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# Chapter

# 1

## General Introduction

Partly based on: *Schistosoma mansoni* egg  
glycoproteins and C-type lectins of host  
immune cells: Molecular partners  
that shape immune responses

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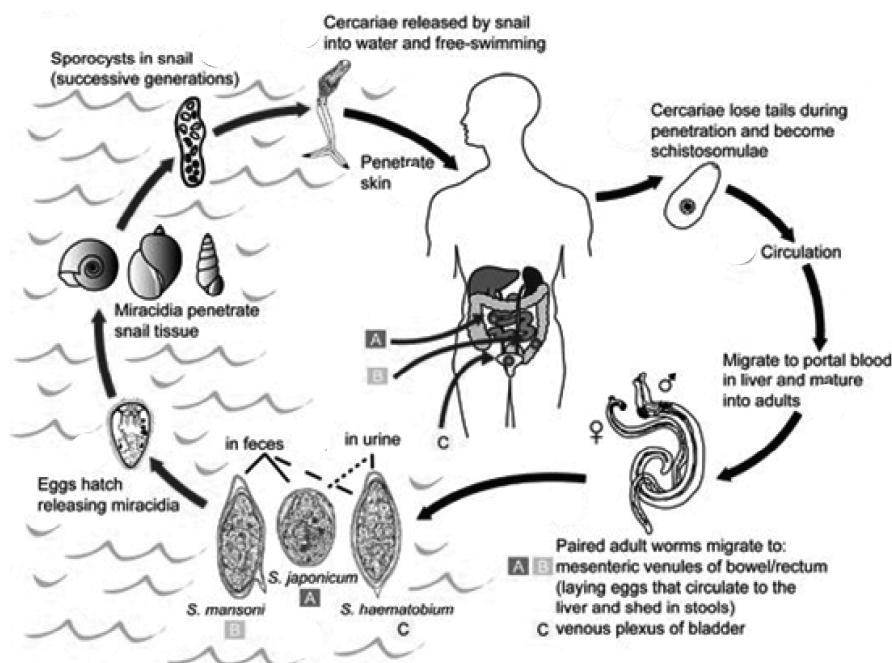
## 1. *Schistosoma mansoni* infection

Schistosomiasis is a chronic, debilitating and poverty-related disease, common in many countries in the tropics and subtropics. Humans can become infected with several species of the parasitic schistosomes, of which *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* are most prevalent. These schistosome species are endemic in different, sometimes overlapping areas in the world and show slightly different pathological and epidemiological characteristics <sup>1</sup>.

Like all schistosomes, the most intensely studied species *S. mansoni* has a complex life cycle that involves sexual reproduction in a definitive mammalian host as well as asexual reproduction in an intermediate host (Figure 1). During the early, acute stages of *S. mansoni* infection in humans, the dominant immune response against the parasite is T helper 1 (Th1)-mediated, hallmarked by high levels of interleukin (IL)-1, IL-6 and interferon (IFN)- $\gamma$  in peripheral-blood mononuclear cells (PBMCs), as well as upregulation of serum levels of tumor-necrosis factor (TNF)- $\alpha$  <sup>2</sup>. In some instances, the acute immune responses result in a disabling fever called “Katayama fever”, which is mostly observed in travelers infected by schistosomes for the first time <sup>3</sup>. Upon deposition of eggs by adult female worms, the immune response drastically alters. Egg-derived molecules downregulate the initial Th1 response and initiate a marked Th2-skewed response <sup>4,5</sup>. In addition, eggs trapped in the liver and other organs induce periovular, Th2-mediated, granulomatous responses, characterized by the presence of alternatively activated macrophages, CD4+ T cells, eosinophils and collagen fibers. In the course of time, a regulatory response develops which downregulates the strong Th2 response, and granulomas around newly deposited eggs decrease in size <sup>1</sup>. The egg-induced tissue granulomas and most notably the associated fibrosis are the main cause of morbidity in *S. mansoni*-infected individuals, as these reactions can lead to severe hepatic, splenic and intestinal damage.

Despite the strong immune responses elicited by schistosomal molecules, generally clearance of schistosomes cannot be achieved by the host, resulting in an ongoing chronic infestation that can last up to several decades. Therefore, treatment with anti-helminthics is required to resolve the infection. Today, the treatment of choice for schistosomiasis is praziquantel <sup>6</sup>. This drug induces severe muscle spasms and paralysis in male adult worms, presumably by increasing the permeability of cell membranes for calcium ions leading to dislodgement and death of worms <sup>7</sup>. The major disadvantage of this and other currently available drugs is that they do not protect individuals from re-infection. Moreover, the drug does not directly reverse damage caused by the host immune reaction against tissue-trapped eggs, and life stages other than mature worms remain unaffected by the drug <sup>8</sup>. Further research on schistosome biology and immunology is vital to develop new drugs and find other more long-

term solutions, such as an effective vaccine. Due to their specific immunological characteristics, schistosomes and schistosome molecules make excellent model systems for studying the development and function of Th2 and regulatory responses. Potentially, these molecules could provide a basis for the development of Th2- and/or Treg-polarizing therapies for the prevention of Th1-mediated autoimmune diseases.



**Figure 1 Life cycle of schistosomes infecting humans.** Cercariae, released from infested water snails, penetrate through the skin of human individuals and shed their tails. The developing schistosomula migrate through the tissue and enter blood vessels via which they are transported to the portal veins, where they mature into adults worms. Upon maturation, female and male worms form pairs and migrate to their final location. The female worms start to produce eggs, part of which enter the lumen wall of the intestine (*S. mansoni* and *japonicum*) or bladder (*S. haematobium*), after which they leave the body via the faeces or urine, respectively. Eggs hatch on contact with fresh water and release miracidia. These motile larvae can infect snails, in which they asexually reproduce into sporocysts and eventually cercariae. (adapted from: <http://www.dpd.cdc.gov/DPDx/HTML/Schistosomiasis.htm>)

## 2. *Schistosoma mansoni* glycoconjugates

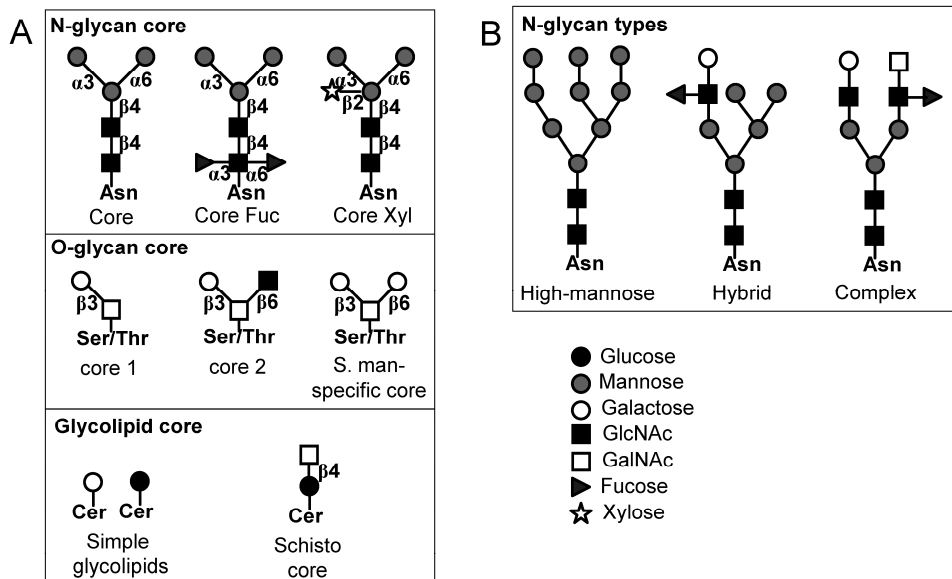
Schistosomal glycoconjugates have been acknowledged to play a major role in the activation and modulation of the immune system during *S. mansoni* infection. Glycosylation is a common feature of proteins and lipids that pass through the rough endoplasmatic reticulum (RER) and the Golgi-complex, and follow the secretory pathway to the cell surface. Proteins can be decorated with N- and O-glycans linked to asparagine and serine/threonine, respectively, which

are formed via a highly regulated cell- and schistosome life stage-specific process involving the concerted actions of glycosyltransferases and other glycosylation-modifying enzyme activities<sup>9</sup>. As is also the case in many other organisms including humans, a *S. mansoni* N-glycan consists of a Man<sub>3</sub>GlcNAc<sub>2</sub> core that can be decorated with specific core-elements (Table 1)<sup>10-14</sup>. The N-glycans can be divided in three major classes, based on different diversifications of N-glycan core; the high-mannose-type, the complex-type and the hybrid-type (Table 1). *S. mansoni* O-glycosylation can consist of short mono- or disaccharides, but also of more complex structures consisting of conventional mucin-type core 1 and 2 structures, as well as of a *S. mansoni* specific novel core (Table 1) decorated with complex antenna structures<sup>10;14-16</sup>. Glycolipids can carry typical simple galactose or glucose-linked ceramides, but also larger, more extended glycolipids based on a so called “schisto-core” occur (Table 1)<sup>17-19</sup>. The non-reducing termini of complex glycan structures on schistosomal glycoproteins and glycolipids are typically made up of (repetitive) Galβ1-4GlcNAc (LacNAc, LN) and GalNAcβ1-4GlcNAc (LacdiNAc, LDN) structures, which can be decorated with one or more fucoses<sup>10-12</sup>. Notably, sialic acids, common terminal monosaccharides of mammalian antennae motifs, are not found on *S. mansoni* glycans.

The precise structural features of schistosome glycans are highly dependent on the life stage and cell type in which they are expressed, as well as on the protein backbone by which they are carried. In the next paragraphs, the general characteristics of the glycoconjugates observed in cercariae and schistosomula (paragraph 2.1), adult worms (paragraph 2.2) and eggs and miracidia (paragraph 2.3), are described, as well as the current knowledge on the immune responses elicited by these structures. Finally, paragraph 2.4 will focus on the glycan-directed antibody responses observed during *S. mansoni* infection.

### 2.1 Glycoconjugates of cercariae and schistosomula

A clear feature of both protein- and lipid-linked glycans in cercariae is the abundance of LN-backbones, of which the immunogenic glycan motif Galβ1-4(Fuca1-3)GlcNAc (Lewis X, LeX) is most observed. LDN-based structures are present as part of the cercarial glycocalyx O-glycans, but in glycolipids and N-glycans they occur only in minor amounts<sup>11;13-15;18</sup>. The majority of cercarial N-glycans are decorated with a core β2-xylose as well as an α6-fucose, while no core α3-fucosylated glycans are detected in this life stage<sup>11</sup>. Interestingly, the cercarial glycocalyx has been reported to carry complex O-glycans with repeating units of unique multi-fucosylated (Fuca1-2Fuca1-3, DF) LDN motifs<sup>16</sup>. The cercarial glycolipids in addition to LeX express the Fuca1-3Galβ1-4(Fuca1-3)GlcNAc (pseudo-LeY) motif<sup>18</sup>, which to date has not been observed in other *S. mansoni* life stages.



**Table 1. *S. mansoni* glycan structures** (A) Core structures as present on *S. mansoni* N-glycans, O-glycans and glycolipids (B) N-glycans can be divided into three classes based on the antenna structures. The high-mannose type glycans carry multiple mannose residues. The complex type glycans carry up to four antenna structures consisting of a wide array of glycan motifs, including the ones displayed in table 2. The hybrid type carries one high-mannose antenna and one complex glycan antenna.

The glycosylation of the schistosomula, which develop after transformation of the penetrating cercariae, is less thoroughly studied. While O-linked and lipid glycosylation have never been analyzed, one mass spectrometric analysis of N-glycosylation of *in vitro* transformed 3-day old schistosomula has been published<sup>13</sup>. In comparison to cercariae, the expression of LN and LeX-containing glycans is reduced, and truncated glycans are more prevalent. Xylosylation of complex glycans is nearly absent, but a major fraction of truncated glycans still carries this motif. Monoclonal antibody (mAb) studies have indicated the presence of LeX, LDN and GalNAc $\beta$ 1-4(Fuca1-3)GlcNAc (LDN-F) on the surface of schistosomula<sup>20,21</sup>. As LDN and LDN-F motifs are not clearly detectable on N-glycans of schistosomula, these might be expressed by O-glycans and/or glycolipids.

Many studies have indicated that *in vitro* killing of cercariae and schistosomula can be initiated by host complement complexes<sup>20;22-25</sup> and antibody-dependent cell mediated cytotoxicity (ADCC)<sup>26-29</sup>. In some instances, the recognition of parasite glycans has been demonstrated to be involved in these immune processes<sup>20;22;29</sup>. The glycocalyx of cercariae seems to activate the alternative pathway of complement binding, leading to efficient killing of a majority of cercariae<sup>22</sup>. Schistosomula on the other hand are less susceptible to killing via this complement pathway<sup>22</sup>, but instead can be targeted via the classical pathway, which requires

antigen-specific antibodies for its initiation<sup>20;24;25</sup>. It was demonstrated that antibodies against LDN in the presence of complement could mediate *in vitro* lysis of schistosomula, indicating that glycan epitopes on the surface of schistosomula could be targets for antibody-mediated complement killing<sup>20</sup>. Killing of schistosomula, at least *in vitro*, can also be mediated by ADCC mechanisms, which involve eosinophils, macrophages or platelets that bind to antigen-specific IgE bound on the surface of the schistosomula<sup>26-28</sup>. In this respect, eosinophil-mediated killing was shown to be dependent on the interaction between LeX structures and selectins, both of which are expressed on eosinophils as well as schistosomula<sup>29</sup>.

Despite the clear evidence that the human immune system has the tools to kill the larval life stages of *S. mansoni*, infected individuals are generally unable to eradicate the parasite. Schistosomes appear to be masters of immune evasion but the exact mechanisms behind this, which involve shedding of the glycocalyx, membrane turnover, acquisition of host molecules and the induction of anti-inflammatory and regulatory immune responses by later life stages, are still elusive.

## 2.2 Glycoconjugates of adult worms

Upon maturation of the larvae into adult worms, xylosylation and  $\alpha$ 3-core fucosylation of N-glycans totally disappears, and LeX structures become less abundant. Instead, N-glycosylation of adult worms is mainly characterized by  $\alpha$ 6-core fucosylated, mono- and di-antennary glycans terminating with LDN<sup>12;13</sup>. Minor glycan subsets on adult worms include diantennary glycans with mixed LDN, LN and LeX termini as well as linear repeats of these structures<sup>12</sup>. Although male and female glycans in general display a similar N-glycosylation profile, subtle differences in the minor glycan subsets are observed, with females expressing more LN/LeX-type glycans, whereas LDN/LDN-F-type glycans are more prevalent in males<sup>30</sup>. MAb stainings revealed that these gender-specific glycans were at least in part found on the tegument, which might have consequences for the type of immune responses elicited by the two sexes. O-glycans could not be directly detected within the adult worm extract also used to characterize the N-glycans<sup>12</sup>, however worms were shown to excrete the highly antigenic circulating anodic antigen (CCA) and circulating cathodic antigen (CAA) from the gut that carry long O-linked carbohydrate chains containing repeats of LeX units and a GlcA-substituted GalNAc polymer, respectively<sup>31;32</sup>. Worm glycolipids have to date been poorly defined in terms of glycosylation. However, using defined anti-glycan antibodies, the presence of (multi-)fucosylated LDN structures including LDN-F and LDN-DF was demonstrated on worm glycolipids<sup>33</sup>, as well as the presence of LeX<sup>34</sup>.

Interestingly, worm glycolipids were demonstrated to induce Th1 responses via modulation of dendritic cells (DCs) *in vitro*, a process which was shown to be dependent on



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fucosylated glycan motifs on the glycolipids as well as DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and TLR4 on DCs <sup>34</sup>. It was hypothesized that DC-SIGN binds the fucosylated structures on glycolipids, thereby facilitating the binding of the lipid entity to TLR4 and the induction of DC maturation via intracellular signaling cascades. This might provide a mechanism via which parasite glycans induce the initial Th1 responses as observed in infected humans and in animal models.

CCA and CAA are excreted in such high levels by the worms that they are detectable in the serum and urine of infected individuals <sup>35</sup>. They are widely used as diagnostic targets in epidemiological studies using specific mAbs that recognize glycan epitopes on these antigens <sup>36</sup>.

### *2.3 Glycoconjugates of eggs and miracidia*

The glycan profile of eggs evidently differs from that of adult worms. Within the N-glycan pool,  $\beta$ 2-core xylosylation as observed in the cercarial stage re-appears, and a set of  $\alpha$ 3-core fucosylated glycans can be detected. As in most other life stages, antenna structures on N-, O- and lipid glycans for a large part consist of fucosylated LN and LDN motifs, including LeX and LDN-F structures <sup>10;17</sup>. Another characteristic feature of egg glycans is the occurrence of multi-fucosylated antenna structures containing the Fuca1-2Fuca1-3 motif <sup>10;14;19</sup>. On glycolipids, these motifs are expressed in the form of repeating -4(Fuca1-2Fuca1-3)GlcNAc $\beta$ 1-units terminating with (Fuca1-2)<sub>0/1</sub>Fuca1-3GalNAc $\beta$ 1- at the non-reducing end.

The N-glycans of miracidia seem to be very similar to the ones found on eggs <sup>13</sup>, which is not surprising as the miracidium constitutes a major part of the mature egg (Figure 2). However, eggs contain certain additional, minor glycan structures which are most likely to be expressed on glycoproteins from the subshell area within the egg, and possibly the egg shell itself <sup>13</sup>. The glycan structures of miracidial O-glycans and glycolipids have not been analyzed yet, but, as observed for N-glycans, are expected to be largely similar to the respective egg glycans.

Schistosome egg glycoconjugates and most notably the secreted egg (glyco)proteins (ES) contain potent immunomodulatory agents. In part 3 of the general introduction, we will focus on *S. mansoni* egg glycoproteins and their interactions with the host immune system.

### *2.4 Anti-glycan antibody responses during S. mansoni infection*

It has long been acknowledged that schistosome glycans are important targets of the strong antibody responses observed during natural and experimental infections <sup>20;37-39</sup>. Antibody responses against many schistosomal motifs including LeX, LDN, F-LDN, LDN-F and LDN-DF and F-GlcNAc <sup>20;39;40</sup> have been found. The most intense antibody responses in humans and primates observed so far are directed against LDN-DF carrying the Fuca1-2Fuca1-3 element and

against Fucal-3GalNAc motifs<sup>41-44</sup>, structures which are abundantly expressed on schistosome glycoconjugates but are uncommon in mammals. However, it should be noticed that the knowledge on antigenic glycans is far from complete due to the limited amount of glycan elements that have been studied. New technologies such as glycan arrays are expected to provide information on antibody responses in schistosomiasis cohorts to a much more complete set of antigenic glycans<sup>45</sup>.

Despite the differential antibody responses observed during *S. mansoni* infection against many parasite glycans, it remains to be established whether these antibodies could generate protective immune responses. In fact, it has been suggested that these high anti-glycan responses may even function as a “smokescreen”, preventing the generation of an effective humoral response<sup>39</sup>. However, certain antibodies against parasite glycans have been reported to confer protection in animal models<sup>46;47</sup>, as well as induce protective responses *in vitro*<sup>20</sup>.

### 3. *Schistosoma mansoni* eggs and the host immune system

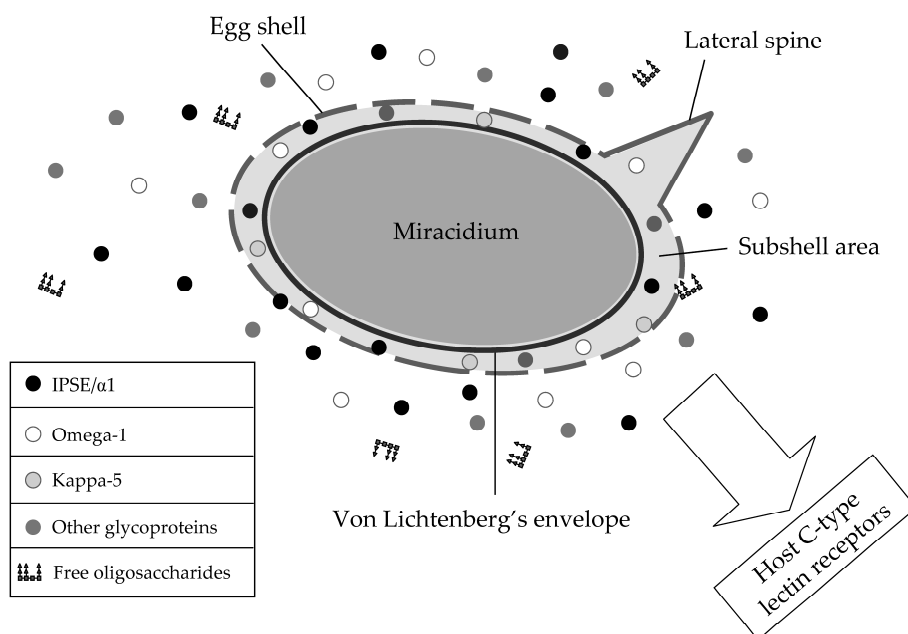
There is increasing evidence that the interplay between egg glycoproteins and C-type lectin receptors (CLRs) on host immune cells plays an important role in shaping immune responses during schistosomiasis. Paragraph 3.1 gives an overview of the molecular aspects of *S. mansoni* egg glycoproteins. Most experiments investigating the immunomodulatory properties of eggs so far have been performed using the complex (glyco)protein preparation SEA (Soluble Egg Antigens), as summarized in paragraph 3.2. The studies in addition indicated that a significant part of the immunological activity associated with these preparations seems to be mediated by the glycans present on SEA glycoproteins (paragraph 3.3). These glycans are thought to dictate interactions with CLRs and mediate antigen uptake by antigen presenting cells (APCs). Paragraph 3.4 gives an overview of the CLRs putatively involved in SEA glycan recognition, while paragraph 3.5 discusses SEA glycans which form possible ligands for these receptors. Importantly, it is still largely unclear which individual glycoproteins within SEA are immunologically active, as discussed in paragraph 3.6.

#### 3.1 Structural aspects of the *Schistosoma mansoni* egg and SEA

*S. mansoni* eggs are released by female worms in an immature form consisting of an egg shell of cross-linked proteins containing an ovum and vitelline cells. While still in the host, the egg matures during 5-6 days by the development of the ovum to a miracidium, and the formation of the Von Lichtenberg's envelope on the inside of the egg shell from which proteins are secreted into the environment through pores (Figure 2)<sup>48;49</sup>. Many of the eggs traverse the gut tissue and

are excreted with the feces, but a significant number get lodged downstream in the liver where they eventually die. The host reacts to eggs and egg products by inducing a Th2-mediated immune response which may lead to granulomatous inflammation and pathological tissue remodeling and fibrosis <sup>50</sup>. It is not clear if particular egg components are critical for the extravasation of the eggs from the blood vessels. Since freshly laid immature eggs are not yet believed to secrete proteins <sup>48;49</sup>, one could hypothesize that the egg shell is involved in this process. More clearly however, the eggs' secretory proteins which are formed in the sub-shell area upon maturation and secreted into the egg's environment <sup>49</sup>, induce a major immune response of the host that appears to be leading to Th2 polarization. To other components of the egg, such as the hatching fluid and most of the soluble SEA glycoproteins, the host will most likely be exposed only when eggs die and fall apart in host tissues.

While a number of recent studies have identified single components of SEA and ES with immunomodulatory properties <sup>51-55</sup>, the vast majority of all molecular studies into the immune mechanisms induced by schistosome eggs have been performed using SEA. SEA is a complex mixture of proteins of a potentially variable composition depending on the developmental stage of the eggs and the solubilisation procedure used. The properties of such preparations have been studied *in vitro*, e.g. in DC/T-cell skewing and signaling assays <sup>54;56-59</sup>, and *in vivo*, e.g. by injections into an array of wild type, transgenic and knock-out mice <sup>4;60;61</sup>.



**Figure 2.** Schematic overview of the mature *S. mansoni* egg and egg components.

Proteomics studies using 2D-gel electrophoresis and mass spectrometry have shown that over a thousand proteins can be detected in SEA, with a broad range of functions on target cells. These can be exerted either inside (e.g. cytosolic and nuclear proteins) or outside (e.g. membrane proteins, secretory proteins) a cell <sup>49;62</sup>. Although few studies have been conducted on individual SEA glycoproteins, it is clear that many SEA components are glycosylated <sup>1;33</sup>. Structural studies on preparations of N- and O-glycans released from the peptide backbones have shown that SEA glycoproteins collectively display a very complex set of glycans, comprising specific schistosome glycans, as well as glycans expressed in the mammalian host (Table 2) <sup>10;14</sup>. Each type of glycan or glycan element can be present on a larger subset of SEA glycoproteins <sup>33</sup>. SEA includes the excretory/secretory (ES) (glyco)proteins, but it does not contain water insoluble glycoconjugates, including membrane glycoproteins and hydrophobic glycolipids, unless specific solubilisation procedures were followed.

### 3.2 Immunomodulatory properties of SEA

SEA consistently is an inducer of Th2 responses in different types of experiments either *in vitro* or *in vivo* and both in humans and in animal models. A central role in the initiation and modulation of these responses is played by APCs such as DCs and macrophages. APCs continuously sample their environment for “danger” signals derived from invading pathogens, upon which they migrate to lymph nodes to instruct T cells to polarize.

Studies of DCs have been instrumental in understanding the polarization of immune responses towards Th2 by SEA <sup>5;56-60</sup>. While DCs fail to show classic signs of maturation when stimulated with SEA <sup>59;60</sup>, *in vitro* experiments show that SEA-primed monocyte-derived human and murine DCs are very potent in polarizing naive Th cells towards a Th2 type <sup>5;56;60</sup>. In accordance with the *in vitro* data, eggs injected into the footpad <sup>4</sup> as well as SEA-primed bone marrow-derived DCs injected intraperitoneally <sup>60</sup> can induce Th2 responses in mice. Apart from inducing Th2 responses, SEA is also able to interfere with Toll-like receptor (TLR)-mediated DC activation <sup>57;59</sup>. SEA can suppress maturation and cytokine production of human and murine DCs induced by the TLR4 ligand LPS and the TLR3 ligand poly-I:C <sup>57;59</sup>. Recently, a single ES glycoprotein omega-1 has been shown to exert similar Th2-inducing and TLR-modulating effects on DCs as does SEA <sup>54;55</sup>, as discussed in paragraph 3.6.

Components of SEA are also suggested to play a role in the initiation and modulation of periovular granulomatous responses observed in host tissues. When eggs lodge into organs such as the liver, host APCs initiate a granulomatous response which is thought to protect the host from overt reactions to egg molecules. In hepatic as well as pulmonary mouse models using antigen-coated Sepharose beads as artificial eggs, SEA has been shown to be a potent inducer of

a granulomatous response <sup>63;64</sup>, giving rise to granulomas with a comparable cellular composition to those around schistosome eggs <sup>63;65</sup>.

### 3.3 Glycan-mediated activities of SEA

Several studies show that the observed immunomodulatory properties of SEA are at least in part dependent on the interaction of glycosylated egg proteins with glycan receptors on APCs. In a mouse model of intranasal sensitization, SEA but not SEA pretreated with metaperiodate could induce Th2-type responses including antigen-specific IgE production and induction of IL-4 <sup>66</sup>. Metaperiodate treatment destroys glycan integrity and functionality by disrupting the ring structure of monosaccharides such as mannose, galactose and fucose, abundant constituents of SEA glycans. Cells from lymph nodes of mice injected with DCs conditioned with SEA produced significantly more IL-4 and IL-5 upon *in vitro* restimulation than lymph node cells from mice that received metaperiodate-treated SEA-pulsed DCs <sup>67</sup>. Production of IFN- $\gamma$  was similar however, indicating that APC-induced Th2 responses require intact glycosylation. Although

Table 2. Typical glycans and glycan elements on *S. mansoni* SEA and ES

Abbreviation	Structure
Lewis X	
LDN	
LDN-F	
F-LDN	
F-LDN-F	
Multi-fucosylated HexNAc	
Core $\alpha$ 3-fucose	
Core $\beta$ 2-xylose	
Major IPSE/ $\alpha$ 1 glycans	

Mannose  
 Galactose  
 GlcNAc  
 GalNAc  
 Fucose  
 Xylose

metaperiodate treatment could theoretically also have a destabilizing effect on the protein backbone and therefore disrupt the integrity of the glycoproteins, support for a role of glycans in Th2-skewing comes from studies using HSA-conjugated LNFPIII. It has been shown that this conjugate of LNFPIII, a pentasaccharide terminating with the LeX epitope, has Th2-skewing properties mimicking those of SEA in the murine model of intranasal sensitization <sup>66,68</sup>. Furthermore, a Dextran-LNFPIII conjugate shows similar DC-modulating effects as SEA <sup>69</sup>, and at least one glycoprotein carrying glycans terminating with LeX has been found in SEA <sup>70</sup>.

SEA glycans may also play a role in the initiation and modulation of perivascular granulomatous responses observed in host tissues. In the hepatic mouse model, it was demonstrated that beads that carry SEA with glycans destroyed by metaperiodate treatment were no longer able to induce granulomas, but elicited only a monolayer of cells comparable to uncoated or albumin-coated beads <sup>65</sup>. A set of model glycoconjugates carrying various synthetic oligosaccharides characteristic for schistosome eggs was tested in the same mouse model. From this set, only LDN- and LN-terminating glycoconjugates were able to induce granuloma formation. In contrast, glycoconjugates with terminal LeX, fucosylated LDN or GlcNAc, elicited only a monolayer of macrophages <sup>71</sup>.

### 3.4 C-type lectin receptors for SEA

Distinctive glycan elements abundantly present on SEA and ES glycoconjugates are recognized by pattern recognition receptors (PRRs) <sup>72,73</sup>. APC are equipped with an array of PRRs, including CLRs and TLRs, in order to recognize and differentiate between pathogens by binding pathogen-associated molecular patterns (PAMPs) and instruct the immune system to mount a dedicated response. Although TLR4-mediated signaling by the LeX-containing glycoconjugate Dextran-LNFPIII has been reported <sup>74</sup>, and LDN, a terminal disaccharide element of SEA glycans, is a ligand for the S-type lectin galectin-3 on macrophages <sup>75</sup>, the CLRs appear to be the most relevant PRRs for schistosome egg glycans. All CLRs carry one or more carbohydrate recognition domains (CRD), which determine the specificity of a CLR for specific pathogen-derived molecules such as polysaccharides, glycolipids and glycoproteins. Binding of such molecules to CLRs typically leads to internalization, processing and antigen-presentation on MHC class I and II molecules. In addition, it was shown that binding of microbial ligands to CLRs can trigger induction of signaling cascades which lead to immune modulation, either by modulating TLR-induced signaling, or by direct induction of gene expression (as reviewed in <sup>76</sup>).

Many glycan structures found in SEA, including LeX, LDN and (multi-) fucosylated LDN, interact with CLRs (Table 3) <sup>59;73;77;78</sup>. It has been shown in an *in vitro* model that DC-SIGN, mannose receptor (MR) and macrophage galactose-type lectin (MGL) are the primary

recognition and uptake receptors on human monocyte-derived immature DCs for SEA<sup>59</sup>. In the same system it was shown that exposure of DCs to SEA leads to Th2 skewing, but it was not demonstrated which SEA glycoproteins and which receptors were precisely involved<sup>59</sup>.

Table 3 Recognition of SEA glycans by C-type lectins

CLR	Expression	General glycan specificity	SEA glycans <sup>a</sup>		SEAs recognition and immunological effects	Refs
			Tested as CLR ligands <sup>b</sup>	Binding		
DC-SIGN	human DC and Mph	Fucose and high-mannose	Lewis X <sup>c</sup>	+	Immature DC recognize SEA via DC-SIGN, MR and MGL	59, 72, 77, 79, 81
			LDN-F <sup>c</sup>	+		
			LDN-DF <sup>c</sup>	-		
			High-mannose N-glycans <sup>d</sup> α3- and α6- core fucoses <sup>d</sup>	+		
L-SIGN	human LSEC	Fucose and high-mannose	Lewis X <sup>d</sup>	-	Synthetic lewis X induces signalling pathways via DC-SIGN	72, 78
			LDN-F <sup>d</sup>	-		
			F-LDN-F <sup>d</sup>	+		
			LDN-DF <sup>d</sup>	-		
SIGNR1	murine Mph and LSEC	Fucose and high-mannose, sialylated lewis Ags	High-mannose N-glycans <sup>c</sup>	+	L-SIGN binds SEA mainly via high mannose structures	87-89
			Lewis X <sup>d</sup>	+		
			High- mannose N-glycans <sup>d</sup>	+		
			High- mannose N-glycans <sup>d</sup>	+		
SIGNR3	specific murine DC and Mph subsets	Fucose and high-mannose	Lewis X <sup>d</sup>	+	No data	86, 88
			High- mannose N-glycans <sup>d</sup>	+		
hMR	human DC and Mph	Fucose, mannose and N-acetylglucosamine	Lewis X <sup>d</sup>	-	Immature DC recognize SEA via DC-SIGN, MR and MGL	59, 80, 93
			Various fucosylated glycans <sup>e</sup> High mannose N-glycans <sup>d</sup>	n.d. +		
mMR	murine DC and Mph	Fucose, mannose and N-acetylglucosamine	Various fucosylated glycans <sup>e</sup> High mannose N-glycans <sup>d</sup>	n.d. +	No data	93
			Lewis X <sup>d</sup>	+		
MGL	human DC and Mph	Terminal N-acetylglucosamine	LDN <sup>c</sup>	-	Immature DC recognize SEA via DC-SIGN, MR and MGL	59, 73
			LDN-F <sup>c</sup>	+		
mMGL1	murine DC and Mph	Terminal galactose	Lewis X <sup>d</sup>	+	No data	97, 98
			LDN <sup>d</sup>	-		
mMGL2	murine DC and Mph	Terminal N-acetylglucosamine	Lewis X <sup>d</sup>	-	No data	97, 98
			LDN <sup>d</sup>	+		
			LDN-F <sup>d</sup>	-		
				-		

<sup>a</sup> for detailed structural information, see table 1 and 2; <sup>b</sup> Many more glycans are present in SEA for which binding has not been determined <sup>c</sup> tested as natural SEA component; <sup>d</sup> tested as model glycoconjugate; <sup>e</sup> Hypothetical binding, not tested; n.d., not determined.

By biochemical binding assays such as ELISA and glycan arrays it has been shown that DC-SIGN binds to many fucosylated motifs including (non-sialylated) Lewis antigens and LDN-F<sup>72,79</sup>. Guo et al. showed that recognition of LeX by DC-SIGN is conferred by binding of the  $\alpha$ 1-3 fucose in a primary binding site of DC-SIGN, while the terminal galactose binds a secondary binding site<sup>72</sup>. This implicates the necessity for a specific presentation and glycosidic linkage of the fucose residue as part of an oligosaccharide to be able to fit the binding sites of DC-SIGN. Indeed,  $\alpha$ 3- and  $\alpha$ 6-linked core fucoses<sup>79</sup> as well as LDN-DF<sup>77</sup> do not seem to be recognized by DC-SIGN. In addition to fucose-containing glycan motifs, DC-SIGN also binds N-linked high mannose-type glycans, as the binding pocket of DC-SIGN is able to bind multiple mannose residues<sup>72,80</sup>. Interestingly, the type of ligand binding to DC-SIGN affects the immunological outcome, as recognition of respectively mannose- or fucose-containing glycans leads to distinct signaling pathways<sup>81</sup>.

L-SIGN, also named DC-SIGNR, is a DC-SIGN homologue that binds multi-fucosylated motifs as well as mannosylated motifs<sup>72,78</sup>. Interestingly, L-SIGN is expressed on liver sinusoidal endothelial cells (LSECs), but not on DCs and macrophages<sup>82</sup>. In the liver, LSECs are part of the internal lining of blood vessels and they express high levels of adhesion receptors and lectins including MR and L-SIGN<sup>83</sup>. LSECs are suggested to mediate the clearance of antigens from the circulation in a similar manner as DCs do<sup>82,84</sup>.

In mice, seven homologues of human DC-SIGN are found that closely resemble DC-SIGN in terms of protein sequence and structure of the CRD domain<sup>85,86</sup>. However, glycan array screening revealed that only SIGNR1 and SIGNR3 share with DC-SIGN the ability to bind both high mannose and fucose-containing glycans<sup>86-88</sup>. SIGNR1 is expressed on murine macrophages and has been shown to bind SEA<sup>89</sup>. Unlike DC-SIGN, SIGNR1 binds sialylated Lewis antigens in addition to unmodified Lewis antigens<sup>87</sup>. Notably, SIGNR1 does not play a critical role during infection, as *S. mansoni*-infected SIGNR1<sup>-/-</sup> mice show an unaltered phenotype in terms of granulomatous responses and pathology<sup>89</sup>. Moreover, in a pulmonary mouse model, the granuloma volume around injected eggs as well as levels of IL-4, IL-10 and IFN- $\gamma$  in lung tissue did not significantly differ between normal and SIGNR1<sup>-/-</sup> mice<sup>89</sup>. SIGNR3 is reported to more closely resemble the glycan specificity of DC-SIGN<sup>86</sup> and it has been shown to mediate endocytosis and signaling<sup>86,90</sup>. However, while DC-SIGN is abundantly expressed on human DCs<sup>91</sup>, SIGNR3 in mice is expressed on small subsets of DCs, macrophages and monocytes in the skin, lungs and lymphoid organs only<sup>92</sup>. Taken together, to date no murine receptor has been found which resembles human DC-SIGN in terms of binding and signaling properties as well as expression on APCs, which may have implications when studying the glycan-dependent immunomodulatory properties of SEA in mice.



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Human MR has a slightly overlapping specificity with DC-SIGN and binds glycans terminating in mannose, fucose and GlcNAc residues <sup>80;93</sup>. The binding motifs for MR have not been reported in detail; however it has been shown that MR is unable to effectively bind LeX residues, which may be caused by the steric hindrance of the galactose residue in the LeX structure <sup>80</sup>. Furthermore, although MR can bind single mannose residues, it binds more complex mannose structures with higher affinity <sup>80;93</sup>. In mice, one clear MR homologue exists which exhibits binding properties comparable to human MR <sup>93</sup>. The expression of MR on macrophages and DCs, as well as the amino acid sequence of the eight CRD domains are well conserved among humans and mice <sup>94;95</sup>.

Finally, MGL is a CLR expressed by immature human DCs and macrophages. In contrast to DC-SIGN, MR and in fact most other CLR, MGL is unable to bind fucose and mannose residues. Instead, MGL has a narrow specificity for non-substituted, terminal  $\alpha$ - and  $\beta$ -linked GalNAc <sup>73</sup>. In mice, two MGL homologues are found, named mMGL1 and mMGL2 <sup>96;97</sup>. Like human MGL, mMGL1 and mMGL2 are expressed by immature DCs and macrophages. However, only mMGL2 shares its narrow glycan specificity for GalNAc with human MGL, whereas mMGL1 primarily binds unsubstituted terminal galactose <sup>97;98</sup>.

### 3.5 Molecularly defined CLR ligands in SEA

It is yet unknown which egg glycans and CLRs are responsible for the modulatory effects of SEA via APCs. The reported importance of DC-SIGN, MR and MGL in the recognition and uptake of SEA <sup>59</sup> would suggest a role for these receptors and specific subsets of SEA as ligands. Many of the glycan motifs recognized by these CLRs have indeed been found to be abundantly present in SEA by chemical structural studies (Table 2). LeX and LDN-F have been shown to be involved in DC-SIGN-SEA binding <sup>77</sup>. Moreover, LDN and LDN-F have been shown to be binding ligands for MGL in SEA <sup>73</sup>, while MR could potentially bind fucosylated LDN motifs. In addition, SEA glycosylation also contains high-mannose N-glycans, which are potential ligands for DC-SIGN, L-SIGN and MR.

The glycosylation of an important immunomodulatory subfraction of SEA, the ES glycoproteins, has also been extensively studied <sup>14</sup>. The major complex N-glycan structures and the O-glycans are rather similar for ES and SEA-released glycans, although high-mannose and truncated N-glycan structures were found to be less abundant in ES glycoproteins than in SEA. While many other helminths also carry potential CLR ligands such as the LDN-F motifs and other fucosylated HexNAc motifs, only *Dictyocaulus viviparus* and schistosomes have been reported to carry LeX motifs <sup>100;101</sup>.

### 3.6 Which individual glycoproteins in SEA are immunomodulatory via CLR?

Although it would be fair to hypothesize that SEA glycoproteins that carry LeX and other CLR ligands are the natural molecules responsible for glycan-induced Th2 induction that has been observed with other LeX-containing glycoconjugates, this has not been proven so far. Most structural information about SEA glycans has been obtained by releasing the glycans from the protein mixtures, SEA or ES, thereby losing all information about which individual proteins and glycans actually combine. To date, only one glycoprotein from *S. mansoni* eggs has been studied in detail in terms of glycosylation. IPSE/ $\alpha$ 1<sup>102</sup> (also named SmEP25<sup>103</sup> / SmCKBP<sup>104</sup>) is a major constituent of ES<sup>62;105</sup> and for the most part carries diantennary N-glycans of the complex-type<sup>70</sup>. The antennae are mainly composed of LeX and LN structures, with a minority composed of (fucosylated) LDN structures. In addition, the innermost core GlcNAc is typically decorated with  $\alpha$ 3/ $\alpha$ 6 difucosylation. IPSE/ $\alpha$ 1 has been shown to exhibit Th2-regulatory properties, as it is capable of inducing IgE-dependent IL-4 in human basophils<sup>52;102</sup> and an innate IL-4 production by murine basophils<sup>106</sup>. However, non-glycosylated recombinant IPSE/ $\alpha$ 1 induced similar effects on basophils as native IPSE/ $\alpha$ 1<sup>106</sup>, indicating that the LeX glycans of IPSE/ $\alpha$ 1 are in this case not playing a critical role. IPSE/ $\alpha$ 1 also seems to play an anti-inflammatory role during granulomogenesis by reducing cellular infiltration into granulomas<sup>104</sup>. This effect is likely to be explained by the chemokine binding properties of IPSE/ $\alpha$ 1, as Smith et al. showed that IPSE/ $\alpha$ 1 can block interaction of chemokines with their host chemokine receptors and inhibit their biological activity *in vivo*<sup>104</sup>, indicating that glycans are probably also not involved in the effects of IPSE/ $\alpha$ 1 on downregulation of granuloma formation.

Another ES glycoprotein, omega-1<sup>107</sup>, harbors potent Th2 skewing activities via modulation of DCs<sup>54;55</sup>. Omega-1-treated DCs are capable of generating Th2 responses in *in vitro* studies<sup>54;55</sup> as well as when transferred into naive mice<sup>55</sup>. Furthermore, omega-1 is a major factor in SEA and ES that conditions DCs for Th2 priming<sup>54;55</sup>, although SEA depleted from omega-1 still harbors Th2-inducing capacities, both *in vitro* and *in vivo*<sup>54</sup>. RNase inhibition experiments indicate that the RNase activity of omega-1 might be involved in Th2 skewing<sup>55</sup>.

A third major *S. mansoni* egg glycoprotein is kappa-5<sup>54</sup>. The function of kappa-5 is unknown, but it is present in high amounts in SEA, and although not produced in the sub shell envelope but by the miracidium, it appears to be a constituent of the hatching fluid. Interestingly, *S. mansoni* infected individuals often mount pronounced IgE responses against this glycoprotein<sup>108</sup>. As opposed to natural kappa-5, recombinant kappa-5 expressed in human embryonic kidney (HEK) cells does not reveal any IgE reactivity. Since mammalian-derived HEK cells have a different glycosylation repertoire from schistosomes, this may point to a role of specific glycans as the IgE target<sup>108</sup>. It has been demonstrated that kappa-5 is the primary *S.*

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*mansoni* SEA constituent that binds to soybean agglutinin (SBA), a lectin specific for terminal  $\alpha/\beta$ -D-GalNAc<sup>108</sup>. This indicates that kappa-5 carries LDN, which is a common *S. mansoni* glycan epitope to which several immunogenic properties have been attributed<sup>20;38;71;75</sup>.

In addition to glycoproteins, *S. mansoni* eggs also excrete a series of multi-fucosylated free glycans<sup>109</sup>, which structurally resemble egg glycolipid glycans<sup>19</sup>. Although this has not been tested so far, it would not be surprising if these oligosaccharides could bind to the same CLRs as the multi-fucosylated glycolipids or glycoproteins. Meyer et al. showed that glycosphingolipids from schistosome eggs were bound by L-SIGN as present on LSECs, presumably via Fuca1-3GalNAc $\beta$ 1-4(Fuca1-3)GlcNAc (F-LDN-F) motifs, and these may have a function in the initial immune recognition of schistosome eggs when they enter the liver<sup>78</sup>. In *in vitro* experiments using PBMCs of donors unexposed to schistosomes, egg glycolipids carrying difucosylated (DF) HexNAc motifs could trigger cytokine responses, whereas worm glycolipids, in which those motifs are absent, could not<sup>110</sup>. The functional consequences of the interaction with CLRs of these glycans and glycolipids may differ from that of (multivalent) protein-conjugated variants. Finally, it might be relevant that the insoluble egg shell also appears to be glycosylated<sup>111</sup>, but it is not yet clear if egg shell-associated glycans could exhibit immunoregulatory properties and what the exact structure of these glycans is.

## 4. Multivalency and molecular presentation of CLR ligands

Conclusions about the immunomodulatory potential of the SEA glycoprotein ligands for CLRs cannot easily be drawn from primary structural data and binding assays. The involvement of DC-SIGN, MR and MGL on DCs in SEA binding and uptake was discovered initially using a system where SEA was covalently coupled to fluorescent beads<sup>59</sup>. Such a system may not be entirely representative for secreted/excreted glycoproteins, such as those that are present in ES, which are not bound to a solid surface, particle or pathogen. Multivalency is an important aspect of CLR binding and subsequent signaling since the spatial presentation of glycans as well as the degree of multimerisation of receptors is of importance to generate stable interactions. The MR for instance is able to recognize oligomannose structures more efficiently than single mannose monosaccharides. This specificity has been shown to be dependent on the presence of multiple CLR domains within a single MR molecule<sup>93;112</sup>. In contrast to MR, most other CLRs contain only one CRD domain, however receptors have been reported to oligomerize, often resulting in enhanced affinity for multivalent ligands<sup>80;113-116</sup>. *In vitro*, soluble DC-SIGN molecules can form tetramers stabilized via an  $\alpha$ -helical stalk, which has been demonstrated to amplify the specificity for high-mannose type glycans on host molecules<sup>113</sup>. In addition, van Liempt et al. studied DC-

SIGN specificity in a glycan array using a bivalent DC-SIGN-Fc molecule <sup>79</sup>. In this assay they found a 4-fold increase in binding affinity of bivalent DC-SIGN-Fc towards N-glycans carrying diantennary LeX or diantennary LDN-F, compared to the respective monovalent trisaccharide determinants. Indeed, for DC-SIGN the manner of presentation of a ligand might influence its affinity due to clustering of this receptor on the cell surface. Immature DCs have been reported to express DC-SIGN in nanometric microdomains, whereas on intermediate DCs, which represents an earlier stage of DC development, DC-SIGN is randomly distributed along the cell surface <sup>115;116</sup>. This clustering of receptors on the cell membrane of immature DCs significantly enhanced binding and internalization of virus particles, while recognition of larger particles remained similar <sup>115</sup>. Due to this DC-SIGN clustering on immature DCs, soluble molecules would be recognized differentially as molecules presented on a solid surface of particles or beads, or at the surface of a cell or on a pathogenic organism. Similarly, multivalency or clustering density will be an important parameter when using synthetic model glycoconjugates to study the immunological aspects of *S. mansoni* glycans, as small carrier-coupled glycans will not necessarily mimic their natural counterparts from schistosome eggs.

## 5. Glycosylation analysis of *S. mansoni* egg glycoproteins

In depth structural information on the glycans of *S. mansoni* egg glycoproteins is commonly generated using mass spectrometry (MS)-based methods. A thorough sample preparation, as described in paragraph 5.1, is an essential part of these methods. A mass spectrometer generally consists of an ionization source, a mass analyzer and an ion detector. The ionization source converts the sample molecules into positively or negatively charged ions. These ions are then directed into a mass analyzer where they are separated according to their mass over charge ( $m/z$ ) ratio and detected to generate a mass spectrum. First choice MS set-ups for the structural analysis of glycan and glycopeptides mixtures are matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-MS (paragraph 5.2) and electrospray-ionisation (ESI)-ion-trap MS, (paragraph 5.3), the latter of which is typically on-line coupled to a liquid chromatography (LC) system in order to separate the sample prior to analysis. Tandem mass analysis (MS/MS) is generally used to further determine the composition and sequence of glycan structures (paragraph 5.4).

Although these MS-based techniques can provide a wealth of structural information, the identity (e.g. galactose vs mannose) and linkage position of the constituting monosaccharides in a glycan can usually not be directly deduced. Additional techniques can be exploited to complement and extend the MS data, which might include the use of specific exo-

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glycosidases, permethylation and anti-glycan antibodies. To generate an overview of the techniques used for the in depth analysis of *S. mansoni* egg glycans, current studies on the glycan analysis of complex egg glycoprotein preparations are summarized in paragraph 5.5, whereas paragraph 5.6 describes a study on the glycosylation of a single egg glycoprotein.

### 5.1 Sample preparation

The majority of *S. mansoni* glycan data is obtained from glycans released from complex total life stage preparations by enzymatic or chemical treatments<sup>10-14,16</sup>. N-glycans are commonly released using PNGase F and/or PNGase A, as these enzymes provide fast and reliable results<sup>117</sup>. PNGase F releases all N-glycans except those containing a fucose  $\alpha$ 3-linked to the innermost GlcNAc of the N-glycan core, while PNGase N cleaves off all N-glycans irrespective of their nature, in each case provided that the glycosylation sites have been made accessible by denaturation or proteolytic digestion of the protein. As the  $\alpha$ 3-core fucose is a common modification in schistosome glycoproteins, sequential treatment with the two enzymes can already provide useful information on the type of glycans present. To facilitate detection and analysis of released glycans, the reducing end of the glycans can be labeled with a fluorescent tag, such as 2-aminobenzoic acid (2-AA) and 2-aminobenzamide (2-AB). For the release of O-glycans, no enzyme is presently available due to the diversity of O-glycan core structures (Table 1). A common and reliable chemical method to release O-glycans from their protein backbone is reductive  $\beta$ -elimination<sup>118,119</sup>.

The analysis of released glycans is an easy and fast way to assess a diversity of glycan structures in complex mixtures. However, crucial information is lost about how individual proteins are glycosylated. Therefore, the direct analysis of glycans still attached to peptides of isolated, native glycoproteins is a recently-emerging technique. Glycoproteins are treated with an appropriate protease (e.g. trypsin) to generate peptides containing a single glycosylation site, and the resulting glycopeptides are analyzed directly by MS. Up till now, such an in-depth glycosylation analysis been carried out for only one schistosome glycoprotein, the ES egg glycoprotein IPSE/ $\alpha$ 1<sup>70</sup>, as discussed in paragraph 5.6.

### 5.2 MALDI-TOF-MS

In MALDI-TOF-MS, a sample is generally co-crystallized with an ultraviolet-absorbing matrix on a target plate, and ionised by a laser pulse. The resulting molecular ions, which are normally singly charged due to the soft ionization method, are then accelerated in an electric field and detected by a TOF mass analyzer. This analyzer detects the time that each ion needs to travel from the source to the detector through a high vacuum tube (time of flight), which can be

directly correlated to their  $m/z$  ratio. Although the exact ionization mechanisms are unclear, it is thought that the matrix absorbs the laser energy and transfers part of the energy to the sample, resulting in the ionization and transfer of the sample to the gas phase<sup>120;121</sup>.

MALDI-TOF-MS is very suitable for the analysis of small amounts of glycans or glycopeptides, as it can detect ions in the femtomole to picomole range. A decent sample clean-up is essential though, as salts or other impurities in the sample can hamper analysis. Low abundant molecules in complex mixtures can however easily be missed due to limitations in the dynamic range of detection. Separation of the mixture (e.g. by using chromatography) prior to measuring can overcome this problem.

### 5.3 ESI-MS

In a standard ESI-MS setting, a solution containing the sample is dispersed via a metal-tipped glass capillary into a fine spray of highly charged droplets. The droplets are accelerated by high voltage and evaporate under a drying gas at atmospheric pressure, creating (multiply) charged molecular ions that can be analyzed by a mass analyzer. Nano-ESI-MS is a variation on ESI-MS for which only very small amounts of sample are required. This technique is more sensitive compared to standard ESI-MS and is more tolerant of salts and impurities<sup>122;123</sup>.

As ESI-MS involves the continuous flow of liquids, it is very suitable for the online coupling to a LC system. This allows easy purification and/or separation of the sample prior to mass analysis. Separation of glycans and glycopeptides is dependent on their retention time on the stationary phase of a LC column. LC methods commonly used for the separation of glycans and glycopeptides are Hydrophilic Interaction Liquid Chromatography (HILIC) and Reverse Phase (RP) chromatography. In HILIC, glycans are retained by hydrogen bonding, ionic interactions and dipole-dipole interactions. These HILIC features enable separation of glycans and glycopeptides on the basis of size, presence of glycan modifications and sometimes even allow separation of isomeric structures. For RP chromatography, retention is primarily based on the hydrophobic properties of the peptide moiety (glycopeptides) or an added fluorescent tag (released glycans). Therefore, glycan variation on a certain glycosylation site, the so-called microheterogeneity, can be easily picked up as glycopeptides with the same peptide moiety but different masses elute at similar time points. Notably, certain monosaccharide moieties slightly influence the retention time, causing small shifts for some glycan variants. By coupling an LC system with (nano-)ESI-MS, low quantity molecules in a complex mixture are more effectively detected, and information based on the LC-separation can be coupled to the ESI-MS results

<sup>124;125</sup>.

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#### 5.4 Tandem mass spectrometry

Many MS instruments have the capacity to determine the composition of glycans by the fragmentation of selected precursor ions, a process which is called tandem MS or MS/MS. The order in which monosaccharides are lost from the parent structure is indicative of the sequence of monosaccharides and the branching pattern within a selected glycan or glycopeptide. Often, amino acid sequence information of the peptide part of glycopeptides can be additionally obtained.

In this thesis, laser-induced dissociation (LID) (for MALDI-TOF) as well as collision-induced dissociation (CID) (for ESI-ion-trap MS) have been employed. In the MALDI-TOF used, the laser-induced tandem MS is based on post-source decay induced by the laser and is usually indicated as MALDI-TOF/TOF <sup>121</sup>. CID-induced fragmentation is performed by colliding a selected ion with inert gas molecules <sup>126</sup>. Various other tandem MS techniques have also been developed to elucidate the composition of glycans and glycoproteins, as reviewed in

<sup>120;127</sup>.

#### 5.5 Glycosylation analysis of complex *S. mansoni* egg preparations

The first MS-based glycosylation analysis of *S. mansoni* egg glycoproteins was performed by Khoo et al. on the complex SEA mixture using fast atom bombardment (FAB)-MS <sup>10</sup>. N-glycans were released from tryptic glycopeptides via sequential digestion with PNGase F and PNGase A, and subsequent reductive elimination released the O-glycans. The released glycan preparations were permethylated to facilitate the determination of branching and glycosidic linkages, and were analyzed with FAB-MS. Using this technique, a sample is mixed with a matrix and bombarded under vacuum with a high energy beam of atoms, generating glycans as well as glycan fragments at the same time. The generated data, combined with accepted models of eukaryotic N-glycan structures, revealed important structural features of SEA glycans, as described in paragraphs 2.3 and 3.5. However, FAB-MS lacks the high sensitivity observed for MALDI-TOF-MS and ESI-MS techniques and is not compatible with tandem MS analysis. Due to these drawbacks, minor glycan structures in complex mixtures can remain undetected, and the complete structural characterization of glycans is not possible. More recently, a pilot study on egg N-glycans, using a MALDI-TOF-MS-based method, validated many of the glycan structures found earlier by Khoo et al. <sup>10</sup>, but in addition found previously unidentified, PNGase A sensitive glycans containing unusual triantennary LDN motifs <sup>13</sup>.

The glycosylation of the secretory ES glycoproteins, a major immunogenic subset of SEA, was extensively analyzed by Jang-Lee et al. <sup>14</sup>. Initial analysis of released, permethylated N- and O-glycans was performed with MALDI-TOF-MS to obtain an overview of glycans within

the released glycan pools. To generate more in-depth, structural information on the observed glycans, the majority of molecular ions were fragmented by MALDI-TOF/TOF as well as ESI-MS/MS. In addition, monosaccharide compositions and glycosidic bond positions were defined using gas chromatography (GC)-MS. Together, this study provides an in-depth overview of major and minor, N- and O-linked glycans present on ES glycoproteins.

### 5.6 Glycosylation analysis of the *S. mansoni* egg glycoprotein IPSE/α1

The reported role of individual glycoproteins and their glycans in the immunoregulatory effects of *S. mansoni* eggs, as outlined in part 3 of the introduction, stresses the need for detailed protein-specific glycosylation analysis. Up to now, only the egg glycoprotein IPSE/α1 has been subjected to such an analysis<sup>70</sup>. For this, IPSE/α1 was trypsinized and resulting glycopeptides were initially analyzed by MALDI-TOF-MS in the positive-ion reflectron mode. Two glycopeptides clusters were found, representing the “microheterogeneity” (glycan variation) of the two N-glycosylation sites of IPSE/α1. Additional analysis demonstrated that within each cluster, mass differences of 146, 162 and 203 Da were present, indicating that the IPSE/α1 glycans differed in the presence of fucose (F), hexose (H) and HexNAc (N) residues, respectively. Based on the deduced masses from MALDI-TOF-MS, the mass of the peptide parts, as well as already existing knowledge on the general structures of *S. mansoni* N-glycans, tentative glycan compositions were generated.

An extensive analysis of the exact glycopeptide structures was generated using tandem MS fragmentation as well as exo-glycosidase treatments. To confirm the characteristics of the peptide part of the glycopeptides, glycopeptides were deglycosylated with PNGase A, after which the amino acid sequence and glycan attachment site was revealed using nano-LC-MS/MS. Tandem mass spectrometry was also used to deduce the antenna composition and core substitutions of the glycan part. However, during fragmentation, fucose rearrangements within the antenna fucoses have been reported for protonated glycoconjugates, potentially leading to misleading fragmentation ions. To validate the fucose localization at the antennae, glycopeptides were treated with β-galactosidase. This enzyme can differentiate between fucosylated and non-fucosylated LN antennae, as it cleaves terminal galactose from LN but not from fucosylated variants such as LeX.

For example, on the basis of the MALDI-TOF data and previous knowledge on *S. mansoni* N-glycans, the major glycan structure H<sub>4</sub>N<sub>5</sub>F<sub>4</sub> on site N80 of IPSE/α1 was suggested to consist of a difucosylated N-glycan core carrying two LeX antennae. The presence of peptide fragments containing a HexNAc with two fucoses (pep-N<sub>1</sub>F<sub>2</sub>) indicated that the core structure indeed was di-fucosylated. β-galactosidase treatment clearly demonstrated that each of the LN



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antennae carry one fucose, as the enzyme was not able to cleave off any galactoses, in line with the presence of two LeX units.

Conclusively, the MS and tandem MS studies on tryptic glycopeptides of IPSE/ $\alpha$ 1 combined with the enzymatic treatment provided a detailed, site-specific glycosylation analysis that is also applicable to other *S. mansoni* glycoproteins.

## 6. Scope of this thesis

Three major, immunogenic egg glycoproteins have been identified; omega-1, IPSE/ $\alpha$ 1 and kappa-5. The studies in this thesis aim to unravel structural and molecular details of the interaction between these three glycoproteins and innate immune cells of the host, with an emphasis on the role of the glycoprotein glycans in the induction of Th2 responses and granulomogenesis.

To generate a better understanding of the interactions between the host and the parasite, it is essential to unravel the structural details of native glycosylation of omega-1, IPSE/ $\alpha$ 1 and kappa-5. The glycans of IPSE/ $\alpha$ 1 have been already described in detail, as outlined in paragraphs 3.6 and 5.6 of this introduction. In **chapter 2 and 3**, we describe the glycosylation of omega-1 and kappa-5 respectively, using an approach combining mass spectrometric techniques and enzymatic treatments.

To induce or modulate immune responses, egg glycoproteins interact with CLRs on APCs such as DCs and macrophages. It was previously shown that SEA glycoproteins interact with the CLRs DC-SIGN, MGL and MR on immature DCs, leading to internalization of glycoproteins. The involvement of these receptors in the recognition of single, native glycoproteins are described in **chapter 4** (kappa-5 and IPSE/ $\alpha$ 1) and **chapter 5** (omega-1). It is shown that specific glycan motifs on the egg glycoproteins determine differential binding to the CLRs.

The importance of glycans in the induction of *S. mansoni* egg-induced Th2 responses and periovular granuloma formation has been acknowledged for years; however the exact native glycoproteins involved in this process remained unknown. Recently, omega-1 has been identified as the major component of the secretory egg glycoproteins that induces Th2 responses via the conditioning of DCs. In **chapter 5**, the underlying characteristics of omega-1 that drive this immunomodulatory effect are identified. Omega-1 glycosylation as well as its RNase activity are both demonstrated to play critical roles. **Chapter 6** describes the identification of kappa-5 as a granulomogenic compound in SEA. Its extraordinary antenna motifs are shown to mediate in

part this immunogenic effect. **Chapter 3** in addition shows that the glycans of kappa-5 are targets of the IgE response observed in *S. mansoni*-infected individuals.

## References

1. Pearce, E.J., MacDonald, A.S. The immunobiology of schistosomiasis. *Nat Rev Immunol* **2002**, 2(7), 499-511.
2. De Jesus, A.R., Silva, A., Santana, L.B., Magalhaes, A., De Jesus, A.A., De Almeida, R.P., Rego, M.A., Burattini, M.N., Pearce, E.J., Carvalho, E.M. Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni. *J Infect Dis* **2002**, 185(1), 98-105.
3. Doherty, J.F., Moody, A.H., Wright, S.G. Katayama fever: an acute manifestation of schistosomiasis. *BMJ* **1996**, 313(7064), 1071-1072.
4. Vella, A.T., Pearce, E.J. CD4+ Th2 response induced by *Schistosoma mansoni* eggs develops rapidly, through an early, transient, Th0-like stage. *J Immunol* **1992**, 148(7), 2283-2290.
5. Jankovic, D., Kullberg, M.C., Caspar, P., Sher, A. Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. *J Immunol* **2004**, 173(4), 2419-2427.
6. Gonnert, R., Andrews, P. Praziquantel, a new broad-spectrum antischistosomal agent. *Z Parasitenkd* **1977**, 52(2), 129-150.
7. Pax, R., Bennett, J.L., Fetterer, R. A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma japonicum*. Naunyn Schmiedebergs *Arch Pharmacol* **1978**, 304(3), 309-315.
8. Doenhoff, M.J., Hagan, P., Cioli, D., Southgate, V., Pica-Mattoccia, L., Botros, S., Coles, G., Tchuente, L.A., Mbaye, A., Engels, D. Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. *Parasitology* **2009**, 136(13), 1825-1835.
9. Varki, A., Esko, J.D., Colley, K.J. Cellular Organization of Glycosylation. **2009**.
10. Khoo, K.H., Chatterjee, D., Caulfield, J.P., Morris, H.R., Dell, A. Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences. *Glycobiology* **1997**, 7(5), 663-677.
11. Khoo, K.H., Huang, H.H., Lee, K.M. Characteristic structural features of schistosome cercarial N-glycans: expression of Lewis X and core xylosylation. *Glycobiology* **2001**, 11(2), 149-163.
12. Wuhler, M., Koeleman, C.A., Deelder, A.M., Hokke, C.H. Repeats of LacdiNAc and fucosylated LacdiNAc on N-glycans of the human parasite *Schistosoma mansoni*. *FEBS J* **2006**, 273(2), 347-361.
13. Hokke, C.H., Deelder, A.M., Hoffmann, K.F., Wuhler, M. Glycomics-driven discoveries in schistosome research. *Exp Parasitol* **2007**, 117(3), 275-283.
14. Jang-Lee, J., Curwen, R.S., Ashton, P.D., Tissot, B., Mathieson, W., Panico, M., Dell, A., Wilson, R.A., Haslam, S.M. Glycomics analysis of *Schistosoma mansoni* egg and cercarial secretions. *Mol Cell Proteomics* **2007**, 6(9), 1485-1499.
15. Huang, H.H., Tsai, P.L., Khoo, K.H. Selective expression of different fucosylated epitopes on two distinct sets of *Schistosoma mansoni* cercarial O-glycans: identification of a novel core type and Lewis X structure. *Glycobiology* **2001**, 11(5), 395-406.
16. Khoo, K.H., Sarda, S., Xu, X., Caulfield, J.P., McNeil, M.R., Homans, S.W., Morris, H.R., Dell, A. A unique multifucosylated -3GalNAc beta 1->4GlcNAc beta 1->3Gal alpha 1- motif constitutes the repeating unit of the complex O-glycans derived from the cercarial glycocalyx of *Schistosoma mansoni*. *J Biol Chem* **1995**, 270(29), 17114-17123.
17. Wuhler, M., Kantelhardt, S.R., Dennis, R.D., Doenhoff, M.J., Lochnit, G., Geyer, R. Characterization of glycosphingolipids from *Schistosoma mansoni* eggs carrying Fuc(alpha1-3)GalNAc-, GalNAc(beta1-4)[Fuc(alpha1-3)]GlcNAc- and Gal(beta1-4)[Fuc(alpha1-3)]GlcNAc- (Lewis X) terminal structures. *Eur J Biochem* **2002**, 269(2), 481-493.

18. Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Lochnit, G., Geyer, R. Schistosoma mansoni cercarial glycolipids are dominated by Lewis X and pseudo-Lewis Y structures. *Glycobiology* **2000**, 10(1), 89-101.
19. Khoo, K.H., Chatterjee, D., Caulfield, J.P., Morris, H.R., Dell, A. Structural characterization of glycopingolipids from the eggs of Schistosoma mansoni and Schistosoma japonicum. *Glycobiology* **1997**, 7(5), 653-661.
20. Nyame, A.K., Lewis, F.A., Doughty, B.L., Correa-Oliveira, R., Cummings, R.D. Immunity to schistosomiasis: glycans are potential antigenic targets for immune intervention. *Exp Parasitol* **2003**, 104(1-2), 1-13.
21. Koster, B., Strand, M.. Schistosoma mansoni: immunolocalization of two different fucose-containing carbohydrate epitopes. *Parasitology* **1994**, 108 ( Pt 4), 433-446.
22. Samuelson, J.C., Caulfield, J.P. Cercarial glycocalyx of Schistosoma mansoni activates human complement. *Infect Immun* **1986**, 51(1), 181-186.
23. Machado, A.J., Gazzinelli, G., Pellegrino, J., Dias, D.S. Schistosoma mansoni: the role of the complement C3-activating system in the cercaricidal action of normal serum. *Exp Parasitol* **1975**, 38(1), 20-29.
24. Moser, G., Wassom, D.L., Sher, A. Studies of the antibody-dependent killing of schistosomula of Schistosoma mansoni employing haptenic target antigens. I. Evidence that the loss in susceptibility to immune damage undergone by developing schistosomula involves a change unrelated to the masking of parasite antigens by host molecules. *J Exp Med* **1980**, 152(1), 41-53.
25. Tavares, C.A., Gazzinelli, G., Mota-Santos, T.A., Dias, D.S. Schistosoma mansoni: complement-mediated cytotoxic activity in vitro and effect of de complementation on acquired immunity in mice. *Exp Parasitol* **1978**, 46(2), 145-151.
26. Glauert, A.M., Butterworth, A.E. Morphological evidence for the ability of eosinophils to damage antibody-coated schistosomula. *Trans R Soc Trop Med Hyg* **1977**, 71(5), 392-395.
27. Pancre, V., Cesbron, J.Y., Joseph, M., Barbier, M., Capron, A., Aurialt, C. IgE-dependent killing of Schistosoma mansoni schistosomula by human platelets: modulation by T cell products. *Int Arch Allergy Appl Immunol* **1988**, 87(4), 371-375.
28. Joseph, M., Capron, A., Butterworth, A.E., Sturrock, R.F., Houba, V. Cytotoxicity of human and baboon mononuclear phagocytes against schistosomula in vitro: induction by immune complexes containing IgE and Schistosoma mansoni antigens. *Clin Exp Immunol* **1978**, 33(1), 48-56.
29. Nutten, S., Papin, J.P., Woerly, G., Dunne, D.W., MacGregor, J., Trottein, F., Capron, M. Selectin and Lewis(x) are required as co-receptors in antibody-dependent cell-mediated cytotoxicity of human eosinophils to Schistosoma mansoni schistosomula. *Eur J Immunol* **1999**, 29(3), 799-808.
30. Wuhrer, M., Koeleman, C.A., Fitzpatrick, J.M., Hoffmann, K.F., Deelder, A.M., Hokke, C.H.. Gender-specific expression of complex-type N-glycans in schistosomes. *Glycobiology* **2006**, 16(10), 991-1006.
31. Van Dam, G.J., Bergwerff, A.A., Thomas-Oates, J.E., Rotmans, J.P., Kamerling, J.P., Vliegthart, J.F., Deelder, A.M. The immunologically reactive O-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke Schistosoma mansoni have Lewis x as repeating unit. *Eur J Biochem* **1994**, 225(1), 467-482.
32. Bergwerff, A.A., Van Dam, G.J., Rotmans, J.P., Deelder, A.M., Kamerling, J.P., Vliegthart, J.F. The immunologically reactive part of immunopurified circulating anodic antigen from Schistosoma mansoni is a threonine-linked polysaccharide consisting of --> 6)-(beta-D-GlcpA-(1 --> 3))-(beta-D-GalpNAc-(1 --> repeating units. *J Biol Chem* **1994**, 269(50), :31510-31517.
33. Robijn, M.L., Wuhrer, M., Kornelis, D., Deelder, A.M., Geyer, R., Hokke, C.H. Mapping fucosylated epitopes on glycoproteins and glycolipids of Schistosoma mansoni cercariae, adult worms and eggs. *Parasitology* **2005**, 130(Pt 1), 67-77.
34. Van Stijn, C.M., Meyer, S., Van den Broek, M., Bruijns, S.C., Van Kooyk, Y., Geyer, R., Van Die, I. Schistosoma mansoni worm glycolipids induce an inflammatory phenotype in human dendritic cells by cooperation of TLR4 and DC-SIGN. *Mol Immunol* **2010**, 47(7-8), 1544-1552.
35. Deelder, A.M., De Jonge, N., Fillié, Y.E., Kornelis, D., Helaha, D., Qian, Z.L., De Calumé, P., Polderman, A.M. Quantitative determination of circulating antigens in human schistosomiasis mansoni using an indirect hemagglutination assay. *Am J Trop Med Hyg* **1989**, 40(1), 50-4.

36. Vermeer, H.J., Van Dam, G.J., Halkes, K.M., Kamerling, J.P., Vliegenthart, J.F., Hokke, C.H., Deelder, A.M. Immunodiagnostically applicable monoclonal antibodies to the circulating anodic antigen of *Schistosoma mansoni* bind to small, defined oligosaccharide epitopes. *Parasitol Res* **2003**, 90(4), 330-336.
37. Kariuki, T.M., Farah, I.O., Wilson, R.A., Coulson, P.S. Antibodies elicited by the secretions from schistosome cercariae and eggs are predominantly against glycan epitopes. *Parasite Immunol* **2008**, 30(10), 554-562.
38. Nyame, A.K., Leppanen, A.M., Debose-Boyd, R., Cummings, R.D. Mice infected with *Schistosoma mansoni* generate antibodies to LacdiNAc (GalNAc beta 1-->4GlcNAc) determinants. *Glycobiology* **1999**, 9(10), 1029-1035.
39. Eberl, M., Langermans, J.A., Vervenne, R.A., Nyame, A.K., Cummings, R.D., Thomas, A.W., Coulson, P.S., Wilson, R.A. Antibodies to glycans dominate the host response to schistosome larvae and eggs: is their role protective or subversive? *J Infect Dis* **2001**, 183(8), 1238-1247.
40. Van Roon, A.M., Aguilera, B., Cuenca, F., Van Remoortere, A., Van der Marel, G.A., Deelder, A.M., Overkleeft, H.S., Hokke, C.H. Synthesis and antibody-binding studies of a series of parasite fuco-oligosaccharides. *Bioorg Med Chem* **2005**, 13(10), 3553-3564.
41. Van Remoortere, A., Hokke, C.H., Van Dam, G.J., Van Die, I., Deelder, A.M., Van den Eijnden, D.H. Various stages of *Schistosoma* express Lewis(x), LacdiNAc, GalNAcbeta1-4 (Fucalpha1-3)GlcNAc and GalNAcbeta1-4(Fucalpha1-2Fucalpha1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. *Glycobiology* **2000**, 10(6), 601-609.
42. Van Remoortere, A., Vermeer, H.J., Van Roon, A.M., Langermans, J.A., Thomas, A.W., Wilson, R.A., Van Die, I., Van den Eijnden, D.H., Agoston, K., Kerekgyarto, J., Vliegenthart, J.F., Kamerling, J.P., Van Dam, G.J., Hokke, C.H., Deelder, A.M. Dominant antibody responses to Fucalpha1-3GalNAc and Fucalpha1-2Fucalpha1-3GlcNAc containing carbohydrate epitopes in Pan troglodytes vaccinated and infected with *Schistosoma mansoni*. *Exp Parasitol* **2003**, 105(3-4), 219-225.
43. Naus, C.W., Van Remoortere, A., Ouma, J.H., Kimani, G., Dunne, D.W., Kamerling, J.P., Deelder, A.M., Hokke, C.H. Specific antibody responses to three schistosome-related carbohydrate structures in recently exposed immigrants and established residents in an area of *Schistosoma mansoni* endemicity. *Infect Immun* **2003**, 71(10), 5676-5681.
44. Kantelhardt, S.R., Wuhler, M., Dennis, R.D., Doenhoff, M.J., Bickle, Q., Geyer, R. Fuc(alpha1-->3)GalNAc: the major antigenic motif of *Schistosoma mansoni* glycolipids implicated in infection sera and keyhole-limpet haemocyanin cross-reactivity. *Biochem J* **2002**, 366(Pt 1), 217-223.
45. De Boer, A.R., Hokke, C.H., Deelder, A.M., Wuhler, M. Serum antibody screening by surface plasmon resonance using a natural glycan microarray. *Glycoconj J* **2008**, 25(1), 75-84.
46. Ko, A.I., Drager, U.C., Harn, D.A. A *Schistosoma mansoni* epitope recognized by a protective monoclonal antibody is identical to the stage-specific embryonic antigen 1. *Proc Natl Acad Sci U S A* **1990**, 87(11), 4159-4163.
47. Bickle, Q.D., Andrews, B.J., Taylor, M.G. *Schistosoma mansoni*: characterization of two protective monoclonal antibodies. *Parasite Immunol* **1986**, 8(1), 95-107.
48. Neill, P.J., Smith, J.H., Doughty, B.L., Kemp, M. The ultrastructure of the *Schistosoma mansoni* egg. *Am J Trop Med Hyg* **1988**, 39(1), 52-65.
49. Ashton, P.D., Harrop, R., Shah, B., Wilson, R.A. The schistosome egg: development and secretions. *Parasitology* **2001**, 122(Pt 3), 329-338.
50. Pearce, E.J. Priming of the immune response by schistosome eggs. *Parasite Immunol* **2005**, 27(7-8), 265-270.
51. Asahi, H., Hernandez, H.J., Stadecker, M.J. A novel 62-kilodalton egg antigen from *Schistosoma mansoni* induces a potent CD4(+) T helper cell response in the C57BL/6 mouse. *Infect Immun* **1999**, 67(4), 1729-1735.
52. Schramm, G., Falcone, F.H., Gronow, A., Haisch, K., Mamat, U., Doenhoff, M.J., Oliveira, G., Galle, J., Dahinden, C.A., Haas, H. Molecular characterization of an interleukin-4-inducing factor from *Schistosoma mansoni* eggs. *J Biol Chem* **2003**, 278(20), 18384-18392.
53. Donnelly, S., Stack, C.M., O'Neill, S.M., Sayed, A.A., Williams, D.L., Dalton, J.P. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. *FASEB J* **2008**, 22(11), 4022-4032.

- 
54. Everts, B., Perona-Wright, G., Smits, H.H., Hokke, C.H., Van der Ham, A.J., Fitzsimmons, C.M., Doenhoff, M.J., Van der Broek, J., Mohrs, K., Haas, H., Mohrs, M., Yazdanbakhsh, M., Schramm, G. Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. *J Exp Med* **2009**, 206(8), 1673-1680.
55. Steinfelder, S., Andersen, J.F., Cannons, J.L., Feng, C.G., Joshi, M., Dwyer, D., Caspar, P., Schwartzberg, P.L., Sher, A., Jankovic, D. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med* **2009**, 206(8), 1681-1690.
56. De Jong, E.C., Vieira, P.L., Kalinski, P., Schuitmaker, J.H., Tanaka, Y., Wierenga, E.A., Yazdanbakhsh, M., Kapsenberg, M.L. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. *J Immunol* **2002**, 168(4), 1704-1709.
57. Kane, C.M., Cervi, L., Sun, J., McKee, A.S., Masek, K.S., Shapira, S., Hunter, C.A., Pearce, E.J. Helminth antigens modulate TLR-initiated dendritic cell activation. *J Immunol* **2004**, 173(12), 7454-7461.
58. Cervi, L., MacDonald, A.S., Kane, C., Dzierszinski, F., Pearce, E.J. Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J Immunol* **2004**, 172(4), 2016-2020.
59. Van Liempt, E., Van Vliet, S.J., Engering, A., Garcia Vallejo, J.J., Bank, C.M., Sanchez-Hernandez, M., Van Kooyk, Y., Van Die, I. *Schistosoma mansoni* soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Mol Immunol* **2007**, 44(10), 2605-2615.
60. MacDonald, A.S., Straw, A.D., Bauman, B., Pearce, E.J. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. *J Immunol* **2001**, 167(4), 1982-1988.
61. Breuilh, L., Vanhoutte, F., Fontaine, J., Van Stijn, C.M., Tillie-Leblond, I., Capron, M., Faveeuw, C., Jouault, T., Van Die, I., Gosset, P., Trottein, F. Galectin-3 modulates immune and inflammatory responses during helminthic infection: impact of galectin-3 deficiency on the functions of dendritic cells. *Infect Immun* **2007**, 75(11), 5148-5157.
62. Mathieson, W., Wilson, R.A. A comparative proteomic study of the undeveloped and developed *Schistosoma mansoni* egg and its contents: the miracidium, hatch fluid and secretions. *Int J Parasitol* **2010**, 40(5), 617-628.
63. Chensue, S.W., Warmington, K., Ruth, J., Lincoln, P., Kuo, M.C., Kunkel, S.L. Cytokine responses during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation. Production of Th1 and Th2 cytokines and relative contribution of tumor necrosis factor. *Am J Pathol* **1994**, 145(5), 1105-1113.
64. Van Marck, E.A., Stocker, S., Grimaud, J.A., Kestens, L., Gigase, P.L., Deelder, A.M. The implantation of sepharose beads in mouse livers as an aid in the study of hepatic schistosomal fibrosis. *Experientia* **1980**, 36(9), 1116-1118.
65. Van de Vijver, K.K., Hokke, C.H., Van Remoortere, A., Jacobs, W., Deelder, A.M., Van Marck, E.A. Glycans of *Schistosoma mansoni* and keyhole limpet haemocyanin induce hepatic granulomas in vivo. *Int J Parasitol* **2004**, 34(8), 951-961.
66. Okano, M., Satoskar, A.R., Nishizaki, K., Abe, M., Harn, D.A., Jr. Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on *Schistosoma mansoni* egg antigens. *J Immunol* **1999**, 163(12), 6712-6717.
67. Faveeuw, C., Angeli, V., Fontaine, J., Maliszewski, C., Capron, A., Van Kaer, L., Moser, M., Capron, M., Trottein, F. Antigen presentation by CD1d contributes to the amplification of Th2 responses to *Schistosoma mansoni* glycoconjugates in mice. *J Immunol* **2002**, 169(2), 906-912.
68. Okano, M., Satoskar, A.R., Nishizaki, K., Harn, D.A., Jr. Lacto-N-fucopentaose III found on *Schistosoma mansoni* egg antigens functions as adjuvant for proteins by inducing Th2-type response. *J Immunol* **2001**, 167(1), 442-450.
69. Thomas, P.G., Carter, M.R., Da'Dara, A.A., DeSimone, T.M., Harn, D.A. A helminth glycan induces APC maturation via alternative NF-kappa B activation independent of I kappa B alpha degradation. *J Immunol* **2005**, 175(4), 2082-2090.
70. Wuhler, M., Balog, C.I., Catalina, M.I., Jones, F.M., Schramm, G., Haas, H., Doenhoff, M.J., Dunne, D.W., Deelder, A.M., Hokke, C.H. IPSE/alpha-1, a major secretory glycoprotein antigen from schistosome eggs, expresses the Lewis X motif on core-difucosylated N-glycans. *FEBS J* **2006**, 273(10), 2276-2292.

71. Van de Vijver, K.K., Deelder, A.M., Jacobs, W., Van Marck, E.A., Hokke, C.H. LacdiNAc- and LacNAc-containing glycans induce granulomas in an in vivo model for schistosome egg-induced hepatic granuloma formation. *Glycobiology* **2006**, 16(3), 237-243.
72. Guo, Y., Feinberg, H., Conroy, E., Mitchell, D.A., Alvarez, R., Blixt, O., Taylor, M.E., Weis, W.I., Drickamer, K. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat Struct Mol Biol* **2004**, 11(7), 591-598.
73. Van Vliet, S.J., Van Liempt, E., Saeland, E., Aarnoudse, C.A., Appelmelk, B., Irinura, T., Geijtenbeek, T.B., Blixt, O., Alvarez, R., Van Die, I., Van Kooyk, Y. Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells. *Int Immunol* **2005**, 17(5), 661-669.
74. Thomas, P.G., Carter, M.R., Atochina, O., Da'Dara, A.A., Piskorska, D., McGuire, E., Harn, D.A. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *J Immunol* **2003**, 171(11), 5837-5841.
75. Van den Berg, T.K., Honing, H., Franke, N., Van Remoortere, A., Schiphorst, W.E., Liu, F.T., Deelder, A.M., Cummings, R.D., Hokke, C.H., Van Die, I. LacdiNAc-glycans constitute a parasite pattern for galectin-3-mediated immune recognition. *J Immunol* **2004**, 173(3), 1902-1907.
76. Geijtenbeek, T.B., Gringhuis, S.I. Signalling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* **2009**, 9(7), 465-479.
77. Van Die, I., Van Vliet, S.J., Nyame, A.K., Cummings, R.D., Bank, C.M., Appelmelk, B., Geijtenbeek, T.B., Van Kooyk, Y. The dendritic cell-specific C-type lectin DC-SIGN is a receptor for *Schistosoma mansoni* egg antigens and recognizes the glycan antigen Lewis x. *Glycobiology* **2003**, 13(6), 471-478.
78. Meyer, S., Tefsen, B., Imberty, A., Geyer, R., Van Die, I. The C-type lectin L-SIGN differentially recognizes glycan antigens on egg glycosphingolipids and soluble egg glycoproteins from *Schistosoma mansoni*. *Glycobiology* **2007**, 17(10), 1104-1119.
79. Van Liempt, E., Bank, C.M., Mehta, P., Garcia-Vallejo, J.J., Kwar, Z.S., Geyer, R., Alvarez, R.A., Cummings, R.D., Kooyk, Y., Van Die, I. Specificity of DC-SIGN for mannose- and fucose-containing glycans. *FEBS Lett* **2006**, 580(26), 6123-6131.
80. Lee, R.T., Hsu, T.L., Huang, S.K., Hsieh, S.L., Wong, C.H., Lee, Y.C. Survey of Immune-related, Mannose/fucose-binding C-Type Lectin Receptors Reveals Widely Divergent Sugar-binding Specificities. *Glycobiology* **2010**.
81. Gringhuis, S.I., Den Dunnen, J., Litjens, M., Van der Vlist, M., Geijtenbeek, T.B. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and *Helicobacter pylori*. *Nat Immunol* **2009**, 10(10), 1081-1088.
82. Bashirova, A.A., Geijtenbeek, T.B., Van Duijnoven, G.C., Van Vliet, S.J., Eilering, J.B., Martin, M.P., Wu, L., Martin, T.D., Viebig, N., Knolle, P.A., Kewalramani, V.N., Van Kooyk, Y., Carrington, M. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *J Exp Med* **2001**, 193(6), 671-678.
83. Knolle, P.A., Gerken, G. Local control of the immune response in the liver. *Immunol Rev* **2000**, 174, 21-34.
84. Karrar, A., Broome, U., Uzunel, M., Qureshi, A.R., Sumitran-Holgersson, S. Human liver sinusoidal endothelial cells induce apoptosis in activated T cells: a role in tolerance induction. *Gut* **2007**, 56(2), 243-252.
85. Park, C.G., Takahara, K., Umemoto, E., Yashima, Y., Matsubara, K., Matsuda, Y., Clausen, B.E., Inaba, K., Steinman, R.M. Five mouse homologues of the human dendritic cell C-type lectin, DC-SIGN. *Int Immunol* **2001**, 13(10), 1283-1290.
86. Powlesland, A.S., Ward, E.M., Sadhu, S.K., Guo, Y., Taylor, M.E., Drickamer, K. Widely divergent biochemical properties of the complete set of mouse DC-SIGN-related proteins. *J Biol Chem* **2006**, 281(29), 20440-20449.
87. Koppel, E.A., Ludwig, I.S., Appelmelk, B.J., Van Kooyk, Y., Geijtenbeek, T.B. Carbohydrate specificities of the murine DC-SIGN homologue mSIGNR1. *Immunobiology* **2005**, 210(2-4), 195-201.
88. Galustian, C., Park, C.G., Chai, W., Kiso, M., Bruening, S.A., Kang, Y.S., Steinman, R.M., Feizi, T. High and low affinity carbohydrate ligands revealed for murine SIGN-R1 by carbohydrate array and cell binding approaches, and differing specificities for SIGN-R3 and langerin. *Int Immunol* **2004**, 16(6), 853-866.

89. Saunders, S.P., Walsh, C.M., Barlow, J.L., Mangan, N.E., Taylor, P.R., McKenzie, A.N., Smith, P., Fallon, P.G. The C-type lectin SIGNR1 binds *Schistosoma mansoni* antigens in vitro, but SIGNR1-deficient mice have normal responses during schistosome infection. *Infect Immun* **2009**, 77(1), 399-404.
90. Tanne, A., Ma, B., Boudou, F., Tailleux, L., Botella, H., Badell, E., Levillain, F., Taylor, M.E., Drickamer, K., Nigou, J., Dobos, K.M., Puzo, G., Vestweber, D., Wild, M.K., Marcinko, M., Sobieszczuk, P., Stewart, L., Lebus, D., Gicquel, B., Neyrolles, O. A murine DC-SIGN homologue contributes to early host defense against *Mycobacterium tuberculosis*. *J Exp Med* **2009**, 206(10), 2205-2220.
91. Geijtenbeek, T.B., Torensma, R., Van Vliet, S.J., Van Duijnhoven, G.C., Adema, G.J., Van Kooyk, Y., Figdor, C.G. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **2000**, 100(5), 575-585.
92. Nagaoka, K., Takahara, K., Minamino, K., Takeda, T., Yoshida, Y., Inaba, K. Expression of C-type lectin, SIGNR3, on subsets of dendritic cells, macrophages, and monocytes. *J Leukoc Biol* **2010**, 88(5), 913-924.
93. Taylor, M.E., Bezouska, K., Drickamer, K. Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannose receptor. *J Biol Chem* **1992**, 267(3), 1719-1726.
94. Harris, N., Super, M., Rits, M., Chang, G., Ezekowitz, R.A. Characterization of the murine macrophage mannose receptor: demonstration that the downregulation of receptor expression mediated by interferon-gamma occurs at the level of transcription. *Blood* **1992**, 80(9), 2363-2373.
95. Linehan, S.A. The mannose receptor is expressed by subsets of APC in non-lymphoid organs. *BMC Immunol* **2005**, 6(1), 4.
96. Higashi, N., Fujioka, K., Denda-Nagai, K., Hashimoto, S., Nagai, S., Sato, T., Fujita, Y., Morikawa, A., Tsuiji, M., Miyata-Takeuchi, M., Sano, Y., Suzuki, N., Yamamoto, K., Matsushima, K., Irimura, T. The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. *J Biol Chem* **2002**, 277(23), 20686-20693.
97. Tsuiji, M., Fujimori, M., Ohashi, Y., Higashi, N., Onami, T.M., Hedrick, S.M., Irimura, T. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. *J Biol Chem* **2002**, 277(32), 28892-28901.
98. Singh, S.K., Streng-Ouwehand, I., Litjens, M., Weelij, D.R., Garcia-Vallejo, J.J., Van Vliet, S.J., Saeland, E., Van Kooyk, Y. Characterization of murine MGL1 and MGL2 C-type lectins: distinct glycan specificities and tumor binding properties. *Mol Immunol* **2009**, 46(6), 1240-1249.
99. Robijn, M.L., Koeleman, C.A., Wuhrer, M., Royle, L., Geyer, R., Dwek, R.A., Rudd, P.M., Deelder, A.M., Hokke, C.H. Targeted identification of a unique glycan epitope of *Schistosoma mansoni* egg antigens using a diagnostic antibody. *Mol Biochem Parasitol* **2007**, 151(2), 148-161.
100. Nyame, A.K., Debose-Boyd, R., Long, T.D., Tsang, V.C., Cummings, R.D. Expression of Lex antigen in *Schistosoma japonicum* and *S.haematobium* and immune responses to Lex in infected animals: lack of Lex expression in other trematodes and nematodes. *Glycobiology* **1998**, 8(6), 615-624.
101. Haslam, S.M., Coles, G.C., Morris, H.R., Dell, A. Structural characterization of the N-glycans of *Dictyocaulus viviparus*: discovery of the Lewis(x) structure in a nematode. *Glycobiology* **2000**, 10(2), 223-229.
102. Schramm, G., Gronow, A., Knobloch, J., Wippersteg, V., Grevelding, C.G., Galle, J., Fuller, H., Stanley, R.G., Chiodini, P.L., Haas, H., Doenhoff, M.J. IPSE/alpha-1: a major immunogenic component secreted from *Schistosoma mansoni* eggs. *Mol Biochem Parasitol* **2006**, 147(1), 9-19.
103. Williams, D.L., Asahi, H., Oke, T.T., da Rosa, J.L., Stadecker, M.J. Murine immune responses to a novel schistosome egg antigen, SmEP25. *Int J Parasitol* **2005**, 35(8), 875-882.
104. Smith, P., Fallon, R.E., Mangan, N.E., Walsh, C.M., Saraiva, M., Sayers, J.R., McKenzie, A.N., Alcamí, A., Fallon, P.G. *Schistosoma mansoni* secretes a chemokine binding protein with antiinflammatory activity. *J Exp Med* **2005**, 202(10), 1319-1325.
105. Cass, C.L., Johnson, J.R., Califf, L.L., Xu, T., Hernandez, H.J., Stadecker, M.J., Yates, J.R., III, Williams, D.L. Proteomic analysis of *Schistosoma mansoni* egg secretions. *Mol Biochem Parasitol* **2007**, 155(2), 84-93.
106. Schramm, G., Mohrs, K., Wodrich, M., Doenhoff, M.J., Pearce, E.J., Haas, H., Mohrs, M. Cutting edge: IPSE/alpha-1, a glycoprotein from *Schistosoma mansoni* eggs, induces IgE-dependent, antigen-independent IL-4 production by murine basophils in vivo. *J Immunol* **2007**, 178(10), 6023-6027.

107. Fitzsimmons, C.M., Schramm, G., Jones, F.M., Chalmers, I.W., Hoffmann, K.F., Grevelding, C.G., Wuhrer, M., Hokke, C.H., Haas, H., Doenhoff, M.J., Dunne, D.W. Molecular characterization of omega-1: a hepatotoxic ribonuclease from *Schistosoma mansoni* eggs. *Mol Biochem Parasitol* **2005**, 144(1), 123-127.
108. Schramm, G., Hamilton, J.V., Balog, C.I., Wuhrer, M., Gronow, A., Beckmann, S., Wippersteg, V., Grevelding, C.G., Goldmann, T., Weber, E., Brattig, N.W., Deelder, A.M., Dunne, D.W., Hokke, C.H., Haas, H., Doenhoff, M.J. Molecular characterisation of kappa-5, a major antigenic glycoprotein from *Schistosoma mansoni* eggs. *Mol Biochem Parasitol* **2009**, 166(1), 4-14.
109. Robijn, M.L., Koeleman, C.A., Hokke, C.H., Deelder, A.M. *Schistosoma mansoni* eggs excrete specific free oligosaccharides that are detectable in the urine of the human host. *Mol Biochem Parasitol* **2007**, 151(2), 162-172.
110. Van der Klei, D., Van Remoortere, A., Schuitemaker, J.H., Kapsenberg, M.L., Deelder, A.M., Tielens, A.G., Hokke, C.H., Yazdanbakhsh, M. Triggering of innate immune responses by schistosome egg glycolipids and their carbohydrate epitope GalNAc beta 1-4(Fuc alpha 1-2Fuc alpha 1-3)GlcNAc. *J Infect Dis* **2002**, 185(4), 531-539.
111. Dewalick, S., Bexkens, M.L., Van Balkom, B.W., Wu, Y.P., Smit, C.H., Hokke, C.H., De Groot, P.G., Heck, A.J., Tielens, A.G., Van Hellemond, J.J. The proteome of the insoluble *Schistosoma mansoni* eggshell skeleton. *Int J Parasitol* **2011**, 41(5), 523-532.
112. Taylor, M.E., Drickamer, K. Structural requirements for high affinity binding of complex ligands by the macrophage mannose receptor. *J Biol Chem* **1993**, 268(1), 399-404.
113. Mitchell, D.A., Fadden, A.J., Drickamer, K. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. *J Biol Chem* **2001**, 276(31), 28939-28945.
114. Lozach, P.Y., Lortat-Jacob, H., De Lacroix, L., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J.L., Arenzana-Seisdedos, F., Altmeyer, R. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* **2003**, 278(22), 20358-20366.
115. Cambi, A., De Lange, F., Van Maarseveen, N.M., Nijhuis, M., Joosten, B., Van Dijk, E.M., De Bakker, B.I., Franssen, J.A., Bovee-Geurts, P.H., Van Leeuwen, F.N., Van Hulst, N.F., Figdor, C.G. Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. *J Cell Biol* **2004**, 164(1), 145-155.
116. De Bakker, B.I., De Lange, F., Cambi, A., Korterik, J.P., Van Dijk, E.M., Van Hulst, N.F., Figdor, C.G., Garcia-Parajo, M.F. Nanoscale organization of the pathogen receptor DC-SIGN mapped by single-molecule high-resolution fluorescence microscopy. *Chemphyschem* **2007**, 8(10), 1473-1480.
117. Geyer, H., Geyer, R. Strategies for analysis of glycoprotein glycosylation. *Biochim Biophys Acta* **2006**, 1764(12), 1853-1869.
118. Huang, Y., Mechref, Y., Novotny, M.V. Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis. *Anal Chem* **2001**, 73(24), 6063-6069.
119. Karlsson, N.G., Packer, N.H. Analysis of O-linked reducing oligosaccharides released by an in-line flow system. *Anal Biochem* **2002**, 305(2), 173-185.
120. Zaia, J. Mass spectrometry and glycomics. *OMICS* **2010**, 14(4), 401-418.
121. Harvey, D.J. Analysis of carbohydrates and glycoconjugates by matrix-assisted laser desorption/ionization mass spectrometry: an update for the period 2005-2006. *Mass Spectrom Rev* **2011**, 30(1), 1-100.
122. Karas, M., Bahr, U., Dulcks, T. Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine. *Fresenius J Anal Chem* **2000**, 366(6-7), 669-676.
123. Harvey, D.J. Proteomic analysis of glycosylation: structural determination of N- and O-linked glycans by mass spectrometry. *Expert Rev Proteomics* **2005**, 2(1), 87-101.
124. Cortes, D.F., Kabulski, J.L., Lazar, A.C., Lazar, I.M. Recent advances in the MS analysis of glycoproteins: Capillary and microfluidic workflows. *Electrophoresis* **2011**, 32(1), 14-29.
125. Kirsch, S., Bindila, L. Nano-LC and HPLC-chip-ESI-MS: an emerging technique for glycobioanalysis. *Bioanalysis* **2009**, 1(7), 1307-1327.
126. Li, B., An, H.J., Hedrick, J.L., Lebrilla, C.B. Collision-induced dissociation tandem mass spectrometry for structural elucidation of glycans. *Methods Mol Biol* **2009**, 534, 133-145.



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127. Wührer, M., Catalina, M.I., Deelder, A.M., Hokke, C.H. Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J Chromatogr B Analyt Technol Biomed Life Sci* **2007**, 849(1-2), 115-128.