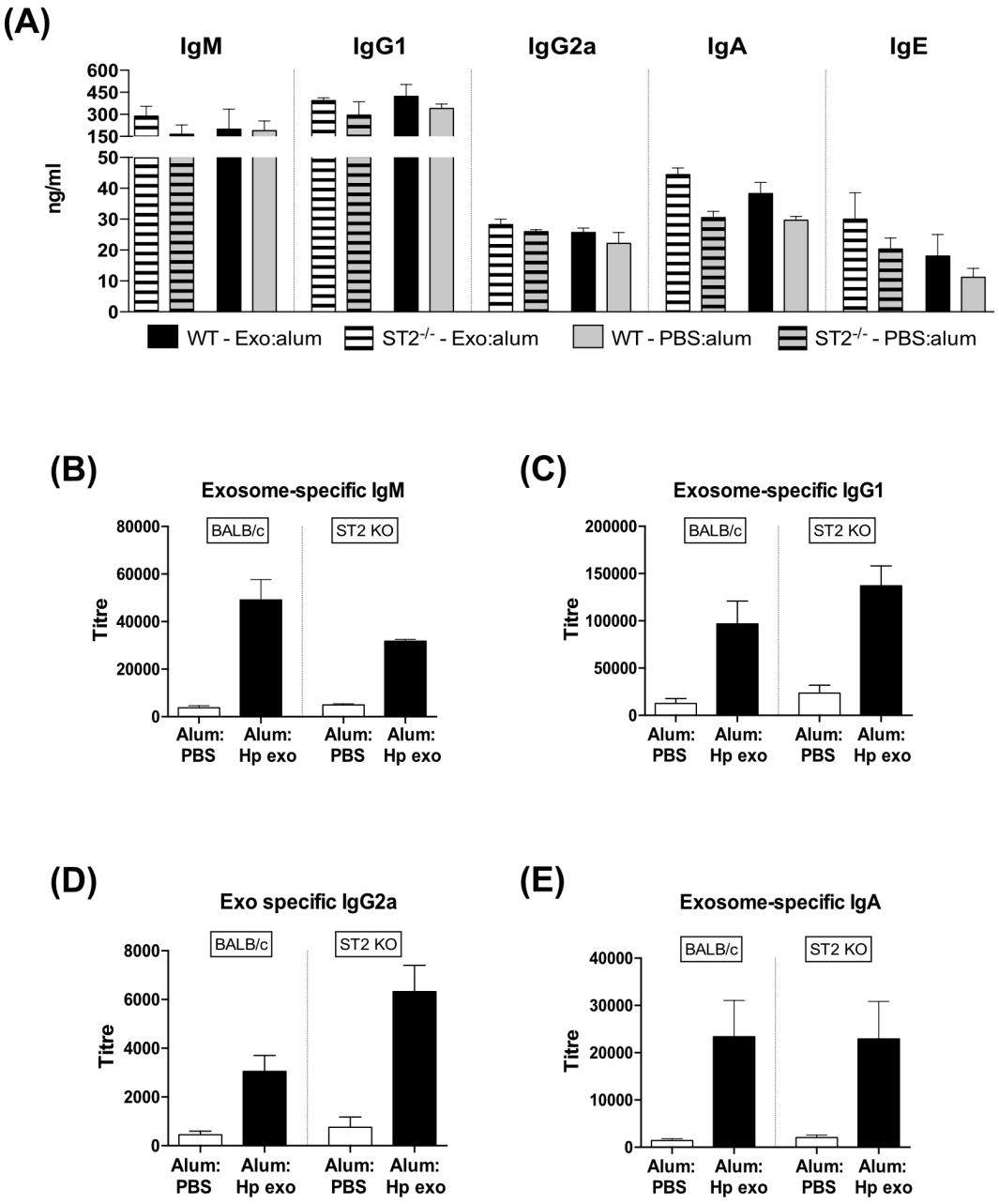
6.2.4 T1/ST2 $^{-/-}$ mice exhibit reduced innate and adaptive cell responses during primary *H. polygyrus* infection

MLNs recovered from *H.polygyrus*-infected BALB/c and ST2$^{-/-}$ mice were isolated at day 28 post-infection. Total numbers of **(A)** macrophages (F4/80+CD11c-MHCII+), **(B)** ILC2s (Lineage-CD4-ICOS+), **(C)** Th cells (CD4+Lineage+) and **(D)** T regulatory cells (CD4+CD25+) were determined by flow cytometry. MLN cells were restimulated with 1 µg/ml exosomes or 2 µg/ml αCD3/CD28 for 72 h and levels of **(E)** IL-13 and **(F)** IFN-γ were measured by ELISA. **(G)** Total macrophages isolated from the peritoneal lavage (gated as above) were quantified by flow cytometry and **(H)** levels of myeloid-derived cytokines; Ym1, RELMα and CCL17 were assessed by ELISA. Data are representative from 1 of 2 experiments, presented as mean values ± SD (n = 5 mice per group; Student's t test). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, *** = p<0.001.



6.2.5 Vaccination against exosomes in T1/ST2 $^{-/-}$ mice does not protect against a subsequent *H. polygyrus* larval challenge

Female T1/ST2 $^{-/-}$ and BALB/c mice were vaccinated with exosomes or PBS in alum adjuvant prior to challenge with 200 *H.polygyrus* L3 larvae (as described in 2.11). **(A)** Eggs/gram faecal matter were calculated on days 14, 21 and 28-post infection. **(B)** Intestinal adult worm burden and **(C)** granulomas were enumerated on day 28. Data are pooled from 2 independent experiments and presented as mean values ± SD (n = 6-7 mice per group; Student's t test). NS indicates a non-significant result. * = p<0.05, ** = p<0.01, **** =p<0.0001



6.2.6 *H.polygyrus* exosomes induce specific antibody responses *in vivo*

Aged matched female BALB/c and ST2$^{-/-}$ mice were vaccinated with exosomes or PBS in alum adjuvant prior to *H.polygyrus* challenge infection (as described in 2.11). **(A)** Total serum IgM, IgG1, IgG2a, IgA and IgE levels in each vaccinated group were determined by ELISA against a standard curve of recombinant mouse IgM, IgG1, IgG2a, IgA or IgE. **(B)** to **(E)** Exosome-specific IgM, IgG1, IgG2a and IgA serum titres from exosome or PBS immunized mice (BALB/c or ST2$^{-/-}$ (KO) as indicated) were measured by ELISA. Data are pooled from 2 experiments, presented as mean values ± SEM (n = 6-7 mice per group).

Table 6.2.5 Parasitology of T1/ST2$^{-/-}$ mice following exosome or HES vaccination and subsequent *H. polygyrus* larval challenge

	Exo/Alum vs PBS/Alum			HES/Alum vs PBS/Alum	
	BALB/c	**ST2 KO**		**BALB/c**	**ST2 KO**
Day 14 Egg counts	72.61%	45.21%		72.61%	45.21%
Day 21 Egg counts	96.21%	88.80%		96.21%	88.80%
Day 28 Egg counts	97.99%	93.25%		97.99%	93.25%
Day 28 Worm burden	**82.08%**	**77.68%**		**82.08%**	**77.68%**

*Percentage reductions in egg/worm burden in exosome vaccinated mice. Each group is pooled from 2 independent experiments (n= 6-7 mice per group). Total HES group data represent one experiment (n=3-4 mice per group)

6.4 Discussion

As detailed previously, exosomes can suppress transcript levels of *il1rl1* (the gene for ST2) in intestinal epithelial cells *in vitro*, and the intensity of its surface expression in ILC2s during an allergic airway response (chapter 5). Data in this chapter illustrate the ability of exosomes, Sup or HES to suppress ST2 expression in alternatively activated macrophages, suggesting an intrinsic mechanism shared amongst the different preparations.

It has been previously shown that signalling through the IL-33 receptor enhances AAMΦ polarization, whereby ST2 is induced in macrophages during type-2 allergic airway responses [62, 417]. However, data in this chapter demonstrate that alternative activation is maintained in wild-type and T1/ST2$^{-/-}$ BMDMs stimulated via the IL-4 receptor-α pathway. In support of this, a recent study has demonstrated that disruption of T1/ST2 signalling had no effect on type-2 mediated responses, including IL-4 release, following challenge by *S. mansoni* [38]. As such, exosomes may modulate ST2 expression to dampen any alarmin responses induced by the parasites during infection. This could be a mechanism used to prevent the activation of ILC2s and/or recruitment of other innate cells during early type-2 immunity (as shown in airway allergy in Chapter 5 and [141]), rather than responses associated with adaptive immunity and wound repair.

Given the correlations between exosome uptake and ST2 expression, it could

be hypothesized that exosomes form a complex with this receptor and be co-internalised. Receptor-mediated endocytosis is a common method of exosome uptake, which is shown across a number of mammalian systems [179], and may be utilized in a similar manner with parasite-derived exosomes. This could also parallel previous data showing the internalization of the mannose receptor (CD206) with *S. mansoni* ES products in macrophages [348]. Despite this, exosome uptake is only modestly reduced in naïve T1/ST2$^{-/-}$ BMDMs, suggesting that, whilst this receptor is not the direct mediator of exosome uptake, deficiency may modulate the total phagocytic capacity of these cells. To support this, there are data demonstrating that IL-33 is shown to enhance the phagocytic activity of macrophages responding to the fungal pathogen *Candida albicans* [418], or during cerebral malaria [419]. However, it was not determined whether uptake of mammalian exosomes was affected in T1/ST2$^{-/-}$ BMDMs, and represents an area that will need to be addressed in future studies, as this could establish whether the IL-33R facilitates uptake of a broader range of vesicles.

Through characterizing the susceptibility of T1/ST2$^{-/-}$ mice to *H. polygyrus* infection, it was also observed that the immune cell repertoires were broadly compromised compared to those isolated from wild-type mice, with MLN cells unable to respond significantly to re-stimulation by exosomes, or through activation by CD3/CD28. The decrease in ILC2s may be a contributing factor in the susceptibility to infection, as previous data has shown that IL-33 supports the expansion of ILC2s and activation of macrophages (dependent on the STAT6/IL-4Rα) during *N. brasiliensis* infection [416]. As ILC2s are a potent source of type-2

cytokines, the low numbers present in T1/ST2$^{-/-}$ mice may correlate to the reduced alternative activation and numbers of macrophages during *H. polygyrus* infection. Although, given recent data demonstrating that IL-33R-driven local proliferation of macrophages can occur independently of IL-4Rα during both filarial nematode infection and airway allergy [435], it may be the combinatorial effect of losing both a prominent source of IL-4, as well as the complete absence of the IL-33R, which contributes to the low numbers of macrophages observed during *H. polygyrus* infection in T1/ST2$^{-/-}$ mice. Furthermore, as macrophages are known play a key role in mediating immunity to *H. polygyrus* [307], this may explain the susceptibility observed in these mice during infection. The overall lower number of granulomas detected in the small intestine of *H. polygyrus* infected T1/ST2$^{-/-}$ mice are consistent with reports showing a reduction of granulomas during *S. mansoni* infection in T1/ST2$^{-/-}$ mice [278]. Both this, and the reduced numbers of both peritoneal and MLN-derived macrophages observed in these mice, may also correlate with data showing that liver granulomas in *L. donovani*-infected mice contained many ST2+ infiltrating macrophages and B cells, whereas T1/ST2$^{-/-}$ mice had a decreased influx of monocytes and polymorpho-nuclear cells to the liver [420].

Unlike the almost sterile immunity induced either in vaccinated wild-type C57BL/6 mice or BALB/c mice, there was no significant protection induced in the same experiment using parasite-susceptible T1/ST2$^{-/-}$ mice. These mice could not control infection following vaccination with exosomes-alum, responding similarly to the control PBS-alum group. These data demonstrate both the essential requirement for IL-33 signalling during resistance to this parasite and the potential role of

exosomes in ST2-mediated anti-parasite responses. This is also relevant given previous work showing that the transfer of IL-33-stimulated macrophages can mediate worm expulsion [400]. Notably, ST2-deficient mice mount antibody responses as powerful as wild-type mice, yet are unable to clear the parasite; hence even in the presence of specific antibody, an IL-33-dependent cell population is necessary for effective immunity.

In conclusion, this chapter reveals that ST2 expression is not required for the development of alternatively activated macrophages, nor the suppressive function of exosomes in this model. However, given the importance of IL-33/ST2 axis during infection [39, 278, 389], it may explain why the parasite targets this pathway with blockade of both the ligand [141] and as I show here, the receptor, to neutralise the host's ability to expel the parasite. Defining the molecular mechanisms that occur during ST2-exosome interactions may be a future objective for therapies that intend to drive innate immunity and expel parasitic infection. It will be particularly interesting to determine whether extracellular-like vesicles secreted by other parasites modulate the IL-33/ST2 axis (owing to their role in susceptibility [34]) or are a specific feature of *H. polygyrus* EVs, especially given recent data showing that total ES from the liver fluke, *Fasciola hepatica*, actually enhanced ST2 expression on peritoneal CD4+ T helper cells [421].

Chapter 7
Final Discussion

The correlative relationship between helminthic burden and autoimmune or inflammatory disease is one that has generated increasing interest in the last ~20 years. The 'hygiene hypothesis' [132] has illustrated the ability of helminths to modulate the host immune system during infection, preventing their clearance, promoting wound repair and, consequently, inducing bystander suppression of autoimmune disease or atopy [380, 422]. The complex mechanisms by which helminths evade host immunity range from shedding of surface antigens [145], to the secretion of a large range of excretory-secretory molecules, which often have potent immunomodulatory properties [391]. Thus far, the literature would suggest that EVs produced from protozoan parasites enhance pathogenesis and suppress host immunity to support parasite survival [188]. It is therefore interesting to note the growing number of publications demonstrating that EVs are also a ubiquitous component of the secretion products of metazoan helminth parasites, including trematode flatworms [220], as well as both human and veterinary nematodes [247, 249, 262, 423, 424]. EVs represent a new type of complex encompassing a set of packaged cargo that has the potential to interfere with host immunity, and represents novel targets for future investigation.

This body of work had two major arcs; the first being to characterize exosomes isolated from *H. polygyrus* excretory-secretory products, investigating their potential origin from the parasite, their antigenic surface markers and

optimizing tools for their detection. The second was to elucidate their role in host-parasite communication: how they interact with host cells, their function during the natural infection, and the effect of parasite-derived exosomes on host immunity.

7.1 Exosomes exhibit broad immunomodulatory functions on host cells correlating to uptake in a dose and time-dependent manner

There is limited literature describing the internalisation and intracellular trafficking of parasite-derived EVs, and the impact this may have on their function within host cells. The uptake studies in Chapter 4 demonstrate that *H. polygyrus* exosomes are internalized by host cells at a similar rate to mammalian exosomes (derived from macrophages or small intestinal epithelial cells). This correlates with other data published by our group, showing that nematode exosomes demonstrate similar fusogenic properties to mammalian exosomes, despite having different biophysical characteristics [350]. However, in order to clarify this further, an assay determining the proportion of vesicles which may be taken up by direct fusion, such as shown in [178], is required in future analyses. I have demonstrated that macrophages preferentially internalize *H. polygyrus*-derived exosomes, most likely through a phagocytic or endocytic pathway that can be blocked by cytochalasin D and enhanced by specific antibodies (presumably through opsonisation). This was validated by confocal microscopy, which revealed the presence of potential endosomal aggregates of *H. polygyrus* EVs within BMDMs after 1 h. Thus, in the early events of uptake parasite-derived EVs are likely still within the endosomal

compartment, which accords with previous observations demonstrating intracellular endosomal aggregates following EV uptake [288, 325]. Whilst uptake might initially be interpreted as a host mechanism for removal of parasite exosomes, I have shown that the nematode-derived exosomes exert functional properties on the recipient macrophages, dependent on the mode of uptake.

7.1.1 Suppression of inflammatory responses to TLR stimulation as a mechanism of host immune evasion

During nematode infection, the breakdown of intestinal integrity can lead to systemic inflammatory or alarmin response through the presence of infiltrating bacteria [307, 308]. In order to regulate this, *H. polygyrus* ES products is shown to modulate immune reactivity during infection [151], for example, preventing responses to TLR stimuli [341, 342]. *H. polygyrus* exosomes can induce similar effects on LPS-mediated macrophage activation, suppressing effector molecules such as iNOS, pro-inflammatory cytokines and downstream signaling molecules such as MyD88. Interestingly, MyD88 is also known mediate innate IL-33-driven responses to helminth infection [389], as it can bind the intracellular Toll/IL-1 receptor (TIR) domain of the IL-33 receptor (ST2). The association of ST2 with this adaptor is consistent with the exosome-mediated suppression of *il1rl1* in both epithelial cells and macrophages. Finally, *H. polygyrus* exosomes also suppress markers of cell death, such as FAS. Interestingly, FAS activation has been shown to enhance LPS-mediated IL-1R1-signalling to promote chronic inflammation [425]. Thus, exosomes can modulate a number of components associated with type-1 immunity to limit inflammation and prevent alarmin responses.

7.1.2 Modulation of type-2 immunity to inhibit host clearance

H. polygyrus EVs generate potent suppression of AAMΦs, modulating type-2 effector molecules, such as RELM-α and Ym1 when administered during or after the onset of alternative activation by IL-4/13. It was rather surprising that exosomes could suppress markers of M2 activation, given their role in tissue repair and mediation of anti-inflammatory responses [58, 347]. However, one of the molecules suppressed, arginase-1, is known to be an essential component of protective immunity to intestinal helminths [115, 122]. The complexity of type-2 immune responses, and the variable roles of type-2 effector molecules [58] may explain why parasites can suppress aspects of this response depending on context or stage of infection. Importantly, treatments that alter the manner of exosome uptake in alternatively activated macrophages, such as cytochalasin D or anti-exosome antibodies, abrogated exosome-suppressive effects in these cells, highlighting the requirement for specific exosome-macrophage interactions during infection. Interestingly, previous data demonstrate that *S. mansoni* ES products can be internalized following CD206 binding on macrophages. This leads to diminished Th2 cell responses and the establishment of a chronic infection [348], and may be a receptor pathway exploited by *H. polygyrus* exosomes.

7.1.3 Small intestinal organoids – bridging the gap between *in vitro* and *in vivo* studies using *H. polygyrus* exosomes

The small intestinal organoid system provides a new and exciting link between *in vitro* and *in vivo* studies using *H. polygyrus* exosomes. During natural infection, it is hypothesized that adult worms release ES (and potentially, exosomes) into the

intestinal environment to induce systemic immune suppression of the host [307]. Exosomes were microinjected into the luminal centre of the organoids in an attempt to replicate these conditions *in vitro*. Each organoid was injected with ~3 ng exosomes (equating to roughly 1 x 10^6 vesicles) and left for a 24 h period. In chapter 3, I show a working model to calculate the number of vesicles potentially secreted by an adult worm over a two-week period (equating to over 7 x 10^5 vesicles per day, per worm). It also important to note that these are just approximations, and are also based on the number of vesicles released during culture, and not what may be released in the intestinal environment. This reflects the difficulties in calculating a physiological dose of exosomes, an issue that is faced by the EV community at large [264]. A further unanswered question remains the potential heterogeneity of parasite exosomes on a per vesicle basis, and whether this is affected by environmental cues or the status of the worms themselves (see future work).

Although the organoid data is preliminary, it suggests potential localization of *H. polygyrus* exosomes to specialized intestinal compartments, such as goblet cells or Paneth cells. Both cell types release anti-microbial effector molecules, such as defensins, mucins and resistin-like molecules, which are shown to which influence parasite viability and expulsion [52, 54, 351]. If exosomes are internalized by these innate effector cells, they could potentially induce suppression of their responses, in a similar fashion to what was observed in macrophages in Chapter 3.

7.2 The immunosuppressive properties of exosomes can be exploited as a therapeutic treatment in airway inflammation

The interactions between host cells and parasite ES offer a potential target for future interventions, aiming to prevent uptake of parasite products or inhibit skewing/modulation of host immunity [391]. As a newly discovered component of *H. polygyrus* ES [243], exosomes were the focus of several studies to determine their contribution to the modulation of airway allergy that has been demonstrated previously by total HES [141, 373]. Exosomes were able to suppress the early cellular responses induced by the airway allergen *Alternaria alternata,* but they did not influence adaptive recall responses to this antigen. Parasite-derived exosomes, used prophylactically or by co-administration, modulated airway eosinophilia and the secretion of type-2 effector molecules, such as Ym1 and RELM-α. Interestingly, this appears to be mediated through ILC2s, given their function as an immediate source of type-2 cytokines, such as IL-5 and IL-13 [40], which promote both innate eosinophil responses [41] and adaptive Th2 responses to *H. polygyrus* [43]. Furthermore, exosomes can also suppress the expression of the IL-33 receptor (IL-33R) on ILC2s. Prospective studies should aim to determine how exosomes induce suppression of ILC2 function, and whether this is linked to modulation of the IL-33R response. This receptor has been shown throughout the thesis to be modulated by exosomes, in intestinal epithelial cells and in activated macrophages. Thus, exosomes, like HES, possess immunomodulatory properties which can be used to suppress host immunity and alarmin responses to fungal allergens. Whether exosomes can replicate the immunosuppressive functions of adult *H. polygyrus*

shown in other models of inflammation, such as type-1 diabetes and colitis [357, 426], remains to be seen.

7.3 *H. polygyrus* exosomes modulate the IL-33R-driven alarmin response to circumvent host immunity

A recurring observation during this study was the modulation of the IL-33R by *H. polygyrus* exosomes both *in vitro* and *in vivo.* As seen in Chapters 4, 5 and 6, parasite-derived exosomes were able to mediate the suppression of *il1rl1,* the gene for IL-33R, in MODE-k cells and primary macrophages, as well as modulating surface expression of T1/ST2 on type-2 innate lymphoid cells *in vivo.* Although this modulation is also induced by HES depleted of exosomes, data in Chapter 6 demonstrated a correlation between ST2 expression and exosome uptake. Thus, suppression of this receptor may be mediated by several different mechanisms in HES. Although exosome modulation of type-2 immunity can occur independently of ST2, this receptor is important for resistance to parasite infection, as demonstrated previously in other helminths [34]. Interestingly, sterile immunity to infection can be induced in wild-type mice by immunisation with an exosome-containing vaccine (Chapter 5). However, the same exosome vaccination in T1/ST2 deficient mice failed to clear the infection. Hence, the activation of IL-33 signalling is essential for immunity to infection, and requires neutralisation of exosomes to take place in wild-type mice for parasite expulsion. In summary, *H. polygyrus* secreted exosomes could

be used by the parasite to circumvent host 'danger' immune responses initiated upon IL-33 receptor signalling that would normally lead to their expulsion.

7.4 Exosomes and exosomal components represent a new target for vaccination against parasitic infection

The ability of parasite-derived exosomes to modulate host immunity [188], they offer an attractive target for vaccination [226]. Exosome-alum adjuvant vaccination induces potent IgG1 antibody responses in mice, which was shown previously to promote resistance during *H. polygyrus* infection [108, 255]. Given the comparable levels of protection elicited by vaccination with either HES, exosomes, or HES-depleted of exosomes, it seems reasonable to suggest that the immunogenic epitopes could be shared amongst all preparations. Using cross-linked immunoprecipitation, I isolated the exosome antigens bound to sera from exosome-immunized mice, and determined their identity via mass spectrometry. This analysis found an enrichment of proteins observed in total exosomes, and it has been interesting to identify exosome antigens that raise antibody responses (shown in Chapter 3). Tetraspanins are transmembrane proteins which are suggested to have important roles in EV biogenesis via cargo selection, targeting and cellular uptake [173]. Notably, a large number of studies have attempted to vaccinate against tetraspanins, or highlighted them as important candidates, in a number of pathogenic diseases, including filariasis [427], schistosomiasis [428], and echinococcosis [429]. Tetraspanin-11, highly enriched on *H. polygyrus* exosomes [219], could potentially be facilitating a number

of the processes that are mentioned above, and deserves further investigation to demonstrate its viability as a target for vaccination.

7.5 Future Directions

Given the youth of the EV field and the starting point of this project, there are a number of areas still to be investigated. Although we have followed a standard regimen for isolating vesicles by ultracentrifugation [251], we may be collecting a heterogeneous population of EVs, potentially consisting of both exosomes and larger microvesicles. Recent work has described the heterogeneity of EVs secreted by *F. hepatica,* isolated by differential ultracentrifugation (with larger vesicles collected from a 15,000 *g* spin, and smaller vesicles with a subsequent 120,000 *g* spin), which are shown to differ both in size and molecular cargo [250]. It is therefore prudent for future studies to attempt either differential centrifugation or sucrose gradient centrifugation with HES, in order to determine whether EVs of different sizes can be isolated. It will be interesting to see whether *H. polygyrus* EVs are similarly diverse to [250], and how this may relate to their function during infection. Another key area of research will concern mechanisms of parasite-derived EV uptake and release, in which data are still limited for both pathogens and mammalian EVs [177, 188, 288]. Tetraspanins offer an attractive target for therapeutic intervention, given their important roles in EV cargo selection, target cell fusion and uptake [173]. This is supported by a recent study which demonstrated that antibodies generated against *O. viverrini* EV tetraspanins blocked subsequent EV uptake into host cells [227]. In

addition to tetraspanins, *H. polygyrus* exosomes also contain a suite of immunogenic molecules that induce host antibody responses, providing a number of vaccine candidates that should be investigated in the future.

Moreover, the intestinal organoid system provides an excellent physiological model for studying localized *H. polygyrus* exosome-host cell interactions, and is likely to provide important information about the intracellular fate of helminth-secreted exosomes. Based on current literature, host immune manipulation appears to be a prevalent function of parasite-derived exosomes [188]. However, it is expected that EVs could also play a prominent role in parasite-to-parasite communication, which has been less well studied to date. It will be fascinating to determine if *H. polygyrus* exosomes possess this function, as previous data have demonstrated the influence of adult worms on the establishment of commensal bacteria in the intestine [135]. Additionally, there may be scope for *H. polygyrus* worms using EVs to communicate with each other, a mechanism which has been reported in protozoan parasites [206, 209, 236] and other microbes (reviewed in [237]). This mechanism has been used by the blood stages of *P. falciparum* [206, 236] to facilitate sexual maturation, and *T. vaginalis* to promote invasion by improving host cell adherence [209]. Future work could examine the properties of *H. polygrus* EVs on different life stages of the parasite, observing whether this improves their function or the potency of infection.

7.6 Conclusions

In conclusion, these results contribute to our current knowledge of exosomes as

functional components of *H. polygyrus* ES, establishing them as an important new vehicle to mediate cross-species communication. The rapidly expanding body of data demonstrating that extracellular vesicles are secreted by diverse parasites brings with it many questions related to how exosomes and other EVs function in host cells. The fundamental interactions between parasite-derived exosomes and host cells that are characterised in this thesis establishes an important framework for future study. Given their biological complexity, a deeper understanding of the different properties of parasite-derived exosomes will be key to determining how these packages of information operate. Suck insights will be crucial to determine how we can interfere with or mimic these processes to treat infectious and inflammatory diseases.

Chapter 8
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Chapter 9

Appendix

9.1 Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity

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Amy H. Buck, Gillian Coakley, Fabio Simbari, Henry J. McSorley, Juan F. Quintana, Thierry Le Bihan, Sujai Kumar, Cei Abreu-Goodger, Marissa Lear, Yvonne Harcus, Alessandro Ceroni, Simon A. Babayan, Mark Blaxter, Alasdair Ivens & Rick M. Maizels

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Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity

Amy H. Buck[1,2], Gillian Coakley[1,2,*], Fabio Simbari[1,2,*], Henry J. McSorley[1,2], Juan F. Quintana[1,2], Thierry Le Bihan[2,3], Sujai Kumar[4], Cei Abreu-Goodger[5], Marissa Lear[1,2], Yvonne Harcus[1,2], Alessandro Ceroni[1,†], Simon A. Babayan[2,†], Mark Blaxter[2,3,6], Alasdair Ivens[2] & Rick M. Maizels[1,2]

In mammalian systems RNA can move between cells via vesicles. Here we demonstrate that the gastrointestinal nematode *Heligmosomoides polygyrus*, which infects mice, secretes vesicles containing microRNAs (miRNAs) and Y RNAs as well as a nematode Argonaute protein. These vesicles are of intestinal origin and are enriched for homologues of mammalian exosome proteins. Administration of the nematode exosomes to mice suppresses Type 2 innate responses and eosinophilia induced by the allergen *Alternaria*. Microarray analysis of mouse cells incubated with nematode exosomes *in vitro* identifies Il33r and Dusp1 as suppressed genes, and Dusp1 can be repressed by nematode miRNAs based on a reporter assay. We further identify miRNAs from the filarial nematode *Litomosoides sigmodontis* in the serum of infected mice, suggesting that miRNA secretion into host tissues is conserved among parasitic nematodes. These results reveal exosomes as another mechanism by which helminths manipulate their hosts and provide a mechanistic framework for RNA transfer between animal species.

[1] Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK. [2] Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK. [3] SynthSys, Centre for Synthetic and Systems Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3BF, UK. [4] Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK. [5] Laboratorio Nacional de Genómica para la Biodiversidad, Langebio-CINVESTAV, Irapuato 36821, Guanajuato, México. [6] Edinburgh Genomics, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK. * These authors contributed equally to this work. † Present addresses: Target Discovery Institute, University of Oxford, Nuffield Department of Medicine Research Building, Oxford OX3 7FZ, UK. (A.C.); Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK (S.A.B.). Correspondence and requests for materials should be addressed to A.H.B. (email: a.buck@ed.ac.uk).

Parasitic nematodes are ubiquitous pathogens of plants and animals, including species that infect over 2 billion people and generally reside in extracellular niches in their hosts. *H. polygyrus* is a parasite related to human hookworm that naturally infects mice, and is in the same nematode clade as *Caenorhabditis elegans*[1]. Within the mouse host, the parasite life cycle is exclusively intestinal: following ingestion, the larvae invade the small intestine, moult into adult worms and emerge into the lumen of the duodenum to mate and to produce eggs expelled in the faeces. The infection induces Type 2 innate and adaptive (Th2) immune responses in parallel with a large expansion of regulatory cells that mediate immunosuppressive effects[2–4], some of which have beneficial properties in allergy and auto-immunity[5]. Immune suppression has been shown to be mediated in part by a suite of immunomodulatory proteins actively secreted by the nematodes[6,7]. Given the burgeoning body of data detailing extracellular small RNAs in mammalian systems, and emerging evidence that these can mediate cell-to-cell communication[8], it is intriguing to think this mechanism could also be used by parasites. Small RNAs derived from bacteria, plants and parasites have been detected in human body fluids[9–12]; however, the mechanism by which these are secreted or excreted is unknown, and the meaning of their extracellular existence unclear[13]. We show here that *H. polygyrus* secretes a specific set of miRNAs and full-length Y RNAs that are stabilized against degradation by encapsulation within vesicles. The vesicles are of intestinal origin and are enriched for homologues of mammalian proteins found in exosomes, including heat shock proteins, tetraspanins and ALIX, a protein associated with exosome biogenesis[14,15] as well as a nematode Argonaute (Ago) protein. Local administration of the nematode exosomes to mice by the intranasal route suppresses Type 2 innate responses and eosinophilia induced by the allergen *Alternaria in vivo*. The nematode vesicles are internalized by mouse intestinal epithelial cells *in vitro* and suppress genes involved in inflammation and immunity, including the receptor for the alarmin IL-33 and a key regulator of mitogen-activated protein kinase (MAPK) signalling, DUSP1. This work identifies exosomes as a new class of immunomodulatory complex produced by helminths and provides the first steps towards a mechanistic framework for RNA-mediated communication between animal species.

Results

Small RNAs in *H. polygyrus* secretory products. Total RNA was extracted from the secretory products of *H. polygyrus* and compared with the profile of small RNAs in adult nematodes, eggs and infective larvae. A heterogeneous population of small RNAs <25 nucleotides (nt) was observed in all samples and several additional species between 25 and 30 nt were apparent in the secretion product (Fig. 1a). Small RNA sequencing (<30 nt) identified miRNAs as the dominant class of secreted parasite small RNA (Fig. 1b) and also identified RNA fragments mapping to nematode stem-bulge RNAs, herein referred to as Y RNAs based on their recognized homology to this class of small RNA[16] (Fig. 1b). In contrast, piRNAs (or '21-U' RNAs) were exclusively identified in the adult library (~14% of reads; Supplementary Fig. 1 and Supplementary Data set 1) with no evidence of secretion. RNAs between 70 and 100 nt were also present in the secreted product (Fig. 1a) and sequencing identified full-length Y RNAs as the major component of this fraction, with two predominant classes of structure identified (Fig. 1c and Supplementary Fig. 2).

A total of 263 'high confidence' miRNAs were classified from the combined libraries based on representation of reads from both 5p and 3p arms of the hairpin and/or homology with known miRNAs in other nematodes (Supplementary Data set 1). Each of these matches DNA sequences in the *H. polygyrus* whole genome currently under assembly. Many also show stage-specific expression patterns: for example, miR-35 family members are exclusively expressed in the egg library, consistent with their functions in embryogenesis[17] (Fig. 2a, boxed). The secretory products are dominated by miRNAs with identical seed sites to mouse miRNAs, many of which are ancient: six are shared among

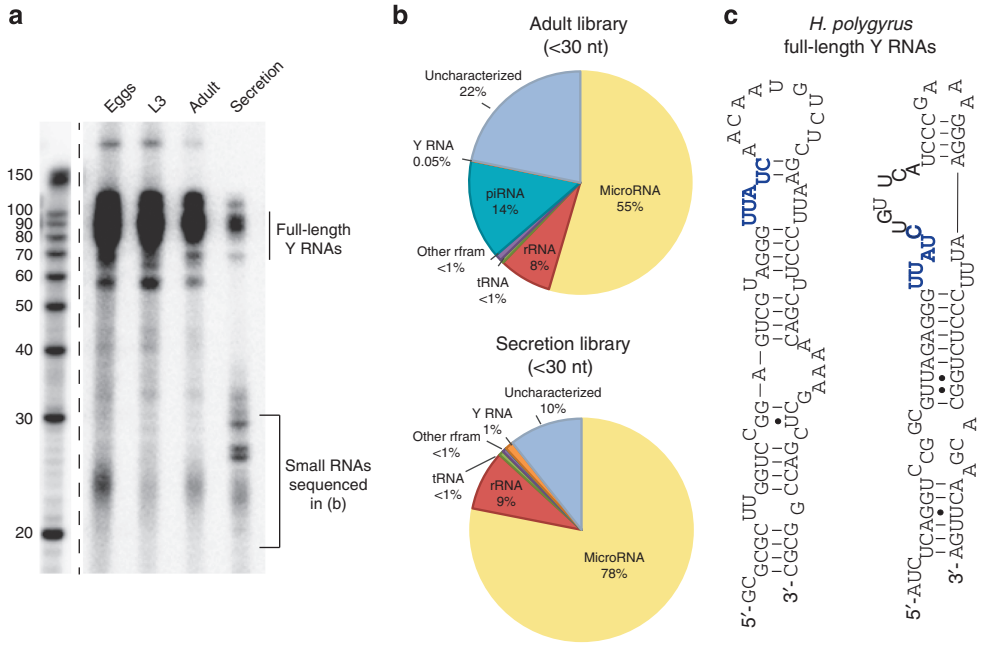

Figure 1 | *H. polygyrus* secretory products contain miRNAs and Y RNAs. (**a**) Size distribution of 3′-end labelled (pCp) total RNA extracted from the life stages (1 μg total RNA) or secretion product of *H. polygyrus* (total RNA from equivalent of 15 μg protein secretion product). (**b**) Proportion of *H. polygyrus* small RNA biotypes (<30 nt) identified in sequencing libraries from adult worms and the secretion product. (**c**) Predicted secondary structures of the two families of Y RNA identified in *H. polygyrus* secretion products based on RNAfold, the conserved UUAUC motif is noted in blue.

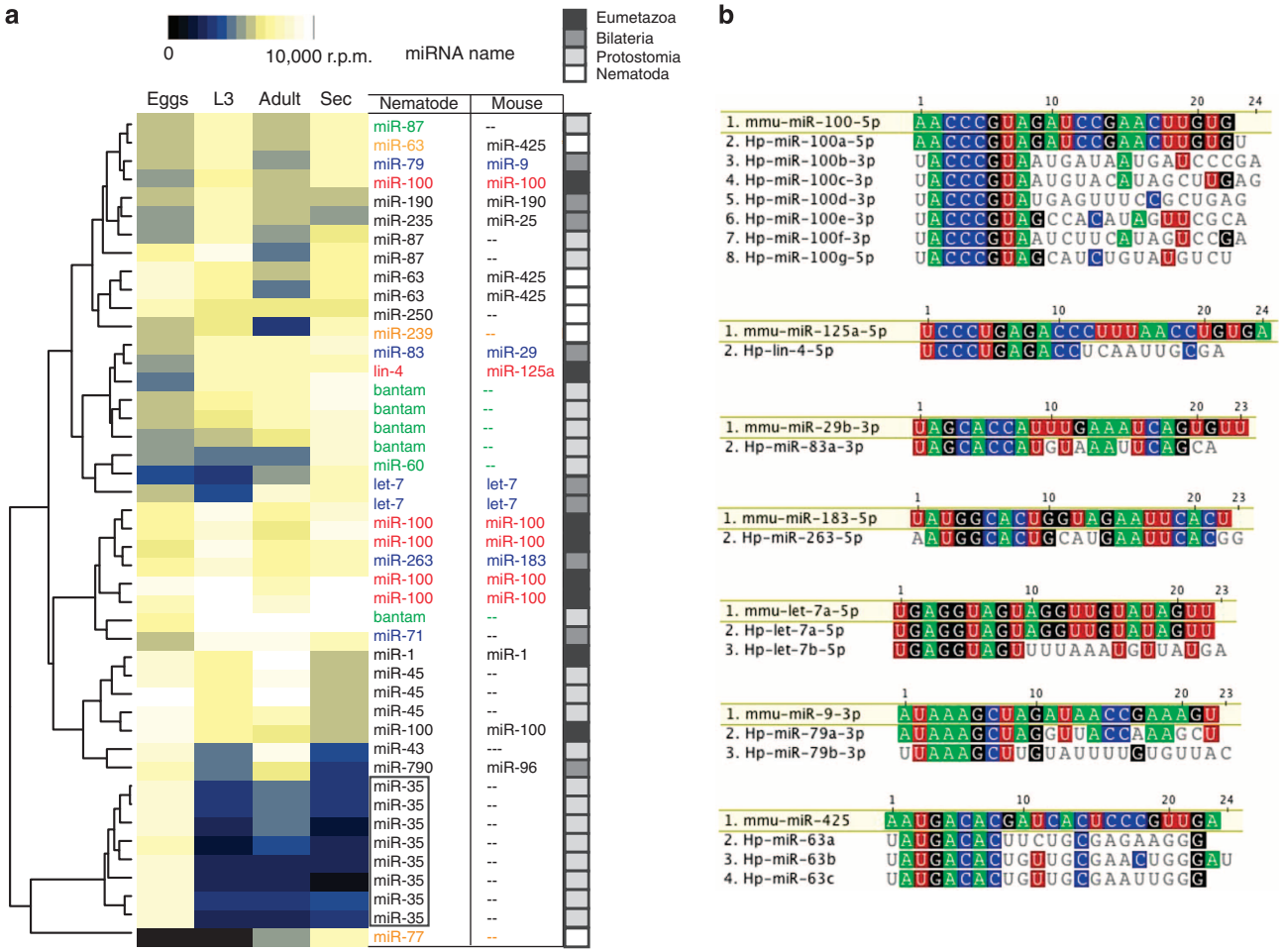

Figure 2 | Many secreted nematode miRNAs have identical seed sites to mouse miRNAs. (**a**) Temporal expression of highly abundant miRNAs (>10,000 reads per million in at least one of the libraries) across the life stages. Nematode and mouse names are listed according to identical seed sites and miRNAs of high abundance in the secretion product are coloured according to their conservation level[18]: Eumetazoa (red), Bilateria (blue), Protostomia (green), Nematoda (orange). (**b**) Sequence alignment of abundant secreted parasite miRNAs that contain identical seed sites between mouse and *H. polygyrus*; all families shown are of common ancestry[18] apart from miR-425/63.

Eumetazoa (lin-4/miR-125 and five miR-100 family members, Fig. 2a, red) and five among Bilatera (miR-79/miR-9, miR-83/miR-29, miR-263/miR-183 and two let-7 family members, Fig. 2a, blue). In addition, five bantam family members dominate the secretory products along with miR-87 and miR-60, which also are shared among Protostomia (Fig. 2a, green) and three miRNAs that evolved in the nematode lineage: miR-63, miR-239 and miR-77 (Fig. 2a, orange). miR-63 shares an identical seed site to mammalian miR-425, although it is not of common ancestry[18], Fig. 2b. On the basis of their sequences, many of the secreted parasite miRNAs could therefore hijack existing mouse miRNA target networks if taken up by host cells.

Nematode vesicles are associated with secreted RNA. In mammalian systems, miRNAs have been found in body fluids in association with specific proteins or in extracellular vesicles[8]. To determine whether these RNAs could be present in vesicles, the *H. polygyrus* secretory products were ultracentrifuged and quantitative reverse transcription–PCR (qRT)–PCR used to measure miRNA levels in the pellet and supernatant, revealing the majority to be present in the pellet (Supplementary Fig. 3). Transmission electron microscopy (TEM) identified vesicle-like structures between 50 and 100 nm in diameter in the pelleted material (Fig. 3a). Label-free quantification of proteins in the vesicles and supernatant by liquid chromatography-electrospray tandem mass spectrometry LC-MS/MS identified 362 proteins, of which 139 were specifically enriched in the vesicle fraction ($P<0.05$, Fig. 3b, Supplementary Data set 2) including homologues of mammalian proteins present in exosomes: heat shock proteins, Rab proteins, tetraspanins[19] and Alix, which is associated with exosome biogenesis[14,15] (Table 1). The venom allergen-like proteins (members of the CAP superfamily, Pfam00188), which were previously identified as the dominant proteins in the *H. polygyrus* secretory products[20], are almost exclusively in the supernatant fraction (Fig. 3b, orange), further demonstrating specificity in the molecular composition of the vesicles and possibly indicating distinct routes of secretion. On this note, nematode intestinal proteins are enriched in the vesicle fraction (Fig. 3b, green and Supplementary Data set 2) and, consistent with an intestinal origin of the nematode exosomes, vesicles of similar size are observed in the intestinal tissue of adult *H. polygyrus* analysed immediately *ex vivo* (Fig. 3c). One Argonaute protein was identified in both vesicle and supernatant fractions (Fig. 3b, red) that belongs to the clade of Worm-specific Agos (WAGO). Phylogenetic analysis suggests that homologues to this WAGO are present in many parasitic nematodes but may have been lost in *Caenorhabditis* (Fig. 3d).

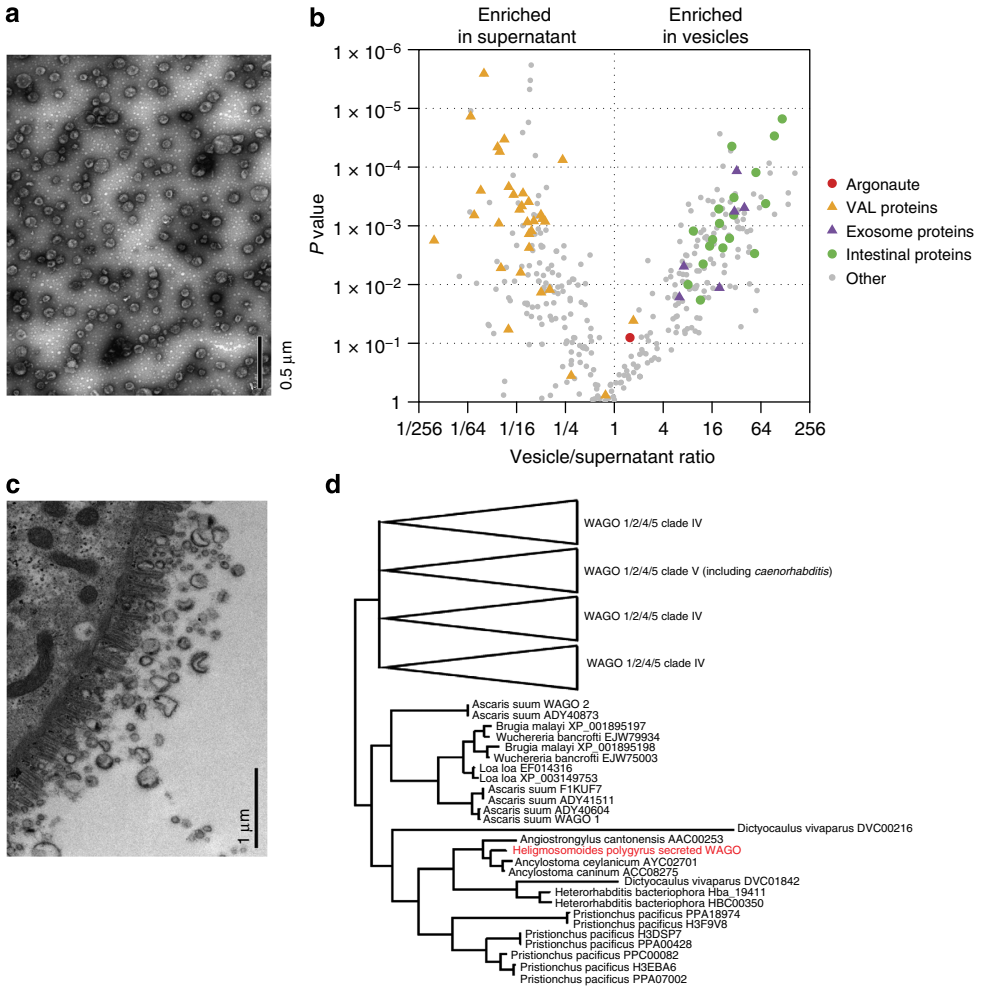

Figure 3 | *H. polygyrus* secretes exosomes of intestinal origin that contain a WAGO protein. (**a**) TEM of purified ultracentrifugation pellet (100 µg ml^{-1} total protein) from *H. polygyrus* secretion product, scale indicates 0.5 µm. (**b**) Scatter plot of proteins enriched in ultracentrifugation pellet or supernatant based on LC-MS/MS, $n=3$, using $P<0.05$ (one-way ANOVA) and FC >1.5 as cutoffs. Noted in the legend are homologues of intestinal nematode proteins (green), mammalian exosome proteins (purple), Venom Allergen-Like (VAL) proteins (orange) and an Argonaute protein (red). (**c**) TEM of adult worm intestine noting vesicles of comparable size to exosomes, scale indicates 1.0 µm. (**d**) Phylogenetic relationship of the secreted Argonaute protein identified in *H. polygyrus* secretion product in relation to other nematode Argonautes. The analysis was performed on the same data set described in ref. 28 with the addition of the *H. polygyrus*-secreted argonaute sequence, using the same method (Bayesian analysis using MrBayes v3.2).

Table 1 | Table of nematode proteins enriched in vesicle fraction that are homologous to mouse proteins associated with exosomes.

Name	Pellet/sup ratio	*P*-value	Organism	Blast *E* value
Tetraspanin-11	40.0	<0.005	*A. suum*	2e − 46
Hsp-70	32.2	<0.005	*D. medinensis*	0.0
Alix	30.2	0.006	*C. elegans*	1e − 79
Rab-11b	19.7	0.011	*S. salar*	1e − 71
Rab-5	7.2	0.005	*C. elegans*	2e − 205
Hsp-90	6.3	0.016	*H. contortus*	0.0

Naming is based on best blast hit and *P* value based on $n=3$.

Nematode RNAs are protected from degradation by exosomes. To determine which RNAs identified in the total secretion product (Fig. 1) are specifically associated with vesicles, small RNA sequencing of replicate vesicle and nonvesicle (supernatant) fractions of the secretion product was carried out. Results from three biological replicates demonstrate that the parasite miRNAs are enriched in the vesicle fractions (75% of reads compared with 10% in supernatant, which is dominated instead by rRNA and Y RNA fragments, Fig. 4a). This analysis also identified three mouse miRNA homologues: miR-193, miR-10 and miR-200, within the top five most abundant secreted miRNAs. These were ranked much lower in the initial Illumina analysis (Supplementary Table 1) likely because of the sequencing bias of the different kits and platforms[21], underscoring the importance of comparing both approaches. Overall the three replicates showed the same profile of miRNAs in each vesicle sample (Supplementary Table 2) and neither vesicle nor supernatant contained intact large ribosomal RNA (Supplementary Fig. 4). Northern blot analysis confirmed the specificity of small RNA biotypes in vesicles versus supernatant, showing miR-100 to be exclusively present in the vesicles and the Y RNA fragment to be exclusively present in the supernatant (Fig. 4b). Notably, on the same blot the full-length Y RNA was detected in the vesicles and both the miRNA and full-length Y RNA were largely resistant to degradation by RNases in untreated samples but became susceptible in the presence of Triton-X-100 (Fig. 4c). Together,

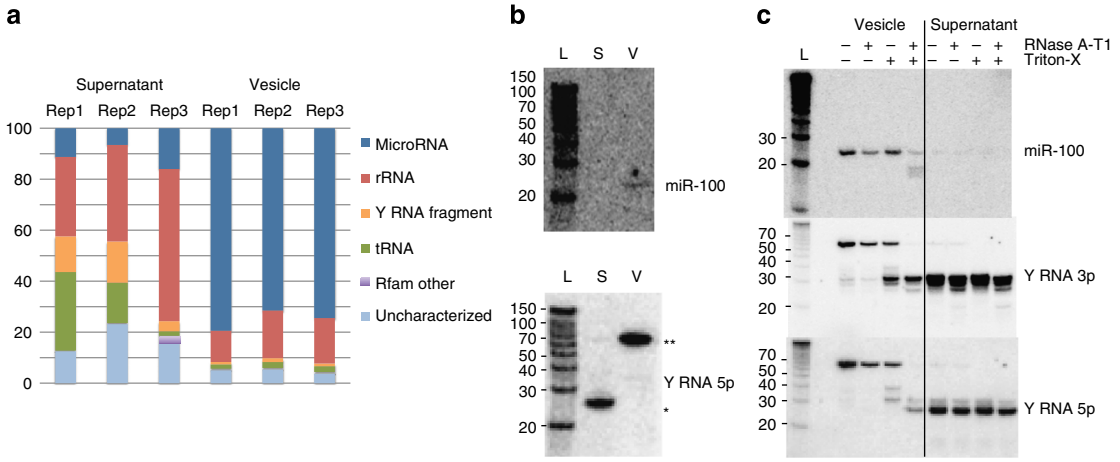

Figure 4 | Secreted miRNAs are protected from degradation through encapsulation within exosomes. (**a**) Classification of *H. polygyrus* small RNAs in the secretion product following ultracentrifugation. (**b**) Northern blot analysis of RNA extracted from ultracentrifuge pellet or supernatant (from equivalent 10 μg protein) using probes complementary to *H. polygyrus* miR-100 or the 5′ arm of nematode Y RNA; * indicates the processed Y RNA and ** indicates the full length Y RNA. (**c**) Northern blot of RNA extracted from the pelleted secretion product following RNase treatment (0.5 Unit RNace-IT, 1 h at 37 °C) in the presence or absence of 0.05% Triton-X-100.

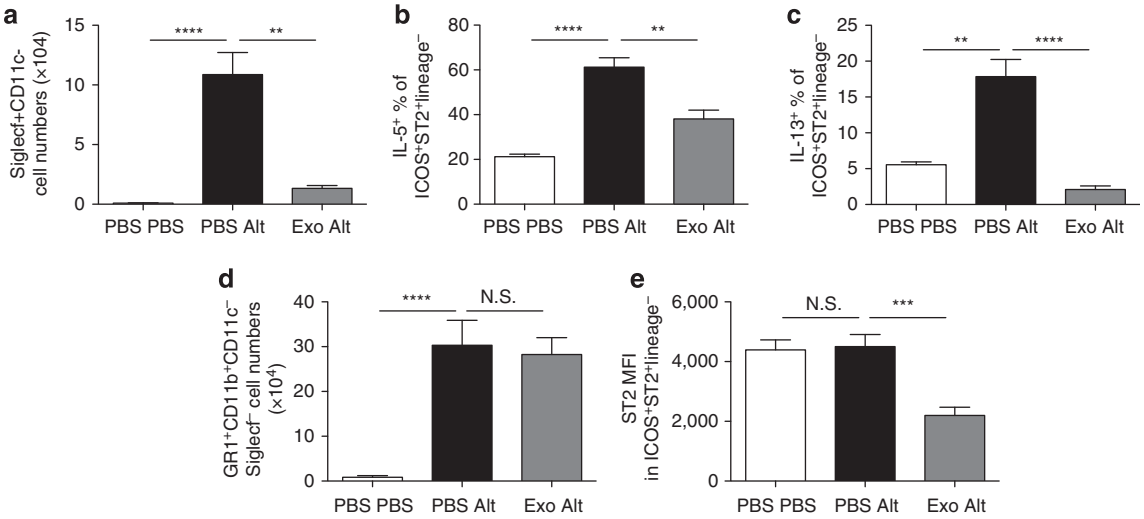

Figure 5 | *H. polygyrus* exosomes suppress a Type 2 innate immune response *in vivo*. *H. polygyrus* exosomes (10 μg) were administered intranasally to BALB/c mice 24 h before administration of 50 μg *Alternaria* extract and a further 5 μg exosomes, or controls that received PBS. (**a**) Siglecf$^+$CD11c$^-$ eosinophils in the bronchoalveolar lavage; (**b**) IL-5 and (**c**) IL-13 expression in PMA/ionomycin-stimulated lineage-negative, ICOS$^+$ST2$^+$ group 2 innate lymphoid cells in digested lung tissue were measured 24 h after *Alternaria* extract administration; (**d**) Gr1$+$CD11b$+$ neutrophils in the same lavage samples; (**e**) the mean fluorescence intensity (MFI) of ST2 (IL33R) staining in ILCs from each group of mice. Data are representative of two independent experiments, $n=4$–6 per group; error bars are mean ± s.e.m. Data analysed by ANOVA and Tukey's post test, ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$.

these results demonstrate that mature miRNAs and full-length Y RNAs are secreted by a parasitic nematode and are protected through encapsulation within vesicles of intestinal origin that share similar size and protein composition to mammalian exosomes.

***H. polygyrus* exosomes suppress innate immunity *in vivo*.** Helminths are well known to suppress pathogenic immune responses in both the gastrointestinal tract and airways[5]. To examine the functionality of the parasite-derived exosomes *in vivo*, they were administered intranasally in combination with extracts of the allergenic fungus *Alternaria*, which induces rapid IL-33 release as part of the Type 2 Th2-like innate immune response that leads to lung eosinophilia[22]. Pre-treatment with parasite-derived exosomes before *Alternaria* extract administration led to a sharp reduction in bronchoalveolar lavage eosinophilia (Fig. 5a), and suppressed expression of the Type 2 cytokines interleukin (IL)-5 and IL-13 by innate lymphoid cells (ILCs; Fig. 5b,c). Neutrophilia, which does not depend on Type 2 cytokines, was undiminished by exosome administration (Fig. 5d). Intriguingly, the overall expression of the IL-33 receptor (also known as ST2) was also suppressed in recipients of exosomes (Fig. 5e).

Internalization of nematode exosomes and RNAs by mouse cells. To determine whether the nematode-derived exosomes can enter mammalian cells, uptake was examined in mouse small intestinal epithelial cells, a cell type that is naturally in direct contact with *H. polygrus in vivo*. Exosomes were labelled with the lipid dye PKH67 and incubated with MODE-K cells *in vitro*.

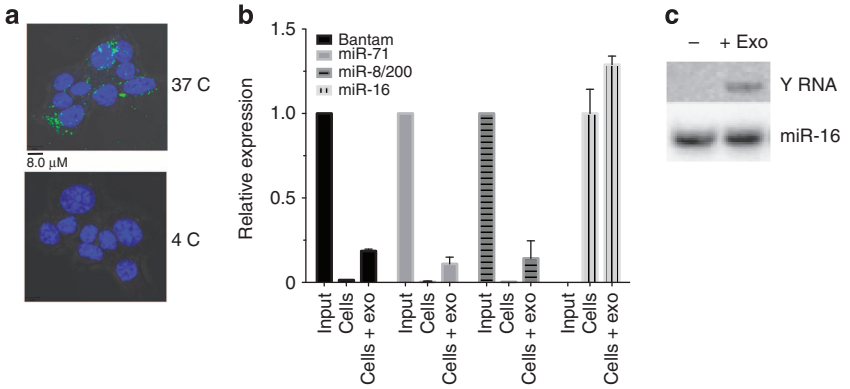

Figure 6 | *H. polygyrus* exosomes and RNAs are internalized by mouse cells. (**a**) Confocal analysis of murine epithelial cells incubated for 1 h with PKH67-labelled *H. polygyrus* exosomes at 37 and 4 °C, scale indicates 8.0 μM. (**b**) Relative expression of parasite-derived miRNAs in murine epithelial cells at 20 h post incubation with 5 μg *H. polygyrus* exosomes following PBS washes. Signal observed in untreated host cells represents the background detection of the probe; for parasite-derived miRNA, the data are normalized to the input detection level of miRNAs in 5 μg of exosomes, whereas miR-16 levels in exosome-treated cells are normalized to untreated cells. (**c**) Northern blot analysis of RNA extracted from murine epithelial cells following 20 h incubation with *H. polygyrus* exosomes (5 μg total protein) compared with untreated cells following PBS washes, using a probe against the loop of the nematode Y RNA or mouse miR-16.

Uptake was analysed by fluorescence-activated cell sorting (FACS) and confocal microscopy. Over 60% of the cells were PKH67-positive after 1 h of incubation with *H. polygyrus* vesicles compared with 1.5% when incubated with background dye (Supplementary Fig. 5). These results are unlikely to be due to nonspecific association with the cell membrane as treatment with trypsin did not eliminate the signal (Supplementary Fig. 5). Confocal analysis confirmed uptake to the cytoplasm and demonstrates that this requires physiological temperature (Fig. 6a). qRT–PCR analysis of the treated cells detects the parasite-specific miRNAs in cells after 20 h of incubation, with no change in the endogenous miR-16 (Fig. 6b). The full-length parasite-derived Y RNA could also be detected by northern blot analysis in cells which were treated directly with exosomes followed by washing (Fig. 6c).

Regulation of mouse genes by nematode exosomes. To determine the function of these vesicles in mouse cells, gene expression analyses were carried out on MODE-K cells following incubation with *H. polygyrus* exosomes. A total of 128 genes were differentially expressed upon treatment (false discovery rate (FDR) $P<0.05$). Relatively subtle changes in gene expression were observed (Fig. 7a); however, the most strongly downregulated gene was *Dusp1* (also known as *MKP-1* in human), a key regulator of MAPK signalling associated with dampening the type 1 pro-inflammatory reaction to Toll like receptor (TLR) ligands. Another gene significantly downregulated by exosomes is *Il1rl1* (also known as *IL33R* in human and so referred to here as *Il33r*), the ligand-specific subunit of the receptor for IL-33, a key alarmin cytokine required for protection against multicellular parasites, which is produced by innate cells to drive early type 2 immune responsiveness[23] and is suppressed by the exosomes in ILCs *in vivo* (Fig. 5e). The effects of the exosomes on *Dusp1* and *Il33r* were validated by RT–qPCR and are unlikely to reflect a nonspecific response to vesicle uptake as exosomes derived from mouse intestinal cells showed similar uptake but did not alter *Dusp1* and *Il33r* levels (Fig. 7b and Supplementary Fig. 5).

There are a number of potential mechanisms that could mediate the decrease in *Dusp1* and *Il33r* levels. The 3′untranslated region (UTR) of *Dusp1* is highly conserved and contains 7mer binding sites for the parasite homologues of mouse miR-200 (aka miR-8) and let-7 as well as a 6mer site for miR-425 (aka miR-63) in between these sites (Supplementary Fig. 6). We therefore examined whether the parasite miRNAs could suppress translation of a reporter vector containing the 3′-UTR of *Dusp1* fused to luciferase. Synthetic parasite miRNAs were transfected into MODE-K cells, resulting in 1.2- to 2.0-fold reduction in luciferase levels for the *Dusp1* reporter but not control (Fig. 7c). Notably, transfection of a cocktail of three of the miRNAs (at the same total RNA concentration) resulted in an increased reduction in luciferase activity (3.1-fold). This is consistent with enhanced repressive effects of miRNA sites in close proximity and suggests that secreted parasite miRNAs could work in cooperation to exert maximal effects on host genes. In contrast, the 3′-UTR of the IL33R-encoding gene *Il1lr1* is not conserved and, although binding sites for some of the secreted miRNAs were identified, we did not observe repression of a *Il1rl1* 3′-UTR reporter by transfection of miR-71, which contained two 7mer sites (Fig. 7c and Supplementary Fig. 6).

Circulating nematode miRNAs in serum. To establish whether the secreted nematode miRNAs naturally circulate in host tissues *in vivo*, we examined serum from mice infected with *H. polygyrus* (which resides in the gut lumen) or the filarial nematode *L. sigmodontis* (which resides in the pleural cavity). No *H. polygyrus* miRNAs were detected in the serum; however, a total of 1,188 reads mapped perfectly and unambiguously to the *L. sigmodontis* draft genome and 761 of these derived from 16 nematode miRNAs (Table 2 and Supplementary Table 3). Although we cannot rule out the possibility that some of the miRNAs in serum could derive from dying worms, the most abundant miRNAs detected are homologues of those found in *H. polygyrus* exosomes, including miR-100, bantam, miR-71 and miR-263 (Table 2, Fig. 8). These data confirm the *in vivo* secretion of parasite miRNAs and are consistent with the idea that exosomes and associated RNAs operate locally in the host's body such that their detection in body fluids will be dictated by the life stage and localization of the parasite in the host.

Discussion

In summary, we have shown that nematode parasite-derived miRNAs and Y RNAs are transported into mammalian host cells via exosomes that regulate host genes associated with immunity and inflammation and suppress an innate Type 2 response *in vivo*. Extracellular vesicles are emerging as a central mechanism for cell-to-cell signalling within mammalian systems,

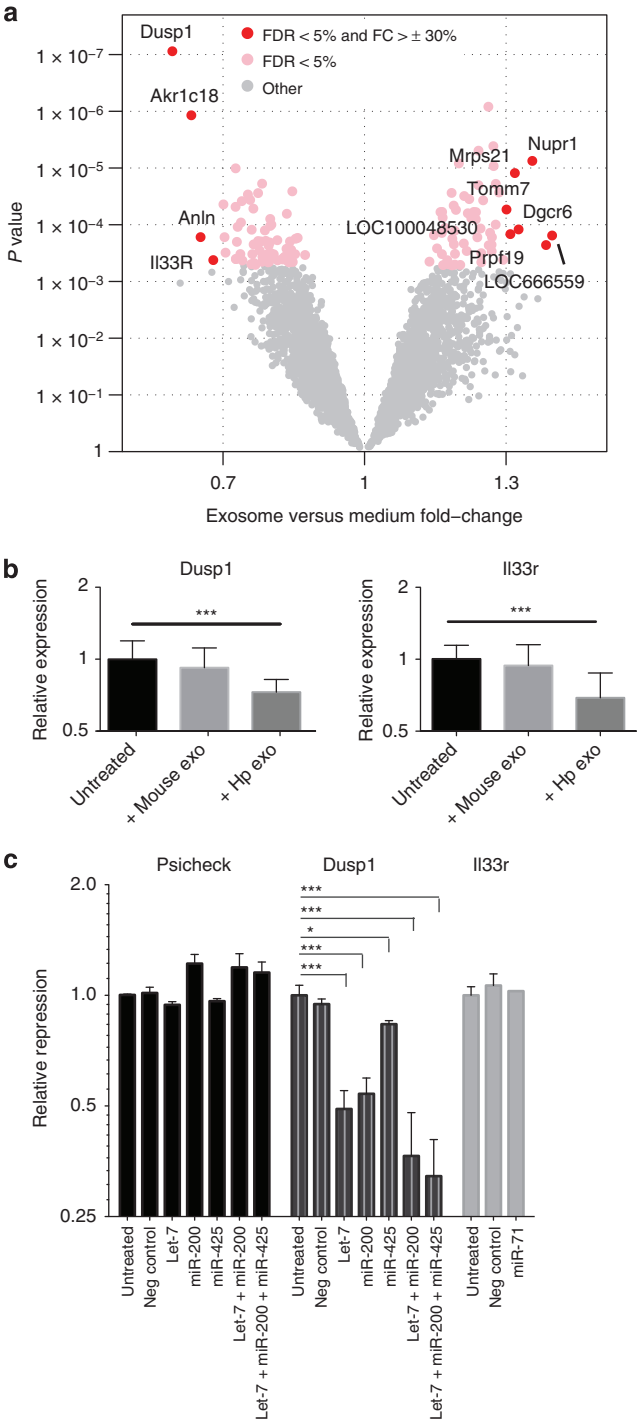

Figure 7 | Mouse *Il33r* and *Dusp1* are suppressed by *H. polygyrus* exosomes and the secreted miRNA repress target sites in *Dusp1*. (**a**) Volcano plot of mouse genes up- or downregulated upon incubation with *H. polygyrus*-derived exosomes; red = FDR $P<0.05$ and FC > 30%. (**b**) Levels of *Dusp1* and *Il1rl1* in mouse epithelial cells (5×10^4) following 48 h treatment with 5 μg *H. polygyrus* exosomes or MODE-K-derived exosomes, $n=8$, error bars are mean ± s.e.m. Data analysed by ANOVA and Tukey's post test, $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$, $^{****}P<0.001$. (**c**) Repression of Psicheck reporter vector containing *Dusp1* or *Il1rl1* 3′UTRs fused to Renilla luciferase by co-transfection with individual or pooled synthetic *H. polygyrus* miRNAs (50 nM), data represent renilla/luciferase ratios, normalized to the values obtained for untreated samples; $n=3$, $^{***}P<0.005$, $^*P<0.05$.

and our report of their secretion by a nematode species is within the setting of vesicle secretion by an increasingly diverse range of pathogens[24–26]. We have demonstrated for the first time that nematode-derived RNAs are a key component within exosomes that can be transferred to host cells. Nematodes are ubiquitous pathogens of both plants and animals and we anticipate that RNA secretion is a conserved phenomenon, supported by the fact that we detect miRNAs from the filarial nematode *L. sigmodontis* in host tissue, consistent with a recent report[12]. In fact, RNA secretion may be a ubiquitous feature across a range of parasites; an initial report suggests that miRNAs are also associated with vesicles in the trematode *Dicrocoelium dendriticum*[27].

Given that many of the nematode miRNAs are homologues to mouse miRNAs, it is tempting to speculate that these could tap into existing miRNA regulatory networks in host cells. In support of this, we show with a reporter assay that three of the secreted nematode miRNAs that have identical seed sites to mouse miRNAs can together downregulate DUSP1 through conserved sites in its 3′UTR. Many questions remain, however, regarding the mechanism by which the nematode miRNAs can operate in host cells. The exosome is a functional ensemble and immune suppression is likely to require a combination of protein and miRNAs for fusion and gene regulation. It will be challenging, therefore, to pin point the individual contributions of each. For example, a nematode Ago protein is secreted with the miRNAs that may be required for functionality. This Ago belongs to the WAGO clade of Agos that evolved in the nematode lineage. The WAGOs mediate diverse RNA interference mechanisms in nematodes and can operate at epigenetic, transcriptional and post-transcriptional levels[28]; it is intriguing to now consider how these possibilities could extend to their hosts.

An exciting finding in this study is the fact that the exosomes can suppress an innate Type 2 response *in vivo*, identifying vesicles as another class of immunomodulator used by the parasite and opening the door to further exploitation of exosomes in a therapeutic context. Our previous work has shown that *H. polygyrus*-secreted material suppresses IL-33 release and it is likely that a combination of soluble proteins and exosomes together suppress this important pathway[22]. From analyses *in vitro* we identify *Il33r* and *Dusp1* as host genes directly suppressed by the exosomes. Although DUSP1 has been broadly viewed as an attenuator of immune activation, it is known to preferentially downregulate IL-6 that has recently been shown to promote susceptibility to *H. polygyrus*[29], while upregulating IL-10, which acts as a broadly immunosuppressive cytokine[30]. Hence, parasite survival is likely to be favoured by reduced DUSP1 levels. Further, DUSP1/MKP-1 dampens the acute inflammatory response to lipopolysaccharide, promoting macrophage arginase expression over nitric oxide synthase[31]. Hence, parasite repression of DUSP1 could block the induction of arginase, a known mediator of killing of *H. polygyrus* in the mouse[32]. These possibilities are now being investigated in our laboratories. Our reporter assays suggest that Dusp1 could be directly targeted by the parasite miRNAs; however, we do not observe repression of Il33r when transfecting the parasite miRNA, miR-71, that is predicted to target its 3′UTR. It may be that additional parasite-derived RNAs or proteins could regulate *Il33r* expression, or that the effect operates indirectly through a separate target gene. For example, reduced expression of *Dusp1* or other regulators of MAPK signalling could result in GATA-2 phosphorylation, which might inhibit its ability to promote *Il33r* transcription[33,34].

Finally, our work has revealed not only secreted miRNAs that are packaged in exosomes but also full-length Y RNAs that are transferred to host cells at an abundance level detectable by northern blot analysis. Y RNAs are not known to function in gene

Table 2 | *Litomosoides sigmodontis*-derived miRNAs found in mouse serum.

Name	Mature sequence	Number of reads (infected)
miR-100a	UACCCGUAGCUCCGAAUAUGUGU	479
miR-86	UAAGUGAAUGCUUUGCCACAGUCU	57
Bantam-a	UGAGAUCAUUGUGAAAGCUAUU	45
Bantam-b	UGAGAUCACGUUACAUCCGCCU	45
miR-100b	AACCCGUAGUUUCGAACAUGUGU	40
miR-71	UGAAAGACAUGGGUAGUGAGACG	32
miR-100c	AACCCGUAGAAUUGAAAUCGUGU	22
miR-50-5p	UGAUAUGUCUGAUAUUCUUGGGUU	10
miR-34-5p	UGGCAGUGUGGUUAGCUGGUUGU	8
miR-263/183	AAUGGCACUAGAUGAAUUCACGG	7
Bantam-c	UGAGAUCAUGCCACAUCCGUCU	4
miR-50-3p	CCAGCAUCUCAGACGUAUCGGC	3
miR-153	UUGCAUAGUCACAAAAGUGAUG	3
miR-87-5p	CGCCUGGGACUUCGACUCAACCU	2
miR-2	UAUCACAGCCAGCUUUGAUGU	2
miR-5866	UUACCAUGUUGAUCGAUCUCC	2

miRNA, micro RNA.
miRNAs that map exclusively to the *L. sigmodontis* but not mouse genome, which were identified in the sera of mice infected with *L. sigmodontis* (40 infective larvae were injected subcutaneously and allowed to migrate to the pleural cavity where they developed naturally for 60 days). The lettering of miR-100 and bantam family members is arbitrary.

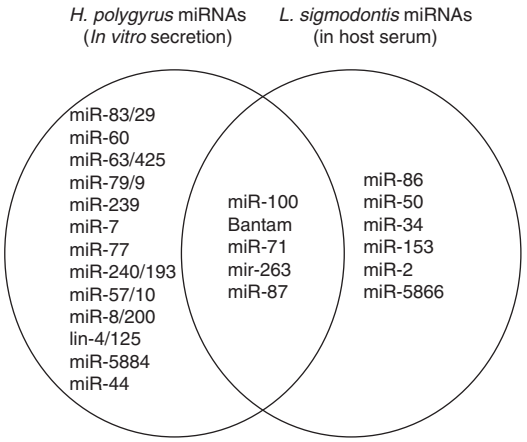

Figure 8 | Venn Diagram of overlap in miRNAs identified in *H. polygyrus* secretion product or serum of mice infected with *L. sigmodontis*. The *H. polygyrus* miRNAs for comparison are taken from Supplementary Table 1 (top 20 most abundant in at least one platform). The miRNAs that are perfectly conserved between nematodes and mice are excluded, since the origin in serum cannot be deduced.

silencing but were recently shown to be packaged into exosomes secreted from dendritic cells[35] and play roles in RNA quality control and DNA replication in humans[36]. Further work is required to understand whether and how each of these classes of secreted parasite RNA can contribute to the capacity of this parasite to manipulate its environment within the host.

Methods

Purification of vesicles from secretion product. For collection of *H. polygyrus* secretion product, CF1 mice are infected with infective-stage larvae by gavage and adult parasites collected from the small intestine 14 days post infection. The worms are maintained in serum-free media *in vitro* as described elsewhere[37]; secretion product is collected every 3 days for a maximum of 3 weeks (samples used here were from the first week of collection) and purified as follows: eggs are removed by spinning at 400 *g* before filtering of the secretion through 0.2-μm filter (Millipore). Filtered media is then processed following a modified protocol from that described in ref. 38, by being spun at 100,000 *g* for 2 h in polyallomer tubes at 4 °C in a SW40 rotor (Beckman Coulter). Pelleted material is washed two times in filtered PBS at 100,000 *g* for 2 h. The supernatant is concentrated using Vivaspin 6 5000 MWCO tubes (Fisher) at 5,000 *g* and washed two times with PBS.

Small RNA library preparation and analysis. For the analysis of small RNAs in the life stages and total secretion products, total RNA was size-selected on 15% denaturing PAGE and libraries prepared from the 18 to 30 nt fraction using Illumina Small RNA preparation kit version 1.5 and sequenced on an Illumina GAIIX instrument in Edinburgh Genomics (http://genomics.ed.ac.uk/). To identify larger RNAs in the secretion product, separate libraries were also prepared for RNA size selected between 60 and 100 nt and sequenced in parallel. For analysis of vesicle and nonvesicle fractions, small RNA libraries were prepared using the TruSeq kit and sequenced on MiSeq platforms, without prior size fractionation of the RNA. All libraries were analysed by first clipping the 3′ sRNA adapter using cutadapt, searching for at least a six-base match to the adapter sequence. For analysis of small RNAs only reads that contained the adapter were 16–40 nt in length and were present at more than two copies were retained for further analysis. For analysis of RNAs >60 nt in the secretion product, sequences present at >100 reads in the library (out of 490,614 reads sequenced) were aligned in Clustalw and manually inspected for sbRNA (Y RNA) content in terms of secondary structure and location of a UUAUC motif in the terminal loop as described in[16] (Fig. 1c and Supplementary Fig. 2). The Y fragments in the small RNA libraries (<40 nt) were then identified based on criteria that they aligned to these full-length Y RNAs.

The draft genome assembly for *H. polygyrus* was created with the CLC *de novo* assembler using two lanes of Illumina GAII data with 50 bp paired-end and 100 bp paired-end reads from Edinburgh Genomics (http://genomics/ed.ac.uk/); the raw and assembled data are available at http://heligmosomoides.nematod.es/. This version was used to map the sequences around small RNA reads to identify structures consistent with miRNA precursors (according to prediction programmes detailed below). Reads matching the genome were aligned to a set of RNA sequences consisting of known *H. polygyrus* 18S, 28S and 5.8S rRNA sequences (Genbank AJ920355.1, AM039747.1 and DQ408618.1:527-678), 5S rRNA from a closely related species (*Trichostrongylus colubriformis*, Genbank U32119.1) and Rfam sequences (version 10, obtained from ftp://ftp.sanger.ac.uk/pub/databases/Rfam/10.0/Rfam.fasta.gz). The best hit with at most two edits was used to classify the reads. Any reads that matched an rRNA or non-microRNA Rfam family were filtered before miRNA analysis. The analysis of piRNAs was carried out with reads that did not match known Rfam classes or miRNAs; initial identification was based on the presence of a 'GUUUCA' between 35 and 65 nt upstream of the 5′ RNA alignment start site[39]. Inspection of the distribution plot identified the region 42–45 nt upstream of the 5′ RNA alignment start site as being the key area for subsequent analyses (Supplementary Fig. 1).

Two miRNA prediction programmes were used to identify miRNAs in the data sets: miRDeep2 (ref. 40) and mireap (http://sourceforge.net/projects/mireap/). Both programmes use miRNA biogenesis to model the expected alignment of sRNA reads to a potential miRNA precursor. For miRDeep2, the following default settings were used: (a) requirement that reads match the genome perfectly, (b) removal of reads that match to more than five places in the genome and (c) cutoff -v 1, (d) the '-s option' was employed, using all mature sequences from mirbase (version 19). The default settings of minimum free energy (< -20 kcal mol^{-1}) and read length (18–30) were employed. In both programmes, precursor predictions with fewer than 10 reads were discarded. Where multiple precursor loci predicted identical mature miRNAs, only the precursor with the largest number of matching reads was reported.

pCp end labelling and northern blot. For 3′ end-labelling, total RNA was extracted from the life stages and secretion product using the miRNAeasy kit (Qiagen): 1 μg total RNA was used from life stages and RNA extracted from a volume of secretion product equating to 15 μg protein (the total RNA concentration was too low to detect by nanodrop or qubit). The 3′-end labelling was carried out at 4 °C overnight in 10 μl using RNA ligase I (NEB) according to the manufacturer's instructions with 3,000 Ci mmol^{-1} ^{32}P PcP (Perkin Elmer). Reactions were quenched by the addition of 2 × loading buffer (8 M urea, 0.5% TBE) and 4 μl run on an 18% PAGE at 350 V for 8 h, which was then visualized by phosphor-imaging using a Typhoon Scanner (GE Healthcare). For northern blot analysis, total RNA was extracted from volumes of vesicle and nonvesicle fractions that contained equivalent protein (10 μg) and then separated by denaturing 15% PAGE, transferred to Hybond-N + membrane (GE Healthcare) and chemically cross-linked as described previously[41]. Blots were prehybridized in PerfectHyb (Sigma) for 1 h at 42 °C before overnight incubation with DNA probes (Invitrogen) that were perfectly complementary to the miRNA or Y RNA: miR-100: 5′-ACACAA GTTCGGATCTACGGGTT-3′, YRNA-5P: 5′-ACCCTACGACTCCGGACCA AGCGCG-3′, YRNA-3P: 5p-GCGCCGGTCGAGCTTTTGTCGAAGGGAAT-3p, Y RNA-loop: 5p-AAGGGAATTCGAGACATTGTTGATAAC-3p. The probes were labelled with T4 PNK (NEB) and 6,000 Ci mmol^{-1} ^{32}P ATP (Perkin Elmer) according to the manufacturers' protocols.

miRNA RT-qPCR. Analysis of miRNA levels in ultracentrifugation fractions was carried out using the miScript system (Qiagen) with unmodified DNA probes

identical to the full-length parasite miRNA (Life Sciences): miR-100: 5′-AACCCG TAGATCCGAACTTGTGT-3′, miR-71: 5′-TGAAAGACATGGGTAGTGAGAC-3′, let-7: 5′-TGAGGTAGTAGGTTGTATAGTT-3′ and miR-60: 5′-TATTATGC ACATTTTCTGGTTCA-3′. For analysis of parasite-derived miRNA levels in host cells, qRT–PCR was carried out using the miRCURY LNA microRNA PCR system (Exiqon) and LNA probes were custom-designed by Exiqon to minimize cross hybridization with mouse sequences, and efficiency of probes was measured between 90 and 100% (data not shown). Analysis of mouse gene expression levels was carried out using the Sybr green I master mix (Roche), with the following primers: gapdh_F: 5′-CATGGCCTTCCGTGTTCCTA-3′, gapdh_R: 5′-GCGGCA CGTCAGATCCA-3′ Dusp1_F: 5′-GTGCCTGACAGTGCAGAATC-3′, Dusp1_R: 5′-CACTGCCCAGGTACAGGAAG-3′, Il33R_F: 5′-AGACCTGTTACCTGGGC AAG-3′, Il33R_R: 5′-CACCTGTCTTCTGCTATTCTGG-3′. Data were collected on a Light Cycler 480 System (Roche) following temperature profiles recommended by each manufacturer. The delta C_t method was used for quantification as described in ref. 11 using GAPDH as the normalizer. Data were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post test and variance within groups assessed by Brown Forsythe test.

LC-MS/MS. Five micrograms of total protein from the secretion product ultracentrifuge pellet or supernatant were loaded on a 12% Tris-Bis NuPAGE gel (Invitrogen) and electrophoresis carried out for 5 min before in-gel digestion as described in ref. 42. Capillary-HPLC-MS/MS analysis was performed using an online system consisting of a micropump (1,200 binary HPLC system, Agilent, UK) coupled to a hybrid LTQ-Orbitrap XL instrument (ThermoFisher, UK). Data were searched using MASCOT Versions 2.4 (Matrix Science Ltd, UK) against an in-house *H. polygyrus* transcriptome assembly of 454 sequences[43] using a maximum missed-cut value of 2. Variable methionine oxidation and fixed cysteine carbamidomethylation were used in all searches; precursor mass tolerance was set to 7 p.p.m. and MS/MS tolerance to 0.4 a.m.u. The significance threshold (p) was set below 0.05 (MudPIT scoring). A peptide Mascot score threshold of 20 was used in the final analysis, which corresponds to a global FDR of 4.6% using a decoy database search. LC-MS label-free quantitation was performed using Progenesis (Nonlinear Dynamics, UK) as described elsewhere[42] where the total number of Features (that is, intensity signal at a given retention time and m/z) was reduced to MS/MS peaks with the charge of 2, 3 or 4+ and we only kept the five most intense MS/MS spectra per 'Feature'. The subset of multicharged ions (2+, 3+ and 4+) was extracted from each LC-MS run. For a specific protein, the associated unique peptide ions were summed to generate an abundance value that was transformed using an ArcSinH function required for the calculation of the P value. A total of 362 proteins were identified in either the supernatant or pellet based on requirement of at least two peptides present; of these, 122 were enriched in the supernatant and 139 in the pellet, while the remaining 101 did not show statistically significant enrichment and were detectable in both samples. The within-group means were calculated to determine the fold change and the transformed data were then used to calculate the P values using one-way ANOVA. Differentially expressed proteins were considered meaningful under the following conditions: detected by two or more peptides, with an absolute ratio of at least 1.5 and $P<0.05$ associated with the protein change. Classification of intestinal proteins is based on homology to proteins identified in other nematodes, described in ref. 44.

TEM. For visualization of the vesicles, the purified ultracentrifuged pellet from *H. polygyrus* secretion product (100 μg ml^{-1} protein concentration) was fixed in 2% paraformaldehyde (PFA), deposited on Formvar-carbon-coated EM grids and treated with glutaraldehyde before treatment with uranyl oxalate and methyl cellulose as described in ref. 38. For analysis of adult *H. polygyrus* parasites, samples were washed with PBS before fixation in 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer overnight. Parasites were rinsed three times with 0.1 M Na cacodylate buffer, and post-fixed in 1% osmium tetroxide for 1 h. After rinsing in 0.1 M Na cacodylate buffer, they were sequentially dehydrated in a graded acetone series. Finally, samples were sequentially incubated for 30 min in an araldite:acetone solution left to evaporate overnight at 60 °C and then embedded in fresh araldite resin and polymerized at 60 °C for 48 h. Ultrathin sections, 60-nm thick, were cut from selected areas, stained in uranyl acetate and lead citrate, and then viewed in a Philips CM120 TEM. Images were taken on a Gatan Orius CCD camera.

Flow cytometry and confocal analyses of uptake. Purified exosomes from *H. polygyrus* or MODE-K cells (measured as 5 μg of total protein) were labelled with 2 μg of PKH67 dye (Sigma) for 5 min at room temperature following the manufacturer's protocol. The staining reaction was stopped by adding an equal amount of 1% bovine serum albumin (BSA), and exosomes were washed in PBS and pelleted by ultracentrifugation (1 h at 100,000 g). A probe solution was prepared with the PKH67 following the same protocol but mixed with PBS solution in the absence of exosomes. MODE-K cells[45] were obtained from Dominique Kaiserlian (INSERM) and grown following the standard protocol in DMEM (Invitrogen) medium supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin–streptomycin (Lonza), 1% L-glutamine (Lonza), 1% non-essential amino acids/sodium pyruvate (Gibco). These were mycoplasma-free based on testing every 4 weeks. On the day of the experiment, cells were seeded in 24-well plates (1×10^5 cells per well) using advanced DMEM serum-free medium (Invitrogen) supplemented with 1% L-glutamine and subsequently incubated (1 h at 37 °C) either in the presence of PKH67-labelled *H. polygyrus*-derived exosomes (5 μg of total protein) or in the presence of the probe alone. After incubation, cells were harvested, washed twice in FACS buffer (PBS 1×, 2.5% FBS, 0.1% BSA, 0.05% NaN_3) and finally resuspended in 500 μl of the same buffer. A subset of the samples were then incubated with 50 ul of 0.25% Trypsin/EDTA (Gibco) for 5 min before analysis (indicated in Supplementary Fig. 5). Samples were analysed using the BD FACS Canto II flow cytometer (BD Bioscience). Data files were acquired from the cytometer, with 5,000 events collected for each tube and the data analysis was performed using the FlowJo software (Tree Star Inc.). For confocal analyses, MODE-K cells were seeded on round microscope cover glasses in 24-well plates (2.5×10^4 cells per well) in media described above. Cells were allowed to attach on to the coverslips overnight and the following day shifted to advanced DMEM medium supplemented with 1% L-glutamine. Cells were incubated (1 h at 37 or 4 °C) either in the presence of labelled exosomes or probe only. After incubation, medium was aspirated, cells were washed twice in PBS and fixed with 4% PFA, with residual PFA quenched with 50 mM glycine. Slide coverslips were washed extensively in PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole-supplemented ProLong Fade Gold (Invitrogen) mounting media. Samples were examined on the Leica SP5 II (Leica Microsystems, lasers exciting at 405 and 488, ×63 objective) using the LAS AP software (Leica). Images were analysed using the Volocity software (Improvision).

Microarray analysis. MODE-K cells were grown in DMEM media as described above and seeded into 24-well plates at 20,000 cells per well. The following day, cells were incubated with *H. polygyrus*-derived exosomes (5 μg total protein per well) for 20 h before washing twice with PBS and total RNA extracted. RNA was prepared for microarray analysis using the Illumina TotalPrep RNA Amplification kit and run on MouseWG-6 v2.0 (Illumina) at the Wellcome Trust Clinical Research Facility (University of Edinburgh). The raw SampleProbeProfile file was processed within R, using 'lumi' and 'lumiMouseAll.db' Bioconductor packages[46–48]. Quality control was performed using Multi-Dimensional Scaling, and one of the control samples that behaved as an outlier was removed. Raw expression values were processed with the Variance Stabilizing Transformation and the Robust Spline Normalization[49]. An InterQuartile Range was calculated across all samples for each probe, and used to select the most variable probe of those that mapped to the same transcript. Probes without a gene or transcript annotation were excluded, leaving a total of 30,708 nonredundant annotated probes. Differential expression was performed using the 'limma' package[50], fitting a linear model for each probe and using an empirical Bayes method to obtain moderated t-statistics. In order to reduce the multiple-test problem and focus on the most interesting genes, 'present' probes with an Illumina detection P value <0.05 in at least three samples were selected, leaving 12,276. The Benjamini and Hochberg method was used to calculate FDRs[51].

***In vivo* analysis of exosome function in *Alternaria* model.** BALB/c mice were bred in-house at the University of Edinburgh and accommodated according to Home Office regulations. Female mice were used when they were 6–10 weeks old. For all experiments presented in this study, the sample size was large enough to measure the effect size. No randomization and no blinding were performed in this study. *H. polygyrus* exosomes (10 μg) were administered intranasally (under isoflurane sedation) in 50 μl PBS, or 50 μl PBS alone to controls, 24 h before intranasal administration of 50 μg *Alternaria* extract with a further 5 μg of exosomes. Mice were killed 24 h after *Alternaria* administration, and bronchoalveolar lavage and lung cell suspensions stained for flow cytometry as described previously[22]. Briefly, cells were counted, then surface stained for Siglecf+ CD11c− (eosinophils) or stimulated with phorbol myristate acetate (PMA) and ionomycin for 4 h in the presence of BrefeldinA and surface stained as negative for lineage markers (CD3/CD4/CD5/CD19/CD11b/CD11c/CD19/GR1) and positive for CD45, ICOS and ST2 (ILC2s), and assessed for staining of IL-5 and IL-13. Samples were acquired on a Becton-Dickinson LSRII flow cytometer.

Data were analysed using Prism 6 (Graphpad Prism, La Jolla, CA, USA). Variance within groups was assessed by Brown Forsythe test and data were log-transformed and analysed by one-way ANOVA, with a Tukey's multiple comparisons post test. Unless otherwise indicated, differences are not significant. ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$, N.S. not significant $P>0.05$.

Luciferase assays. The 3′UTRs of Dusp1 and Il33r were cloned behind *Renilla* luciferase in the Psicheck2 vector (Promega) at NotI and XhoI restriction sites as described in ref. 41 using the following primers: Psi-Dusp_F: 5′-CTTTAC TCGAGAGGTGTGGAGTTTCACTTGC-3′, Psi-Dusp_R: 5′-CTTTAGCGGC CGCAGCTACAAACCTACACTGGC-3′, Psi-Il33r_F: 5′-CTTTACTCGAGGA CTGTGTGTTGTAGCTTGG-3′, Psi-Il33r_R: 5′-CTTTAGCGGCCGCCAGA GGGAGGCTTTATAAGG-3′.

For reporter assays, 15,000 cells were reverse transfected into a 96-well plate with 0.3% lipofectamine (Invitrogen) and 50 ng of each Psicheck reporter in the absence or presence of 50 nM synthetic miRNA mimic (Thermofisher). Luciferase

measurements were carried out at 48 h post transfection, using the Dual Glo Luciferase assay system (Promega) and Luminensence measured on a Varioskan plate reader (Thermofisher). Data shown in Fig. 7b represent $n=3$ replicates (separate transfection experiments of the MODE-K cell line) measured in parallel to control for consistent Renilla and Luciferase ratios using the same kit; data were analysed by one-way ANOVA, with a Dunnett's post test, $^{****}P<0.0001$, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$.

MicroRNA target prediction. A custom Perl script was used to identify seed-matching sites for the *H. polygyrus* miRNAs identified by sRNA-Seq. All the 3′UTR sequences corresponding to probes on the microarray were scanned, and the results were passed to the TargetScan (v6.2) context scores Perl script. Targets for each miRNA were ranked by 'Context + Scores'. Conservation scores for relevant 3′UTRs (Dusp1 and Il33r) were obtained from the UCSC Genome Browser, using the 'rtracklayer' package to access the 'phastCons60wayPlacental' table[52,53].

Sequencing analysis of serum from infected mice. F1 mice were infected with *H. polygyrus* (400 L3 larvae introduced by oral gavage) and serum collected on day 14 post infection. The presence of adult parasites was confirmed by visual inspection of the mouse gut lumen. Six-week-old BALB/c mice were infected with *L. sigmodontis* (subcutaneous inoculation of 40 L3), and gel-separated serum (BD Microtainer) was collected by arterial exsanguination at 60 days post infection, which was confirmed by detection of adult worms in the mouse pleural cavity and microfilarie in peripheral blood. For library preparation, 200 µl of serum was extracted with the miRNAeasy kit (Qiagen) and libraries generated following the Trueseq protocol and sequenced on the Illumina Rapid HighSeq in Edinburgh Genomics. Data were processed as described above and analysed for perfect alignment to the mouse or *L. sigmodontis* genome (reads mapping to both genomes were not analysed). Reads that aligned were then categorized by matches to Rfam or prediction as miRNAs with miRdeep2, as described in ref. 11.

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Author contributions

A.H.B. designed and carried out RNAseq experiments and validation, identified vesicles in secretion, co-designed and contributed to analysis of serum and proteomic data, co-supervised functional and uptake assays and wrote the paper; G.C. co-designed and carried out functional analyses, *in vivo Alternaria* extract experiments and vesicle detection in nematodes; F.S. designed and carried out uptake and functional assays; S.K. carried out genome alignment/annotation; H.J.McS. co-designed and performed *in vivo Alternaria* extract experiments; J.Q. prepared and co-analysed serum libraries, T.L.B. carried out LC-MS/MS and analysis; C.A.-G. analysed microarray data and target prediction; M.L. purified secretion material and supported functional studies; Y.H. generated worm and secretion samples; A.C. supported uptake and reporter assays; M.B. oversaw genome assembly and phylogenetic analyses of AGO protein; S.A.B. performed the *L. sigmodontis* infections and tissue sampling; A.I. analysed small RNAseq data; R.M.M. supplied *H. polygyrus* life stage material, contributed to analysis of proteomic data, co-designed immunological experiments and edited the manuscript.

Additional information

Accession codes: The sequencing and microarray data from this study have been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession codes GSE55978 and GSE55941. The novel miRNA sequences identified in this study have been deposited in miRBase and the official naming is provided in Supplementary Data 1. The genomic information for *H. polygyrus* and *L. sigmodontis* is available at http://www.nematodes.org/genomes/heligmosomoides_polygyrus/ and http://nematodes.org/genomes/litomosoides_sigmodontis/.

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9.2 Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections

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Gillian Coakley, Rick M. Maizels & Amy H. Buck

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Review

Exosomes and Other Extracellular Vesicles: The New Communicators in Parasite Infections

Gillian Coakley,[1,2] Rick M. Maizels,[1,2] and Amy H. Buck[1,2,*]

Extracellular vesicles (EVs) have emerged as a ubiquitous mechanism for transferring information between cells and organisms across all three kingdoms of life. In addition to their roles in normal physiology, vesicles also transport molecules from pathogens to hosts and can spread antigens as well as infectious agents. Although initially described in the host–pathogen context for their functions in immune surveillance, vesicles enable multiple modes of communication by, and between, parasites. Here we review the literature demonstrating that EVs are secreted by intracellular and extracellular eukaryotic parasites, as well as their hosts, and detail the functional properties of these vesicles in maturation, pathogenicity and survival. We further describe the prospects for targeting or exploiting these complexes in therapeutic and vaccine strategies.

Host–Parasite Interactions: Do We Know it All?

More than 1 billion people worldwide are burdened by parasitic disease, including malaria [1] and neglected tropical diseases such as leishmaniasis, Chagas disease and helminthiases (http://whqlibdoc.who.int/hq/2012/WHO_HTM_NTD_2012.1_eng.pdf), with most prevailing in developing regions such as eastern Asia, sub-Saharan Africa, and the Americas [2]. The prospects for drug resistance are alarming, with an increasing incidence in livestock that highlights a potential threat to the human population through zoonotic transmission as well as having strong economic and social implications [3]. There is a clear need for more efficacious therapies, which require an improved understanding of how these parasites adapt to, and manipulate, their host environment. Most parasites at some stage in their life cycle rely on the ability to communicate with one another and with their hosts, but the mechanisms underpinning this communication are still coming to light. Research in this area has largely focused on the soluble proteins secreted by parasites, many of which down-modulate the host immune response (reviewed in [4,5]). For example, in the case of helminths, the egg stage of *Schistosoma mansoni* secretes an omega-1 glycoprotein, demonstrated in several studies to promote type 2 helper (Th2) skewing of dendritic cells (DCs) and T cells during infection [6,7]. The immunomodulatory lipoprotein antigen B is secreted by *Echinococcus granulosus* and facilitates Th2 polarization and limits migration of neutrophils to the site of infection [8]. The ES-62 protein from *Acanthocheilonema viteae* has potent anti-inflammatory properties on mast cells [9]. Protozoan parasites similarly secrete a range of immunomodulatory molecules; for example, *Trypanosoma cruzi* mucins have been associated with suppression of active T cell immune responses by inducing arrest in the cell cycle [10]. Secreted parasite proteins have also been proposed to be involved in metabolic adaptation to the host environment [11] and tissue invasion, where proteases play a major role [12]. In the past 5 years, EVs have been revealed as another component of parasite secretion

Trends

EVs are secreted by most or all organisms and are emerging as a ubiquitous and functionally diverse type of host–pathogen interaction.

EVs contain a diverse suite of molecules including proteins, lipids, and nucleic acids, some of which are known to have immunomodulatory properties.

Parasite-derived EVs can communicate information and transfer genetic material to host cells or other parasites.

Host-derived EVs can play a key role in host defense and are candidates for generating a vaccine against pathogenic infection.

[1]Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK
[2]Centre for Immunity, Infection, and Evolution, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK

*Correspondence: a.buck@ed.ac.uk (A.H. Buck).

products that provide a previously unrecognized mechanism to package and protect a set of parasite cargo for uptake and integration into other cells. EVs are known to play a role in communication and genetic exchange between microbes [13]. The functional niches in which EVs operate in eukaryotic parasites and other pathogens are still emerging and are summarized in Table 1.

Table 1. Proposed Functions of Pathogen or Host-Derived Exosomes during Infection[a]

Pathogen	EV origin	Host or parasite?	EV target	Functional response	Effector mechanism	Refs
Protozoa						
Leishmania amazonensis	Macrophages exposed to promastigotes	Host	Monocytes and/or macrophages	Promotion of Th1 responses for parasite elimination	Naïve macrophages are primed to release IL-12, IL-1β, and TNF	[46]
Leishmania donovani	Promastigotes	Parasite	Monocytes and/or macrophages	Invasion and persistence within host cells and delivery of virulence factors	*Leishmania* EF-1α and GP63 activate host PTPs in monocytes responding to IFNγ. GP63 can also influence exosome cargo selection and inhibit host miRNA processing.	[35–37,39,40,42]
		Parasite	Immune cells, including macrophages	Induction of *Leishmania* peptide-carrying exosomes from monocytes	Overall increase in IL-8 secretion by macrophages, which promotes neutrophil recruitment. Induces release of IL-10 in human monocytes while suppressing release of TNF.	[35–37]
Leishmania major	Promastigotes	Parasite	Monocytes/macrophages	Invasion and persistence within host cells and delivery of virulence factors	*Leishmania* EF-1α and GP63 activate host PTPs in monocytes responding to IFNγ.	[39,41]
		Parasite	Immune cells, including macrophages and T cells	Increased disease exacerbation and Th2 polarization *in vivo*	Increase in the number of IL-4-producing $CD4^+$ T cells/ decrease in the number of IFNγ-producing $CD4^+$ T cells	[37]
Leishmania mexicana	Macrophages exposed to promastigotes	Parasite	Macrophages	Immunomodulation of host signaling events promoting parasite survival	Upregulation of Adora2a by parasite-derived GP63 contained within host exosomes	[40]
Plasmodium berghei	Infected erythrocytes	Host	Macrophages	Activate systemic inflammation and T cell priming	Via MyD88/TLR4 pathway and CD40/CD40L interactions	[77]
Plasmodium falciparum	Infected erythrocytes	Parasite	Monocytes and macrophages	Transfer of parasite material and parasite dissemination	Innate cell activation. Cytokine induction in macrophages (IL-6, IL-12, IL-1β, and IL-10) in a dose-dependent manner.	[49]
		Parasite	Infected erythrocytes	Commitment of asexual parasites to gametocytes	Transfer of genetic information between parasites and budding of EVs via PfPTP2	[49,50]
Plasmodium vivax	Platelets, erythrocytes, leukocytes	Host	Human immune cells, erythrocytes, endothelial cells	Higher acute fever and greater duration of malaria symptoms in human patients	Unknown mechanism	[78]
Trichomonas vaginalis	Mature parasites	Parasite	Ectocervical cells	Limit neutrophil migration to site of infection	Parasite-derived exosomes downregulate IL-8 secretion in ectocervical cells	[54]

Table 1. (continued)

Pathogen	EV origin	Host or parasite?	EV target	Functional response	Effector mechanism	Refs
		Parasite	Weakly adherent strains of the parasite	Promote adherence of weakly adherent strains and increase their virulence	Unknown mechanism	[54]
Trypanosoma brucei	Procyclic forms of the parasite (pathogenic in bloodstream)	Parasite	Host cells	Improved entry into host cells, enhanced parasite survival	Abundance of parasite-derived proteases (e.g., oligopeptidase B) favors parasite invasion	[51–53]
Trypanosoma cruzi	Trypomastigotes	Parasite	$CD4^+$ T cells and macrophages	Th2 polarization leading to parasite dissemination and enhanced parasite survival	Increase in IL-4 and IL-10 secretion and reduction in iNOS expression in $CD4^+$ T cells and macrophages	[34]
	Infected lymphocytes, monocytes and erythrocytes	Parasite	Recipient immune cells and monocyte-derived complement factors	Parasite invasion of host cells and inhibition of complement-induced parasite elimination	Plasma membrane-derived vesicles containing surface TGF-β, which promotes entry into host cells	[47,48]
Fungi						
Cryptococcus neoformans	Exosomes secreted during the fungal cell phase	Pathogen	Host cells – unknown	Promote colonization of infected tissues	Release virulence factors – glucosylceramide and GXM	[72]
		Pathogen	Macrophages	Stimulate fungal killing	Enhanced IL-10 and TGF-β secretion and increased nitric oxide production by macrophages	[75]
Malassezia sympodialis	Yeast – skin-living flora component	Pathogen	PBMCs	Exacerbation of atopic dermatitis	Promote IL-4 and TNF secretion from PBMCs	[74]
Paracoccidioides brasiliensis	Yeast phase exosomes	Pathogen	Immune cells	Potential to skew to a suppressive Th2 response	Enriched in α-Gal, which may bind host lectins potentially improving infectivity by fungi	[69]
Helminths						
Heligmosomoides polygyrus	Intestinal tract of adult nematode	Parasite	Intestinal epithelial cells of the host	Suppress classical inflammation and danger responses, promoting parasite survival	Suppression of host targets including IL-33R and DUSP1	[59]
Schistosoma japonicum	Adult worms	Parasite	Macrophages	Polarization of host macrophages to M1 phenotype	Unknown mechanism	[58]

[a]Details in each column (from left to right) describe: the parasite species, the life stage and/or cellular origin of the EV, the proposed beneficiary (host or parasite), the proposed target and functional outcome, the mechanistic data in support of this function, and the primary literature reference.

Exosomes and Other Extracellular Vesicles: Origins and Functions

In mammalian systems EVs represent a mechanism of cell-to-cell communication through the direct stimulation of cells by receptor-mediated contact and/or through the transfer of genetic material, proteins, and lipids. Several distinct types of EV have been described, including those derived from the endocytic pathway, exosomes, versus those derived from shedding of the plasma membrane. We refer to the latter as microvesicles but note that these have been called by many names in the literature, including ectosomes, plasma membrane-derived vesicles, and microparticles [14]. Exosomes are endocytic vesicles approximately 40–100 nm in size that are released from most cell types [15]. Their biogenesis is initiated by inward budding of multivesicular endosomes (Figure 1). Consequently, exosomes express markers of their parent cells, but are also specifically enriched in other molecules associated with their biogenesis or that are selectively packaged into them; for example, by the endosomal sorting complexes required for

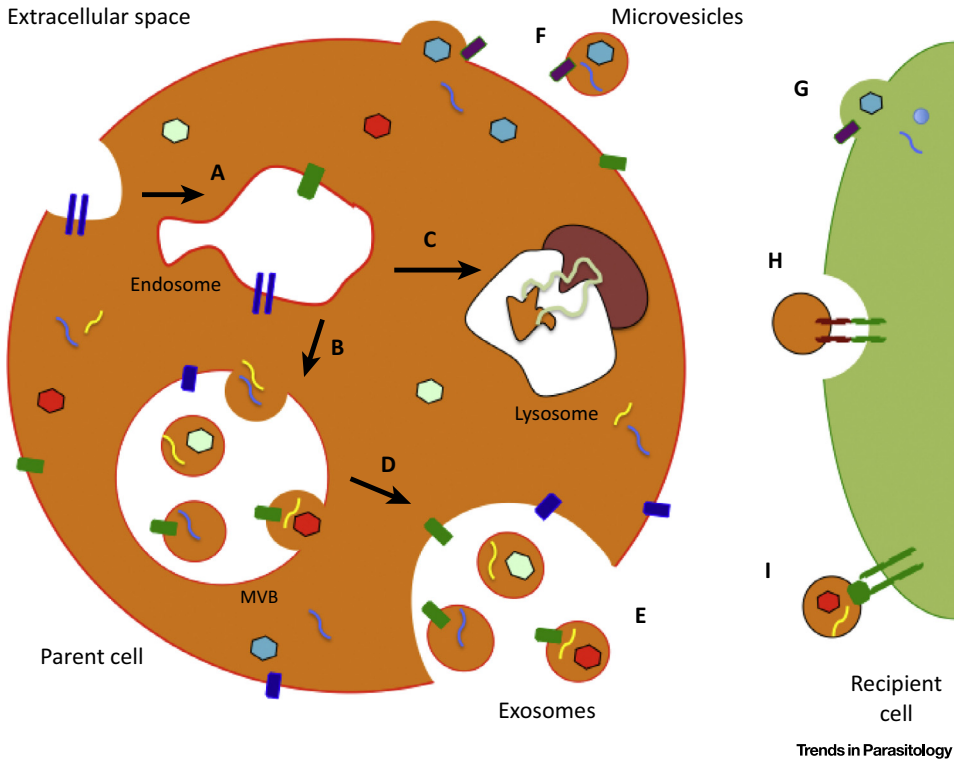

Figure 1. The Biogenesis and Transfer of Different Extracellular Vesicles (EVs). (A) Early endosome formation within the parent cell, surrounded by a range of different bioactive molecules [e.g., nucleic acids, proteins, lipids (denoted by different colors and/or shapes)]. (B) On development into a late endosome, inward budding allows capture of some of the host cell cytosolic contents in intraluminal vesicles (ILVs). The late endosome is also referred to as a multivesicular body (MVB). (C) Some mature MVBs fuse with the hydrolytic lysosome, where the vesicle cargo is subsequently degraded. (D) MVBs can also fuse directly with the plasma membrane, releasing their ILVs, now known as exosomes, into the extracellular space. (E) Release of exosomes into the extracellular environment. (F) Other microvesicles are released into the extracellular space following direct budding from the host cell plasma membrane. There are at least three mechanisms by which EVs interact with recipient cells: (G) direct fusion with the plasma membrane of the recipient cell; (H) receptor-mediated endocytosis following receptor–ligand interactions between EVs and the recipient cell; and (I) signaling via direct interactions of receptor and ligand on the recipient cell surface.

transport (ESCRT) pathway (reviewed in [16]). Microvesicles can be difficult to distinguish from exosomes, but are generally up to 1 μm and bud from the plasma membrane, incorporating certain lipids, surface proteins, and other molecules before fission [17]. As reports in the literature do not always identify vesicular origin, here we refer to parasite exosomes or 'exosome-like vesicles' if they have been described as such in the primary literature or parasite microvesicles if they are suggested to derive from the plasma membrane, or 'EVs' if the origin is unclear.

In recent years, the literature surrounding EV function has exploded as their ubiquity in many biological and disease contexts has been realized [18]. Historically, these were first identified in reticulocytes as a mechanism to release transferrin receptors during maturation [19,20] and then became of interest to immunologists as they contain MHCs and can present antigens [21]. However, following the report that functional mRNAs and miRNAs are transferred between mast cells via exosomes [22], there was further momentum in studying EVs as a mechanism of cell–cell communication. In this context, they have been shown to have various functions in immune cell activation and suppression [23,24] and are also proposed to play a role in disease development and tissue homeostasis, [25]. An ever-expanding literature has also demonstrated various roles of EVs in diseases including cancer, since tumors also secrete these vesicles with

oncogenes [26], such as those seen in gastrointestinal stromal tumor cell lines [27]. Exosomes and other EVs are now part of larger clinical initiatives to test their properties in drug delivery, their use as diagnostic biomarkers, and their potential as therapeutics. While most of this work has focused on oncology [28,29], these vesicles also have exciting implications across a range of infectious diseases [30]. Here we detail the recent literature describing their roles in eukaryotic parasite infection, focusing on the communicative relationship between parasites and hosts. Furthermore we highlight the importance of EVs in the future identification of novel therapeutic targets and the development of vaccine strategies.

Intracellular Protozoan Parasites: Host Manipulation by EVs

Several protozoan parasites have been shown to release exosomes and/or microvesicles, including *Leishmania* species [31] and *T. cruzi* [32–34], the parasites that cause human leishmaniasis and Chagas disease, respectively. Seminal reports showed that promastigote and amastigote forms of *Leishmania donovani* and *Leishmania major* can release exosomes that are detected in host cells and selectively induce IL-8 secretion from macrophages [35,36] (Figure 2A). The subsequent chemokinetic recruitment of neutrophils has been proposed as a 'Trojan horse' effect, whereby *Leishmania* can invade these cells and gain access to macrophages on phagocytosis of the infected neutrophils [37,38]. *Leishmania* exosomes have also been shown to induce the release of the immunosuppressive cytokine IL-10 and inhibit the

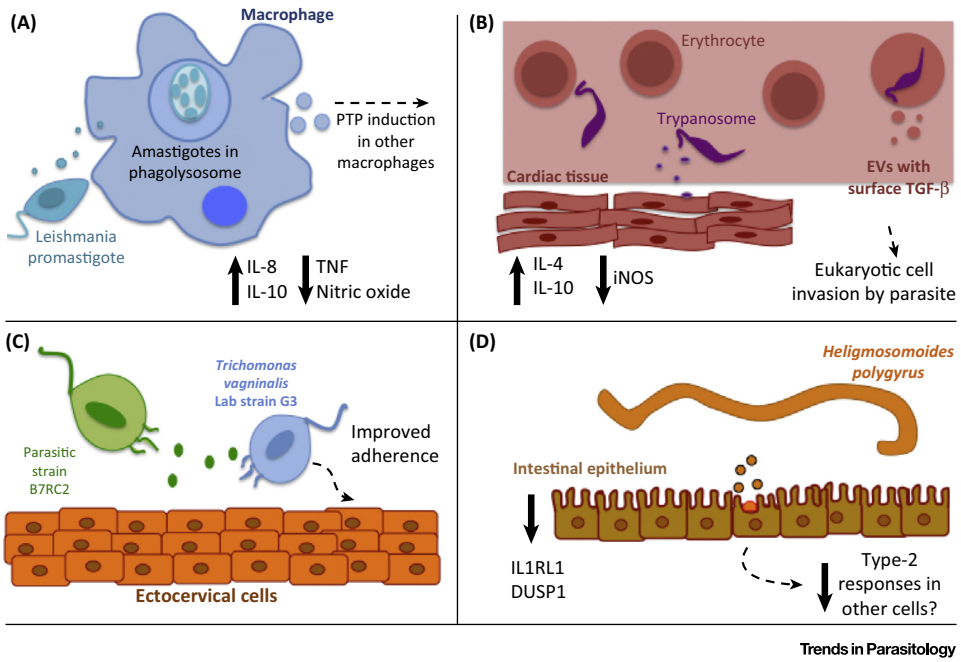

Figure 2. Schematic Representation of the Different Functions of Parasitic Extracellular Vesicles (EVs). (A) *Leishmania* spp. promastigotes release exosomes, which can modulate immune properties of monocytes, shown by an increase in the production of IL-8 and IL-10 and a decrease in tumor necrosis factor (TNF) and nitric oxide [35–37]. Infected monocytes also release exosomes that have immunomodulatory properties in recipient cells (indicated by broken line), such as the induction of protein tyrosine phosphatases (PTPs) and changes in gene expression [39,41]. (B) *Trypanosoma cruzi* trypomastigote-shed microvesicles can induce type 2 helper (Th2) polarization [seen by an increase in IL-4 and IL-10 and a decrease in inducible nitric oxide synthase (iNOS)] and invasion of cardiac tissue (indicated by broken line) [34]. Infected erythrocytes and lymphocytes release microvesicles containing surface transforming growth factor beta (TGF-β) [47]. (C) The extracellular protozoan *Trichomonas vaginalis* secretes exosomes, which can promote better adherence of weaker strains to ectocervical cells [54]. (D) Adult *Heligmosomoides polygyrus* worms secrete exosomes as part of their excretory–secretory product in the lumen of the small intestine. These are potentially taken up by intestinal epithelial cells, where they modulate gene expression of the mitogen-activated protein (MAP) kinase regulatory phosphatase gene *dusp1* and the IL-33 receptor (ILRL1) and can have downstream suppressive effects on antiparasite type 2 responses [59].

inflammatory cytokine tumor necrosis factor (TNF) in human monocyte-derived DCs in response to interferon gamma (IFNγ). Pretreatment of mice with exosomes derived from *L. major* and *L. donovani* resulted in exacerbated infection and pathogenesis *in vivo*, associated with enhanced IL-10 production and a skewed Th2 response, preventing parasite expulsion as a type 1 response is normally required for clearance [37]. Specific components of *Leishmania* exosome cargo have also been identified and shown to be involved in immunomodulation, including elongation factor 1 alpha (EF-1∝) and the membrane-bound metalloprotease GP63 [39]. These have both been associated with a depression in signalling events during a proinflammatory IFNγ response by monocytes (and potentially subsequent Th1 polarization) [36,40]. GP63 is also associated with numerous downstream modulatory effects during *Leishmania* infection, including the modulation of inflammation by activating macrophage protein tyrosine phosphatase (PTP) signaling. This metalloprotease has also been shown to impact protein sorting into exosomes and to inhibit miRNA processing in host cells by targeting the endoribonuclease DICER [39,41,42].

At least two types of EV have been identified from the infective (metacyclic trypomastigote) and noninfective (epimastigote) forms of *T. cruzi* parasites; both forms release microvesicles from the plasma membrane as well as exosomes presumed to derive from the endocytic pathway [32]. Following their initial identification [43], these EVs were further shown to contain a cohort of proteins associated with immune modulation and virulence and include the homolog to the multifunctional metalloprotease GP63, described above [32]. Notably, following inoculation of the parasite microvesicles and subsequent infection with *T. cruzi*, mice develop heightened cardiac parasitism and increased inflammatory infiltrates associated with higher levels of IL-4 and IL-10 [34]. These cytokines induce the polarization of a Th2 response as well as lower levels of inducible nitric oxide synthase (iNOS) in the tissue, suggesting that these microvesicles may serve to promote parasite dissemination and enhance survival (Figure 2B). Acid phosphatases involved in the adherence and infection of various trypanosome strains have also been shown to be present in the microvesicles [44].

In addition to the direct secretion of exosomes and microvesicles by these parasites, both *Leishmania* spp. and *T. cruzi* induce the release of exosomes from the cells that they infect. A study of *Leishmania mexicana*-treated macrophages *in vitro* showed that exosomes released from infected cells are capable of inducing phosphorylation of signaling proteins and significantly upregulating immune-related genes including adenosine receptor 2a (Adora2a) on macrophages [40]. Interestingly, Adora2a receptor activation on these cells by *Escherichia coli*, another pathogen that drives type 1 immune responses, has been associated with increased IL-10 and down-modulated TNF [45]. Conversely, a recent study suggests that exosomes from *Leishmania amazonensis*-infected macrophages can prime other naïve macrophages to initiate antiparasitic Th1 responses through the release of the inflammatory cytokines IL-12, IL-1β, and TNF [46]. *T. cruzi* also induces the release of microvesicles from infected host cells, including lymphocytes and monocytes *in vitro* and erythrocytes *in vivo*. These microvesicles express surface transforming growth factor beta (TGF-β), which has been shown to facilitate eukaryotic cell invasion by the parasite and leads to maturation and continuation of the life cycle [47]. The microvesicles also protect extracellular life cycle stages of *T. cruzi*, including epimastigotes from the vector and trypomastigotes from ruptured cells, from complement-mediated attack, thus facilitating parasite invasion of host cells [48]. More specifically, monocyte-derived microvesicles develop a complex with the complement C3 convertase C4b2a on the parasite surface, limiting the interaction with its substrate C3. The inhibition of this crucial step prevents complement-mediated lysis, opsonization, and the release of anaphylatoxins, subsequently leading to increased parasite survival [47]. In an analogous manner, erythrocytes infected with the malaria parasite *Plasmodium falciparum* produce microvesicles that enhance dose-dependent secretion of proinflammatory cytokines such as IL-1β, IL-6, and IL-12 from monocytes following

phagocytosis [49]. In the context of infection, it has been hypothesized that these cytokines may aid endothelial cell activation and erythrocyte sequestration. As with many immunomodulatory mechanisms, however, it can be difficult to distinguish whether vesicle secretion by host cells during infection is controlled by the host and/or the parasite, as both may benefit. This is discussed further later in this review.

Interspecies Communication between Intracellular Protozoan Parasites

In addition to manipulation of the host immune response, EVs can also mediate intercellular communication between parasites. It has been reported that microvesicles traffic between *P. falciparum*-infected erythrocytes and increase the commitment of asexual parasites to the sexual stages, gametocytes, to promote transmission [49,50]. Furthermore, it is suggested that EVs (described by Regev *et al*. as 'exosome-like' [50]) secreted by red blood cells following infection with transgenic *P. falciparum* parasites can rescue parasitic growth by transferring DNA encoding a drug resistance marker to other *P. falciparum* in infected cells under conditions of drug selection. Thus, genetic material can be transferred between the infected erythrocytes via EVs, and this may also contribute to the sexual development mentioned above. This pathway has been shown to be dependent on trafficking mechanisms that transport parasite-encoded proteins to the host-erythrocyte membrane through membranous structures called Maurer's clefts in infected erythrocytes [50].

This is one of the few examples to date of vesicle involvement in parasite-to-parasite communication (a further example is provided below in the case of the extracellular parasite *Trichomonas vaginalis*). This is likely to represent a bias in the literature, which focuses largely on the immunomodulatory properties of parasite secretion products. In the microbial context, it is well established that secreted vesicles play a role in microbe–microbe communication and genetic exchange (reviewed in [13]). Many gaps remain in our understanding of how different eukaryotic parasites communicate with one another to regulate aspects of their life cycles, including reproduction or commitment to transmission stages. It will be interesting to see whether this is a functional niche occupied by EVs that extends beyond malaria parasites.

Extracellular Protozoan Parasites: Communication within Their Environment

An obvious function of EVs in extracellular pathogens is their ability to protect cargo and move this into host cells. However, mechanistic aspects of this are not understood, including whether and how there is specificity in the uptake by certain cell types, whether the parasite cargo is recognized by the host immune system, and how communication is conducted between two phylogenetically distant species. Among extracellular protozoan parasites, comparative analysis of the secretome of *Trypanosoma brucei* subspecies, the causative agent of African sleeping sickness, identified several exosome-associated proteins such as enolase, heat-shock protein 70, and the clathrin heavy chain. Different members of the metallopeptidase family are also found in the secreted microvesicles and may serve as potential drug targets or even diagnostic biomarkers during stages of African trypanosomiasis [51,52]. Complimentary studies on the *T. brucei* secretome also demonstrate the presence of 50–100-nm microvesicles budding from the plasma membrane of the infective parasite [53]. The parasitic protozoan *T. vaginalis*, which can cause infertility through sexual transmission, has been shown to release functional exosomes that can play a role in both parasite-to-parasite and parasite-to-host communication [54]. Virulence products are present within the exosomes that are able to specifically downregulate IL-8 secretion by ectocervical cells (potentially limiting neutrophil migration to prevent pathogen clearance). Furthermore, preincubation with exosomes released from a more adherent strain of the parasite, B7RC2, can induce better adherence of weaker strains, such the laboratory strain G3, in a dose-dependent fashion, which is not seen in the converse scenario (Figure 2C). The mechanisms underpinning these effects and the cargo within the exosomes involved are not yet known.

Extracellular Parasites: Interactions at the Cell-to-Parasite Interface

Helminth worms are ubiquitous pathogens of plants and animals that have coevolved with their hosts for hundreds of millions of years and use sophisticated mechanisms for manipulating them [55]. It has only recently been demonstrated that these complex parasites also secrete exosomes, and potentially other classes of EV, into the environment that can be internalized by host cells. Electron microscopy images of EVs derived from diverse helminths are shown in Figure 3, including studies in the trematodes *Fasciola hepatica* and *Echinostoma caproni*, which release EVs that can be detected on the tegumental surface. Marcilla *et al.* [56], showed that these EVs are internalized by rat intestinal epithelial cells *in vitro* and contain protein homologs of proteins found in mammalian exosomes. Notably, earlier work examining the glycocalyx of *S. mansoni* cercariae demonstrated the potential presence of structures similar to multivesicular bodies adjacent to the schistosomula tegument [57]. A recent study has detailed the presence of exosome-like vesicles secreted by *Schistosoma japonicum* adults that were shown to induce macrophage polarization to a M1 phenotype, thereby highlighting the potential immunomodulatory properties of *Schistosoma*-derived exosomes and their potential role during infection [58].

We recently demonstrated that the gastrointestinal nematode *Heligmosomoides polygyrus* secretes exosomes that are internalized by host cells (Figure 2D). These are enriched in specific proteins, including those associated with exosome biogenesis (e.g., alix, enolase, HSP70), as well as many proteins of unknown function and contain miRNAs and other classes of noncoding RNA [59]. The presence of an Argonaute protein and small RNAs within nematode exosomes may suggest the existence of cross-species RNA interference, although the mechanism of this remains unknown. Several of the *H. polygyrus* exosome-derived miRNAs have target sites in the 3′ untranslated region (3′UTR) of the mouse *dusp1* gene, which encodes a mitogen-activated protein (MAP) kinase regulatory phosphatase. We showed that transfection of three nematode-derived miRNAs could suppress a luciferase reporter containing the 3′UTR of DUSP1. Although relatively little is known about this phosphatase in helminth infection, DUSP1$^{-/-}$ macrophages have previously been shown to have sustained IL-10 expression in the presence of helminth cystatins [60]. IL-10 is an immunoregulatory cytokine that could prevent an antiparasitic or inflammatory response and promote parasite longevity within the host [61,62]. We further

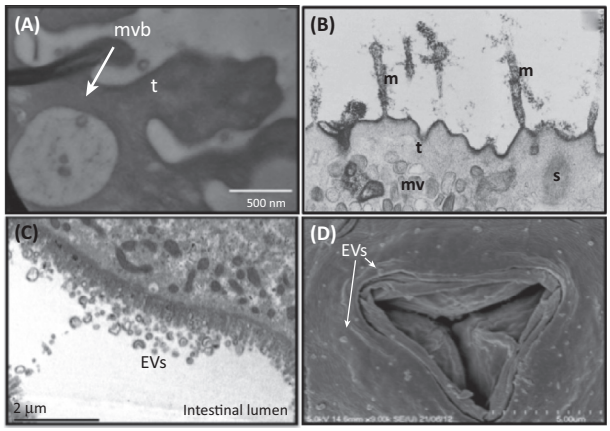

Figure 3. Electron Micrographs Demonstrating Exosome-Like Vesicles Derived from Extracellular Helminths. (A) Presence of exosome-like vesicles contained within the multivesicular body (MVB) on the tegument of *Echinostoma caproni*. Reproduced, with permission, from [56]. (B) Potential MVBs close to the tegumental surface of *Schistosoma mansoni* cercariae, microvilli (m), tegument (t), spines (s), and multilaminate vesicles (mv) are noted. Reproduced, with permission, from [57]. (C) Cross-section of *Heligmosomoides polygyrus* adult worms revealing numerous potential extracellular vesicles (EVs) within the intestinal lumen. Reproduced, with permission, from [59]. (D) Anterior opening of *H. polygyrus* covered in structures similar in size to exosomes, labeled as EVs.

demonstrated that the *H. polygyrus* exosomes could suppress an inflammatory airway response *in vivo*: during the first 24 h of an innate atopic 'danger' response to the fungus *Alternaria alternata in vivo*, *H. polygyrus* exosomes block activation of type 2 innate lymphoid cells and have downstream effects on eosinophilic recruitment. Furthermore, *H. polygyrus* exosomes suppressed expression of IL1RL1/ST2 (the IL-33-specific receptor subunit) following treatment *in vitro* in intestinal epithelial cells and *in vivo* during the allergic asthma response to *Alternaria*. As the IL-33 ligand–receptor interaction is known to be important in antihelminthic responses [63,64], these data suggest the ability of *H. polygyrus* exosomes to modulate aspects of the host cell response to prevent pathogen clearance. A previous report demonstrated that the model free-living nematode *Caenorhabditis elegans* releases peptide-containing exosomes using a defined apical secretion pathway [65] and it is expected that exosomes may be used by all nematodes, either as a mechanism of cell-to-cell communication within the organism or, when exported outside the organism, as a mode of communication with other species.

In addition to the above reports, analyses of the secretion products of other helminths suggest the presence of exosome-associated proteins, including CD63-like tetraspanins from the cestode *E. granulosus* [66]. Tetraspanins have been implicated in the formation and targeting of exosomes to recipient cells [67]. Interestingly, tetraspanins have independently been suggested as promising targets for vaccination against another parasite, *Echinococcus multilocularis*, the causative agent of alveolar echinococcosis [67,68]. This suggests that targeting exosomes and their surface proteins may provide an important antiparasite vaccination strategy.

EVs from Microorganisms and Ectoparasites: More Players at the Extracellular Surface

Other eukaryotes, such as the pathogenic fungus *Paracoccidioides brasiliensis*, release highly immunogenic EVs that are detectable in the sera of paracoccidioidomycosis patients [69]. One such immunogenic epitope is the cellular membrane carbohydrate galactose-α-1,3-galactose (α-Gal), which is not found in human cells. Although α-Gal-enriched EVs may generate a robust immune response in the host, they are suggested to be beneficial to the pathogen, both by binding to host lectins and, potentially, by stimulating a suppressive type 2 response. This is in accordance with previous literature showing that α-Gal-enriched *T. cruzi* exosomes are able to stimulate IL-4/IL-10 expression in cardiac tissue and splenocytes [34]. Many types of opportunistic fungi, including *Cryptococcus neoformans*, *Candida albicans*, and *Histoplasma capsulatum*, release EVs [70], which have been suggested to contain virulence-associated factors including polysaccharides and lipids (reviewed further in [71]). The EVs released by *C. neoformans*, for example, are enriched in virulent capsular components such as glucosylceramide and glucuronoxylomannan (GXM) [72]. Interestingly, a recent study has shown the importance of phospholipid translocases (flippases) in *C. neoformans* exosome packaging and transport, whereby mutant Apt1 flippase-knockout fungi have diminished levels of GXM and are consequently unable to successfully colonize the lung and brain of infected mice [73]. Furthermore, the yeast *Malassezia sympodialis*, a component of natural human flora, is able to release EVs capable of generating IL-4 and TNF secretion from peripheral blood mononuclear cells, enhancing an inflammatory response in patients afflicted with atopic dermatitis [74]. Fungus-released EVs may also induce antimicrobial activity by host cells: EVs released by *C. neoformans* are taken up by macrophages *in vitro* and stimulate TNF, IL-10, TGF-β, and nitric oxide production [75].

A recent study in the argasid tick, *Ornithodoros moubata*, suggests that some immunomodulatory proteins may be secreted in arthropod saliva, and it is tempting to speculate that EVs would also be found in this environment. Proteomics of the tick saliva reveal several exosome-associated proteins (e.g., aldolase, enolase) as well as anti-inflammatory lipocalins, which serve as scavengers of leukotrienes, and adenosine nucleotides at the location of the bite [76]. It is

clear that we are only at the beginning of many new discoveries with extracellular parasites and the functionally diverse EVs they might secrete. There are a growing number of reports containing proteomic matches to exosome proteins in parasite secretomes and this should cement the idea that these are probably used by most, or all, pathogens at some stage in their life cycle. The effects that these EVs may exert at this interface will be of particular importance in the context of antiparasite treatment, and conversely, based on the ability to suppress an innate immune response [59], they may also be useful tools to ameliorate inflammation-associated disease [4].

Host Exosomes in the Context of Pathogen Infection: A Useful Therapeutic Strategy?

As parasites have evolved to secrete exosomes that are able to effectively interact with the host, it is only logical that the host would also use this pathway as a defense mechanism. During infection with a rodent malaria parasite, *Plasmodium berghei*, plasma cell-derived microvesicles induce CD40 on antigen-presenting cells, generating a potent inflammatory response through potential T cell priming and effector initiation [77]. Subsequently, macrophage activation may be responsible for clearance of the parasite. This is further supported by studies in *Plasmodium vivax* infection in humans, whereby immune cell-derived microvesicles are associated with greater acute inflammation in the pursuit of parasite eradication [78]. These mechanisms can be exploited in a therapeutic context; for example, murine reticulocytes infected with the nonlethal *Plasmodium yoelii* X strain can significantly attenuate pathogenesis when transferred into mice that are then infected with the lethal strain *P. yoelii* XL [79]. On a separate note, intestinal epithelial cells were shown to increase the release of antimicrobial peptide-containing exosomes in response to *Cryptosporidium* infection, which is driven by enhanced toll-like receptor 4 signaling following recognition of the protozoan parasite [80]. The facultative intracellular bacterium *Mycobacterium tuberculosis* induces exosome release from infected macrophages, which consequently promotes recruitment of lymphocytes through heightened inflammatory chemokine secretion (such as RANTES and MIP-1α) [13,81]. Exosomes derived from *Mycobacterium bovis*-infected macrophages are able to promote DC activation as well as generating an antibacterial T cell response *in vivo* [82].

Host-derived exosomes also play important roles in antigen presentation. DCs pulsed with *Toxoplasma gondii* antigens are able to induce both a systemic and a local humoral response against the parasite *in vivo*, thereby serving as an efficient vaccine against toxoplasmosis [83,84]. Similar results are seen in a vaccine trial with *L. major*-pulsed DC exosomes, showing that DC-derived exosomes are able to mediate protective Th1 immunity against cutaneous leishmaniasis in a cell-independent manner [85]. Importantly, several studies have emerged using DC-derived exosomes for protection against common livestock parasites. Vaccination of chickens with *Eimeria* parasite antigen-loaded DC exosomes was able to successfully ameliorate symptoms of avian coccidiosis caused by several species (*Eimeria tenella*, *Eimeria maxima*, and *Eimeria acervulina*) as well as reduce mortality rates [86].

Concluding Remarks and Future Perspectives

From this review, it is clear that exosomes and other EVs can be used by both parasite and host to influence the outcome of an infection. Vesicles can function by transmitting signals between parasites, from parasite to host, or from host to the environment for antigen presentation and other aspects of host defense. The ability of vesicles to transport and deliver diverse populations of molecules in a specific package might occupy a range of niches in biology. There has been a surge of reports in the past 5 years detailing the presence of parasite-derived vesicles and it seems likely that this will only increase with the appreciation that all organisms are likely to secrete these [13]. Based on the literature, immune manipulation appears to be a prevalent

function of parasite-derived exosomes, which feeds into numerous cell-to-cell interactions within the human body [87]. However, it is expected that EVs could also play a prominent role in parasite-to-parasite communication, which has been less well studied to date (see Outstanding Questions Box). The molecules within exosomes that mediate their functions require further investigation. We and others have detailed the small RNAs present in pathogen-derived exosomes [59,88–91] and previous reports have shown the functionality of exosomal RNA in an immune context [24,92,93]. One concern in this field at present, however, is the lack of quantitative data to determine the abundance and stoichiometry of RNA within EVs and whether this is sufficient for effective gene silencing under physiological conditions [94]. Intriguingly, we found that an Argonaute protein is also secreted with exosomes derived from *H. polygyrus*, and it could be expected that ribonucleoprotein complexes, rather than individual molecules, might underpin functionality. In addition to nucleic acids, there are many immunomodulatory proteins in exosomes, [87,95–97], as well as lipids that might have immunomodulatory properties [98]. During the preparation of this manuscript, two additional papers demonstrated EV secretion by helminths: the liver fluke *Opisthorchis viverrini* [99] and pig whipworm *Trichuris suis* [100]. Chaiyadet *et al*. [99] show that EVs produced by *O. viverrini* drive IL-6 production and proliferation of human cholangiocytes, and may link to the chronic periductal fibrosis associated with this pathogen. Additionally, they demonstrate that uptake of these EVs by host cells is blocked by Ab directed against a surface tetraspanin. A deeper understanding of the biochemical properties of exosomes will be key to interrogating how these complicated packages of information operate and how we can interfere with or mimic these processes to treat infectious disease.

Outstanding Questions

How are the diverse combinations of molecules packaged into EVs integrated in a functional response in recipient cells?

Are all parasite EVs recognized by the host immune system or are they able to escape this?

How heterogeneous are the EVs secreted by parasites and is it possible that these have multiple targets and functions?

How is EV packaging and release regulated and can this be targeted as a therapeutic strategy?

What proteins are bound to the RNAs within EVs and how would these integrate into a functional RNAi pathway inside recipient cells?

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9.3 Host parasite communications—Messages from helminths for the immune system

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Gillian Coakley, Amy H. Buck & Rick M. Maizels

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Host parasite communications—Messages from helminths for the immune system
Parasite communication and cell-cell interactions

Gillian Coakley[a], Amy H. Buck[a], Rick M. Maizels[b,*]

[a] Institute for Immunology and Infection Research, and Centre for Immunity, Infection and Evolution, School of Biological Sciences, University of Edinburgh, UK
[b] Wellcome Trust Centre for Molecular Parasitology, Institute of Infection, Immunology and Inflammation, University of Glasgow, 120 University Place, Glasgow, G12 8TA, UK

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ABSTRACT

Helminths are metazoan organisms many of which have evolved parasitic life styles dependent on sophisticated manipulation of the host environment. Most notably, they down-regulate host immune responses to ensure their own survival, by exporting a range of immuno-modulatory mediators that interact with host cells and tissues. While a number of secreted immunoregulatory parasite proteins have been defined, new work also points to the release of extracellular vesicles, or exosomes, that interact with and manipulate host gene expression. These recent results are discussed in the overall context of how helminths communicate effectively with the host organism.

1. Introduction

Helminth parasites generally establish long-term infections in their host, reflecting their ability to drive a new physiological and immunological homeostasis that best accomodates the invader [1]. Over eons of evolutionary time, parasites have developed a remarkable suite of finely-tuned molecular adaptations that manipulate, inhibit or activate different host cells or pathways in order to maximise parasite success [2,3]. In this review, we discuss some of the more recent and exciting developments that shed light on the molecular pathways of host-parasite communication.

Helminths are parasitic worms belonging to the lower invertebrate phyla of nematodes (roundworms) and platyhelminths (flatworms). A wide variety of helminth species are able colonise an extraordinary array of niches and host organisms, in each case circumventing host defence and expulsion mechanisms. Interestingly, the strategy of helminths is not to outpace the immune system through rapid multiplication or antigenic variation, but to manipulate and modulate immunity in order to defuse immune defences, meaning the host fails to eliminate the parasites [4]. Helminths essentially take hold by stealth, first inactivating host detection systems that would otherwise raise the alarm, and then effectively tolerizing the immune system to parasite antigens, and in doing so, also dampening responses to bystander antigens in allergy or autoimmunity [5].

The softly–softly strategy of helminths has implications for how they communicate with their hosts and the immune system of their host, suggesting that there must be a continual dialogue to maintain the state of tolerance. Because infection comprises relatively stable populations of long-lived parasites, it is logical to deduce that the dialogue is conducted by products continuously released from live parasites that address different specific components of the immune system [2]. This notion is supported by observations that most of the immunomodulatory effects of helminth infections are reversed following drug-mediated parasite clearance [6–8].

Correspondingly, much attention has been paid to the "excretory-secretory" (ES) antigens of helminths, a pragmatic approach to collect mixtures of released proteins that dates back over 60 years [9]. Much more recently, of course, the application of genomics, transcriptomics and mass spectrometry has transformed our understanding of these complex and heterogeneous preparations by defining the individual molecular components that parasites release to modify their environment [2]. As discussed below, these products are not only proteins, but also include glycans, lipids and nucleic acids, in particular miRNAs, as well as small molecules and metabolites, and are released in a variety of "packages", including lipid vesicles.

* Corresponding Author.
E-mail address: rick.maizels@glasgow.ac.uk (R.M. Maizels).

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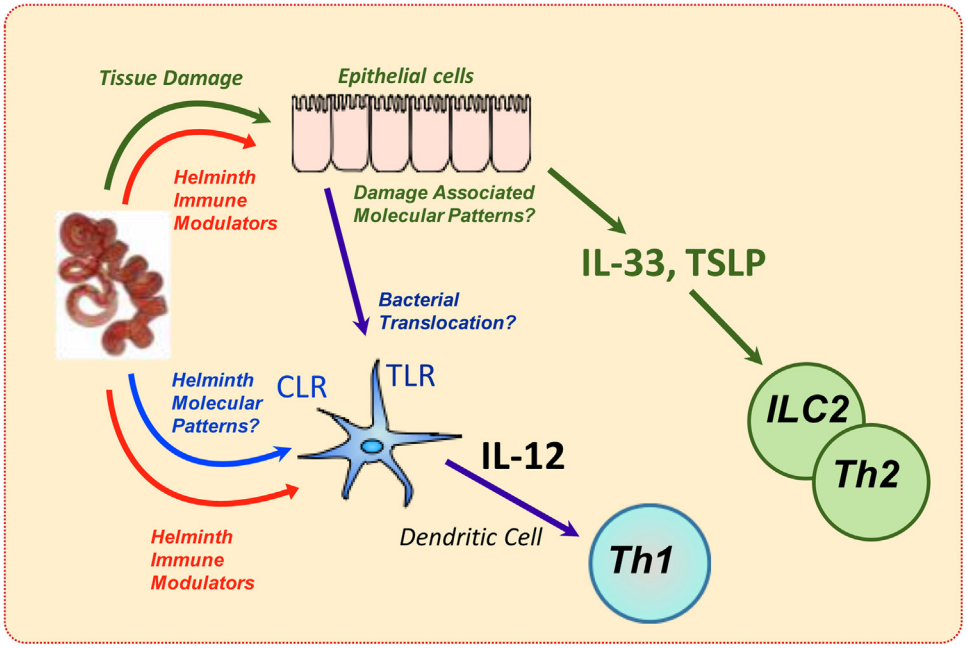

Fig. 1. Pathogen recognition systems in helminth infection. Innate mechanisms respond to tissue injury with release of alarmins (eg IL-33, TSLP) which can initiate a type 2 response; helminths can block alarmin release or receptors for alarmins such as ST2 (the IL-33R). Pathogen associated molecular patterns may also be recognised eg by Toll Like Receptors (TLRs) or C-type lectin receptors (CLRs), and these molecular patterns may be directly presented by helminths, or indirectly through bacteria translocating through injured epithelium. In the latter case, the Th1 response driven by IL-12 is blocked by helminth secreted immune modulators.

2. Host recognition of parasites

The first encounter between parasite and host generally entails breaching of a barrier surface (such as skin or intestinal epithelium) that provokes release of 'alarmins' [10] and recognition of the invader by pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) that drive inflammatory cytokine production. Alarmins, closely associated with helminth-mediated tissue damage, include IL-33 and TSLP [11,12], which both promote a Type 2 pro-allergic and anti-helminth mode of the immune response. However, helminths can partially or entirely circumvent this threat (Fig. 1); for example, the response of dendritic cells (DCs) to TLR ligation is effectively negated by products from *Nippostrongylus brasiliensis* and other helminths, with IL-12 production being especially inhibited [13–17] while epithelial cell release of IL-33 is directly blocked by products released by *Heligmosmoides polygyrus* [18]. As discussed in the following section, some of the molecular mediators responsible for blocking innate activation are now being defined.

The archetypal PRRs react to microbial products such as LPS and lipoteichoic acid by triggering production of pro-inflammatory cytokines, such as IL-12, that drive the Th1 response. The consistent ability of varied helminth products to suppress IL-12 release following TLR stimulation may be a mechanism aimed not so much at blocking anti-parasite immunity, but at avoiding collateral inflammation at barrier sites where, for example, bacterial translocation could accompany helminth invasion. Whilst the central role of TLRs in pathogen pattern recognition by the host is now well understood, it is surprising that no parallel recognition system has yet been defined for Th2-inducing organisms such as helminths. However, a few TLR ligands from helminths have been described, including from *Schistosoma mansoni* both the lysophosphatidylserine glycolipid [19] and RNA activating TLR3 [20], and other receptor systems such as the C-type lectin receptors (CLRs) may fulfill the role of innate recognition in other settings [21–23].

3. Protein-mediated interactions

The first level of parasite communication with the host can be considered to be simple protein–protein interactions in the extracellular milieu, either with fluid phase host components, or exposed receptors on host cell surfaces. For example, *H. polygyrus* secretes a functional mimic of the immunomodulatory cytokine TGF-β, which ligates mammalian surface receptor and transduces a suppressive signal to T cells (Johnston et al., submitted for publication). Space precludes further discussion of the many individual proteins now found to be involved in host-helminth interactions, but perhaps the most intriguing are members of the CAP superfamily (Pfam00188) which are greatly expanded across all helminth parasite lineages [24,25], and highly represented in the secreted protein compartments [26,27]. One member of this family from *Necator americanus* (a hookworm) was one of the first to be characterised functionally as NIF, a secreted inhibitor of integrin binding that blocks neutrophils [28].

While functional assignments for members of the CAP gene family other than NIF are scarce, it is interesting to note that in a plant parasitic nematode, a homologue binds to a tomato plant innate defence protein, disabling resistance pathways and promoting infection [29]. Thus, helminth secreted proteins are not necessarily limited to interactions at the host cell surface, but can perform functions within host cells, raising the question of how they may enter the cell.

3.1. Intracellular action of helminth proteins

Two well-studied helminth glycoproteins are known to enter host cells and mediate profound biological effects. The *Schistosoma mansoni* egg-derived glycoprotein ω1 is a ribonuclease bearing Lewis X glycan side chains, that bind to surface lectin of dendritic cells, mediating uptake into the cell, resulting in the protein moiety acting to block protein synthesis [30,31]. DCs pretreated with ω1 are also switched into the type 2 immune pathway, activating naive T cells to become Th2 effector cells.

A different mediator is the predominant secreted glycoprotein of the filarial nematode *Acanthocheilionema viteae.* This product, ES-62, is a 62-kDa component bearing N-linked phosphorylcholine (PC) sidechains. Through interaction with surface TLR4, ES-62 enters the cell, and in the intracellular milieu the PC moiety interrupts the downstream signalling of both the B cell receptor and TLR4, effectively inhibiting cell activation [32]. A further example is the FheCL1 cysteine protease from *Fasciola hepatica,* which degrades TLR3 in host macrophages thereby inhibiting activation; although TLR3 is an intracellular pathogen sensor, FheCL1 is able to enter the endosome to degrade the receptor in situ [17].

A separate pathway is targetted by the filarial cystatin molecule CPI-2. This protein has two inhibitory sites which target conventional cysteine proteases, and asparaginyl endopeptidase (AEP) respectively [33]. Human B cells exposed to CPI-2 from *Brugia malayi* (a human filarial parasite) are no longer able to process protein antigen for presentation to T cells, a pathway dependent on AEP activity in the endosome [33]. Further studies on a closely related cystatin from *A. viteae* show that this protein is taken up by mouse macrophages and activates ERK and p38 kinases, resulting in the production of immunoregulatory IL-10, in a manner linked to the phosphorylation of the CREB and STAT3 signalling factors [34].

Many other products have been shown to modulate intracellular signalling in host cells, although the mode of entry is not always understood. For example, the ALT-2 protein is derived from an abundant larval transcript of the filarial parasite *B. malayi*. The effect of this protein is seen when added to macrophages, or introduced into the macrophage via transfection of the intracellular protozoan *Leishmania mexicana*, in the induction of the signalling proteins GATA-3 and SOCS-1, which act to induce type 2 responses and dampen IFN-γ dependent inflammatory signals in the cell [35].

3.2. Discovery of exosomes

It is now becoming increasingly apparent that extracellular vesicles, and exosomes in particular, play a key role in cellular communication. Exosomes are nanovesicles around 50–100 nm in size that are secreted by virtually all cells to facilitate the transfer of selected cargo, mainly lipids, proteins and RNA species, whilst retaining phenotypic markers from their cell of origin [36,37]. Exosomes develop within a cell by inward budding of multi-vesicular endosomes, and thus contain components of the parental cell, such as RNAs or proteins, that may be trafficked into the same compartment. The discovery of extracellular vesicles from kinetoplastids, fungi and bacteria drove the theory that exosome-mediated communication could operate on a cross-species platform, whereby parasite-derived exosomes could interact with, and potentially modulate, the host immune system [38]. Only recently have exosomes been recognised as integral products from extracellular organisms like helminths [38,39].

It has recently been discovered that parasitic helminths produce exosomes. This was initially reported in the excretory-secretory components of the trematodes, *Echinostoma caproni* and *Fasciola hepatica*, which infect the gastrointestinal tract and liver respectively [40], and in the nematode *H. polygyrus,* which infects the small intestine [41]. Data from the trematode studies further suggests that ES-derived exosomes are capable of reaching the host environment, as they appear to be found intact on the parasites' tegument. Further support of this is demonstrated by the internalisation of helminth exosomes by host intestinal epithelial cells, suggesting their capacity for cross-phylum communication between helminths and mammals.

The formation of exosomes by helminths had originally been established in free-living nematodes, with the demonstration that *Caenorhabditis elegans* use a novel secretion pathway from the apical membrane, to co-secrete multivesicular bodies, containing exosome-like vesicles, with peptides that normally promote cuticle development. [42]. Exosomes from helminths and protozoa appear to share many specific markers, with those known to be present in mammalian exosomes, such as Heat-shock protein 70 (HSP70), endosomal sorting components e.g. Alix, and surface tetraspanins including CD9 and CD63 [37]. For example, it was shown that whilst exosomes secreted by *Leishmania*-infected macrophages undergo a series of phenotypic changes following infection, they still retain some typical exosome markers, including TsG101, Alix and CD63 [43]. Additionally, transcriptomic analysis of the cestode, *Echinococcus granulosus,* revealed the existence of other CD63-like tetraspanin family members [44]. Tetraspanins

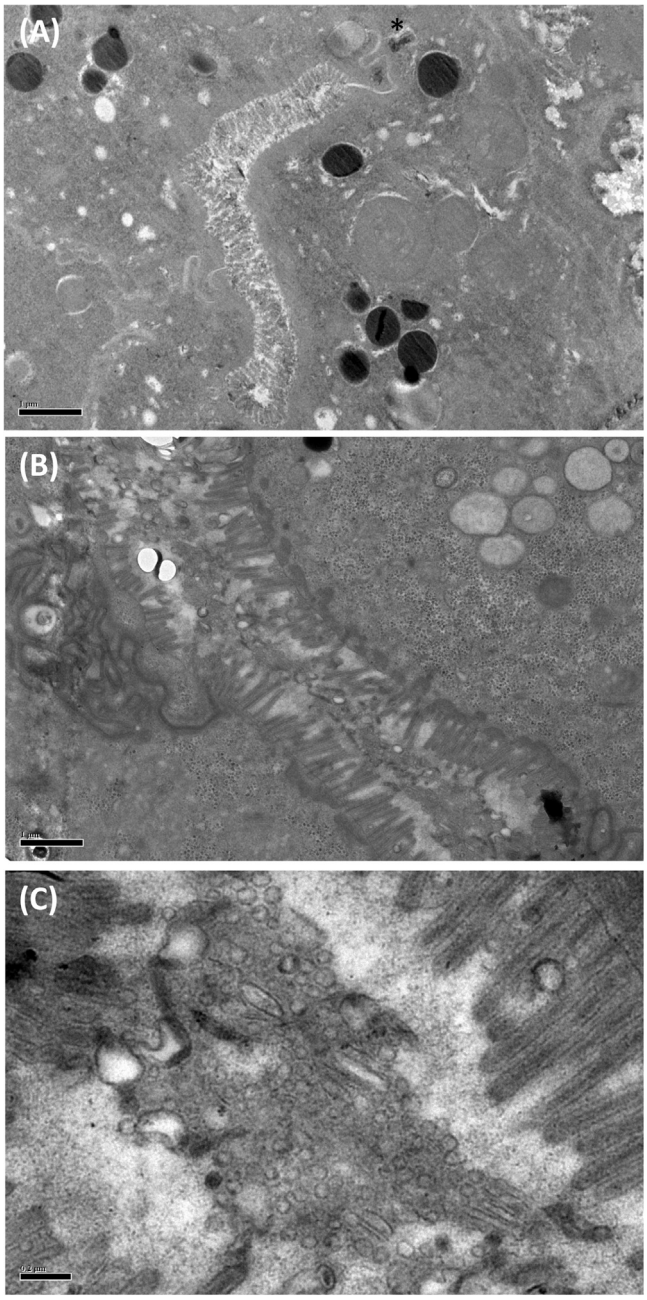

Fig. 2. Proposed route of secretion of exosomes by the nematode *H.polygyrus*. (**A**) Low-power micrograph of the intestinal tract of adult *H. polygyrus* showing brush border epithelium, as well as ducted secretory gland (marked with asterisk). (**B**) Higher power image *of H.polygyrus* intestinal ultrastructure, with (**C**) zoom of the luminal contents containing a large number of vesicles and macromolecular structures consistent with the presence of exosomes.

were independently selected as target candidates for vaccination against *Echinococcus multilocularis,* another tapeworm which causes alveolar echinococcosis, a highly fatal disease dominating parts of Siberia, Central Europe and China [45,46]. The focus on a tetraspanin-targeting vaccine is also being explored against the human pathogen *S. mansoni* [47,48].

Previously, we have shown the ability of *H. polygyrus*, a murine gastrointestinal nematode, to secrete exosomes that contain multiple miRNA species, as well as a significant number of proteins, representing approximately 10% of the total protein secretion of an adult worm [41]. Proteomic comparison of the secreted products represented in the soluble and vesicular fractions separate by ultacentrifugation also demonstrated enrichment of a number of key components within the exosomes. Interestingly, some of these were proteins which have previously been located at the apical membrane of intestinal epithelial cells of *C. elegans*; electron microscopy also recorded multi-vesicular bodies in the intestinal tissues of *H. polygyrus* adults and exosome-like structures released into the lumen [41], strongly suggesting that the parasite releases exosomes from its alimentary tract (Fig. 2).

Functionally, we were also able to show the immunomodulatory capacity of exosomes derived from extracellular helminths. When given prophylactically, *H. polygyrus* exosomes suppress the innate immune response to the fungus *Alternaria alternata*, commonly associated with respiratory allergies, primarily through the modulation of type 2 innate lymphoid cells (ILC2s) [41]. Activation of ILC2s normally drives eosinophilia through the release of IL-5, which is blocked by parasite exosomes (Fig. 3). Moreover, *H.polygyrus* exosomes have been shown to reduce the expression of IL1RL1/ST2 transcript, both *in vitro* and *in vivo* in murine cell populations. This gene encodes the IL–33 receptor, and is required for the type 2 immune response to be initiated by ILC2s, consistent with the observed protection from allergic inflammation conferred by exosomes *in vivo*. The importance of the IL-33 ligand-receptor axis in anti-parasite responses has also been well-documented [18,49]. Thus, our data demonstrated the ability of *H. polygyrus*-derived exosomes to avoid parasite clearance by modulating this key aspect of the host immune response.

Parallel studies on the digenean trematode cattle parasite, *Dicrocoelium dendriticum*, also found exosomes to be released into culture medium, and to contain over 80 protein components as well as at least 30 miRNA species with identity or near-identity to known sequences [50]. Although no functional tests were reported, the authors highlighted the commonality with the major Schistosome miRNAs Bantam, miR-10 and miR-3479 that are detectable biomarkers in the plasma of infected hosts [51].

Most recently, Nowacki et al. described 30–100 nm exosome-like vesicles secreted by *S. mansoni* schistosomulas that are enriched in specific non-coding RNAs and proteins [52]; over 200 miRNAs were identified as well as 20 tRNA-derived small RNAs and over 100 proteins. In addition, it was shown that the L3 infective stage of *B. malayi* secrete 50–120 nm vesicles rich in miRNA species, and a protein complement that included not only classical exosome-associated products, but those with potential to interfere with host cell responses, such as Cathepsin L [53]. Significantly, the infective stage was found to be much more prolific exosome producers than the adult worm stage, possibly reflecting the demands of transition from vector to host at this point in the life cycle. Sotillo et al. further reported that adult *S. mansoni* worms release 50–130nm-sized exosome vesicles, containing over 80 identifiable proteins 5 of which are tetraspanins and an abundant saposin-like protein [54]. These authors also highlighted that a number of known Schistosome vaccine candidate antigens, including the tetraspanins discussed above, are prominent components of the exosomes. In the related parasite, *S. japonicum*, Wang et al. reported that 30–100 nm vesicles released by adult worms cultured in vitro for 5 h, detectable upon ultracentrifugation of the culture medium [55]. These authors also found that *S. japonicum* exosomes stimulated the murine macrophage-like cell line RAW264.7 to produce nitric oxide alongside other indicators of a Type 1 pathway, although in this study the protein cargo of the exosomes was not identified. The presence of many key proteins, as well as RNA species, in the secreted vesicles highlights both the complexity and diversity of cargo within exosomes, with a correspondingly wide range of potential interactions within recipient cells [56].

A broader scope for helminth exosomes has also emerged from analyses of the liver fluke *Opisthorchis viverrini*, a trematode prevalent in parts of South-East Asia where it is causally linked to cholangiocarcinoma (bile duct cancer). As with the species described above, secretory material contained exosomes (measuring in this case 40–180 nm), with a similar spectrum of associated proteins including tetraspanins [57]. Some exosome-associated proteins were also found in the bile fluid of infected hosts. Exosome entry into host cells was blocked with anti-tetraspanin antibody, arguing that this protein is likely to be exposed on the vesicular surface as found for mammalian exosomes. Most significantly, *O. viverrini* exosomes were found to stimulate cell proliferation in a human cholangiocyte cell line, and to also induce their production of the pro-inflammatory cytokine IL-6 in a manner that was partly inhibitable by anti-tetraspanin antibody. Taken together, these data make a strong case that *O. viverrini* drives potentially tumorogenic changes in the host bile duct that could account for the carcinogenic effects of infection with this parasite.

3.3. Helminth miRNAs in exosomes

It has been well documented that non-coding RNAs, and microRNAs in particular, transfer between cells and organisms through their encapsulation within exosomes and other extracellular vesicles [58]. Indeed, this provides a mechanism for protecting RNAs from degradation when outside of the cell, and presumably enables an uptake pathway to deliver RNA to the appropriate cellular compartment in the recipient. Several of the studies discussed above identified small RNAs within parasite-derived exosomes, including those from the nematodes *B. malayi* [53] and *H. polygyrus* [41], and the trematodes *D. dendriticum* [50] *and S. mansoni* [52].

In the case of *H. polygyrus,* we were able to show a suite of RNA species packaged within exosomes, including miRNAs such as let-7, miR200 and bantam [41], which could suppress the mouse phosphatase Dusp1 using a reporter assay. New data identifying extensive miRNA repertoires in parasitic helminths are now becoming available, although the distribution of these within secretory exosomes has in most cases yet to be established.

Most importantly, definitive evidence for helminth-derived miRNAs acting on host genes remains to be obtained; however, the circumstantial evidence remains enticing; not only are extensive seed sequences shared between helminth and host miRNAs, but the miRNA-rich exosomes (of *H. polygyrus* at least) also carry the worm Argonaut protein [41,59], suggesting that a functional package for gene repression is being delivered to the target cells.

4. Small molecule interactions

Increasing attention is being paid to how small molecules, metabolites, hormones and molecular cues, are intimately involved in intercellular communication. For example, the short-chain fatty acids (SCFAs, butyrate, acetate and proprionate) are not produced by the mammalian organism, but are derived from commensals at levels that promote regulatory T cells [60]; hence dysbiosis can be pathogenic due to disruption of this pathway [61,62]. Interestingly,

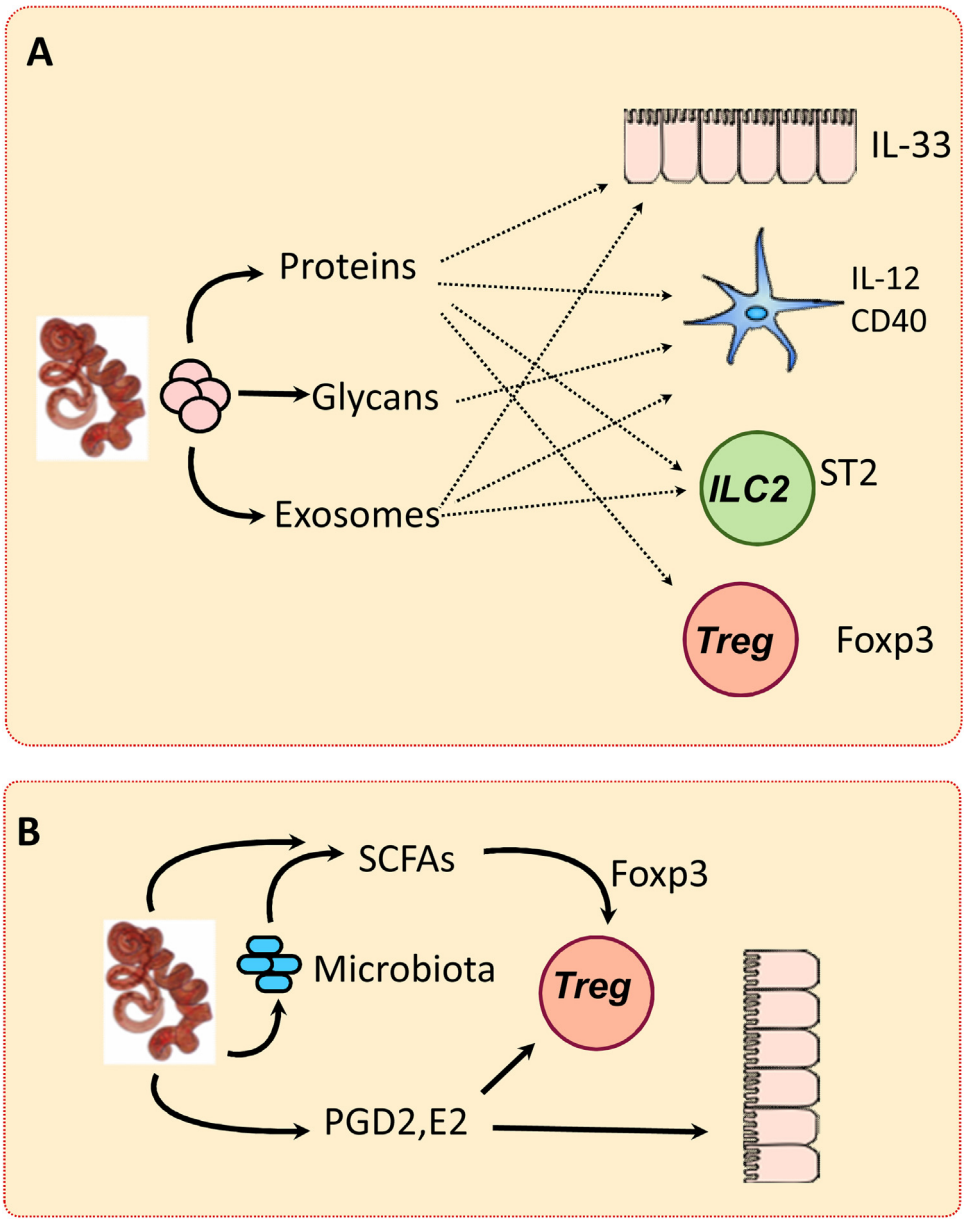

Fig. 3. Helminths release diverse molecular species to communicate with host cells, including proteins, glycans and exosome components, including miRNAs (**A**). They also produce short chain fatty acids (SCFAs) and promote commensal microbes which release SCFAs, expanding the Foxp3+ Treg population (**B**).

helminths can also synthesise these compounds, [63] as well as promote the commensal bacteria able to produce significant quantities of SCFAs [64].

Other small molecules include prostaglandins D2 and E2 produced by filarial parasites *B. malayi* [65] and *Onchocerca volvulus* [66], and by the skin-invasive cercariae of *S. mansoni* [67]. In addition to small molecules and metabolites, helminths also directly modify host-derived small ligands such as acetylcholine (through acetylcholinesterase [68]), platelet-activating factor (PAF hydrolase [69]) and ATP (apyrase [70]), among many others, a discussion of which are beyond the scope of the current review.

5. Interactions through the microbiome

Helminth parasites, particularly in the intestinal tract, share their niche with a myriad of micro-organisms, principally hundreds of bacterial species known as the microbiota [71–73]. Notably, helminth infections depend to a great extent on the presence of these commensals: for example, in the absence of caecal bacteria, *Trichuris* eggs do not hatch in the intestine [74]. Most studies of the microbiota in mice infected with intestinal helminths have found significant and occasionally sweeping changes in the species composition, particularly among *Bacteriodes* and *Lactobacillus* populations [71–73]. Recently, it was found that BALB/c mice infected with *H. polygyrus* showed expansion of the *L. taiwanensis* species, and that the degree of colonisation with this bacterium positively correlated with both adult worm numbers and the level of Treg activation [75]. Interestingly, if mice were given *L. taiwanensis* prior to receiving *H. polygyrus* larvae, they were rendered more susceptible to infection, establishing a mutual promotion between the bacteria and helminth organisms.

It has also been suggested that the immune modulatory effects of helminth infection may be mediated in part indirectly, through altering the intestinal microbiome. To date, intriguing experiments have been reported in which the intestinal contents of infected mice (containing bacteria but also a range of host and parasite products)

are able to dampen the allergic response when transferred to recipient mice [64]. It will be fascinating to analyse this effect in more detail, particularly if as with *L. taiwanensis*, an individual bacteria is found responsible.

Significantly, a recent report has shown that faecal miRNAs derived from intestinal epithelial cells can influence the microbiome, potentially through direct interactions with bacterial genes [76]. These miRNAs were suggested to be present in extracellular vesicles, raising the possibility that both host and helminths could modulate the microbiome through this novel mechanism, and indeed as mentioned below, that host exosomes could impact on the helminth organisms parasitising the intestinal tract.

6. Two way street – helminths listening to their host

While the focus of this review has been on how helminths deliver messages to the host immune system, there are some intriguing examples of how helminths also detect and respond to host immune status. Classic studies on *N. brasiliensis* found that the adult worms adapt to an immunised host by switching expression levels and isoforms of secreted acetylcholinesterase [77]. More recently, detection of host cytokines has been found in Schistosomes, which require the presence of host TNF to mature to egg laying [78] and filarial parasites responding to high IL-5 levels *in vivo* by accelerating their maturation and production of offspring [79]. An example of a helminth receptor able to ligate a host cytokine was established in the case of the TGF-β family receptor of *S. mansoni* [80].

An intriguing possibility that extracellular vesicles from the host provide a channel of communications that influence the helminth parasites, although as yet there are no reports of parasites being directly receptive to vesicle-mediated signals. However there is a growing literature demonstrating the use of host-derived extracellular vesicle impact on defence against pathogens. For example, exosomes derived from IFN-α stimulated cells were able to induce antiviral activity and limit viral replication in recipient infected cells [81,82]. Furthermore, human semen exosomes have also been implicated in resistance to HIV-1 following their uptake into naïve cells by reducing viral fitness [83]. Another example of exosome-mediated host defence is demonstrated during the innate response to protozoan parasite *Cryptosporidium parvum*. TLR4-mediated activation of the host epithelium induces the release of antimicrobial peptide-containing exosomes that limit infectivity of the pathogen in the intestinal environment [84]. The development of a directed anti-pathogen response by host exosomes has also been explored for use in a more clinical setting, in which host exosomes collected from parasite antigen-primed dendritic cells induce protection from different protozoan infections, including *Toxoplasma gondii* [85] and *Leishmania major* [86].

7. Conclusions and outlook

Helminths have accompanied a vast range of host species throughout evolution, developing sophisticated pathways of communication with, and even control of, the immune system of their hosts. The rapid discovery that many helminth species have the ability to release exosomes to mediate cross-phylum interactions speaks to the importance of this pathway in host-parasite biology. In this new light, we now see how the large extracellular parasites, classified as helminths, may be able to "reach in' to the intracellular machinery of host cells, modifying their behaviour in ever more remarkable ways. As exosome uptake is not necessarily receptor-dependent, it is difficult for the host to evolve counter-measures to block parasite exosome effects, while it would be relatively easy for the parasite to exploit exosome traffic for effective interference molecules, from proteins and enzymes to small RNAs and other modifiers of gene expression. Furthermore, these vesicles offer a robust vehicle for parasites that may have to deliver their 'message' through extracellular spaces of very different nature, and quite possibly through cells and tissues too.

Greater understanding of helminth exosomes, however, should direct us to ways of neutralising their effects, building on our existing knowledge of immunomodulatory proteins and glycans. For example, exosomes may constitute good vaccine targets, if we can generate antibody responses to key surface membrane components that are required for cell entry. In addition, new drug targets may emerge from defining the pathways required for exosome biogenesis in helminths, and/or the events within the host cell that follow helminth exosome uptake. Hence, a new window has opened not only on how helminths defeat the immune system, but on how we can turn the tables and defeat the strategy of the helminth.

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