Schistosoma mansoni extracellular vesicles and their impact on the immune system: glycosylated messengers in host-pathogen communication

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“We’re all here to do what we’re all here to do. I’m only interested in one thing, Neo, the future. And believe me, I know – the only way to get there is together.”
– The Oracle (from The Matrix, 1999)

Chapter 1

General introduction

1

Introduction

Schistosoma and host immune modulation

The parasitic trematode (flatworm) *Schistosoma (S.) spp.* affects over 250 million people in poor, rural communities of the tropics and subtropics, especially where access to safe drinking water and adequate sanitation is lacking[1]. Once *S. mansoni* worms establish their residence in the blood vessels of the hepatic portal system (see Figure 1 for the full life-cycle), they can persist there for many years or even decades[2]. This demonstrates that schistosomes have a remarkable capacity to evade both humoral and cellular immune responses. Because parasitic worms (also called helminths) have co-evolved with humanity, they have developed evasion mechanisms alongside the maturation of human innate and adaptive immune responses, which makes them experts in immune modulation[3].

The skin invasion of the cercariae, the infectious larvae of *Schistosoma*, will trigger keratinocytes to release damage-associated molecular patterns (DAMPs). Together with the pathogen-associated molecular patterns (PAMPs) of the parasite itself, these DAMPs will activate the first line of defense of the immune system. Cells such as monocytes, macrophages and dendritic cells (DCs) can recognize DAMPs and PAMPs via their pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs)[3]. The skin penetration of the schistosomula leads to infiltration of polymorphonuclear cells (e.g. neutrophils, eosinophils) and mononuclear cells (e.g. monocytes, macrophages, DCs) and a local release of pro-inflammatory and anti-inflammatory cytokines[4]. This initial phase of infection (up to week 8) includes responses that characterize T helper 1 (Th1), Th2, and regulatory profiles[5–7]. An important manner in which the schistosomula elicit these immune responses, is *via* the release of excretory/secretory (ES) products[8]. Additional immune evasion strategies of the schistosomula includes expression of enzymes that can degrade DAMPs and compositional changes in their outer membrane to evade complement activation[9].

Once matured into adult worms, the immune responses observed shift towards a dominant Th2 response. This is mostly caused by the worms' production of eggs and the egg ES components, such as omega-1[10]. However, single-sex infections in mice have shown that adult worms can also induce Th2 skewing before deposition of eggs[11]. Eventually, when the infection becomes chronic (>12 weeks), the Th2 reactivity to the parasite decreases. This Th2 dampening is suggested to be attributed by the production of anti-inflammatory cytokines by regulatory T cells (Tregs) and B (Breg) cells, and macrophages, and induced T cell anergy by alternatively activated macrophages[12]. Adult worm extracts are able to induce pro- and anti-inflammatory responses by immune cells isolated from chronically

Figure 1. Life Cycle of schistosomes.

The intermediate host of this parasites, a freshwater snail, releases larvae (cercariae) into the water, which can infect the human host via skin penetration. Once inside, these cercariae will transform into juvenile worms that are called schistosomula. The schistosomula will migrate in the bloodstream towards the liver, where they will dwell in the hepatic portal vein and surrounding blood vessels. The eggs produced by the females of the dioecious worm pairs leave the body via the feces (*S. mansoni* and *S. japonicum*) or urine (*S. haematobium*) so that the larvae inside the egg, the miracidium, can be released upon fresh water contact and infect a snail host again. However, not all eggs find their way out, and if not, they can cause a major immunological response and organ damage, which is the basis of schistosomiasis pathology that can end up killing the host.

infected mice[13] and infected humans[7], but there is limited data on actively released adult worm ES products that affect cytokine production by host immune cells directly[14]. Like the schistosomula, the adult worm ES contains many other factors to sustain life in their host by promoting anti-coagulation, reduction of oxidative stress, and complement degradation[3,8].

Thus, schistosomes, like other helminth, are able to modulate immune mechanisms in the host. The alteration of pro-inflammatory responses and enhancement of the regulatory immune network can temper overreactive immunity. Notably, with the disappearance of most parasitic infections in industrialized countries, the incidence of inflammatory disorders and allergies in these countries simultaneously increased[15]. This observation gave rise to the "hygiene hypothesis" or "old friends hypothesis", which suggests that altered early-life exposure to microbes and/or helminths impacts the training of the regulatory arm of the immune system[16–19]. Indeed, many rural helminth-endemic areas have a low incidence of allergic and auto-immune disorders, but this is not consistent in urbanized areas, suggesting the influence of yet different environmental factors[17,18,20]. The host may rely on parasite-induced immunomodulation for a balanced immune development, but whether this is sufficient to prevent the onset of immune-mediated diseases depends on various factors, such as the parasite species, environmental conditions, and host characteristics (e.g. genetics)[21]. Therefore, understanding the strategies of immune modulation by helminths on the molecular level can aid in the development of novel treatments for immunopathologies without the harmful consequences associated with parasitic infections.

To date, there is an increase in knowledge on the molecular mechanisms of immune modulation by ES components released by *Schistosoma*[14]. Extracellular vesicles (EVs) are constituents of the parasites' ES and it is expected that EVs also play a role in host immune modulation, a function that has been attributed to EVs from other helminths[22].

Extracellular vesicles released by schistosomes

Almost all cells of eukaryotes and prokaryotes release EVs[23]. EVs are generally 50–200 nm sized vesicles enclosed by a phospholipid-bilayer membrane and contain various molecules, including (glyco)proteins, (glyco)lipids, metabolites, and nucleic acids. There are two major EV biogenesis pathways: they can be formed by inward budding of multivesicular bodies (MVBs) inside the cell and released after fusion of the MVB with the plasma membrane; and EVs can be released into the extracellular space by budding off the plasma membrane itself (Figure 2, left panel)[24]. Their molecular composition is highly heterogenous, differs per cell type,

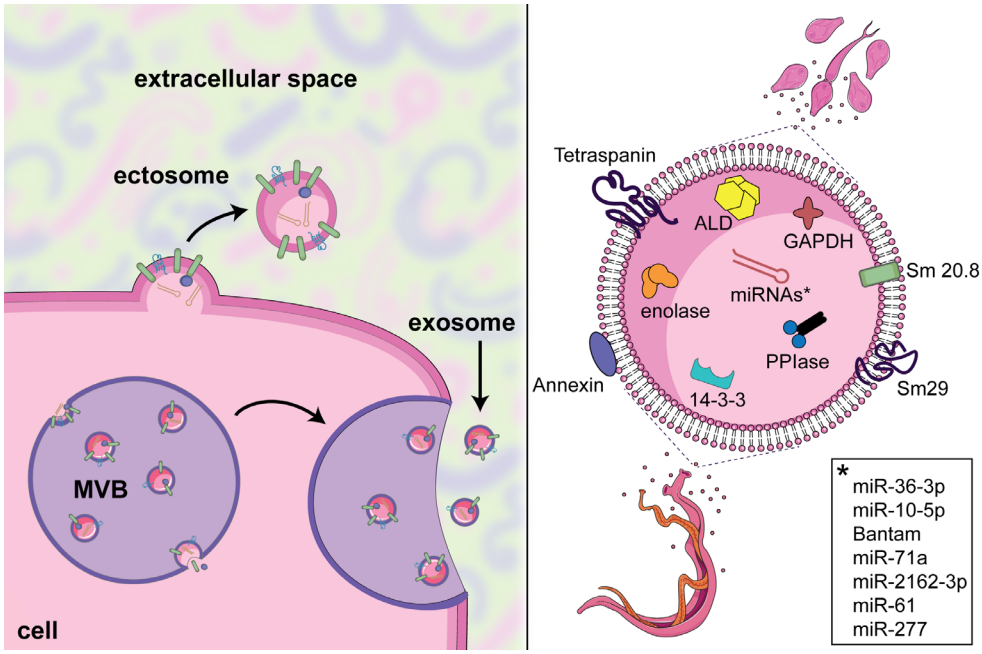

Figure 2. Release of EVs from *S. mansoni* schistosomula and adult worms.

Left panel: schematic overview of EV release by a cell. Exosomes are formed by inward budding of a multivesicular body (MVB) inside the cell and are released after fusion of the MVB with the plasma membrane. Ectosomes or microvesicles are released into the extracellular space by budding off the plasma membrane. EVs is an umbrella term for all vesicles released in the extracellular space.

Right panel: Selection of most frequent reported proteins and RNAs associated with EVs released by *S. mansoni* schistosomula and adult worms[30–33,62]. ALD, Fructose-bisphosphate aldolase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PPIase, Peptidyl-prolyl cis-trans isomerase.

and depends on the current status (e.g. cell cycle stage, environmental conditions, stress) of the EV-releasing cell. This heterogeneity and their small size makes EVs technically challenging to study. Thus far, many efforts have been made in the mammalian EV field to optimize EV isolation protocols to separate them from equally sized lipoprotein particles and non-EV particles, such as large protein complexes[23]. The three most commonly used EV isolation/separation techniques are differential centrifugation followed by ultra centrifugation with speeds above 100,000 × *g*, size exclusion chromatography (SEC), and density gradients[25]. In general, obtaining a highly pure EV population requires multiple steps that result in a low EV yield, which will limit following applications to study EV characteristics

and/or function. Yet, there is no golden standard and overall each EV population of interest and research question might require different technical approaches[23].

EVs play a role in cell-to-cell communication by transferring their contents to other cells on both local and systemic levels[26]. In mammals, EVs are involved in many processes and have the ability of regulating immune responses[27]. In the last decade, EVs have emerged as a compelling communication mechanism between parasites and their host[28,29]. Thus far, proteome and transcriptome studies have been reported of EVs released by *S. mansoni* schistosomula[30] and adult worms[31–33] (Figure 2, right panel), *S. japonicum* adult worms[34–37] and eggs[38], and *S. haematobium* adult worms[39]. In addition, one lectin microarray study showed that adult worm EVs from *S. mansoni* contain glycans[40]. Yet, there is little literature on the direct effect of schistosome EVs on immune cells[22]. Thus far, crude EV pellets of *S. japonicum* adult worm EVs skewed macrophages towards an M1 phenotype[34] and interacted with peripheral blood derived monocytes and T cells[36]. For *S. mansoni*, adult worm derived EVs are shown to be taken up by a monocyte cell line, but there is no report on subsequent immune responses[41]. *S. mansoni* EVs and EV-associated molecules are also explored *in vivo* as vaccination strategy against schistosomes[42,43]. There currently is no effective vaccine to prevent schistosomiasis and utilization of EVs may reveal additional insights[44]. Thus far, immunization of mice with *S. mansoni* egg EVs was shown to reduce worm and egg burden after challenge with cercariae[45]. In addition, vaccination with recombinant Tetraspanin (TSPs) proteins, abundant proteins in adult worm EVs[31,33], reduced egg burden in mice after challenge[39]. These studies provide evidence that schistosome EVs influence host immunity, but the exact mechanisms or cells involved remain unknown.

S. mansoni glycoconjugates and lectin receptors

An important part of the immune modulation by schistosomes is *via* their ligation of glycosylated ES products to CLRs or other glycan-binding proteins (lectins). CLRs constitute a large family of proteins that have a carbohydrate recognition domain (CRD) which predominantly depends on calcium (Ca^{2+}) for binding their glycan ligand[46]. The ligands for lectins can be N-linked (carbohydrate chain is attached to Asn) or O-linked (carbohydrate chain is attached to Ser/Thr) glycans of glycoproteins, or glyco(sphingo)lipids (glycan attached to ceramide). CLRs are often expressed on antigen presenting cells (APCs), such as DCs, to discriminate between non-self, altered self (e.g. tumors) and self[47]. On these APCs, CLR activation by PAMPs can influence adaptive immunity *via* direct activation of signaling pathways or through crosstalk with other PRRs, in which activated signaling pathways can be glycan-specific[48]. Furthermore, CLRs can mediate endocytosis and internalize (glycosylated) molecules for processing towards the

antigen loading machinery[49]. CLRs may recognize glycans that are pathogen specific but can also bind to glycan structures found in both pathogens and mammalian cells[50].

Schistosomes have a conserved glycosylation machinery that is similar to other multicellular organisms[51]. Their glycans are constructed from the same monosaccharides as their mammalian host, with the exception of sialic acids, which are abundant in mammalian, but lacking from helminth glycans[52]. Schistosome-derived glycans include both motifs that are rarely observed within mammals (e.g. fucosylation of terminal GalNAcβ1–4GlcNAc (LacDiNAc or LDN) and/or unusual core modifications that are common in plants[52]) or that are expressed in their mammalian host more commonly, but in specific organs or cells (e.g. Galβ1–4(Fucα1–3)GlcNAc (Lewis X or Le^X))[47]. Detailed mass spectrometry analyses have revealed the glycan motifs expressed in each life stage of *S. mansoni*[53]. However, most studies on schistosome-CLR interaction have been performed with egg-derived soluble antigens (SEA), which contain many glycosylated proteins[54]. For example, egg-derived glycoprotein omega-1 is internalized by monocyte-derived DCs (moDCs) *via* the mannose receptor (MR)[55]. Previous reports have illustrated that glycans on ES components released by cercariae/schistosomula can bind to the MR[56] and DC-SIGN[57] (Figure 3). Adult worm-derived glycans, mainly structures containing a terminal LDN motif, have been shown to bind to macrophage galactose-type lectin (MGL)[58], soluble Galectin-3[59], and the mouse homologue of DC-SIGN[60]. Still, in most of these studies on adult worm- and schistosomula-derived glycans, subsequent immune responses upon CLR binding were not investigated.

Glycosylation of schistosome EVs and their interaction with immune cells: the knowledge gap and thesis outline

Helminth released EVs form a relatively young field of research[28], as well as EV glycosylation[61]. Schistosomes release EVs, but there is limited to no knowledge on their glycosylation[40], their interaction with immune cells[22], and how host immune cells respond to this interaction. In this thesis, we aim to elucidate the glycosylation of EVs released by *S. mansoni* schistosomula and adult worms. Additionally, we explore the interaction of these EVs with CLRs. Subsequently, we study the effects these EVs have on host immune cells and their cytokine responses. To investigate EV-associated glycans and their functionality, we also had to overcome methodological challenges in EV isolation.

Chapter 2 is a narrative review, outlining the current understanding of immune modulation by pathogen EV-associated molecules, including EVs from parasites, bacteria, and fungi. In **Chapter 3**, we set out experiments to optimize the protocol

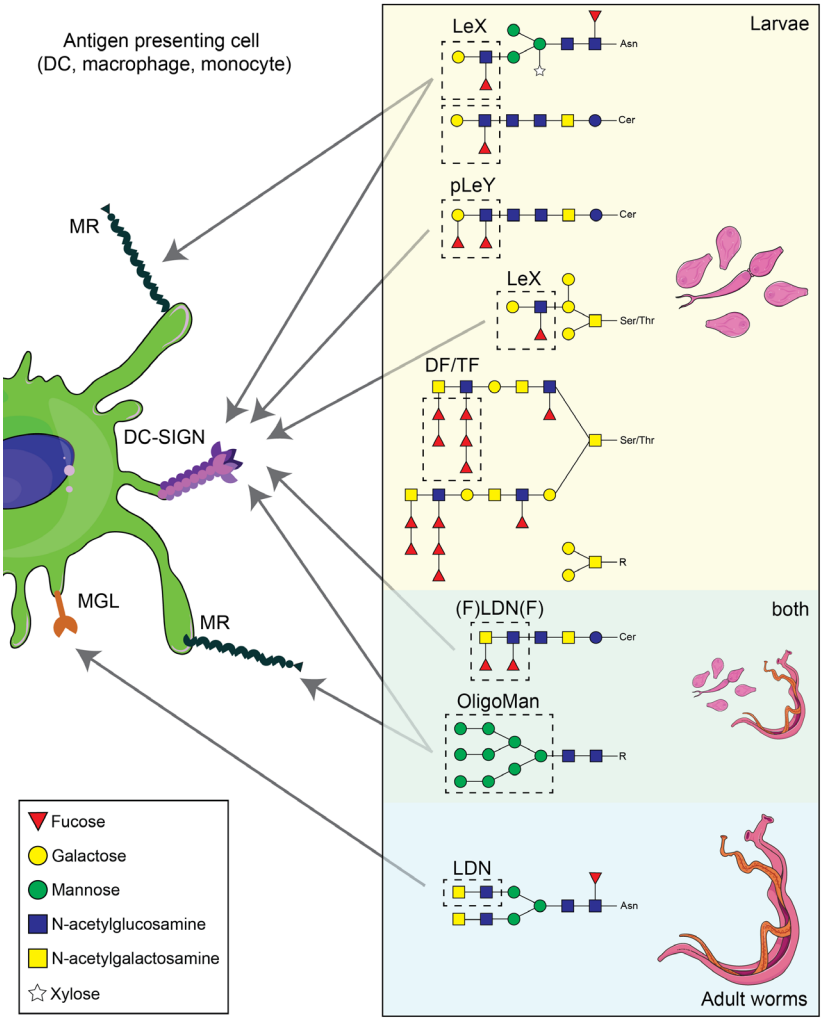

Figure 3 – Examples of major glycan motifs found in larvae (cercariae/ schistosomula) and adult worms and possible interactions of these structures with host C-type lectin receptors on human antigen presenting cells.

Le^X (Lewis X, Galβ1-4(Fucα1-3)GlcNAc-), pLe^Y (pseudo Lewis Y, Fucα1-3Galβ1-4(Fucα1-3)GlcNAc-), F-LDN-F (Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1-), and OligoMan (Oligomannoside, Man9GlcNAc2) motifs can be recognized by DC-SIGN[48,63,64]. Oligomannoside and LeX (less strongly) can bind to the MR[56]. LDN (LacDiNAc, GalNAcβ1-4GlcNAcβ1-) is a ligand for the MGL[58]. Glycan overview adapted from Hokke & van Diepen[52]. DF/TF; Fucα1-2Fucα1-3-/ Fucα1-2Fucα1-2Fucα1-3-; DC, dendritic cell; MR, mannose receptor; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; MGL, macrophage galactose-type lectin.

for the isolation and purification of EVs from *Schistosoma* adult worms, using density gradients as opposed to SEC. In the context of a limited availability of parasites, we also study the influence of density gradient volume on the overall amount of isolated EVs. Next, in **Chapter 4**, we characterize the glycan profile of adult worm EVs in detail by mass spectrometry. We compare the adult worm EV-associated glycans to the glycan motifs associated with schistosomula EVs. In addition, we investigate whether differences in glycosylation between adult worm and schistosomula EVs have consequences for the interaction of these EVs with CLRs MGL and DC-SIGN. In **Chapter 5** we explore the effect of adult worm ES and EVs on the induction of cytokine release (i.e. both IL-10 and IL-6) by B cells *in vitro*. This study includes both mouse splenic B cells as well as human peripheral blood B cells. The direct interaction of EVs with the B cells is investigated by microscopy using an EV-associated *Schistosoma* tetraspanin protein. Finally, **Chapter 6** addresses the ability of schistosomula EVs to activate human moDCs. In addition, this chapter describes mass spectrometry characterization of schistosomula EV-associated protein and lipid glycans. We furthermore investigate the role of EV-associated glycans on schistosomula EVs in the interaction with the C-type lectin receptor DC-SIGN on moDCs. In conclusion, **Chapter 7** summarizes and discusses the main findings of this thesis and includes suggestions for future research on *Schistosoma* EVs to elucidate EV-mediated pathogen-host communication.

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