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

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Chapter 7

Summarizing discussion and future perspectives

Introduction

Parasites evolved together with their hosts over millions of years. Some of them, like *Schistosoma mansoni*, have gained ways to survive within the venous system of the host for prolonged times. Part of the survival strategies of schistosomes have evolved to combat or evade the innate and adaptive immune responses deployed by the host. Elucidating how these developed immunomodulatory mechanisms work can aid the understanding of our immune system. By studying how parasites evade the immune system, we might discover how we can steer immune responses for our own benefit in immunological diseases[1,2].

Decades of research on *S. mansoni* substantiated that these organisms release products, so called 'excretory/secretory products' (ES), that can influence host immunity[3]. Each life stage: schistosomula larvae, adult worms, and eggs, release a different set of ES products, each with different effects on host immune responses[4]. ES contains various types of molecules such as metabolites, peptides, lipids, proteins, including glycoconjugates thereof, with a unique glycosylation profile linked to each life stage[5]. And it is known that these glycans play an important part in immune recognition and regulation[6].

A little over 10 years ago, extracellular vesicles (EVs) were first described to be part of helminth parasites' released products[7,8]. The first publications of EVs released by *S. mansoni* schistosomula and adult worms followed soon and reported on their protein and RNA content[9,10]. During this time, when the field of helminth-derived EVs was still in its infancy, our research group started the ground work on EVs released by schistosomes that led to the work described in this thesis. Here, the aim was to explore the glycosylation of *S. mansoni* EVs and their interactions with and effects on host immune cells. In this discussion, I will reflect on the results obtained along this explorative journey and on the missing links that can be the focus for future endeavors.

Schistosoma mansoni EVs: what is their cellular source?

Schistosomes are multicellular organisms and thus far, the exact cellular source(s) of the EVs isolated from *in vitro* cultured helminths remains unknown[11]. As described in Chapter 2, release of vesicle-like structures by *Schistosoma* parasites has been visualized well before EVs became a main focus of research[12,13]. Since then, only limited studies have shown or suggested possible origins for helminth released EVs, such as the adult worm tegument (its outer layer)[8] and their intestinal lumen[14]. For *Schistosoma* larvae, the EV-associated protein SmLEV1 was detected in high concentration in the pre-acetabular glands and on the parasites' surface[15], suggesting that these are potential sites of origin for schistosomula EVs. Our findings on the EV-associated glycans that are presented in this thesis

(Chapter 4 and 6) provide additional information on the possible EV-releasing source in the parasites.

The GalNAcβ1–4GlcNAcβ1 (LacDiNAc / LDN) structure that was substantially present on the adult worm EVs has been reported to be present in or on the worms' tegument and excretory system[16]. Additionally, lectin staining suggested that the O-linked α-GalNAc (Tn antigen), which is another glycan we expect to be present on the EV surface, is also found in or on the tegument[17]. Through visualisation by monoclonal antibodies, fucosylated LDN variants, including GalNAcβ1-4(Fucα1-3) GlcNAcβ1 (LDN-F), Fucα1-3GalNAcβ1-4GlcNAcβ1 (F-LDN) and Fucα1–3GalNAcβ1–4(Fucα1–3)GlcNAcβ1 (F-LDN-F) have been found in the parenchyma (F-LDN, LDN-F, F-LDN-F)[16,18], the excretory system (LDN-F, F-LDN-F), and in the gut (LDN-F)[16] of adult worms. The detection of these structures partly depended on whether the worms were frozen or fixed. Detection in the gut and secretory system of LDN-F was only visible in frozen sections and not when fixed with a solution that contained ethanol[16]. Furthermore, the F-LDN and F-LDN-F traced in parenchymal ducts disappeared after chloroform/methanol treatment[18]. The disappearance of glycans after such treatments suggests that these fucosylated LDNs are most likely attached to lipids. Indeed, mass spectrometry of adult worm extracts show exclusively fucosylated LDN motifs in the glycolipid profile[5]. Interestingly, however, we could not detect any glycolipids in worm EVs and also western blots of the EVs gave no signal for F-LDN and F-LDN-F. We could detect LDN-F by western blot in highly weight bands, but the LDN-F motif was not among the abundant peaks in the N-glycan spectra. Concluding from these data, the majority of the adult worm EVs would be most likely released from their tegument and not the parenchyma surrounding the gut. This is also substantiated by high abundance of the EV-associated protein Tetraspanin-2 (TSP2)[10] in the surface tegument of adult worms[19].

Glycan motifs we detected on the schistosomula EVs, mainly Galβ1-4(Fucα1-3) GlcNAc (Lewis X or Le^X) and highly fucosylated glycolipids, have also been detected on cercariae and schistosomula[16,20]. Le^X was mostly localized to the oral sucker in cercariae[16] and in the oral sucker area of schistosomula 3 hour post-transformation. Expression of Le^X became visible in the whole schistosomula 24 hours or later after cercarial transformation[20]. Additionally, detection of fucosylated LDNs or mono- and multifucosylated GlcNAc with monoclonal antibodies showed these motifs on the whole surface of both cercariae and schistosomula. One antibody that binds F-LDN, LDN-F, and F-LDN-F showed presence of these glycan structures on the whole schistosomula surface in both fixed and live parasites. In contrast, an antibody that was more specific for LDN-F only detected this motif on fixed parasites, suggesting that fixation might have

exposed underlying glycans[20]. Interestingly, the same LDN-F targeting antibody showed limited LDN-F presence in schistosomula EVs. These data suggest that isolated EVs from schistosomula are most likely released from their tegument surface. However, we cannot exclude that the cercarial oral sucker and acetabular glands release EVs that contributed to the complete EV pool isolated from the medium after 72 hours culture. To study this further, one can utilize the fact that cercarial acetabular gland atrophies between 48–72 hours post infection (e.g. transformation)[21]. If a substantial amount of EVs are released by the acetabular gland, its atrophy may lead to a reduction of these EVs released in culture. Thus, comparing the glycosylation profile of isolated EVs from <24 hours cultured schistosomula versus EVs released between 72 to 96 hours post transformation will illustrate what proportion was acetabular gland-derived in our 0-72 hours collected EVs. This information is important to further understand in which stage of transformation and development the schistosomula release EVs with the immunomodulatory effects that we have observed for our isolated schistosomula EVs on monocyte-derived dendritic cells (moDCs).

Our data supports that adult worm and schistosomula EVs originate from the parasites' tegument. For the adult worm EVs, this is substantiated by the presence of specific glycans (e.g., LDN and the Tn antigen) and the abundance of TSP2 on the tegument surface, which we also found on the EVs. Schistosomula EVs are likely released from their tegument surface because of the EV-association of many fucosylated glycans, including Le^X, that is similar to the glycans on the surface of cercariae and schistosomula. However, further evidence is required since the tegument as EV source remains under debate[22]. Here I will propose and illustrate three methodologies for exploration. The first would be to incubate live worms or schistosomula with a TSP2 or glycan-targeting antibody and isolate their released EVs at different time points for detection. Most ideally, the antibody is conjugated to a fluorescent molecule, so that the presence of the antibody on the EV can be shown easily either by high resolution flow cytometry[23] or super resolution microscopy[24]. The possibilities for this labeling will expand with the availability of glycan targeting nanobodies[25]. Nanobodies are small fragments of heavy-chain-only antibodies of camelids. Because they are smaller than intact immunoglobulins, they allow for a higher spatial resolution[26] and reduce multiplex bias when aiming to detect more than one target[27]. The second approach extends the use of labeling molecules by targeting differentially expressed proteins or glycans along the parasites' anatomy. Subsequent single EV measurements could point towards a more detailed localization of EV releasing cells. These regions could then be further examined by (cryo) transmission electron microscopy (TEM), targeting the same molecules with gold-nanobodies[28] to identify the true origin

of schistosome released EVs. Thirdly, the location of EV release may be studied by taking advantage of the use of host lipids by helminths for the construction of lipid membranes[29,30]. It has been shown that when feeding fluorescent lipid analogues to *Anisakis spp.* and *Trichuris suis* larvae in culture, they integrated this lipid in their released EVs[31]. Previous lipid staining of schistosomes also showed that there was specificity of labeled phosphatidylcholine analogues to the surface, gut and acetabulum[32], but these experiments have not been done in relation to released EVs. Using different lipid formulations that are incorporated in different cellular locations within the parasite, one could study which EV is derived from which location based on EV fluorescence.

Knowing the exact cellular source of the EVs can provide valuable insights in how schistosomes employ EVs in the host. For example, if EVs are released from the schistosomula acetabular glands, it might suggest that these EVs are involved in enzymatic digestion of extracellular matrix components during skin penetration[33]. And when EVs are released from the tegument, either from schistosomula or adult worms, these might contribute to disable host defense mechanisms as complement and immunoglobulins[21]. In addition, EVs from the adult worm tegument might play a role in the inhibition of blood coagulation, since ecto-enzymes as alkaline phosphatase are present on the tegument surface[34] and have been detected in adult worm EVs[10,35]. Furthermore, uncovering which cells of schistosomes release EVs that can modulate host immune responses might also direct us to the cells that release well-known immunomodulatory ES components[36]. In future perspective when helminth derived (stem) cell lines will become available, which would overcome many challenges in helminth EV research[37], it would be advantageous that collected EVs from the cell cultures are close to identical to the EVs released by the whole organism. For this, we need to know the cell source of which the EVs in whole parasite cultures derive from. Ultimately, deep understanding of helminth EV release may reveal possible new therapeutic targets to combat schistosome infections.

Surface structures on schistosome EVs: unique, helminth specific, or common across species?

One of the characteristic features of the EVs isolated from the schistosomula were their long hair-like surface structures (Chapter 6). To date, the specific molecular makeup of these structures is still unclear. Hence, I want to emphasize that the comparisons in this paragraph will be based on visual features. Similar surface protruding structures have been reported on adult worm EVs released by *Hymenolepis diminuta*, *Ascaris suum*, *H. polygyrus bakeri*, and *Oesophagostomum dentatum*[37], but they seem different from to the ones on schistosomula EVs since

the structures on the EVs from these other parasites are less "fluffy" and much shorter in length. In Chapter 4 we mentioned that the presence of EV surface protrusions, as we also observed for *S. mansoni* adult worms, may be a hallmark for pathogen-derived EVs, because they have mainly been reported for EVs from helminths[37], bacteria[38,39], fungi[40], and EVs from mammalian cells transfected with nematode or viral membrane proteins[41]. However, short protruding surface structures have also been detected on EVs from human primary tracheobronchial cell cultures[42,43] and BEAS-2B lung epithelial cells[44]. It is possible that more mammalian or pathogen-derived EVs contain similar filamentous structures as those present on the schistosomula EVs. This characteristic may be easily missed, as the only way to clearly visualize them is by high quality cryo TEM, which is not available in all laboratories. We were prompted to use cryo TEM, as we observed a discrepancy in size measurements between classic TEM and Nanoparticle Tracking Analysis (NTA) (Chapter 6). Of note, only half of the schistosomula EVs we analyzed by cryo TEM contained the hair-like structures. This could imply a difference in cell type or cell compartment origin of the EVs (from multivesicular body or from plasma membrane). Considering the clear morphological differences between the two schistosomula EV populations with or without the surface structures, it is tempting to assume that these EVs have distinct functional roles within the host. For now, it seems that the schistosomula EV hair-like surface structure is outstanding in their "fluffy" morphology and extended length, but not completely unique. This raises the question about their biological significance. Since we isolated EVs from 0-72 hours cultured schistosomula post-transformation, and schistosomula have been reported to leave the skin 48 to 72 hours post infection[21], their EVs might play a role during the skin stage and/or the lung stage. Below, I will briefly discuss how these structures could be connected to schistosomula survival strategies in both the skin and the lung and describe two experimental directions in which these structures could be further elucidated.

In the first three hours after invading the skin, the schistosomula will shed its cercarial glycocalyx. This layer of proteins and glycans protected the cercariae against osmotic pressure when it was still in water[21]. In the skin, however, it is a prime target for the complement system[45]. Interestingly, this glycocalyx looks very similar to the EV surface structures[13]. Thus, it seems likely that some of the EVs and their structures serve as decoys to attract complement until the schistosomula has reorganized its membrane, which expresses various molecules that can disarm complement and immunoglobulins[46]. This could be examined by incubating schistosomula EVs with normal human serum (NHS) or heat-inactivated NHS and subsequent detection of activated complement component C9[47]. Actual binding of complement to the structures could subsequently be explored by TEM

and gold-labelled anti-C9. Checking complement binding of EVs released by schistosomula after different timepoints following cercarial transformation will provide additional knowledge on whether it is the glycocalyx like structure that serves as this scavenger function on the EVs.

From the skin, the schistosomula will migrate to the lungs where they reside for several days[21]. Here, the visual similarity between the schistosomula EV structures and the airway epithelial EV structures offers an interesting case for assessment of functionality. The airway EVs are highly enriched in mucins, which are known to be heavily O-glycosylated[48]. Within the airways, mucins are part of the innate immune defense system to protect against pathogens and environmental toxins[44]. Part of these toxins are reactive oxygen species (ROS). ROS plays an important role in the lung stage of *Schistosoma* infections[49,50], where schistosomula switch to anaerobic metabolism and scavenger molecule release to survive[21]. Another molecule that can scavenge ROS are proteoglycans, which are similarly heavy glycosylated proteins as mucins. Glycosaminoglycans have been shown to be modified or degraded by several ROS species after direct contact[51]. Thus, it might be that the long hair-like structures we observe on the surface of schistosomula EVs are similar scavenger glycans. Whether these are actually mucins is currently unknown, as there are no reports on mucin expression by schistosomula[21]. However, based on the many glycans we found in schistosomula EV preparations, we do assume that these surface structures consist of glycans, with or without a protein backbone. One way to test if the schistosomula EV structures would be relatively similar tot proteoglycans, is to check by TEM whether the structures are degraded after incubating the EVs with ROS, such as H_2O_2[52]. Another interesting factor in the lungs that combats pathogens are surfactants, which include C-type lectin receptors (CLRs) from the collectin family[53]. One of these, surfactant protein (SP)-D, has been shown to bind to the oral sucker of *S. mansoni* cercariae and the complete surface of the schistosomula, most likely via F-LDN and F-GlcNAc[54]. We detected similar glycan structures on the schistosomula EVs (Chapter 4 and 6), which suggest another molecular scavenger function of released schistosomula EVs. Whether SP-D binds to schistosomula EVs and whether this would colocalize with fucosylated glycans on the surface could be examined using super resolution microscopy.

My suggestions thus far mainly included the possible scavenger functions of the EV hair-like structures to protect the schistosomula from harmful host defense molecules. However, both in the skin and in the lung, immunomodulation by the parasite is crucial for its survival[55,56]. Therefore, we should also consider that these surface structures increase the interaction with host antigen presenting

cells, like DCs and macrophages, either by itself or via a scavenged host molecule. There are two experimental directions to get more information on these unique EV structures and their function in immune modulation: determining their exact composition or trying to separate EVs with the structures from EVs without. Hereafter, examples for both options will be further elaborated.

To determine the structures' molecular makeup, we already performed an initial experiment were schistosomula EVs were treated with PNGase F (to release N-glycans) or very briefly with proteases. However, no differences in the structures were observed by cryo TEM after both treatments (unpublished data). This experiment should be repeated with longer treatments and various proteases to really exclude the presence of a protein backbone. In addition, albeit technically challenging[57], more effort can be made to investigate the presence or absence of O-glycans, as the larval stage of schistosomes expresses various O-glycans[6,58]. To examine whether the highly abundant glycolipids are part of these hair-like structures, one could culture the schistosomula in presence of different lipid formulations to possibly alter the EV glycolipid composition. When the composition of the structures is known, subsequent functional experiments can be performed in which EVs with removed or altered surface structures can be compared to the 'original' EVs. These could be a repetition of the moDC experiments presented in Chapter 6 to study the role of these structures in the observed dependance on DC-SIGN interaction and the enhancement of pro- and anti-inflammatory cytokines. However, with this approach, there will still be the contribution of the EVs without the structures, as they remain most likely unaltered. Addressing this issue seamlessly transitions us towards the second experimental direction: separate the two population of EVs.

To separate EVs with and without the hair-like surface structures we can utilize the possible difference in biochemical characteristics between the two. Our density gradient experiments presented in Chapter 3 show that schistosomula EVs float to a higher equilibrium density in a sucrose gradient compared to EVs from adult worms, but both life stage EVs behave similar in iodixanol gradients. This different behavior in a high- (sucrose) or isosmotic (iodixanol) solutions may be due to different interaction of the schistosomula surface structures with surrounding solutions. Factors to consider for separation, besides size, include surface charge[59] and hydrophobicity[60]. For example, to separate negatively charged plasma EVs from positively charged lipoprotein particles, researchers applied a dual-mode chromatography based on size and ion exchange[61]. Additionally, a hybrid chromatography with separation on charge, size and glycan-binding has been illustrated[62]. Logically, when knowing a specific ligand (protein or glycan motif) that is exclusively expressed on the surface structures, or exclusively on

the surface of the EVs without structures, one can easily remove this population by an affinity-based method[63]. Once separation succeeds, the isolated EV population can be tested for their immunomodulatory effects on host cells. Unraveling the function and molecular configuration of the schistosomula EV structures may increase our understanding of how schistosomes deploy their vesicles during the initial stage of infection, either as scavengers and/or immunomodulators. Furthermore, we might be able to use this knowledge by adding similar structures to therapeutic nanoparticles[64].

Host molecules associated with schistosome EVs: absent, overlooked, or culture condition dependent?

To be able to attribute molecular characteristics or functions to EVs, the populations of isolated EVs must be free from contaminating molecules (Chapter 2). These can include EVs that are present in cell culture supplements such as FCS, large (nucleo)protein complexes that are co-isolated in size-based methods, or lipoprotein particles that end up at similar gradient densities[65]. Schistosomes can be kept alive in culture for a few days in medium without red blood cells or FCS. This limits the extent to which EV isolates of cultured schistosomes contain contaminants and EVs derived from these mammalian sources. Still, we needed to separate EVs from non-EV molecules released by the parasite. In Chapter 3, we presented an optimized density gradient separation protocol to minimize EV loss of the limited schistosome material and to reliably characterize and study the effect of these EVs. However, it could be that some host materials that are not present in culture would be normally utilized by the parasite for its metabolism, development, and immune evasion. These host molecules could also end up in their released EV and possibly affect their interaction with host cells. Furthermore, there is a recent interest and growing body of evidence in the EV field that mammalian EVs in the blood spontaneously adsorb proteins from the protein-rich blood matrix[66–69]. This 'protein corona' around the EV, either strongly or loosely bound to the surface, can contain lipoproteins, immunoglobulins, enzymes, and larger protein complexes and aggregates and can affect the bioavailability and bio-distribution of EVs[66]. Since schistosomes reside in the blood, similar interaction of the EV surface with blood components would be conceivable. In case host molecules are incorporated in the EVs and/or bind to the EVs after their release, both could have an impact on the EVs' characteristics and function. This raises the question whether the EV populations we studied is similar to in the *in vivo* situation in terms of EV-associated glycans, the cellular interaction of the EVs with host cells, and immune responses induced by schistosome EVs. In the following two paragraphs I will discuss what is currently documented on host

molecules associated to or interacting with schistosome EVs and what should be considered in follow-up investigations. In the final two paragraphs of this section, I will describe the current information regarding *in vivo* released *Schistosoma* EVs and give suggestions on how this field can move forward.

Within our glycomics data presented in Chapter 4 and 6, we did not detect any sialic acids. The presence of sialylated glycans would automatically indicate the presence of glycans from the host, as the molecular machinery essential for their biosynthesis is absent in schistosomes[6]. Contradictory, another research group did detect host derived sialic acids in their *S. mansoni* adult worm EV preparations[70]. A likely explanation for this difference is that the other group cultured their worms in the presence of EV-depleted FCS, which is shown never to be fully EV-free[71]. Indeed, the authors reported in a subsequent publication that the majority of sialic acid glycans were linked to bovine proteins[72]. However, it remains unknown whether these bovine proteins were present in or on the adult worm EVs or on co-isolated bovine EVs that remained in the EV-depleted FCS. In addition, studies on the proteome of adult worm EVs did[73] or did not[10] detect host proteins in their EV preparations, but it is unknown whether this was depending on culture conditions. A third proteomics study did not report whether they searched their data for host proteins in adult worm EVs[35]. There is sufficient data on adsorption of host proteins in the worm's tegument as one of their survival strategies[21], mainly via its own surface receptors that can bind host antigens[74]. This could suggest that if EVs are collected from adult worms briefly after perfusion, there is a higher chance of finding possible host molecules associated to the EVs than in prolonged cultures in absence of host material. To study this, one could repeat the experimental design described for exploring the tegument as EV source, and incubate freshly obtained adult worms with anti- or nanobodies targeting host molecules, such as IgG or complement components[74,75] or lectins targeting sialylated glycans[70,76]. This approach is preferred over host molecule detection after EV isolation, to avoid the contribution of possible host molecules on EVs in regurgitated gut material[77]. Subsequent EV isolations will inform on the presence of these host molecules on the EV surface. By collecting EVs over time, both with and without re-incubating the worms with host-derived blood, we can better understand the dynamics of host proteins associated with released EVs. When host proteins on EVs subside over time in blood and serum free cultures, it would be worth to incubate immune cells with the early and the late released EVs to study differences in immune responses in presence or absence of EV-associated host proteins. However, this approach only provides answers when the rest of the *Schistosoma* EV composition does not change over time.

An additional element of interest would be to compare the interaction of

schistosome EVs with blood components or serum of uninfected versus infected animals/individuals. It has been reported that *S. mansoni* adult worm EVs can elicit a humoral response in mice[72]. Furthermore, pre-incubation of adult worm EVs with serum from rabbits immunized with recombinant *Schistosoma* specific TSP2 and TSP4 inhibited their uptake by endothelial and monocytic cell lines[78]. However, in our experiments where we preincubated adult worm EVs with antibodies targeting TSP2, we did not observe any reduction in interaction with B cells (Chapter 5). Finally, anti-sera from rats immunized with *H. polygyrus* EVs were reported to increase the uptake of EVs in murine bone marrow derived macrophages[79]. The authors additionally showed that incubation with anti-sera directed internalized EVs more towards degradation in lysosomes, thereby reducing the immunomodulatory ability of these EVs. Altogether, these data suggest that host components in blood may very well interact with schistosome released EVs. However, changes in EV capacity for immune modulation upon interaction with host molecules might be cell type dependent and should be further explored. Eventually, the ideal goal would be to have isolated schistosome EVs from parasite cultures for *in vitro* stimulations that are like those presented to the host cells *in vivo*. For that we need to know how to optimize parasite culture conditions and most of all what the surface molecules and molecular cargo are of *in vivo* released EVs.

Thus far, only a few research groups searched for schistosome-derived EVs in blood of infected patients[80], hamsters[81], and mice[73]. However, these studies did not report the detection of parasite-derived EVs (or separation from mammalian EVs) and it remains unclear whether the parasite molecules isolated from the blood is EV-associated. This is in part due to the absence of confirmation that the detected schistosome-derived miRNAs are EV-associated and that the EVs are parasite derived[73,80]. Hence, solid evidence of *Schistosoma* EVs released *in vivo* is, to the best of my current understanding, still lacking. There is, however, one study that compared *in vitro* released *Echinococcus granulosus* EVs to *in vivo* EVs in fluid from parasite harboring cysts in infected people[82]. Results showed major differences in protein amount and content between the two EV sources, which is not surprising considering that the cyst fluid is surrounded by a parasite-derived germinal layer and its fluid also contained many host-derived materials, including lipoprotein particles, immunoglobulins, and complement[82]. This example demonstrates how difficult it can be to separate parasite EVs from host EVs from host body fluids. Yet, there might be an overlooked solution to discriminate parasite EVs from host EVs. As presented in Chapter 5, adult worm EVs could be detected in contact with mouse and human B cells by targeting them with an antibody against *S. mansoni* TSP2. This antibody did not bind to the host tetraspanins, indicating that

the existence of schistosome EVs *in vivo* can be supported by detection of worm specific proteins that are enriched in EVs, such as TSP2[10] or SmLEV1[15]. Antibodies against these proteins should be tested for their suitability to trace parasite EVs in blood cells or tissue sections of infected animals. Another strategy could be to use these antibodies to immunocapture schistosome EVs from plasma of infected individuals. These immunocaptured EVs could subsequently be characterized and compared with similarly isolated EVs from parasite cultures to further understand possible differences between *in vitro* and *in vivo* released EVs. It is important to keep in mind that potential differences between *in vitro* and *in vivo* isolated EV populations may be caused by differences in (re-)uptake of selective EV subsets by host cells and parasites *in vitro* or *in vivo*. Additional *in vitro* studies from cultured parasites should be performed to show that there are indeed specific schistosome EV subsets that target distinct cell types.

A more challenging, yet promising approach to trace EVs released *in vivo* is the use of gene manipulation or transgenesis of schistosomes[83]. Interference RNA (via double stranded DNA) or mRNA addition via electroporation have been shown effective to transiently alter schistosomula and adult worms genetically in culture[84–86]. Moreover, with the rise of CRISPR-Cas as gene editing tool, schistosomes can now be genetically modified[87,88]. This is heritable when done in the egg zygote, but so far there are no reports on this being implemented. Hence, there could be an opportunity to generate a genetically modified *S. mansoni* strain that expresses TSP2 and/or smLEV1 coupled to a fluorescent molecule like GFP. If multiple colors could be linked in one transgenic parasite, and each color linked to a different EV-associated molecule that is enriched in a specific stage of schistosome development, this would greatly enhance our understanding of EV targeting by schistosomes *in vivo*. While it may still take a long time before we succeed in generating such transgenic parasites, it is surprising that the approach of targeting parasite-specific molecules is not utilized more often and pursued in the helminth research. Overall, the molecular differences of proteins between parasite and host is a huge advantage over the mammalian EV field when aiming to detect EVs derived from a specific source *in vivo*[89].

Cellular targets of schistosome EVs: more than immune cells?

One objective in this thesis was to study the interaction of *Schistosoma* EVs with host immune cells. We have shown in Chapter 4 that EVs from adult worms and schistosomula can interact *via* EV-associated glycans with CLRs MGL and DC-SIGN on CLR-expressing cell lines, respectively. In Chapter 5 we provided evidence of interaction between adult worm EVs and mouse and human B cells. Lastly, in Chapter 6 we demonstrated that the internalization of schistosomula

EVs by human moDCs is mostly DC-SIGN dependent. Other research has shown that *S. mansoni* adult worm EVs can be internalized by primary mouse CD4 T cells[90] and THP1 monocytes[78] and *S. japonicum* adult worm EVs can interact with mouse peripheral blood monocytes and T cells[91]. However, none of these studies have investigated possible EV-receptor interaction. There are various CLRs on monocytes, macrophages, DCs, neutrophils, and B cells[92], and all these cells are worth investigating to see whether they can interact with schistosome EVs *via* their associated glycans. However, for this part of the discussion, I want to shift the reflection to cells beyond blood immune cells that the parasites may also encounter in the host, as they might be equally important targets for the parasite EVs. Other cells that have been shown to be affected during a schistosome infection, or when stimulated with ES material, include keratinocytes[93], lung endothelial cells[94], and liver cells[95]. EVs from *Echinostoma caproni*[8], *Heligmosomoides polygyrus*[14], *Nippostrongylus brasiliensis*[96], and *Trichuris muris*[97], all interact with intestinal epithelial cells, and *Opisthorchis viverrini* EVs can interact with cholangiocytes (cells of the bile duct)[98]. However, studies on the interaction of schistosome EVs with host cells other than phagocytes and lymphocytes is limited. Thus far, there is one study showing interaction of *S. mansoni* adult worm EVs with an endothelial cell line[78], one study on *S. japonicum* adult worm EVs that interacted with murine liver cells (NCTC cell line)[99] and two studies on *S. japonicum* egg-derived EVs that were suggested to be internalized by murine liver cells (HEPA1-6 cell line)[100] and human liver stellate cells (LX-2 cell line)[101]. I hereafter will further discuss cells in the liver and endothelial cells as potential target cells for schistosome EVs and use our current understanding of the EV-associated glycans as starting point to future directions to explore.

Adult worms reside in the portal vein, meaning that almost everything they will release will travel to or through the liver. In Chapter 4, we described that adult worm released EVs mainly contain LDN motifs on their surface N-glycans, but also contain the O-glycan Tn antigen. Using CRL expressing cell lines, we discovered that these EVs mainly interacted with the MGL receptor and not so much with DC-SIGN. The MGL receptor is exclusively expressed on tolerogenic DCs and macrophages and it mainly recognizes GalNAc residues, which includes sialylated and non-sialylated Tn antigen and LDN[102]. The alternative name for the MGL is DC-ASGPR, as the MGL gene is in the same cluster as the asialoglycoprotein receptor (ASGPR)[103]. The ASGPR is nearly exclusively expressed on hepatocytes in the liver and recognizes N-glycans terminating in βGal or βGalNAc[103]. Thus, based on our findings that the surface glycans on the adult worm EVs contain terminal βGalNAc, the ASGPR is a potential receptor for these EVs in the liver. In the next paragraph, I will hypothesize how targeting of the ASGPR by worm EVs

can be linked to current *in vivo* data and what *in vitro* studies can be performed to investigate this interaction.

The general function of ASGPR in the liver is to clear proteins, lipoprotein particles[104], and mammalian EVs after they are desialylated, leading to exposed βGal[105]. The ASGPR also takes up senescent, desialylated platelets which subsequently leads to increased numbers of platelet producing megakaryocytes[106] and impairment of this platelet clearance reduces the levels of platelet activity[106]. During human schistosome infections, increased levels of HDL particles can be observed in plasma[107] as well as lower platelet levels, and the blood needs longer time to coagulate[108]. It is tempting to suggest that competitive binding of worm EVs to the ASGPR plays a role in these HDL and platelet changes. Altering the lipoprotein particle levels might be a strategy of the parasite to obtain the lipids it cannot synthesize itselve[29,30]. And reduced amounts of platelets that do not coagulate quickly, benefit both the adult worms and its host, as this lowers the chance on thrombotic complications induced by the worms' presence[109]. One initial experiment should first confirm the interaction of adult worm EVs with the ASGPR on cultured hepatocytes. Thereafter, the subsequent effect on levels of lipoprotein particles or senescent platelets left in the medium can be studied[110,111]. The next step would be to inject the EVs in liver organoids[112] to study the amount of EVs that interact with each individual cell type within the liver. Since the liver resident macrophages (Kupffer cells) also express an LDN binding CLR[113], there might be a functional significance when adult worm EVs have a preferential targeting to the hepatocytes *via* ASGPR or to the liver Kupffer cells.

In addition to the liver, the interaction of schistosome EVs with endothelial cells are worth exploring further[78]. Endothelial cells not only modulate metabolic homeostasis, vascular permeability, coagulation, and cell extravasation, they also participate in both innate and adaptive immune responses[114]. They express several TLRs, can upregulate MHC II and co-stimulatory molecules as OX40 ligand, CD80 and CD86, and release pro- and anti-inflammatory cytokines and chemokines. Kifle *et al*, have shown that incubation of HUVECs with adult worm EVs led to differential expression of genes associated with intravascular parasitism, such as increased IL-6 and chemokines and downregulation of coagulation[78]. They suggested a role for schistosome TSP2 and TSP4 in the adult worm EV uptake by blocking these EV-associated proteins with antibodies[78]. Still, this might not be the only interaction these EVs can have with endothelial cells. There are currently two CRLs described that can be found in endothelial cells: CLEC1A and CLEC8A. These receptors are actually C-type Lectin-Like receptors (CTLRs) and do not need calcium to interact with glycans and can also bind proteins and/or lipids[115]. It might be that EV-glycosylation is redundant for the EVs to interact

with endothelial cells. Yet, both receptors might still play a role in targeting and/or affecting endothelial cells, which I will elaborate on next.

CLEC1A (or CLEC-1) in endothelial cells is mainly present intracellularly, but expression is increased upon IL-10 and TGFβ stimulation[116]. Once activated, it can dampen Th17 responses, however, the exact ligand for this effect remains unreported[115]. Th17 responses play a role in the development of severe schistosomiasis pathology[117], thus reduction of these responses *via* CLEC1A activation would benefit the host and thus the parasite. In human lung transplants, decreased expression of CLEC1A is a predictive marker for the development of graft rejection[118]. I speculate that increased and activated CLEC1A could benefit tolerance to the "non-self" parasites in the blood. For future experiments, the possible interaction with CLEC1A could be studied by incubation of EVs in the presence or absence of IL-10 and TGFβ to increase CLEC1A expression on the endothelial cells. Subsequent changes in mRNA of the CTLR overexpressing cells may also indicate whether uptake of the EVs effects endothelial functions.

The other CTLR, CLEC8A, also called lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) recognizes oxidized and acetylated LDL and is mainly studied in the context of atherosclerosis[115]. LOX-1 belongs to the 'scavenger receptor' family[119] and it has been shown that scavenger receptors on endothelial cells mediate EV uptake *in vivo*[24,120]. However, it remains unknown whether glycosylation is essential for this scavenger receptor-mediated uptake, and there is currently no mention in the literature on interaction of *Schistosoma* secretions with scavenger receptors. Of note, *Schistosoma* adult worm EVs contain their own scavenger receptor, namely the CD36-like class B scavenger receptor[35]. It has been reported that *S. japonicum* eggs use the CD36-like protein to bind and internalize HDL to get essential cholesteryl esters for egg embryo development[121]. Schistosome adult worm CD36-like receptor has been shown to bind human LDL[122], and LDL binding has also been observed on the worms' tegument[123]. It is therefore possible that the CD36-like scavenger receptor on adult worm released EVs bind to LDL and facilitate uptake by LOX-1 on endothelial cells *via* the scavenged LDL. This seems likely as also mammalian EVs are reported to be prone for binding to LDL[110]. For involvement of LOX-1, schistosome EVs can be pre-incubated with various lipoprotein particles that have been oxidized or not to see whether this influences their interaction with endothelial cells. Finally, we should consider that uptake of adult worm EVs by endothelial cells can happen *via* their TSPs, their associated glycans, or indirectly by scavenging host (lipoprotein) particles, which all may lead to subsequent different downstream effects and this should be explored further.

The survival of schistosomes in the host depends not only on their

immunomodulatory strategies. The interaction with or mimicking of host molecules, modulation of complement, and prevention of blood coagulation, all contribute to a prolonged infection without death of the parasite or killing of its host[124]. Cells in the liver and endothelial cells play a role in pathogen recognition and forward this information towards the immune system. Schistosomes need to restrict these signals to mask their presence, possibly by their released EVs. Increasing our knowledge on how schistosome EVs affect these host cells, could reveal how to uncloak them and make them more visible to the immune system, which could be beneficial for future vaccination strategies[125].

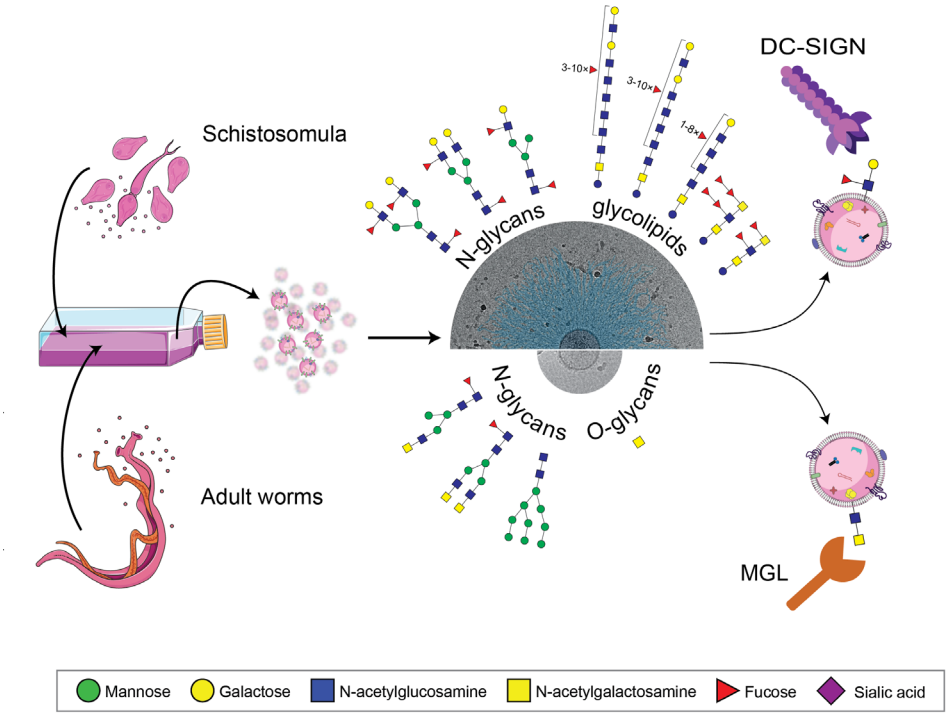
Schistosome induced immune responses: what is the significance of EVs and their cargo?

Schistosoma spp. are master regulators of their hosts' immune system, which is partly achieved through targeted release of specific immunomodulatory components[1,21,36]. We and others have provided evidence that schistosome released EVs can affect host immune responses[80,91,126], which adds up to the reports on other helminth EVs with similar capacity[127]. In Chapter 5, we showed that highly purified adult worm EVs are able to induce the release of IL-10 by mouse splenic B cells and IL-6 and IL-10 by peripheral blood B cells from humans. This corresponds with the observation that single sex male infections in absence of eggs, which are strong inducers of B cell IL-10 release[128], also led to increased IL-10 producing B cells[129]. In Chapter 6 we demonstrated that EVs released by the schistosomula augment moDC activation, leading to more CD86, CD80, and PD-L1 expression and increased IL-12 and IL-10 release. These findings corresponded with previous publications on responses in the skin upon cercarial infection and *in vitro* stimulations with schistosomula ES[56,130,131]. However, many of these induced immune responses cannot be exclusively appointed to EVs, because other ES components[132–135] and EV depleted ES (Chapter 5) show similar capacities. Furthermore, it remains unknown whether observed immune responses are depending on EV-receptor interaction or that the delivery of the EV luminal cargo plays a significant role. In the next two paragraphs, I will propose some experimental approaches to investigate the functional differences between EVs and non-EV ES components on immune responses and to study the contribution of EV-surface molecules versus their luminal cargo.

One benefit of EVs would be that EVs allow the parasite to deliver a complete set of molecules to recipient cells all at once, and this combination could have more effect on immune responses than just one molecule present in the ES. In addition, as shown for DC-SIGN, some receptors preferentially interact with bigger particles compared to smaller single proteins[136–138], which may eventually

lead to a different uptake route and effect of EVs compared to smaller ES components. So how to study the difference of immune responses between EVs and non-EV ES components released by the parasite? In chapter 5, we found that it is very difficult to completely deplete the ES from EVs using differential ultracentrifugation. Therefore, it might be an option to use a trans-well system, where parasites will be placed on the top and cells in the bottom, with a Matrigel or PEGDA hydrogel instead of a permeable membrane. Parasites release their products in the top well, and depending on the type of gel, EVs can (Matrigel) or cannot (PEGDA hydrogel) pass through, while the smaller ES products can still pass both gel types[139]. This way, the effect of ES without EVs and ES with EVs can be examined. Another strategy would be to put more effort in separating the EVs from the ES[63], for example by immunocapture of EVs. However, for this, it is important to know which EV-associated molecule (protein or glycan) is not present as non-EV ES component. Although proteomics have been performed on schistosome ES[140] (containing EVs) and EVs[10,73,35] separately, there are no reports on comparative analysis of the protein content or glycosylation profile between EV-enriched and EV-depleted ES from the same preparation. In addition, it would also be worth investigating overlapping proteins detected in both EV preparation and as non-EV-associated proteins in the ES. When making these as recombinant proteins[132,141], they could be added to immune cells as extracellular protein, or be incorporated in an EV mimic[142] to study whether the association with EV-like particles influences the downstream effects.

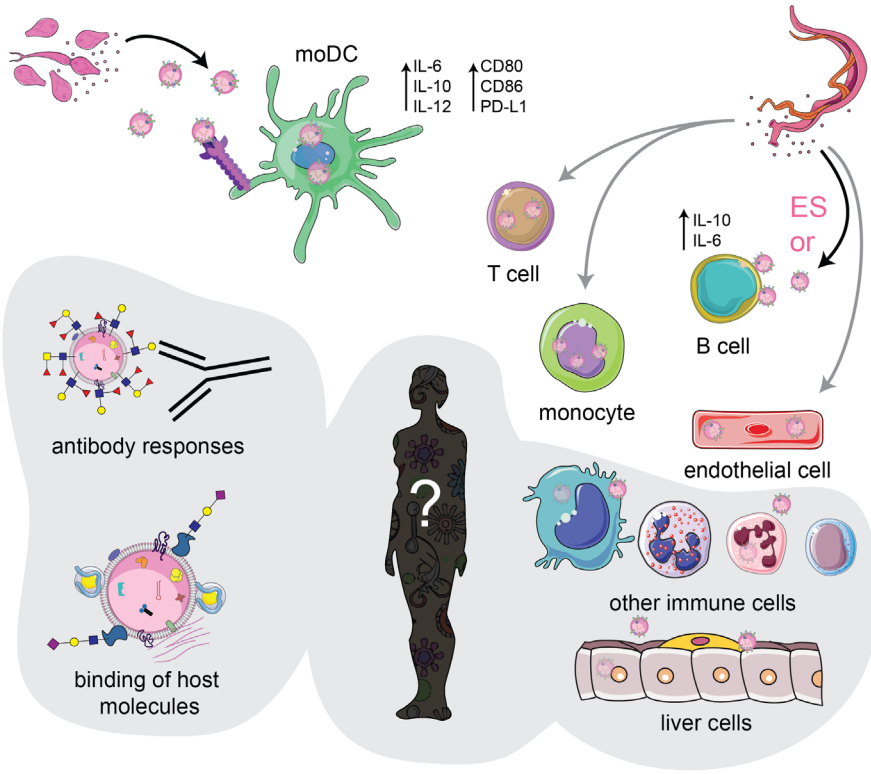
Another potential benefit for schistosomes to release EVs in addition to other ES components is the protection of luminal EV cargo, like RNAs, from degrading enzymes. Several studies have proposed that micro(mi)RNA associated with schistosome EVs have an effect on immune responses[90,91,99-101]. However, these studies often showed immune effects by cells that were transfected with miRNAs that were previously detected in unpurified EV preparations. Together with the helminth EV community, we have proposed the proper controls for the assessment of EV-associated molecules, including nucleic acids, in a recent position paper[37]. In addition to RNAs, luminal proteins have been described for adult worm EVs, but their importance in EV function was not investigated[35]. Thus, there is currently no solid evidence that immune effects are induced by EV surface molecules interacting with cellular receptors, by their luminal cargo that is delivered into the cell, or both. Investigating this, however, is currently hampered by technical difficulties. For example, altering EV cargo with either biogenesis inhibitors or disruption techniques also changes other EV characteristics that influence cellular interaction[143,144]. It may be easier to investigate the role of the EV surface, in our case its surface glycans interacting with CLRs, and their downstream effect on



Main findings presented in this thesis

- Adult worm EVs can be separated from non-EV ES components using density gradients (Chapter 3)
- Schistosomula and adult worm EVs both have a glycosylation profile that corresponds with their own life stage, yet show different enrichments (Chapter 4 and 6)
- Adult worm EVs interact with the MGL receptor and schistosomula EVs with DC-SIGN (Chapter 4)
- Adult worm EVs interact with B cells and induce cytokine release by these cells (Chapter 5)
- Schistosomula EVs interact specifically with DC-SIGN on human moDCs (Chapter 6)
- Schistosomula EVs increase immune responses by human moDCs (Chapter 6)

Summarizing overview of current understanding of the glycosylation of EVs from *S. mansoni* schistosomula and adult worms, the interaction of these EVs with host cells and EV-induced immune responses



Follow up questions

- What are the cellular sources of the schistosome EVs?
- What are the structures on the schistosome EV surface, and are these structures common for EVs across species?
- Can host molecules bind to schistosome EVs and what would be its impact on the function of these EVs?
- What other host cells can schistosome EVs interact with?
- What is the role of EVs within ES induced immune responses by schistosomes?
- Are schistosome EVs released *in vivo* and do they share chacteristics with *ex vivo* released EVs?
- Can schistosomula EVs induce antibody reponses within the host?

Text boxes describe the main findings presented in this thesis and the follow up questions. More details in text. Uptake of adult worm EVs by T cells, monocytes, and endothelial cells have been presented elsewhere[78,90].

immune cells without the presence of other EV-associated molecules (e.g. luminal cargo). For this, we can utilize systems like *Nicotiana benthamiana* plants, that are already used to reconstruct native helminth glycoproteins[145]. In addition, it has been shown that EVs can be isolated from *N. tabacum*[146]. It would be interesting to examine the possibilities of generating plant EVs containing the surface glycans we found on schistosomula or adult worms. With these transgenic plant EVs it might be possible to reveal whether immune cell targeting and effector functions by schistosome EVs are depending on their associated surface glycans or their additional cargo.

Elucidating the role of EVs for schistosome induced immune responses will tell us whether it is useful to focus research efforts towards EVs or whether we should keep including the non-EV released ES components in future studies on immune regulation by this parasite. Furthermore, when the parasite seems to depend largely on these vesicles, either for immune evasion, immune modulation, and/or anti-coagulation, they would offer a new target for drug treatments and vaccines.

Schistosome EVs and host immunity: inspiration for vaccine development

Schistosomiasis still affects hundreds of millions of people. Annual mass drug administration in affected communities provides treatment, but does not prevent reinfection[147]. Therefore, researchers are aiming to develop a vaccine against schistosomes for decades, with four candidates now on clinical trials, but it might still be a long way till a safe and sufficiently effective vaccine against schistosomes becomes available[148]. One of these vaccines that is currently being tested is recombinant smTSP2[149], a protein highly present on schistosomula and adult worm EVs[10] (Chapter 3). EVs are already explored as vaccine candidates against cancer, viral infections, and non-viral infections such as bacteria and parasites[150]. Several studies in mice have shown that administration of EVs isolated from *ex vivo* helminth cultures, often in presence of an adjuvant, can reduce infection readouts upon parasite challenge[79,151–154]. Only one of these studies included *S. mansoni* EVs, which was a subcutaneous injection of a crude preparation of egg-derived EVs that reduced worm and egg counts and granuloma formation upon cercarial challenge[154]. Interestingly, unpublished data on administration of isolated *S. mansoni* adult worm EVs did not affect subsequent infection challenge and readouts in mice, despite the presence of TSP2 in these EVs[155]. It is tempting to suggest that this might be because of the LDN and Tn antigen containing glycan motifs on adult worm EVs that favor tolerance via MGL activation. Thus far, there are no reports on schistosomula EVs and their *in vivo* effect on host

immunity. Therefore, in this final section of the discussion, I will advocate that the unexplored glycosylated schistosomula EVs can be an inspiration for future vaccine development.

In a recent study, researchers extensively tested 96 recombinant antigens found in proteomics and transcriptional data from the schistosomula stage[156]. These antigens included surface proteins as well as various secreted proteins. They tested all 96 as vaccine candidates in a mouse infection model, however, none showed strong protection against subsequent schistosome infection[156]. However, since the recombinant proteins were generated in human embryonic kidney (HEK) cells, these antigens missed one important native feature: helminth glycosylation. Studies in "self-curing" rhesus macaques have shown that these macaques generate high IgG titers against (multi-)fucosylated terminal LDN and Le^x motifs upon schistosome infection[157]. Furthermore, baboons, which are natural hosts for schistosomes like humans, develop protective anti-glycan antibody responses when vaccinated with radiation-attenuated cercariae[158]. The developed antibodies by the vaccinated baboons mainly targeted multi-fucosylated motifs on O-glycans and glycosphingolipid glycans[159]. We have found Le^x and multi-fucosylated motifs to be associated with schistosomula EVs (Chapter 6). Thus, these data suggests that schistosomula EV-associated glycans could induce a (protective) humoral response.

One approach to study the influence of schistosomula EV-associated glycans on antibody responses is to vaccinate an animal model with isolated schistosomula EVs. A second approach is to generate *N. benthamiana* plant EVs exposing N-glycans consisting of multifucosylated LDN and Le^x motifs and test these as vaccine strategy in animal models. An additional important factor for these types of studies is the choice of adjuvant. It has been shown that synthetic nanoparticles provide additional adjuvant properties and can enhance immunity[160,161]. For example, soluble worm antigens loaded into mesoporous silica nanoparticles stimulated a stronger immune response than worm antigens in combination with a more conventional aluminum salt adjuvant[162]. Membrane-encapsulated nanoparticles are currently explored as therapeutic agents[163], such as coating nanoparticles with bacterial outer membrane vesicles[164]. Thus, a similar nanoparticle coating could be explored using schistosomula EVs or the plant EVs with specific schistosomula glycans, thereby providing a stronger adjuvant to stimulate immunity. Finally, this could also be combined with loading a specific mRNA within the nanoparticles, a novel vaccine technology that has advanced rapidly in the past years[165]. The mRNA could code for one of the receptors expressed on the worm surface that plays a role in binding to or inactivating host molecules[22,74]. When these worm proteins can be targeted by antibodies, thereby inhibiting their function, this

could potentially make the worm less capable to evade host immune responses and increase parasite killing.

Native EVs release by the parasite might not yield the required immunity to offer full protection. However, utilization of EVs to deduce the exact molecules needed for a strong humoral response against the parasite may bring us closer to developing an effective vaccine against schistosomes.

In conclusion

The work presented in this thesis lays the foundation for deepening our fundamental knowledge on EV isolation methods, EV-glycosylation, and EV-mediated host immune responses by schistosomes. The current age is represented by technological advances for EV isolation of EV subsets[63], single EV measurement techniques[166–168], more sensitive omics approaches[169], and *in vivo* EV tracking[120]. Combining these advancements with the development of transgenic parasites[88] and controlled human *Schistosoma* infection trials[170,171] will open up a range of future possibilities to uncover the true function of schistosome released EVs. With the increasing amount of data and a rise of machine learning tools[172–174], and the realization that there is common ground between helminths and cancer (glycan) mediated immunotolerance[175], multidisciplinary collaborations are crucial to accelerate novel discoveries that will benefit the health of many individuals.

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