Schistosoma mansoni egg glycoproteins:

glycan structures & host immune responses

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

Moniek HJ Meevissen, Maria Yazdanbakhsh and Cornelis H Hokke

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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 [1].

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)-γ in peripheral-blood mononuclear cells (PBMCs), as well as upregulation of serum levels of tumor-necrosis factor (TNF)-α [2]. 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 [3]. 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 [4;5]. 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 [1]. 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 [6]. 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 [7]. 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 [8]. 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.

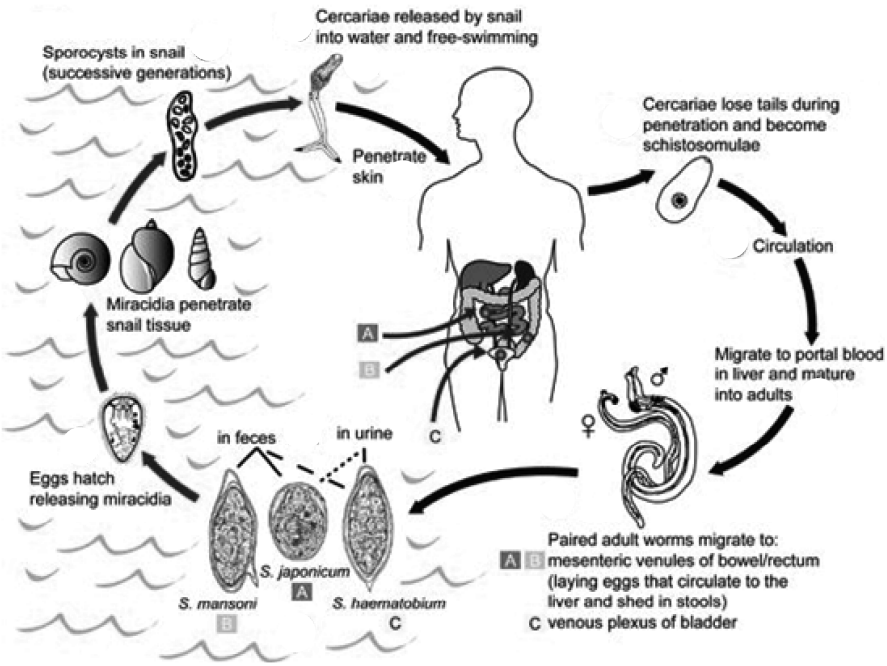


Figure 1 Life cycle of schistosomes infecting humans. Cercariae, released from infested water snails, penetrate through the skin of human individuals and shed their tails. The developing schistosomula migrate through the tissue and enter blood vessels via which they are transported to the portal veins, 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 [9]. As is also the case in many other organisms including humans, a *S. mansoni* N-glycan consists of a $Man_3GlcNAc_2$ core that can be decorated with specific core-elements (Table 1) [10-14]. 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 [10;14-16]. 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) [17-19]. 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 [10-12]. 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(Fucα1-3)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 [11;13-15;18]. 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 [11]. Interestingly, the cercarial glycocalyx has been reported to carry complex O-glycans with repeating units of unique multi-fucosylated (Fucα1-2Fucα1-3, DF) LDN motifs [16]. The cercarial glycolipids in addition to LeX express the Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (pseudo-LeY) motif [18], which to date has not been observed in other *S. mansoni* life stages.

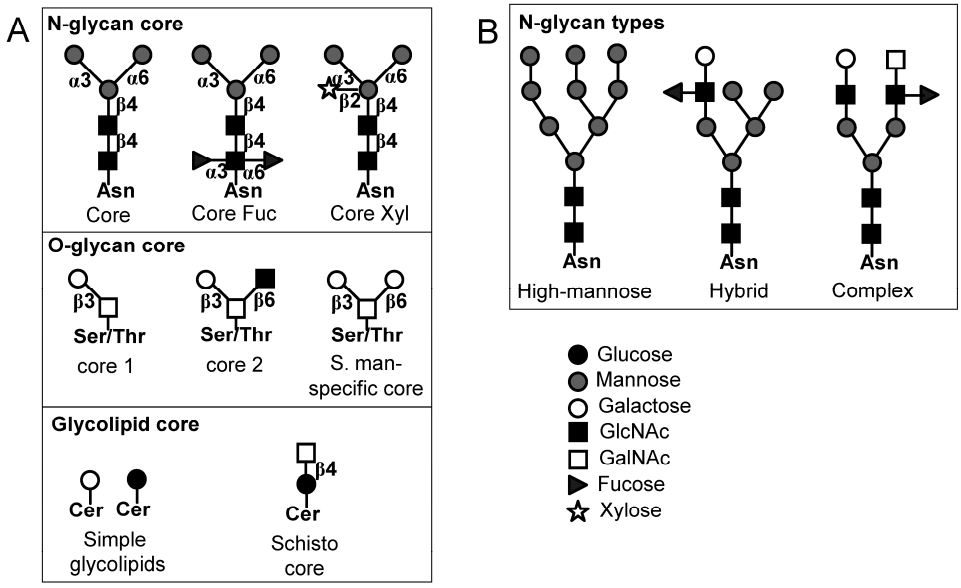


Table 1. *S. mansoni* glycan structures (A) Core structures as present on *S. mansoni* N-glycans, O-glycans and glycolipids (B) N-glycans can be divided into three classes based on the antenna structures. The high-mannose type glycans carry multiple mannose residues. The complex type glycans carry up 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 [13]. 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 GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) on the surface of schistosomula [20;21]. 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 [20;22-25] and antibody-dependent cell mediated cytotoxicity (ADCC) [26-29]. In some instances, the recognition of parasite glycans has been demonstrated to be involved in these immune processes [20;22;29]. The glycocalyx of cercariae seems to activate the alternative pathway of complement binding, leading to efficient killing of a majority of cercariae [22]. Schistosomula on the other hand are less susceptible to killing via this complement pathway [22], but instead can be targeted via the classical pathway, which requires

antigen-specific antibodies for its initiation [20;24;25]. 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 [20]. 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 [26-28]. 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 [29].

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 α3-core fucosylation of N-glycans totally disappears, and LeX structures become less abundant. Instead, N-glycosylation of adult worms is mainly characterized by α6-core fucosylated, mono- and di-antennary glycans terminating with LDN [12;13]. Minor glycan subsets on adult worms include diantennary glycans with mixed LDN, LN and LeX termini as well as linear repeats of these structures [12]. 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 [30]. 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 [12], 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 [31;32]. 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 [33], as well as the presence of LeX [34].

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

fucosylated glycan motifs on the glycolipids as well as DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and TLR4 on DCs [34]. 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 [35]. They are widely used as diagnostic targets in epidemiological studies using specific mAbs that recognize glycan epitopes on these antigens [36].

2.3 Glycoconjugates of eggs and miracidia

The glycan profile of eggs evidently differs from that of adult worms. Within the N-glycan pool, β2-core xylosylation as observed in the cercarial stage re-appears, and a set of α3-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 [10;17]. Another characteristic feature of egg glycans is the occurrence of multi-fucosylated antenna structures containing the Fucα1-2Fucα1-3 motif [10;14;19]. On glycolipids, these motifs are expressed in the form of repeating -4(Fucα1-2Fucα1-3)GlcNAcβ1-units terminating with $(Fuc\alpha1\text{-}2)_{0/1}Fuc\alpha1\text{-}3GalNAc\beta1$- at the non-reducing end.

The N-glycans of miracidia seem to be very similar to the ones found on eggs [13], 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 [13]. 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 [20;37-39]. Antibody responses against many schistosomal motifs including LeX, LDN, F-LDN, LDN-F and LDN-DF and F-GlcNAc [20;39;40] have been found. The most intense antibody responses in humans and primates observed so far are directed against LDN-DF carrying the Fucα1-2Fucα1-3 element and

against Fucα1-3GalNAc motifs [41-44], 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 [45].

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 [39]. However, certain antibodies against parasite glycans have been reported to confer protection in animal models [46;47], as well as induce protective responses *in vitro* [20].

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) [48;49]. 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 [50]. 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 [48;49], 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 [49], 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 [51-55], 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 [54;56-59], and *in vivo*, e.g. by injections into an array of wild type, transgenic and knock-out mice [4;60;61].

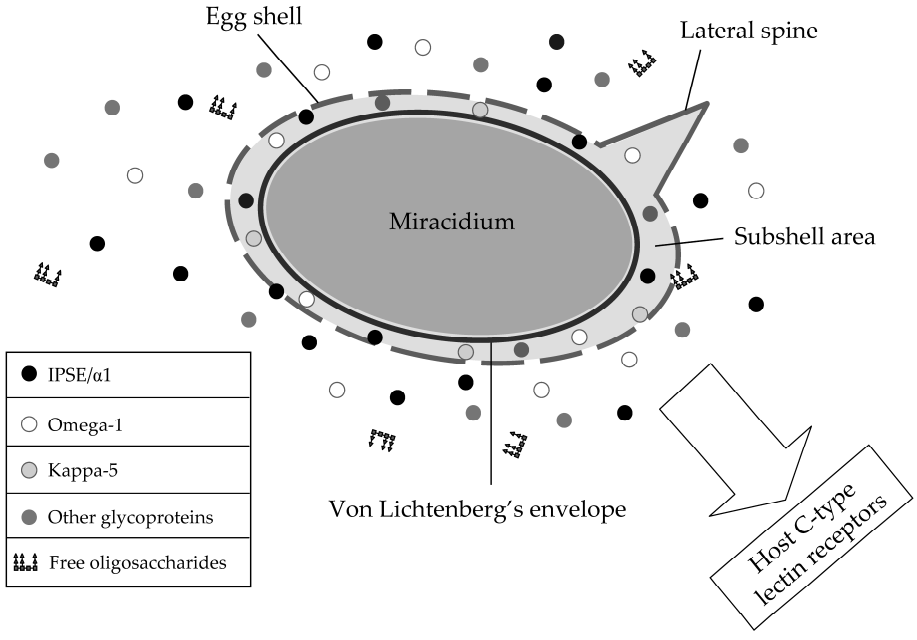


Figure 2. Schematic overview of the mature *S. mansoni* egg and egg components.

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 [49;62]. Although few studies have been conducted on individual SEA glycoproteins, it is clear that many SEA components are glycosylated [1;33]. 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) [10;14]. Each type of glycan or glycan element can be present on a larger subset of SEA glycoproteins [33]. 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 [5;56-60]. While DCs fail to show classic signs of maturation when stimulated with SEA [59;60], *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 [5;56;60]. In accordance with the *in vitro* data, eggs injected into the footpad [4] as well as SEA-primed bone marrow-derived DCs injected intraperitoneally [60] 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 [57;59]. 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 [57;59]. Recently, a single ES glycoprotein omega-1 has been shown to exert similar Th2-inducing and TLR-modulating effects on DCs as does SEA [54;55], as discussed in paragraph 3.6.

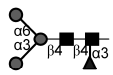
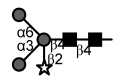
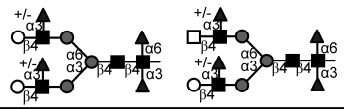
Components of SEA are also suggested to play a role in the initiation and modulation of periovular granulomatous responses observed in host tissues. When eggs lodge into organs such as the liver, host APCs initiate a granulomatous response which is thought to protect the host from overt reactions to 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

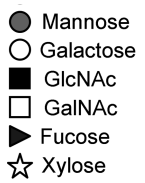
a granulomatous response [63;64], giving rise to granulomas with a comparable cellular composition to those around schistosome eggs [63;65].

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 [66]. 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 [67]. Production of IFN-γ 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 α3-fucose	
Core β2-xylose	
Major IPSE/α1 glycans	



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 [66;68]. Furthermore, a Dextran-LNFPIII conjugate shows similar DC-modulating effects as SEA [69], and at least one glycoprotein carrying glycans terminating with LeX has been found in SEA [70].

SEA glycans may also play a role in the initiation and modulation of periovular 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 [65]. 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 [71].

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) [72;73]. 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 [74], and LDN, a terminal disaccharide element of SEA glycans, is a ligand for the S-type lectin galectin-3 on macrophages [75], 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 [76]).

Many glycan structures found in SEA, including LeX, LDN and (multi-) fucosylated LDN, interact with CLRs (Table 3) [59;73;77;78]. 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 [59]. 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 [59].

Table 3 Recognition of SEA glycans by C-type lectins

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

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

By biochemical binding assays such as ELISA and glycan arrays is has been shown that DC-SIGN binds to many fucosylated motifs including (non-sialylated) Lewis antigens and LDN-F [72;79]. Guo et al. showed that recognition of LeX by DC-SIGN is conferred by binding of the α1-3 fucose in a primary binding site of DC-SIGN, while the terminal galactose binds a secondary binding site [72]. 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, α3- and α6-linked core fucoses [79] as well as LDN-DF [77] 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 [72;80]. 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 [81].

L-SIGN, also named DC-SIGNR, is a DC-SIGN homologue that binds multi-fucosylated motifs as well as mannosylated motifs [72;78]. Interestingly, L-SIGN is expressed on liver sinusoidal endothelial cells (LSECs), but not on DCs and macrophages [82]. 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 [83]. LSECs are suggested to mediate the clearance of antigens from the circulation in a similar manner as DCs do [82;84].

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 [85;86]. 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 [86-88]. SIGNR1 is expressed on murine macrophages and has been shown to bind SEA [89]. Unlike DC-SIGN, SIGNR1 binds sialylated Lewis antigens in addition to unmodified Lewis antigens [87]. Notably, SIGNR1 does not play a critical role during infection, as *S. mansoni*-infected SIGNR1-/- mice show an unaltered phenotype in terms of granulomatous responses and pathology [89]. Moreover, in a pulmonary mouse model, the granuloma volume around in jected eggs as well as levels of IL-4, IL-10 and IFN-γ in lung tissue did not significantly differ between normal and SIGNR1-/- mice [89]. SIGNR3 is reported to more closely resemble the glycan specificity of DC-SIGN [86] and it has been shown to mediate endocytosis and signaling [86;90]. However, while DC-SIGN is abundantly expressed on human DCs [91], SIGNR3 in mice is expressed on small subsets of DCs, macrophages and monocytes in the skin, lungs and lymphoid organs only [92]. 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.

Human MR has a slightly overlapping specificity with DC-SIGN and binds glycans terminating in mannose, fucose and GlcNAc residues [80;93]. 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 [80]. Furthermore, although MR can bind single mannose residues, it binds more complex mannose structures with higher affinity [80;93]. In mice, one clear MR homologue exists which exhibits binding properties comparable to human MR [93]. 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 [94;95].

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 α- and β-linked GalNAc [73]. In mice, two MGL homologues are found, named mMGL1 and mMGL2 [96;97]. 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 [97;98].

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 [59] 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 [77]. Moreover, LDN and LDN-F have been shown to be binding ligands for MGL in SEA [73], 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 [14]. 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 [100;101].

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/α1 [102] (also named SmEP25 [103] / SmCKBP [104]) is a major constituent of ES [62;105] and for the most part carries diantennary N-glycans of the complex-type [70]. 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 α3/α6 difucosylation. IPSE/α1 has been shown to exhibit Th2-regulatory properties, as it is capable of inducing IgE-dependent IL-4 in human basophils [52;102] and an innate IL-4 production by murine basophils [106]. However, non-glycosylated recombinant IPSE/α1 induced similar effects on basophils as native IPSE/α1 [106], indicating that the LeX glycans of IPSE/α1 are in this case not playing a critical role. IPSE/α1 also seems to play an anti-inflammatory role during granulomogenesis by reducing cellular infiltration into granulomas [104]. This effect is likely to be explained by the chemokine binding properties of IPSE/α1, as Smith et al. showed that IPSE/α1 can block interaction of chemokines with their host chemokine receptors and inhibit their biological activity *in vivo* [104], indicating that glycans are probably also not involved in the effects of IPSE/α1 on downregulation of granuloma formation.

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

A third major *S. mansoni* egg glycoprotein is kappa-5 [54]. 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 [108]. 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 [108]. It has been demonstrated that kappa-5 is the primary *S.*

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

In addition to glycoproteins, *S. mansoni* eggs also excrete a series of multi-fucosylated free glycans [109], which structurally resemble egg glycolipid glycans [19]. 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 Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) motifs, and these may have a function in the initial immune recognition of schistosome eggs when they enter the liver [78]. 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 [110]. 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 [111], 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 [59]. 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 [93;112]. 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 [80;113-116]. *In vitro*, soluble DC-SIGN molecules can form tetramers stabilized via an α-helical stalk, which has been demonstrated to amplify the specificity for high-mannose type glycans on host molecules [113]. In addition, van Liempt et al. studied DC-

SIGN specificity in a glycan array using a bivalent DC-SIGN-Fc molecule [79]. 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 [115;116]. 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 [115]. 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-

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 [10-14;16]. N-glycans are commonly released using PNGase F and/or PNGase A, as these enzymes provide fast and reliable results [117]. PNGase F releases all N-glycans except those containing a fucose α3-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 α3-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 β-elimination [118;119].

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. trypsine) 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/α1 [70], 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 [120;121].

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 [122;123].

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 [124;125].

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 [121]. CID-induced fragmentation is performed by colliding a selected ion with inert gas molecules [126]. Various other tandem MS techniques have also been developed to elucidate the composition of glycans and glycoproteins, as reviewed in [120;127].

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 [10]. 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. [10], but in addition found previously unidentified, PNGase A sensitive glycans containing unusual triantennary LDN motifs [13].

The glycosylation of the secretory ES glycoproteins, a major immunogenic subset of SEA, was extensively analyzed by Jang-Lee et al. [14]. 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 [70]. 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 $H_4N_5F_4$ 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_1F_2) indicated that the core structure indeed was di-fucosylated. β-galactosidase treatment clearly demonstrated that each of the LN

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/α1 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/α1 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 emphasisis 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/α1 and kappa-5. The glycans of IPSE/α1 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/α1) 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.

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Chapter 2

Structural characterization of glycans on omega-1, a major *Schistosoma mansoni* egg glycoprotein that drives Th2 responses

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Abstract

Soluble egg antigens (SEA) of the human parasite *Schistosoma mansoni* are among the strongest natural stimuli of Th2 responses. Omega-1, a major glycoprotein in SEA, initiates these characteristic Th2 responses through conditioning of dendritic cells (DCs). In view of the reported immunomodulatory potential of SEA glycans, we have investigated omega-1 glycosylation, using an approach combining mass spectrometric (MS) techniques and enzyme treatments at the glycopeptide level. We demonstrate that omega-1 has two fully occupied N-glycosylation sites, each mainly carrying core-difucosylated diantennary glycans with one or more LeX motifs in the antennae. Using a specific approach of nanoscale LC-MS(/MS) and MALDI-TOF(/TOF)-MS in combination with exo-glycosidase treatments of tryptic glycopeptides, we were able to provide a detailed, site-specific glycosylation analysis of a single, native *S. mansoni* glycoprotein. The obtained knowledge of the glycans present on omega-1 contributes to a full understanding of the mode of action of this immunomodulatory glycoprotein.

Introduction

Schistosomiasis, a disease caused by infection with parasitic flatworms, is a major public health burden in many developing countries in the tropics and sub-tropics. Schistosome larvae released from infested water snails penetrate through the skin of the host, and the developing schistosomes migrate towards the mesenteric venules of the bowel (*Schistosoma mansoni* and *S. japonicum*) or the venous plexus of the bladder (*S. haematobium*), where adult female and male worms pair and produce hundreds of eggs per day. Instead of being excreted with the feces or urine to continue the schistosome life cycle, many of the eggs get trapped inside organs where they induce granulomatous immune reactions, the main cause of morbidity associated with schistosomiasis [1].

During *S. mansoni* infection, an initial T helper 1 (Th1)-mediated response to the parasite shifts to a highly Th2-polarized immune response. This shift coincides with the start of egg production by the mature worms, suggesting that egg-derived molecules are capable of inducing the Th2-polarization [2]. Indeed, schistosome eggs [3], soluble egg antigens (SEA) [4;5], and more specifically a subfraction of SEA formed by the excretory/secretory (ES) egg proteins [6] are highly potent inducers of a Th2 immune response. Recently, the major single factor from ES that is responsible for Th2-priming *in vitro* and *in vivo* has been identified as omega-1, a glycoprotein with RNase activity and a major constituent of ES [7;8]. *In vitro,* omega-1 conditions human monocyte-derived dendritic cells (DCs) to drive Th2-polarization, while SEA depleted from omega-1 shows a significantly reduced activity [6;9]. Furthermore, when injected in IL-4 dual reporter mice, omega-1 is sufficient for Th2-induction *in vivo*. Although is has been suggested that the RNase activity of omega-1 may be involved in its capacity to drive Th2-polarization [9], total SEA requires intact glycosylation to be able to induce Th2-type responses including antigen-specific IgE production and induction of IL-4 and IL-10 [5]. Moreover, synthetic conjugates of the Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX) element occurring in SEA have been shown to harbor Th2-promoting properties [10;11]. These observations suggest that glycans or glycan motifs carried by SEA glycoproteins such as omega-1 play a role in the mechanisms by which they exert their Th2-inducing activity.

Schistosome egg glycosylation has been studied extensively, mainly by mass spectrometric analysis of released N- and O-glycans. A wide spectrum of complex and partly stage-specific structures has been found [12-15]. Glycoprotein glycans from SEA comprise high mannose, complex-type and truncated N-glycans which are predominantly α3/α6-difucosylated at the Asn-linked GlcNAc, potentially accompanied by a core-xylose [12]. The non-reducing N-glycan termini are for a large part composed of Galβ1-4GlcNAc (LacNAc, LN), LeX and (mono-

or di-fucosylated) GalNAcβ1-4GlcNAc (LacdiNAc, LDN) motifs [12;13]. Egg glycoprotein O-glycans are mainly based on the mucin-type core 1 (Galβ1-3GalNAc) and core 2 (Galβ1-3(GlcNAcβ1-6)GalNAc) structures, but also a *S. mansoni*-specific Galβ1-3(Galβ1-6)-GalNAc core was found [12,14], while O-glycan termini were mainly composed of LeX, LN and (multi-fucosylated) LDN motifs. Recently, the glycans released from the ES fraction of *S. mansoni* eggs were analyzed, showing that these display N-glycan α3/α6-core fucosylation and terminal LN, LeX and (fucosylated) LDN motifs as main features [15].

Although it is currently debated exactly which proteins make up the ES fraction [16-18] of *S. mansoni* eggs, it is clear that multiple N- and O-glycosylated proteins are present [16]. The presence or absence of specific glycans may confer antigenic properties to individual proteins in ES and influence binding to C-type lectin receptors (CLRs) on immune cells, such as dendritic cell-specific intercellular adhesion molecule-2-grabbing non-integrin (DC-SIGN), mannose receptor (MR) or macrophage galactose-type lectin (MGL) [19-22]. Therefore, it would be appropriate to analyze the glycosylation of ES in a protein-specific fashion, instead of by releasing glycans from the protein mixture and thus losing crucial information about how individual proteins are glycosylated. To date, only in the case of IPSE/α1 [23], another major component of ES, has a protein-specific in-depth glycosylation analysis been carried out [24].

In view of the immunomodulatory properties of omega-1 and the lack of structural information about omega-1 glycans, we performed a detailed analysis of omega-1 glycosylation using nanoscale LC-MS(/MS), and MALDI-TOF(/TOF) MS measurements of tryptic glycopeptides in combination with exo-glycosidase treatments. We show that omega-1 has two fully occupied N-glycosylation sites, which carry mainly core-difucosylated diantennary glycans with one or two terminal LeX motifs. These data provide a molecular basis for investigations on functional properties of omega-1 glycosylation and shed light on the structural details of schistosome egg glycan antigens.

Results

Initial analysis of omega-1 glycosylation

Omega-1 is produced in the subshell area of *S. mansoni* eggs where also IPSE/α1 is produced [8,24]. To investigate whether omega-1, like IPSE/α1, carries LeX motifs [24], purified omega-1 (Figure 1A) was subjected to Western blot analysis. The anti-LeX mAb 128-4F9-A binds to omega-1 (Figure 1B), demonstrating the presence of LeX. Treatment of omega-1 with α-fucosidase to remove terminal α1-3,4-linked fucoses (omega-1 fuco, Figure 1B) strongly reduces binding of the antibody, confirming the presence of the fucosylated LeX motif on omega-1. For subsequent

in depth glycosylation analysis of omega-1, the purified glycoprotein was subjected to SDS-PAGE and the gel piece containing omega-1 was cut, reduced, alkylated and treated with trypsin to generate (glyco)peptides. Extracted (glyco)peptides were subjected to nano-RP-LC-MS analysis. Omega-1 has two concensus glycosylation sites at residues N71 (tryptic peptide Q69-R77) and N176 (tryptic peptide E172-R183) [8]. In the LC chromatogram, based on the observation of the glycosylation marker ions at *m/z* 366 (LN antenna) and *m/z* 512 (LeX antenna), three glycopeptide regions were registered at time windows 7.4-9.3, 14.3-16.9 and 19.3-20.9 minutes (data not shown). The first and second glycopeptide clusters are indistinguishable in terms of glycan heterogeneity and both arise from peptide Q69-R77. Evidently, the glutamine at the amino terminus of the second cluster had undergone cyclization, forming a pyroglutamate, leading to a mass shift of *m/z* - 5.3 $[M+3H]^{3+}$ compared to the glycopeptide cluster with native N-terminal glutamine, and a shift in elution time due to an increase in hydrophobicity upon cyclization. The nano-LC-mass spectrum belonging to the first cluster (site N71) is shown in Figure 2A. The nano-LC-mass spectrum of tryptic glycopeptides containing site N176 which eluted at 19.3-20.9 minutes is shown in Figure 2B.

The glycans of both glycosylation sites are composed of variable numbers of hexoses (H), *N*-acetylhexosamines (N) and fucoses (F), as reflected by corresponding mass differences

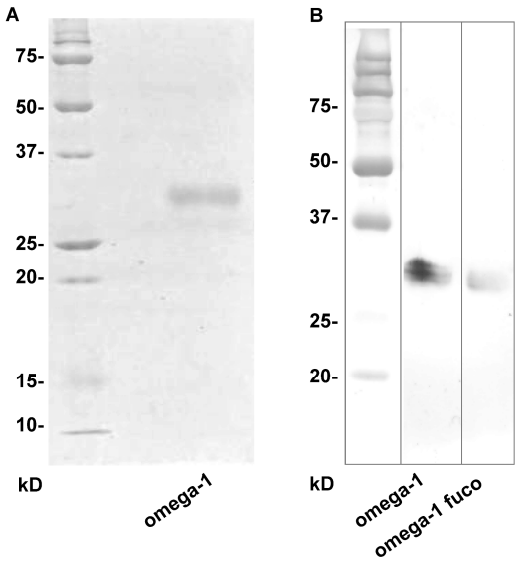


Figure 1. Visualization and characterization of omega-1. Omega-1 was separated under reducing conditions by SDS-PAGE and stained with Colloidal blue (A). For testing the presence of terminal LeX, omega-1 and omega-1 treated with α1-3,4 fucosidase (Omega-1 fuco) were subjected to Western blot using a monoclonal antibody against LeX (128-4F9-A) (B).

(162, 203, and 146 Da, respectively) between the registered glycopeptides. For glycopeptides containing site N71 (Figure 2A), the assumption of a Q69-R77 peptide moiety decorated with an N-glycan leads for the major glycopeptide species ($[M+3H]^{3+}$ at *m/z* 1080.5) to the deduced glycan composition $H_5N_4F_4$. This composition may represent an N-glycan with two LeX antennae and core-difucosylation, which has also been found on IPSE/α1 [15;24]. Other major signals include N-glycans composed of H_5N_4 and H_4N_5 substituted with two to six fucoses, suggesting a high abundance of multi-fucosylated LN and, to a lesser extent, LDN antennae. Tryptic glycopeptides covering site N176 (Figure 2B) were found to contain glycans which were similar in composition to those of site N71. However, ratios of glycan variants were significantly different for the two sites (Figure 2). In general, glycopeptides of site N176 showed a lower degree of fucosylation than those of site N71. Non-glycosylated forms of the peptides Q69-R77 and E172-R183 were not detected by LC-MS, indicating that in both peptides the N-glycosylation sites are fully occupied (data not shown).

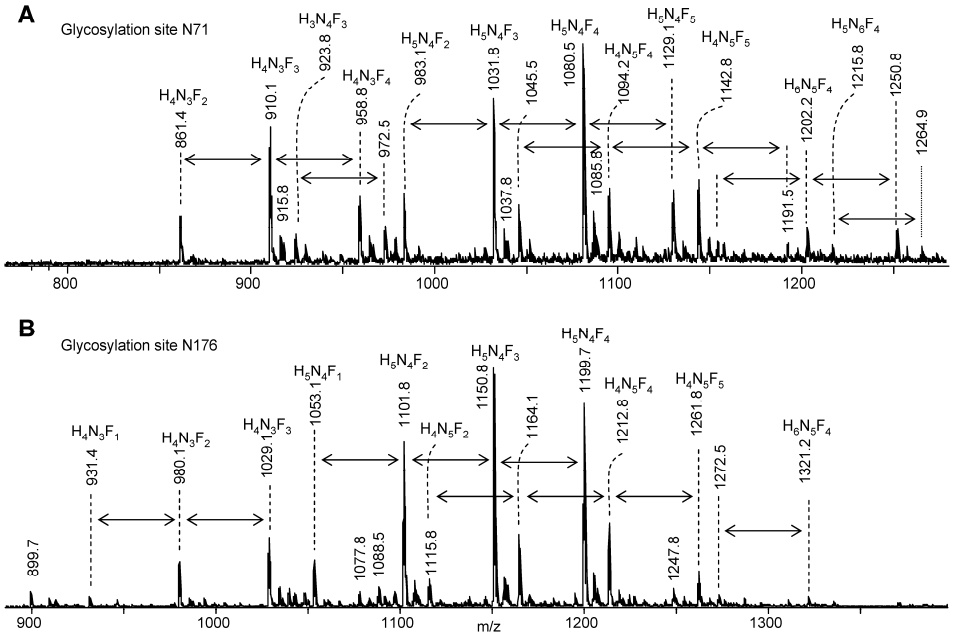


Figure 2. nano-LC-MS of glycopeptides from a tryptic digest of omega-1, covering glycosylation sites N71 (A) and N176 (B). Signals are triple positively charged and labeled with monoisotopic masses. Compositions of the glycan moieties are given in terms of hexose (H), *N*-actetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows.

N-glycosylation analysis of glycopeptide E172-R183

To confirm the tentative assignment of the glycopeptide clusters presented in Figure 2, the tryptic glycopeptides were subjected to enzymatic treatment with β-galactosidase and/or α-fucosidase and analyzed by MALDI-TOF-MS (Figures 3 and 4). Selected untreated and α-fucosidase treated glycopeptides were in addition subjected to fragmentation using MALDI-TOF/TOF-MS to further substantiate the structural assignments. A set of illustrative glycopeptide fragmentation spectra is shown in Figures 5-7 and Supplementary figures 1 and 2. For the other glycopeptides, the fragmentation data are summarized in Table 1 (untreated) and Supplementary table 1 (α-fucosidase treated). First, we confirmed that the glycopeptides assigned to site N176 (Figure 2B, 3A, and 4A) comprised the peptide moiety E172-R183: for the glycopeptide at *m/z* 3594.7 (Figure 3A), a characteristic MALDI-TOF/TOF-MS pattern of fragment ions was observed (Figure 5), including *m/z* 1370.2 (protonated peptide-NH_3), *m/z* 1470.2 (^{0,2}X-ring fragmentation of the innermost *N*-acetylglucosamine), *m/z* 1736.3 (protonated peptide with *N*-acetylglucosamine and one fucose) and *m/z* 1882.2 (protonated peptide with *N*-acetylglucosamine and two fucoses). This indicates a peptide mass of 1387.3 Da $[M + H]^+$ and a glycan moiety of $H_5N_4F_4$. The deduced peptide mass of 1387.3 Da fits the theoretical mass of the tryptic peptide E172-R183 of 1388.6 Da $[M + H]^+$. In addition, peptide fragments in the low mass region provided a GSANCIR sequence tag that further confirms the assignment. For the other fragmented glycopeptides belonging to the peptide moiety E172-R183 containing glycosylation site N176, similar signal patterns were observed (Figures 6 and 7, Supplementary figures 1 and 2 and Table 1). The combined data from enzymatic treatments and MALDI-TOF/TOF-MS analysis showed that $H_5N_4F_4$ on N176 is an N-glycan constructed of a trimannosyl core with two fucoses on the innermost *N*-acetylglucosamine and two fucosylated LN antennae. Based on the reactivity of omega-1 with an anti-LeX antibody (Figure 1B) and previously published data on the glycosylation of ES proteins including IPSE/α1 [15;24], the fucosylated LN antennae were interpreted as LeX motifs. Both enzyme treatments (Figures 3 and 4) and fragmentation data (Figure 5) are consistent with this interpretation. The galactose β1-4 linkage in a LeX trisaccharide is not cleavable by β-galactosidase because of the 3-substitution of GlcNAc by fucose. In line with the assumption that the $H_5N_4F_4$ species contains two LeX antennae, β-galactosidase treatment did not lead to the disappearance of the signal at *m/z* 3594.7 and at the same time no increased signal at *m/z* 3432.6 was observed (Figures 3A and B). On the other hand, when the glycopeptides were treated with α-fucosidase from *Xanthomonas manihotis*, the fucoses in the LeX motif were removed (loss of signals at *m/z* 3594.7 ($H_5N_4F_4$) and *m/z* 3448.6 ($H_5N_4F_3$); Figure 4A and B), yielding a set of signals at *m/z* 3302.7, 3156.1 and 3010.1 from $H_5N_4F_2$, $H_5N_4F_1$ and H_5N_4 glycoforms, respectively (Figure 4B). Treatment with β-

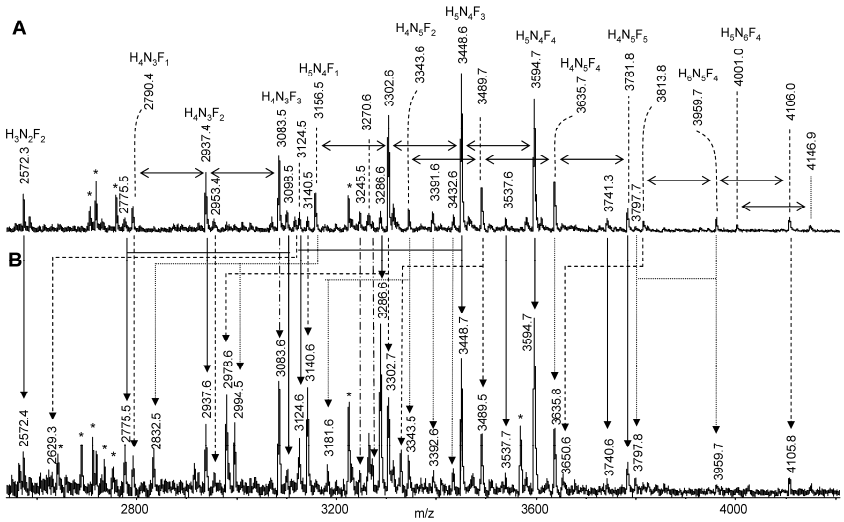


Figure 3. β-galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N176 were analyzed by MALDI-TOF-MS before (A) and after (B) treatment with β-galactosidase. Signals are singly positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-actetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Non-glycopeptide signals are marked with asterisks (*).

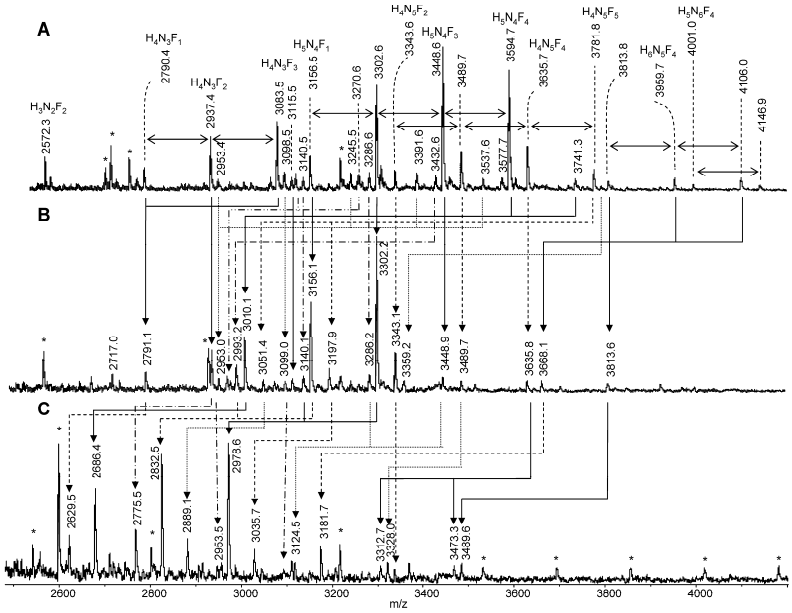


Figure 4. α-fucosidase and β-galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N176 were analyzed by MALDI-TOF-MS before treatment (A), after treatment with α-fucosidase only (B) or in combination with β-galactosidase (C). Signals are singly positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-actetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Non-glycopeptide signals are marked with asterisks (*).

galactosidase subsequent to this fucosidase treatment leads to a complete removal of the antenna galactoses from each of the $H_5N_4F_2$, $H_5N_4F_1$ and H_5N_4 species (shift of two times 162 Da; e.g. the signal at *m/z* 3302.2 in Figure 4B shifts to *m/z* 2978.6 in Figure 4C). The resistance of the $H_5N_4F_4$ glycopeptide to β-galactosidase treatment and the gained susceptibility after α-fucosidase treatment further supports our proposal that the terminal fucosylated structures are LeX motifs. To corroborate this, a MALDI-TOF/TOF-MS fragmentation spectrum of the E172-R183-$H_5N_4F_4$ glycopeptide was recorded (Figure 5) in which the losses of 511 Da and 673 Da (signals at *m/z* 3083.3 and 2920.7) from the parent ion are observed, corresponding to the loss of a LeX ($H_1N_1F_1$) and a LeX-Man fragment ($H_2N_1F_1$), respectively. In addition, a core cleavage pattern with an intense signal at *m/z* 1882.2 (peptide-N_1F_2) (Figure 5) is observed, indicating that two fucose residues in the $H_5N_4F_4$ species are linked to the core GlcNAc residue. The lower intensity signal at *m/z* 1736.3 (peptide-N_1F_1), is interpreted as the result of a chitobiose cleavage in combination with loss of a core fucose. This interpretation was further confirmed by MALDI-TOF/TOF-MS of the E172-R183-$H_5N_4F_2$ glycopeptide species which is formed after the α-fucosidase treatment (Supplementary figure 1). In this spectrum, signals derived from antenna fragmentations are observed at *m/z* 2937.1 and 2774.9 (loss of H_1N_1 and H_2N_1, respectively), but no loss of $H_1N_1F_1$ is observed, indicating that all antenna fucoses had been removed. In addition, the core fragmentation pattern including an intense signal at *m/z* 1882.4 (peptide-N_1F_2) accompanied by a minor signal at *m/z* 1736.4 (loss of one fucose from peptide-N_1F_2), a pattern highly similar to that present in Figure 5, indicates that two core fucoses remain.

A second abundant signal in the MALDI-TOF MS spectrum in Figures 3A and 4A is that of glycopeptide E172-R183-$H_5N_4F_3$. Our data indicate that the $H_5N_4F_3$ species carry two LN antennae and comprise two differentially fucosylated isomers. A fraction of the E172-R183-$H_5N_4F_3$ glycopeptides is resistant to β-galactosidase treatment (part of the signal at *m/z* 3448.6 in Fig. 3A remains in Fig. 3B), which, as previously explained for $H_5N_4F_4$, indicates the presence of two LeX antennae. The other fraction of the glycopeptides exhibited the loss of one hexose after β-galactosidase treatment (loss of 162 Da, resulting in a signal at *m/z* 3286.6 in Figure 3B), which suggests the presence of one non-fucosylated and one fucosylated LN. Based on these results, we suggest that $H_5N_4F_3$ on site N176 consists of two isomeric glycans, one containing two LeX antennae and a mono-fucosylated core, while the other carries one LeX and one LN antenna and two core fucoses. The assumed presence of LeX and LN motifs on the isomers was verified using MALDI-TOF/TOF-MS (Figure 6). The fragmentation spectrum reveals the loss of 511 Da and 673 Da (signals at *m/z* 2937.9 and 2775.5) from the parent ion, corresponding to the loss of a LeX ($H_1N_1F_1$) and a LeX-Man ($H_2N_1F_1$) moiety, respectively. Furthermore, the observed losses of 365 Da and 527 Da (signals at *m/z* 3083.8 and 2921.1) indicate the presence of LN (H_1N_1) and

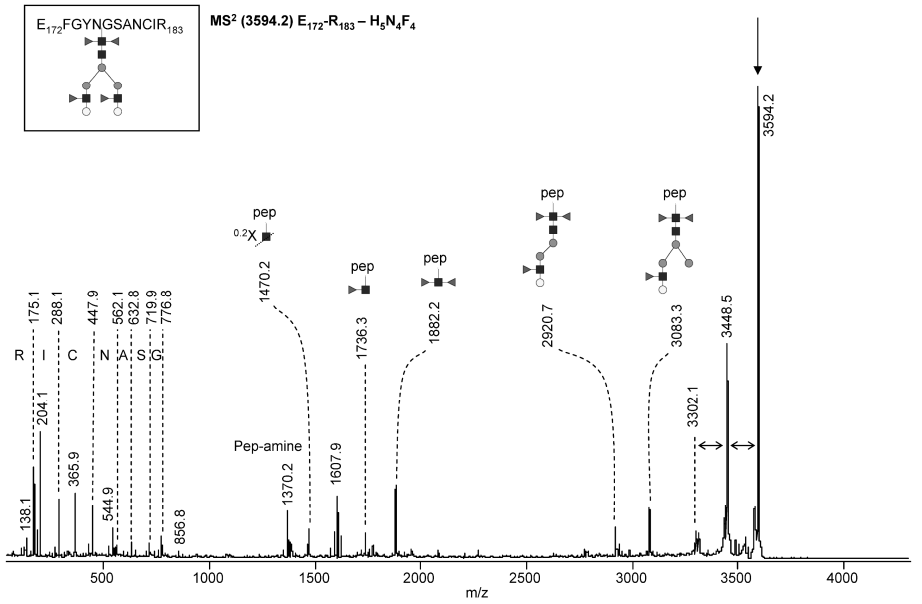


Figure 5. MS/MS of peptide E172–R183 carrying $H_5N_4F_4$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1. The single-headed arrow indicates the precursor ion. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; dark circle, mannose.

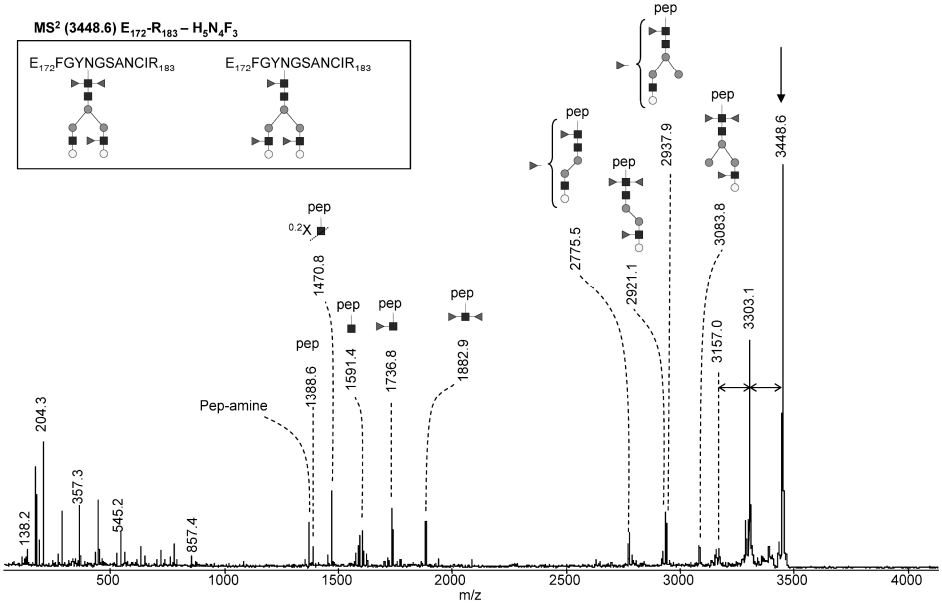


Figure 6. MS/MS of peptide E172–R183 carrying $H_5N_4F_3$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; dark circle, mannose.

LN-Man (H_2N_1) antennae, respectively, in one of the isomers. In addition, a core cleavage pattern is observed (Figure 6) which clearly differs from the pattern characteristic of core-difucosylation (Figure 5 and Supplementary figure 1). In Figure 6, the signal at *m/z* 1736.8 (pep+N_1F_1) is more intense than the signal at *m/z* 1882.9 (pep+N_1F_2). By comparison with the core cleavage patterns in Figure 5 and Supplementary figure 1, the pattern in Figure 6 can only partially be explained as resulting from an isomer with difucosylated core (left structure in Figure 6). The fragment ion at *m/z* 1736.8 (pep+N_1F_1) is in addition formed by chitobiose cleavage of the second isomer having a monofucosylated core (right structure in Figure 6).

The composition of the $H_4N_5F_4$ variant on glycosylation site N176 indicates the presence of an N-glycan with two fucoses at the Asn-linked GlcNAc, combined with one LeX and one fucosylated LDN antenna, LDN being a common structural element in helminth glycans [28]. The $H_4N_5F_4$ fragmentation data (Supplementary figure 2) are in line with this interpretation. Signals derived from the loss of N_2F_1 (*m/z* 3081.8) and $H_1N_1F_1$ (*m/z* 3124.7) from the precursor ion are observed, as well as the characteristic core fragmentation pattern indicative of a difucosylated core GlcNAc. The mass spectrometric data do not allow differentiation between Fucα1-3GalNAcβ1-4GlcNAc (F-LDN) and GalNAcβ1-4(Fucα1–3)GlcNAc (LDN-F) structures. However, using synthetic F-LDN and LDN-F glycoconjugates [29], the α-fucosidase from *Xanthomonas manihotis* was shown to cleave only the fucose α1-3 linked to the GlcNAc in LDN-F, but not the α1-3 linkage between the fucose and GalNAc in F-LDN (data not shown). α-Fucosidase treatment of omega-1 glycopeptides (Figure 4B) results in removal of antenna fucoses from $H_4N_5F_4$, as the signal at *m/z* 3635.7 in Figure 4A almost completely shifts to signals at *m/z* 3051.4, 3197.1 and 3343.1, representing glycans with one LN and one LDN motif and zero, one or two core fucoses, respectively (Supplementary table 1). Similar shifts were observed for other glycans that carry fucosylated LDN based on their monosaccharide composition (Figure 4B). Therefore, we propose that some glycoforms of omega-1 carry LDN-F motifs, but F-LDN motifs do not occur.

Another representative example of a fragmentation is given by the MALDI-TOF/TOF mass spectrum of E172-R183-$H_6N_5F_5$ which carries one LeX and one dimeric LeX antenna accompanied by two fucoses at the core (Figure 7). The presence of the dimeric LeX motif was demonstrated by a high signal at *m/z* 3082.4 after fragmentation, indicative of the loss of two interlinked LeX elements. The results of the successive treatment of α-fucosidase and β-galactosidase support this interpretation. α-Fucosidase treatment of $H_6N_5F_5$ glycopeptides results in the loss of up to three antenna fucoses (signal at *m/z* 4106.0 in Figure 4A shifts to *m/z* 3813.6 and 3668.1 in Figure 4B). After subsequent treatment with β-galactosidase, the signals at *m/z* 3668.1 and 3813.6 (Figure 4B) shift to *m/z* 3343.1 and 3489.6 respectively (Figure 4C), indicative

Table 1. Omega-1 tryptic glycopeptides

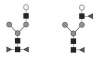
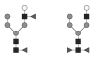
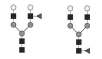
Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
Peptide E_{172}FGY**N**GSANCIR_{18}				
$H_3N_2F_2$	2572.3 (β-gal: 2572.4; -0 Hex) 858.1 $[M+3H]^{3+}$; 1286.6 $[M+2H]^{2+}$	3	1591.4 (pep^1+N_1) 1882.9 ($pep^1+N_1F_2$) FSI-MS/MS: 366.1 (H_1N_1); 528.2 (H_2N_1); 690.3 (H_3N_1)	Trimannosyl core, ^{3,6}F 
$H_3N_3F_2$	2775.5 (β-gal: 2777.5; -0 Hex)	1		HexNAc antenna, ^{3,6}F 
$H_4N_3F_1$	2790.4 (β-gal: 2791.4; -0 Hex, and 2629.3; -1 Hex)	1	1590.6 (pep^1+N_1) 1736.4 ($pep^1+N_1F_1$) 2279.8 ($pep^1+H_3N_2$) -$H_1N_1F_1$	Trimannosyl core, ^{3,6}F 
$H_4N_3F_2$	2937.4 (β-gal: 2937.6; -0 Hex, and 2775.5;-1 Hex); 979.8 $[M+3H]^{3+}$	6	1590.9 (pep^1+N_1) 1737.9 ($pep^1+N_1F_1$; 1.5) 1883.2 ($pep^1+N_1F_2$; 1.0) 2265.0 ($pep^1+H_2N_2F_1$) - $H_2N_1F_1$ 2426.5 ($pep^1+H_3N_2F_1$) - $H_1N_1F_1$ 2572.3 ($pep^1+H_3N_2F_2$) - H_1N_1 ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 868.8 ($pep^1+N_1F_1$; $[M+2H]^{2+}$)	Mixture: 1 Lewis X, monofucosylated core; and: 1 LN, ^{3,6}F  2.5% 3.5%
$H_5N_3F_1$	2953.4 (α-fuc: 2953.0; -0 Fuc)	1		1 LN, mono-fucosylated core 
$H_4N_3F_3$	3083.5 (β-gal: 3083.6; -0 Hex); 1028.5 $[M+3H]^{3+}$	7	1591.4 (pep^1+N_1)1737.2 ($pep^1+N_1F_1$; 0.33)1883.3 ($pep^1+N_1F_2$, 1.0) 2410.8 ($pep^1+H_2N_2F_2$) -$H_2N_1F_1$2426.5 ($pep^1+H_3N_2F_1$) -$H_1N_1F_2$2572.7 ($pep^1+H_3N_2F_2$) -$H_1N_1F_1$ ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 941.9 (pep^1+N1F2; $[M+2H]^{2+}$)	1 Lewis X and ^{3,6}F 
$H_5N_3F_2$	3098.5 (β-gal: 3099.5; -0 Hex, and 2937.6; -1 Hex)	2		Mixture: 1 LN with core and/or antenna fucosylation  0.5% 1.5%
$H_6N_3F_1$	3115.5 (α-fuc: 3115.1; -0 Fuc)	1		1 LN, mono-fucosylated core
$H_3N_4F_3$	3124.5 (α-fuc: 2978.5; -1 Fuc)	2		1 fucosylated LDN, ^{3,6}F
$H_4N_4F_2$	3140.5 (α-fuc: 3140.1; -0 Fuc)	1		1 LN and 1 HexNAc, ^{3,6}F 
$H_5N_4F_1$	3156.5 (β-gal: 2994.5; -1 Hex, and 2832.5; -2 Hex); 1052.8 $[M+3H]^{3+}$	4	1591.4 (pep^1+N_1) 1737.2 ($pep^1+N_1F_1$) 2483.8 ($pep^1+H_3N_3$) -$H_2N_1F_1$ 2629.1 ($pep^1+H_3N_3F_1$) -H_2N_1 2646.4 ($pep^1+H_4N_3$) -$H_1N_1F_1$ 2792.0 ($pep^1+H_4N_3F_1$) -H_1N_1 ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 868.8 ($pep^1+N_1F_1$; $[M+2H]^{2+}$)	Mixture: biantennary LN with one fucoses at the core or antenna  2.5% 1.5%
$H_5N_3F_3$	3245.5 (β-gal: 3245.5; -0 Hex)	2	1881.3 ($pep^1+N_1F_2$)	1 Lewis X, ^{3,6}F 
$H_3N_4F_4$	3270.6 (β-gal: 3270.5; -0 Hex; α-fuc: 2978.5)	1		^{3,6}F
$H_4N_4F_3$	3286.6 (β-gal: 3286.6; -0 Hex, and 3124.6; -1 Hex)	2	1592.0 (pep^1+N_1) 1737.0 ($pep^1+N_1F_1$; 1.0) 1883.3 ($pep^1+N_1F_2$; 1.0) 2630.2 ($pep^1+H_3N_3F_1$) -$H_1N_1F_2$ 2773.7 ($pep^1+H_3N_3F_2$) -$H_1N_1F_1$ 2919.9 ($pep^1+H_3N_3F_3$) -H_1N_1	1 Lewis X or fucosylated GlcNAc, ^{3,6}F  1.0% 1.0%

Table 1. Omega-1 tryptic glycopeptides, continued

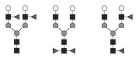
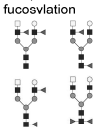
Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
Peptide E_{172}FGY**N**GSANCIR_{18}				
$H_5N_4F_2$	3302.6 (β-gal: 3302.7; -0 Hex, 3140.6: -1 Hex, and 2978.6: -2 Hex); 1101.5 $[M+3H]^{3+}$	12	1592.0 (pep^1+N_1) 1737.0 ($pep^1+N_1F_1$; 1.0) 1883.3 ($pep^1+N_1F_2$; 1.0) 2630.2 ($pep^1+H_3N_3F_1$) -$H_1N_1F_2$ 2773.7 ($pep^1+H_3N_3F_2$) -$H_1N_1F_1$ 2919.9 ($pep^1+H_3N_3F_3$) -H_1N_1	Mixture: biantennary LN with core and/or antenna fucosylation  4.5% 4.0% 3.5%
$H_4N_5F_2$	3343.6 (β-gal: 3343.5; -0 Hex, and 3181.6; -1 Hex)	2	1590.3 (pep^1+N_1) 1736.3 ($pep^1+N_1F_1$; 1.0) 1882.4 ($pep^1+N_1F_2$; 1.0) 2628.7 ($pep^1+H_3N_3F_1$) -$H_2N_1F_1$ 2775.1 ($pep^1+H_3N_3F_2$) -H_2N_1 2789.7 ($pep^1+H_4N_3F_1$) -$H_1N_1F_1$ 2937.1 ($pep^1+H_4N_3F_2$) -H_1N_1 ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 941.9 ($pep^1+N_1F_2$; $[M+2H]^{2+}$)	Mixture: biantennary (1 LN, 1 LDN) with core and/or antenna fucosylation  1.0% 1.0%
$H_5N_3F_4$	3391.6 (β-gal: 3391.6; -0 Hex)	2	1591.3 (pep^1+N_1) 2880.4 ($pep^1+H_4N_3F_3$) -$H_1N_1F_1$ 3080.4 ($pep^1+H_4N_3F_3$) -H_1F_1	1 Lewis X and 1 fucosylated mannose, ^{3,6}F 
$H_4N_4F_4$	3432.6 (β-gal: 3432.6; -0 Hex)	1		1 Lewis X and 1 fucosylated HexNAc, ^{3,6}F 
$H_5N_4F_3$	3448.6 (β-gal: 3448.7; -0 Hex, 3286.6; -1 Hex); 1150.2 $[M+3H]^{3+}$	16	1591.4 (pep^1+N_1) 1736.8 ($pep^1+N_1F_1$; 1.3) 1883.0 ($pep^1+N_1F_2$; 1.0) 2629.4 ($pep^1+H_3N_3F_1$) -$H_2N_1F_2$ 2775.5 ($pep^1+H_3N_3F_2$) -$H_2N_1F_1$ 2789.9 ($pep^1+H_4N_3F_1$) -$H_1N_1F_2$ 2921.1 ($pep^1+H_3N_3F_3$) -H_2N_1 2937.8 ($pep^1+H_4N_3F_2$) -$H_1N_1F_1$ 3083.8 ($pep^1+H_4N_3F_3$) -H_1N_1 ESI MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 942.0 ($pep^1+N_1F_2$; $[M+2H]^{2+}$)	Mixture: biantennary LN with core and antenna fucosylation  8.0% 8.0%
$H_4N_5F_3$	3489.7 (β-gal: 3489.5; -0 Hex, and 3327.7; -1 Hex); 1163.8 $[M+3H]^{3+}$	4	1591.5 (pep^1+N_1) 1736.7 ($pep^1+N_1F_1$; 0.33) 1883.3 ($pep^1+N_1F_2$; 1.0) 2774.8 ($pep^1+H_3N_3F_2$) -$H_1N_2F_1$ 2816.9 ($pep^1+H_2N_4F_2$) -$H_2N_1F_1$ 2937.0 ($pep^1+H_4N_3F_2$) -N_2F_1 2976.9 ($pep^1+H_3N_4F_2$) -$H_1N_1F_1$ 3080.4 ($pep^1+H_4N_3F_3$) -N_2 3124.1 ($pep^1+H_3N_4F_3$) -H_1N_1 ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$); 553.3 (N_2F_1)	Mixture: biantennary (1 LN, l LDN) with one antenna fucose, ^{3,6}F  1.5% 2.5%
$H_5N_3F_5$	3537.6 (β-gal: 3537.7; -0 Hex)	1		Lewis X, ^{3,6}F
$H_5N_4F_4$	3594.7 (β-gal: 3594.7; -0 Hex); 1198.9 $[M+3H]^{3+}$	14	1590.9 (pep^1+N_1) 1736.3 ($pep^1+N_1F_1$; 0.33) 1882.2 ($pep^1+N_1F_2$; 1.0) 2774.2 ($pep^1+H_3N_3F_2$) -$H_2N_1F_2$ 2920.7 ($pep^1+H_3N_3F_3$) -$H_2N_1F_1$ 2936.5 ($pep^1+H_4N_3F_2$) -$H_1N_1F_2$ 3083.3 ($pep^1+H_4N_3F_3$) - H_1N_1F,ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 942.0 ($pep^1+N_1F_2$; $[M+2H]^{2+}$)	2 Lewis X, ^{3,6}F 
$H_4N_5F_4$	3635.7 (β-gal: 3635.8; -0 Hex); 1212.5 $[M+3H]^{3+}$	5	1590.4 (pep^1+N_1) 1736.3 ($pep^1+N_1F_1$; 0.33) 1882.3 ($pep^1+N_1F_2$; 1.0) 2774.3 ($pep^1+H_3N_3F_2$) -$H_1N_2F_2$ 2920.8 ($pep^1+H_3N_3F_3$) -$H_1N_2F_1$ 2936.5 ($pep^1+H_4N_3F_2$) -N_2F_2 3081.8 ($pep^1+H_4N_3F_3$) -N_2F_1 3124.7 ($pep^1+H_3N_4F_3$) -$H_1N_1F_1$ ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$); 553.2 (N_2F_1)	1 Lewis X, 1 fucosylated LDN and ^{3,6}F 

Table 1. Omega-1 tryptic glycopeptides, continued

Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
Peptide E_{172}FGY**N**GSANCIR$_{18}$				
$H_5N_4F_5$	3741.0 (β-gal: 3740.6; -0 Hex)	1	1590.8 (pep^1+N_1) 1737.5 (pep^1+N_1F_1; 0.12) 1883.4 (pep^1+N_1F_2; 1.0) 2937.5 (pep^1+$H_4N_3F_2$) -$H_1N_1F_3$ 3082.4 (pep^1+$H_4N_3F_3$) -$H_1N_1F_2$	1 Lewis X and 1 pseudo LewisY, ^{3,6}F 
$H_4N_5F_5$	3781.8 (β-gal: 3781.6; -0 Hex)	2	1591.1 (pep^1+N_1) 1737.5 (pep^1+N_1F_1; 0.2) 1882.9 (pep^1+N_1F_2; 1.0) 2923.1 (pep^1+$H_3N_3F_3$) -$H_1N_2F_2$ 3083.8 (pep^1+$H_4N_3F_3$) -N_2F_2 3109.1 (pep^1+$H_2N_4F_4$) -$H_2N_1F_1$	1 Lewis X and 1 difucosylated LDN, ^{3,6}F 
$H_5N_5F_4$	3797.7 (β-gal: 3979.8; -0 Hex; α-fuc: 3359.2; -2 Fuc)	1		^{3,6}F
$H_6N_5F_3$	3813.8 (β-gal: 3650.6; -1 Hex)	1		Mixture: biantennary (1 LN, 1 di LN) with core and antenna fucosylation  One of several possible isomers is shown
$H_6N_5F_4$	3959.7 (β-gal: 3959.7; -0 Hex, and 3797.7; -1 Hex)	1	1592.4 (pep^1+N_1) 1737.5 (pep^1+N_1F_1; 0.33) 1883.8 (pep^1+N_1F_2; 1.0) 2776.4 (pep^1+$H_3N_3F_2$) -$H_3N_2F_2$ 2940.2 (pep^1+$H_4N_3F_2$) -$H_2N_2F_2$ 3085.2 (pep^1+$H_4N_3F_3$) -$H_2N_2F_1$ 3302.1 (pep^1+$H_5N_4F_2$) -$H_1N_1F_2$ 3450.7 (pep^1+$H_5N_4F_3$) -$H_1N_1F_1$ 3596.0 (pep^1+$H_5N_4F_4$) -H_1N_1	Mixture: biantennary (1 LN, 1 di-LN) with core and antenna fucosylation  One of several possible isomers is shown.
$H_5N_6F_4$	4001.0	1	1592.7 (pep^1+N_1) 1737.5 (pep^1+N_1F_1; 0.25) 1884.7 (pep^1+N_1F_2; 1.0) 2939.9 (pep^1+$H_4N_3F_2$) -$H_1N_3F_2$ 3302.5 (pep^1+$H_5N_4F_2$) -N_2F_2 3448.9 (pep^1+$H_5N_4F_3$) -N_2F_1 3593.3 (pep^1+$H_5N_4F_4$) -N_2	Mixture: 1 LN and LN-LDN or 1 LDN and di-LN, with core and antenna fucosylation  One of several possible isomers is shown
$H_6N_6F_5$	4106.0 [(β-gal: 4105.8; -0 Hex)	1	1590.4 (pep^1+N_1) 1882.3 (pep^1+N_1F_2;) 2920.5 (pep^1+$H_3N_3F_3$) -$H_3N_2F_2$ 3082.4 (pep^1+$H_4N_3F_3$) -$H_2N_2F_2$ 3299.3 (pep^1+$H_5N_4F_2$) -$H_1N_1F_3$ 3447.5 (pep^1+$H_5N_4F_3$) -$H_1N_1F_2$ 3596.3 (pep^1+$H_5N_4F_4$) -$H_1N_1F_1$	1 Lewis X, 1 di-Lewis X, ^{3,6}F 
$H_5N_6F_5$	4146.9	1	1591.4 (pep^1+N_1) 1737.5 (pep^1+N_1F_1; 0.1) 1883.3 (pep^1+N_1F_2; 1.0) 3083.8 (pep^1+$H_4N_3F_3$) -$H_1N_3F_2$ 3303.6 (pep^1+$H_5N_4F_2$) -N_2F_3 3447.8 (pep^1+$H_5N_4F_3$) -N_2F_2 3594.7 (pep^1+$H_5N_4F_4$) -N_2F_1	Mixture: 1 LN and LN-LDN or 1 LDN and di-LN, with core and antenna fucosylation  One of several possible isomers is shown
Peptide Q_{69}P**N**CTGSLR$_{77}$				
$H_4N_3F_2$	861.4 $[M+3H]^{3+}$ (β-gal: 861.4; -0 Hex, 807.7; -1 Hex)	4	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$); 528.2 (H_2N_1) 618.2 (pep^2+N_1; $M+2H]^{2+}$); 691.4 (pep^2+N_1F_1; $[M+2H]^{2+}$); 764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Mixture: 1 LN with core and/or antenna fucosylation  3.0% 1.0%
$H_4N_3F_3$	910.1 $[M+3H]^{3+}$ (β-gal: 910.1; -0 Hex)	10	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$) 691.4 pep^2+N_1F_1; $[M+2H]^{2+}$) 764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	1 Lewis X, ^{3,6}F 
$H_5N_3F_2$	915.8 $[M+3H]^{3+}$ (β-gal. 915.8, -0 Hex, 807.7; -1 Hex)	2		Mixture: 1 LN with core and/or antenna fucosylation 

Table 1. Omega-1 tryptic glycopeptides, continued

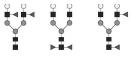
Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
Peptide Q_{69}PNCTGSLR$_{77}$				
$H_3N_4F_3$	923.7 $[M+3H]^{3+}$ (β-gal: 923.7; -0 Hex)	2		
$H_4N_4F_2$	929.8 $[M+3H]^{3+}$	1		
$H_4N_5F_4$	958.8 $[M+3H]^{3+}$ (β-gal: 958.8; -0 Hex)	5	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$)691.4 pep^2+N_1F_1; $[M+2H]^{2+}$)764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Lewis X, ^{3,6}F
$H_5N_3F_3$	964.4 $[M+3H]^{3+}$ (β-gal: 964.4; -0 Hex)	2		1 Lewis X, ^{3,6}F 
$H_3N_4F_4$	972.5 $[M+3H]^{3+}$ (β-gal: 972.5; -0 Hex)	3		^{3,6}F
$H_4N_4F_3$	977.8 $[M+3H]^{3+}$ (β-gal: 977.8; -0 Hex)	1		1 Lewis X and 1 HexNAc, ^{3,6}F 
$H_5N_4F_2$	983.1 $[M+3H]^{3+}$ (β-gal: 983.1; -0 Hex, 929.8; -1 Hex, 875.1; -2 Hex)	5	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$)691.4 pep^2+N_1F_1; $[M+2H]^{2+}$)764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Mixture: biantennary LN with core and/or antenna fucosylation  1.0% 2.0% 2.0%
$H_3N_5F_3$	991.4 $[M+3H]^{3+}$ (β-gal: 991.5; -0 Hex)	1		
$H_5N_4F_3$	1031.8 $[M+3H]^{3+}$ (β-gal: 1031.8; 0 Hex, 977.8; 1 Hex)	12	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$)764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Mixture: biantennary LN with core and antenna fucosylation  8.0% 4.0%
$H_4N_5F_3$	1045.5 $[M+3H]^{3+}$ (β-gal: 1045.5; -0 Hex, 991.5; -1 Hex)	5	ESI-MS/MS: 366.1 (H_1N_1); 407.2 (N_2); 512.2 ($H_1N_1F_1$); 553.2 (N_2F_1)764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Mixture: Lewis X or fucosylated LDN. ^{3,6}F  2.0% 3.0%
$H_5N_4F_4$	1080.5 $[M+3H]^{3+}$ (β-gal: 1080.5; -0 Hex, 1026.4; -1 Hex)	16	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$); 658.2 ($H_1N_1F_2$); 691.4 (pep^2+N_1F_1; $[M+2H]^{2+}$); 764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	Mixture: 2 Lewis X, ^{3,6}F or 1 LN with two fucoses, ^{3,6}F  14.0% 2.0%
$H_6N_4F_3$	1086.2 $[M+3H]^{3+}$ (β-gal: 1086.2; -0 Hex)	4	ESI-MS/MS. 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$)	Lewis X, ^{3,6}F
$H_4N_5F_4$	1094.2 $[M+3H]^{3+}$ (β-gal: 1094.1; -0 Hex)	5	ESI-MS/MS: 366.1 (H_1N_1); 407.2 (N_2); 512.2 ($H_1N_1F_1$); 553.2 (N_2F_1)	1 Lewis X, 1 fucosylated LDN and ^{3,6}F 
$H_5N_5F_3$	1099.8 $[M+3H]^{3+}$ (β-gal: 1099.8; -0 Hex)	2		
$H_5N_4F_5$	1129.1 $[M+3H]^{3+}$ (β-gal: 1129.1; -0 Hex)	5	ESI-MS/MS: 366.1 (H_1N_1); 512.2 ($H_1N_1F_1$); 658.2 ($H_1N_1F_2$); 764.4 (pep^2+N_1F_2; $[M+2H]^{2+}$)	1 Lewis X and 1 difucosylated LN, ^{3,6}F 
$H_4N_5F_5$	1142.8 $[M+3H]^{3+}$ (β-gal: 1142.8; -0 Hex)	6	ESI-MS/MS: 366.1 (H_1N_1); 407.2 (N_2); 512.2 ($H_1N_1F_1$); 553.2 (N_2F_1); 658.2 (H1N1F2); 699.2 (N2F2) 764.4 (pep^2+N1F2; $[M+2H]^{2+}$)	Mixture: 1 LN and 1 LDN with 3 antenna fucoses, ^{3,6}F  3.0% 3.0%
$H_5N_5F_4$	1148.5 $[M+3H]^{3+}$ (β-gal: 1148.2; -0 Hex)	1		
$H_6N_5F_3$	1153.5 $[M+3H]^{3+}$ (β-gal: 1099.8; -1 Hex)	1		Mixture: biantennary (1 LN, 1 di-LN) with core and antenna fucosylation

Table 1. Omega-1 tryptic glycopeptides, continued

Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
Peptide Q_{69}**PN**CTGSLR_{77}				
$H_4N_5F_6$	1191.5 $[M+3H]^3$	1		
$H_6N_5F_4$	1202.2 $[M+3H]^{3+}$ (β-gal: 1202.5; -0 Hex, 1148.2; -1 Hex)	2		Mixture: biantennary (1 LN, 1 di-LN) with core and antenna fucosylation
$H_5N_6F_4$	1215.8 $[M+3H]^{3+}$	1		Mixture: 1 LN and LN-LDN or 1 LDN and di-LN, with core and antenna fucosylation
$H_6N_5F_5$	1250.8 $[M+3H]^{3+}$ (β-gal: 1250.8; -0 Hex)	2		Mixture: 1 LN, 1 di-LN with core and antenna fucosylation
$H_5N_6F_5$	1264.9 $[M+3H]^{3+}$	1		Mixture: 1 LN and LN-LDN or 1 LDN and di-LN, with core and antenna fucosylation

a Glycan compositions are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F).
b Monoisotopic masses of glycopeptide precursors are given throughout. Glycopeptides are singly positively charged, unless specified otherwise. Singly-charged glycopeptide species were registered by MALDI-TOF-MS, and multiply charged species were detected by LC-ESI-MS. For many glycopeptides, masses after β-galactosidase (β-gal) and/or α-fucosidase (α-fuc) treatment are listed and the loss of n hexose or n fucose is concluded (respectively - n Hex and - n Fuc).
c Relative peak height is expressed as a percentage of cumulative peak height per site.
d Fragment ions were determined by MALDI-TOF/TOF-MS unless specified otherwise (ESI-MS/MS indicates analysis by nano-HPLC ESI-ion trap-MS/MS); pep1 corresponds to peptide E172FGYNGSANCIR183 containing glycosylation site N176; pep2 corresponds to peptide Q69PNCTGSLR77 containing glycosylation site N71.
e Proposed glycan structures are deduced from glycopeptide MS after enzyme treatments and fragmentation data. Triangle, fucose; light circle, galactose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose. Glycan isomer percentages are calculated using the relative peak heights of glycopeptides after β-galactosidase treatment and/or relative peak heights of peptides after fragmentation.

of the loss of two galactoses. This observation is in line with the occurrence of one non-susceptible internal galactose in the proposed structure of $H_6N_5F_5$. In addition to the aforementioned signals, a signal at *m/z* 3181.7 appears, which might be interpreted as the loss of three galactoses from the signal at *m/z* 3668.1. However, we believe that the latter signal is not derived from fucosidase/galactosidase treatment of $H_6N_5F_5$, but arises from $H_4N_5F_2$ (*m/z* 3343.1 in Figure 4B), which represents a core difucosylated glycopeptide with one LN and one LDN antenna. Upon β-galactosidase treatment, the $H_4N_5F_2$ glycoform loses one galactose residue from the LN antenna, thereby giving rise to a signal at *m/z* 3181.7 in Figure 4C, derived from the similar intense signal at *m/z* 3343.1 in Figure 4B.

In a similar manner, combination of fragmentation analysis by MALDI-TOF/TOF-MS and α-fucosidase/β-galactosidase treatment led to structure proposals for most other glycoforms observed for the E172-R183 peptide. The data are summarized in Table 1 and Supplementary table 1.

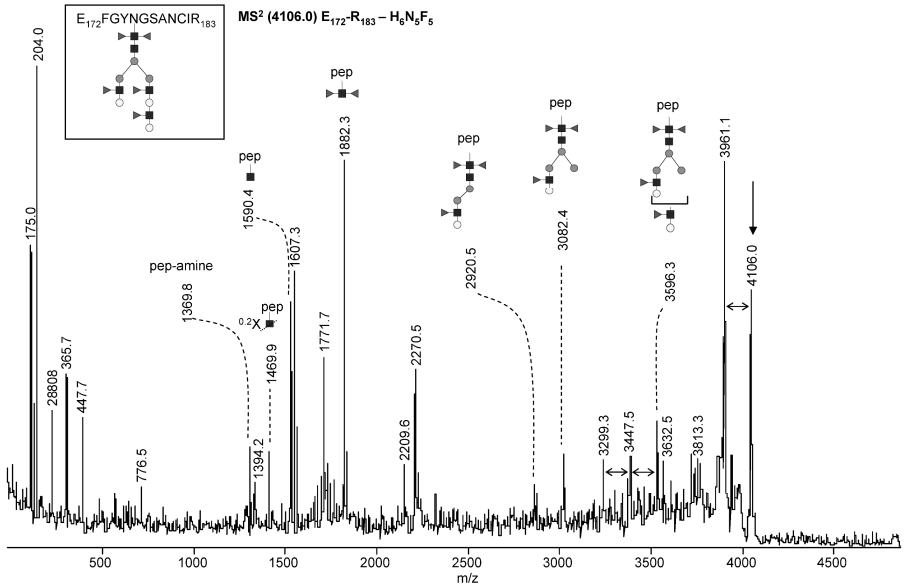


Figure 7. MS/MS of peptide E172–R183 carrying $H_6N_5F_5$ N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; dark circle, mannose.

N-glycosylation analysis of glycopeptide Q69-R77

Unlike from the E172-R183 glycopeptides, signals from peptide Q69-R77 containing the glycosylation site N71 could not be detected by MALDI-TOF-MS. The most abundant Q69-R77 glycopeptide, carrying a $H_5N_4F_4$ glycan (Figure 2A), would correspond to a $[M+H]^+$ ion of a theoretical mass of 3095.4 Da, but this was not observed in the spectrum in Figure 3A, nor were any other signals detected that would correspond to glycopeptides from Q69-R77. Therefore, with the specific aim of further characterizing the glycosylation of the Q69-R77 glycopeptides, untreated and β-galactosidase-treated glycopeptides from omega-1 were also measured by nano-LC-MS (Figure 8) and LC-MS/MS (Figure 9 and Table 1). Registered masses, fragmentation data as well as deduced glycan compositions for all the detected glycopeptide species are given in Table 1.

For the most abundant glycopeptide of Q69-R77 carrying the N-glycan $H_5N_4F_4$, the main structure is composed of a trimannosyl core with two fucoses attached to the innermost *N*-acetylglucosamine and two LeX antennae (left structure in Figure 9), using similar reasoning as for the $H_5N_4F_4$ glycan on site N176. However, ~15% of the $H_5N_4F_4$ glycans on site N71 contain a non-fucosylated LN antenna, as demonstrated by the removal of one hexose after β-

galactosidase treatment (*m/z* 1080.5 $[M+H]^{3+}$ in Figure 8A shifts to *m/z* 1026.4 in Figure 8B). This indicates that two fucoses are located at the other LN branch. To verify this, the $H_5N_4F_4$ glycopeptide was fragmented using nano-LC-MS/MS (Figure 9). Losses of 365 Da and 657 Da (signals at *m/z* 1437.6 and 1291.6) were observed from the parent ion, which corresponds to the loss of LN (H_1N_1) and difucosylated LN ($H_1N_1F_2$), respectively. Notably, fragmentation data of protonated glycans and glycopeptides may be compromised due to the occurrence of fucose rearrangements, possibly contributing to the generation of the above described fragments. This phenomenon is commonly seen in the analysis of N-glycans by MALDI as LC-MS fragmentation [30]. However, based on the results from the β-galactosidase treatment, it was clearly demonstrated that a small part of $H_5N_4F_4$ glycans on site N71 contain one LN and one difucosylated LN antenna, a glycan structure which was not observed for $H_5N_4F_4$ on site N176. In analogy with this, we propose for glycopeptide Q69-R77 containing an N-glycan with composition $H_4N_5F_5$ a similar structural assignment as for $H_4N_5F_4$, being one isomer with a difucosylated core in combination with one mono-fucosylated LDN and one difucosylated LN antenna, and another with a difucosylated core and one LeX and one difucosylated LDN antenna (Table 1).

For most of the other N-glycans at site N71, similar information was obtained, as summarized in Table 1. Most of the glycan structures at site N71 were identical to the structures of glycosylation site N176, however in some cases with differences in fucosylation as described above. The main differences between the sites are in the relative abundance of glycan structures, with site N71 containing a higher amount of multi-fucosylated structures compared to site N176.

General overview of omega-1 glycosylation

An overview of the most abundant glycosylation forms of omega-1, combining the data from glycosylation sites N71 and N176, is given in Table 2. Notably, the abundance of individual glycan structures was calculated using signal intensities derived from LC-MS data for site N71 (Figure 10A) and MALDI-TOF-MS data for site N176 (Figure 3A), which might have implications for the comparability of the two sites. On the other hand, when comparing the LC-MS derived data (Figure 2A) and MALDI derived data (Figure 3A) from site N176, the ratio of the high intensity signals are very comparable, although the low abundant species show some deviations.

The majority of the glycan structures on the two sites exhibit two fucoses on the innermost *N*-acetylglucosamine and one or two LeX antennae. In view of the documented immunogenic properties of LeX and core fucosylation [10;31], we estimated the abundance of these structures on omega-1 using the most abundant glycans as markers (Table 2).

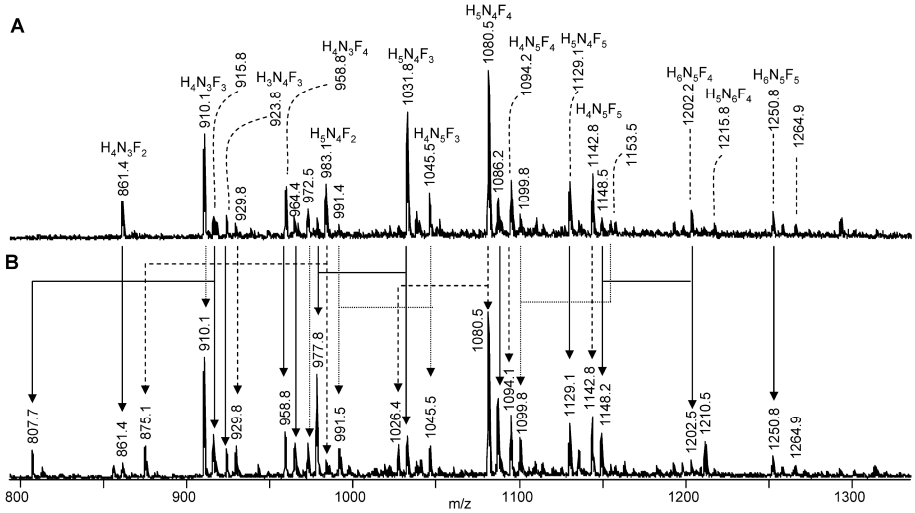


Figure 8. β-galactosidase treatment of omega-1 glycopeptides. Omega-1 was subjected to reduction and alkylation, digested with trypsin and the resulting (glyco)peptides of glycosylation site N71 were analyzed by nano-LC-MS before (A) and after (B) treatment with β-galactosidase. Signals are triple positively charged and labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), N-acetylhexosamine (N) and fucose (F).

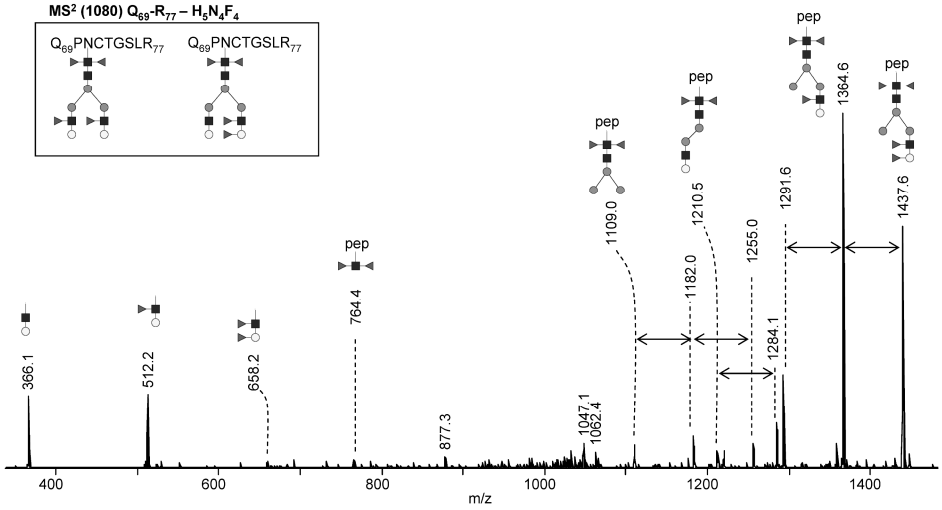


Figure 9. MS/MS of peptide Q69–R77 carrying $H_5N_4F_4$ N-glycans. The MS/MS spectrum was acquired by nano-LC-MS/MS of a tryptic digest of omega-1. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; dark circle, mannose.

One omega-1 glycan carries an average of 1.3 LeX motifs, meaning that on average 2.6 LeX units are present on the omega-1 glycoprotein. To estimate the occurrence of core fucosylation, the data from the α-fucosidase treatment of site N176 were used. As evident from the mass spectra in Figure 4B and 4C, recorded after the removal of antenna fucoses, the vast majority of N176 glycans carry one or two core fucoses. This conclusion was verified by MALDI-TOF/TOF-MS for the high abundant glycans (Table 2). We calculated that 60 % of the glycans at N176 carry two fucoses at the core, 30 % carry one core fucose and 10 % have no core fucosylation. For site N71, when focusing on the twelve most abundant glycan structures of site N71 (Table 2), we calculated that 74 % of these glycans carry two core fucoses, 10 % carry one core fucose and 16 % have no core fucosylation. As already reported for the egg and ES glycoprotein mixture [12;15], the observed difucosylated core structures are expected to be comprised of α1-3,α1-6-linked fucoses. It remains unclear whether the fucose at the mono-fucosylated core is α1-3 or α1-6 linked.

To conclude, the analysis of tryptic glycopeptides by MALDI-TOF/TOF-MS and nano-LC-MS/MS, in combination with β-galactosidase and/or α-fucosidase treatment reveal that the majority of N-glycans of omega-1 express two fucoses on the innermost *N*-acetylglucosamine and contain one or two terminal LeX motifs. Less abundant antenna structures include LN and difucosylated LN, non, mono- and difucosylated LDN and (fucosylated) LN and LDN repeats.

Discussion

Omega-1 is a single glycoprotein in *S. mansoni* SEA that harbors the potent Th2-inducing properties characteristic for this complex antigen mixture [6;9]. Glycans from schistosome egg glycoproteins are ligands for multiple CLRs present on immune cells [20;32], and these glycans appear to be involved in immunomodulatory activities attributed to SEA [5;10]. Prompted by these observations and the lack of structural information about omega-1 glycans, we performed a site-specific analysis of the glycosylation pattern of omega-1.

This study reveals that omega-1 has two fully occupied N-glycosylation sites. Both sites display similar glycan heterogeneity, but with different relative abundances of the individual glycans present (Figure 2 and Table 2). All glycans on omega-1 are of a diantennary complex type, and most display an α3 and/or α6 fucosylation of the innermost *N*-acetylglucosamine (Tables 1 and 2). LeX was found to be the major antenna motif, but also other terminal glycan elements were found to be expressed on omega-1, including LDN-F, tandem repeats of LeX, and difucosylated LN and LDN. In addition, we searched the LC-MS-derived data for O-glycosylated peptides, focusing on O-glycosylation marker oxonium ions at *m/z* 204 (HexNAc antenna), *m/z* 350 (fucosylated HexNAc antenna), *m/z* 407 (LDN antenna), *m/z* 496 (difucosylated HexNAc

antenna), *m/z* 512 (LeX antenna) and *m/z* 699 (difucosylated LDN antenna). Using these marker ions, we could not find any indications for O-linked glycosylation on omega-1.

The occurrence of these types of N-glycan elements in a schistosome egg glycoprotein is in line with data generated by non-protein-specific analysis of the N-glycans of *S. mansoni* egg glycoproteins in SEA [12] as well as in ES [15], the latter being a mixture of excretory/secretory glycoproteins of which omega-1 is a major component [16;17]. Simultaneous expression of LDN- and LN-based antenna elements has been reported in the egg glycoprotein mixtures as well as in the protein-specific glycosylation analysis of IPSE/α1 [24]. Based on the LN and LDN backbones,

Table 2. Schematic representation of the most abundant N-glycans detected on omega-1

Glycans[a]	relative abundance on site N176 (%)[b]	relative abundance on site N71 (%)[b]	relative abundance on Omega-1 (%)
	14	14	14
	7	10	9
	8	8	8
	8	4	6
	5	5	5
	4	2	3
	1	5	3
	4	2	3
	4	1	2.5
	2	3	2.5
	2	3	2.5
	2	3	2.5

a Triangle, fucose; light circle, galactose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose.

b Numbers are based on glycopeptide signal intensities in MALDI-TOF-MS (site N176) or LC-MS (site N71), in combination with signal intensities from fragmentation and enzyme data for isomeric structures.

both LeX and LDN-F antigens are present on omega-1, most likely due to action of a LeX-type fucosyltransferase (FucT) in *S. mansoni* eggs that is also capable of transferring fucose to the GlcNAc residue in LDN in addition to that in LN [33]. We also detected difucosylated LN and LDN variants on omega-1. The F-LDN-F element has been previously described in schistosome glycoproteins and glycolipids [12;15;26]. In contrast, the difucosylated LN element has not been detected before in schistosome eggs or ES. This element likely represents the pseudo-LeY motif (Fucα1-3Galβ1-4(Fucα1-3)GlcNAc) as has previously been described in a major fraction of *S. mansoni* cercarial glycolipids [34]. Hypothetically, a difucosylated LN motif also may contain the Fucα1-2Fucα1-3 sequence. This disaccharide sequence is abundantly present on schistosome egg O-glycans [15], glycolipids [26] and free excretory oligosaccharides [35], but only in the context of chito-oligomeric or LDN backbones. We did not find indications for the occurrence of the Fucα1-2Fuc element on omega-1. With respect to the biosynthesis of the different fucosylated elements present in omega-1, some may be formed by the action of the same FucT (e.g. LeX and LDN-F [33]), as has also been suggested for the formation of the Fucα1-3GalNAc linkage in F-LDN-F and the Fucα1-3Gal in pseudo-LeY [34;36]. Still, at least 4 different FucTs appear to be active at the site of production of omega-1, and taking into account the occurrence of the other fucosylated motifs in *S. mansoni* eggs and other life stages, it is not surprising that at least 20 FucT genes are present in the *S. mansoni* genome [37], some of which display highly regulated expression levels by transcriptome analysis [38]. It is unclear at present what the precise acceptor and linkage specificities of these FucTs are, as current annotation based on sequence homology is not sufficient to classify these FucTs.

With respect to the overall set of N-glycans found on ES glycoproteins [15], truncated and high-mannose type glycans as well as core xylosylation observed in a minor set of ES glycoproteins were not found for omega-1. On the other hand, the glycosylation of IPSE/α1, the only other *S. mansoni* egg glycoprotein of which specific glycosylation data is available to date, appears to be highly homologous to omega-1 [24]. IPSE/α1 is produced in the sub-shell area of the schistosome egg, at the same location as omega-1, which suggests that these two proteins, both major constituents of *S. mansoni* ES, are subjected to the same glycosylation machinery. According to Mathieson et al., at least four other major glycoproteins are present in *S. mansoni* egg ES, including thioredoxin and three micro-exon-gene (MEG) proteins [18], and it would be interesting to see if these proteins carry the O-glycans as well as the subset of N-glycans found in ES, but not present on omega-1 and IPSE/α1. In this respect, it must be noted that proteomic analysis of ES by another group led to the identification of 188 proteins [16] instead of only a few [18]. This discrepancy may be explained by the different methods used for the preparation of ES. Furthermore, while Cass et al. analyzed the whole ES mixture [16], Mathieson and Wilson

separated ES proteins on a 2-DE gel and merely investigated the spots visible after Sypro Ruby staining, possibly missing low-abundant proteins [18]. However, in spite of the large differences between the protein contents of ES fractions, in both cases omega-1 and IPSE/α1 were identified as the most abundant ES proteins, underlining also the relative abundance of the glycans they carry in the overall ES glycome.

The finding that omega-1 contains the LeX motif is of importance. In the context of a LNFPIII conjugate, LeX has previously been shown to induce Th2 responses in a mouse model following intranasal immunization [10]. Furthermore, it can stimulate murine DCs *in vitro* towards a type 2 DC capable of inducing the development of Th2 cells from naive mouse-derived CD4+ T cells [11]. Based on these data, it would be tempting to attribute the recently reported Th2-polarizing properties of omega-1 to its LeX structures [6;9]. However, IPSE/α1, which carries virtually identical LeX-containing glycans to omega-1, is not able to induce the strong DC-mediated type 2 responses observed for omega-1 in the same assay [6;24], indicating that in this case also properties provided by the underlying protein backbone play a role. Interestingly, Steinfelder et al. recently demonstrated in an *in vitro* bystander polarization assay that upon treatment with diethyl pyrocarbonate, a chemical which irreversibly modifies histidine residues, omega-1 is no longer able to condition DCs for Th2-induction [9]. Clearly, the possible contributions of the glycan and protein parts to the immunological properties of omega-1 need to be further investigated. In this respect, it is interesting to note that fucosylated glycan structures abundantly present on omega-1, are recognized by CLRs such as DC-SIGN and MR [19;39], which have been reported to mediate internalization of SEA constituents by DCs [20].

The detailed examination of the site-specific glycosylation of omega-1 was made possible by an approach based on MALDI-TOF(/TOF)-MS and nano-LC-MS/(MS) analysis. Importantly, by combining the MS analyses with specific exo-glycosidase treatments, we could validate structural assignments of important glycan elements, exclude potential false interpretations of the MS/MS spectra due to frequently occurring fucose rearrangements [30], and thus provide a solid basis for the identification and characterization of the glycan structures. This methodology, based on gel-derived tryptic glycopeptides, is also easily applicable to many other glycoproteins as it does not require extensive or complicated sample workup.

Materials and Methods

Purification of omega-1

Omega-1 was purified from SEA as previously described [7,23,25]. In short, SEA was prepared from *Schistosoma mansoni* eggs and fractionated by cation exchange chromatography. Omega-1,

together with IPSE/α1 a constituent of the highly cationic egg fraction CEF6, was separated from IPSE/α1 by affinity chromatography using specific anti-IPSE/α1 monoclonal antibodies coupled to an NHS-HiTrap Sepharose column according to the manufacturer's instructions (GE Healthcare, Little Chalfont, UK).

α -Fucosidase treatment for Western blot analysis

Antigens were dissolved in 10 µl 100 mM sodium phosphate buffer, pH 5.0 and treated with α-1-(3,4)-fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma, Zwijndrecht, The Netherlands) overnight at 37 °C.

To confirm removal of fucoses, omega-1 was reduced by incubation with 0.025 volumes of 200 mM dithiothreitol for 30 minutes at 60°C, after which the antigen solution was mixed with 0.075 volumes of 200 mM iodoacetamide and incubated for 30 minutes in the dark at room temperature. Trypsin (Promega, Leiden, The Netherlands) was added to the sample at a 1:100 trypsin/antigen ratio and incubated overnight at 37°C. After incubation, the digestion was quenched with 1 µl of 5% trifluoroacetic acid. Tryptic digests were measured using MALDI-TOF-mass analysis in the positive ion reflectron mode (results not shown).

Western blot analysis

1 µg of untreated and fucosidase-treated omega-1 was subjected to SDS-PAGE under reducing conditions on a 12% gel using the Mini-Protean Cell system (Bio-rad, Veenendaal, The Netherlands). The proteins were transferred onto a nitrocellulose membrane in a Bio-rad Criterion Blotter system according to manufacturer's instructions. The protein blots were stained as described previously [26]. In short, blots were blocked with BSA and incubated with the primary anti-carbohydrate mAb 128-4F9-A, which binds to LeX antennae. Blots were subsequently incubated with AP-labeled goat-anti-mouse IgG (Caltag; Invitrogen, Breda, The Netherlands) and stained with NBT/BCIP.

Sample preparation for mass spectrometric analysis

10 µg of omega-1 were separated on a 12% polyacrylamide gel by SDS-PAGE electrophoresis under reducing conditions using the Mini-Protean Cell system (Bio-rad) and stained with Colloidal blue (Invitrogen, Groningen, The Netherlands) according to manufacturer's recommendations. The stained omega-1 band (31 kD) was excised and stored in 1% acetic acid at 4°C. The gel piece containing omega-1 was reduced, alkylated and digested with 0.015 µg

trypsin (Promega), as previously described [27]. After digestion, peptides were extracted twice with 20 µl of 0.1% TFA. Extracts were pooled and stored at -20 °C.

Exo-glycosidase treatment for mass spectrometric analysis

Tryptic glycopeptides were treated with β-galactosidase from bovine testis (1 mU; Sigma, Zwijndrecht, the Netherlands) in 10 µl 100 mM sodium phosphate buffer, pH 5.0, for 24 hours at 37°C. α-Fucosidase treatment was performed on tryptic glycopeptides with α1-(3,4)-Fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 24 hours at 37°C. Part of the α-fucosidase-treated glycopeptides was subsequently treated with β-galactosidase for 24 hours at 37°C.

MALDI-TOF(/TOF) MS

Total tryptic digest of omega-1, as well as glycopeptides after β-galactosidase and/or α-fucosidase treatment, were purified by Zip-Tip µ-C18 following the manufacturer's instructions (Millipore, Billerica, MA, USA), eluted with 10 $mg \cdot ml^{-1}$ DHB in 50% acetonitrile, 0.1% trifluoroacetic acid onto a MALDI target plate and air dried. MALDI-TOF(/TOF)-MS data were obtained using an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) equipped with a LIFT-MS/MS facility. Spectra were acquired in the positive ion reflectron mode. For fragment ion analysis in the tandem time-of-flight (TOF/ TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by metastable decomposition of the precursor in the field-free drift region were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. MS/MS spectra with fragment ions showing a high mass deviation were subjected to internal calibration using chitobiose cleavage products as calibrant signals.

Nano-HPLC ESI-ion trap-MS(/MS)

Total tryptic digest of omega-1, as well as glycopeptides after β-galactosidase treatment, were applied to a reverse-phase column (PepMap, 3 µm, 75 µm·100 mm; Dionex /LC Packings, Amsterdam, the Netherlands) using an Ultimate 3000 nano-LC system (Dionex /LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water) at a flow rate of 200 $nL \cdot min^{-1}$. After injection of the sample, elution conditions were switched to 10% solvent B (95% acetonitrile, 0.1% formic acid), followed by a gradient to 60% B in 45 min and a subsequent isocratic elution of 10 min. The eluate was monitored by absorption at 215 nm.

The LC column was coupled to an Esquire HCT-Ultra ESI-ion trap-MS (Bruker-Daltonics, Bremen, Germany) equipped with an online nanospray source operating in the positive-ion mode. For electrospray (1100–1250 V), electropolished, stainless steel LC/MS emitters (150 µm OD, 30 µm ID) from Proxeon A/S (Odense, Denmark) were used. The solvent was evaporated at 175ºC employing a nitrogen stream of 7 L·min^{-1}. Ions from *m/z* 500 to *m/z* 1800 were registered in the MS mode. When operated in the auto MS/MS mode, registering ions from *m/z* 140 to 2200, each MS scan was followed by the acquisition of MS/MS spectra of up to three of the most abundant ions in the MS spectrum.

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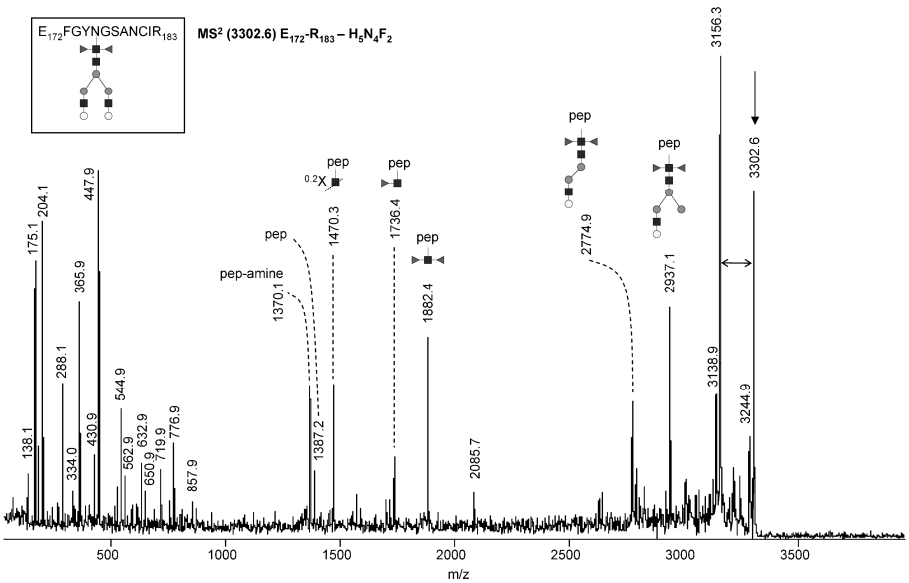
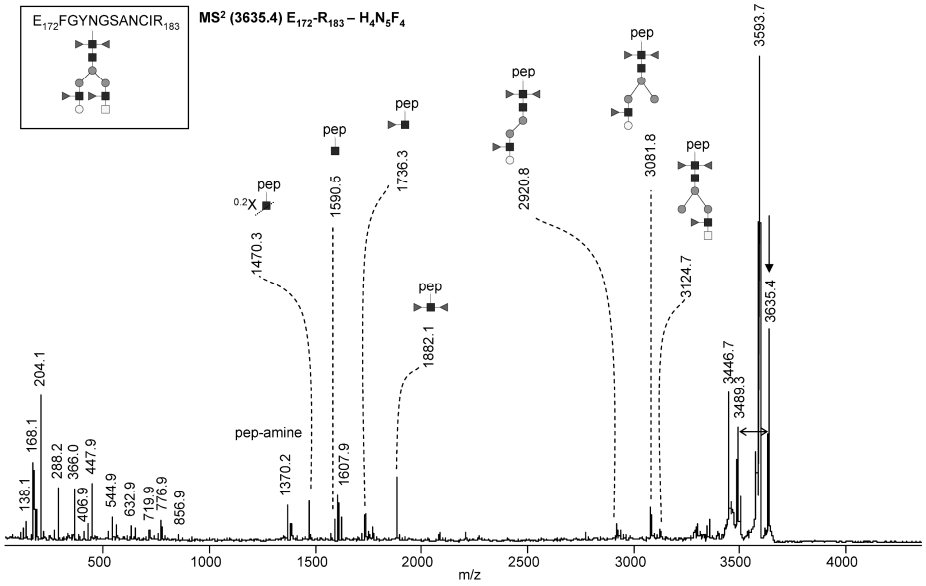
Supplementary figures



Supplementary figure 1. MS/MS of peptide E172–R183 carrying H5N4F2 N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1 treated with α-fucosidase. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; dark circle, mannose.



Supplementary figure 2. MS/MS of peptide E172–R183 carrying H4N5F4 N-glycans. The MS/MS spectrum was acquired by MALDI-TOF/TOF-MS of a tryptic digest of omega-1. The single-headed arrow indicates the parent ion. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light circle, galactose; dark square, *N*-acetylglucosamine; light square, *N*-acetylgalactosamine; dark circle, mannose.

Supplementary Table 1. Omega-1 glycopeptide species detected after trypsin digestion and α-fucosidase treatment

Glycan composition[a]	Glycopeptide signal (*m/z*)[b]	Relative peak height (%)[c]	Fragment ions[d]	Proposed structures[e]
$H_4N_3F_1$	2791.1 [M+H]$^+$ (β-gal: 2629.5; -1 Hex)	3		1 LN, 1 core fucose
$H_4N_3F_2$	2937.4 [M+H]$^+$ (β-gal: 2775.5; -1 Hex)	7	1736.5 (pep^1+N_1F_1; 0.5) 1882.6 (pep^1+N_1F_2; 1.0) 2572.2 (pep^1+$H_3N_2F_2$) - H_1N_1	1 LN, ^{3,6}F 
$H_5N_3F_1$	2953.4 [M+H]$^+$ (β-gal: 2953.6, -0 Hex)	2		Mono-fucosylated core
H_5N_4	3010.5 [M+H]$^+$ (β-gal: 2686.4; -2 Hex)	10	1590.7 (pep^1+N_1) 2483.2 (pep^1+H_3N_3) - H_2N_1 2645.4 (pep^1+H_4N_3) - H_1N_1	2 LN, no core fucosylation 
H_4N_5	3051.4 [M+H]$^+$ (β-gal: 2889.1; -1 Hex)	2		1 LN, 1 LDN, no core fucosylation 
$H_5N_3F_2$	3099.0 [M+H]$^+$ (β-gal: 3099.1; -0 Hex)	1		1 LN, ^{3,6}F
$H_6N_3F_1$	3115.1 [M+H]$^+$	2		Mono-fucosylated core
$H_4N_4F_2$	3140.1 [M+H]$^+$ (β-gal: 2978.6; -1 Hex)	3		1LN and 1 HexNAc, ^{3,6}F
$H_5N_4F_1$	3156.6 [M+H]$^+$ (β-gal: 2832.5; -2 Hex)	17	1590.6 (pep^1+N_1) 1736.6 (pep^1+N_1F_1) 3629.3 (pep^1+$H_3N_3F_1$) - H_2N_1 2791.4 (pep^1+$H_4N_3F_1$) - H_1N_1	2 LN, mono-core fucosylated 
$H_4N_5F_1$	3197.9 [M+H]$^+$ (β-gal: 3035.7; -1 Hex)	4	1736.7 (pep^1+N_1F_1) 2628.4 (pep^1+$H_3N_3F_1$) - H_1N_2 2790.7 (pep^1+$H_4N_5F_1$) - N_2 2833.4 (pep^1+$H_3N_4F_1$) - H_1N_1	1 LN, 1 LDN, mono-core fucosylated 
$H_4N_4F_3$	3286.2 [M+H]$^+$ (β-gal: 3124.5; -1 Hex)	3		
$H_5N_4F_2$	3302.6 [M+H]$^+$ (β-gal: 2978.6; -2 Hex)	30	1736.4 (pep^1+N_1F_1; 0.4) 1882.4 (pep^1+N_1F_2; 1.0) 2774.9 (pep^1+$H_3N_3F_2$) - H_2N_1 2937.1 (pep^1+$H_4N_3F_2$) - H_1N_1	2 LN, ^{3,6}F 
$H_4N_5F_2$	3343.1 [M+H]$^+$ (β-gal: 3181.7; -1 Hex)	7	1590.8 (pep^1+N_1) 1736.9 (pep^1+N_1F_1; 0.33) 1882.7 (pep^1+N_1F_2; 1.0) 2936.3 (pep^1+$H_4N_3F_2$) - N_2 2979.2 (pep^1+$H_3N_4F_2$) - H_1N_1	1 LN, 1 LDN, ^{3,6}F 
$H_5N_4F_3$	3448.9 [M+H]$^+$ (β-gal: 3125.5; -2 Hex)	3		
$H_4N_5F_3$	3489.7 [M+H]$^+$ (β-gal: 3327.9; - 1 Hex)	1		1LN, 1 fucosylated LDN, ^{3,6}F 
$H_4N_5F_4$	3635.8 [M+H]$^+$ (β-gal: 3473.3; -1 Hex, and 3312.7; -2 Hex)	2		^{3,6}F
$H_6N_5F_2$	3668.1 [M+H]$^+$ (β-gal: 3343.1; -2 Hex)	2		1 LN, 1 di-LN, ^{3,6}F
$H_6N_5F_3$	3813.6 [M+H]$^+$ (β-gal: 3489.6; -2 Hex)	1		^{3,6}F

a Glycan compositions are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F).

b Monoisotopic masses of glycopeptide precursors are given throughout. Glycopeptides are singly positively charged, as registered by MALDI-TOF-MS. For many glycopeptides, masses after β-galactosidase (β-gal) treatment are listed and the loss of n hexose is concluded (- n hex).

c Relative peak height is expressed as a percentage of cumulative peak height.

d Fragment ions were determined by MALDI-TOF/TOF-MS. Pep1 corresponds to peptide E_{172}FGYNGSANCIR$_{183}$ containing glycosylation site N176.

e Proposed glycan structures are deduced from glycopeptide MS after enzyme treatments and fragmentation data. Triangle, fucose; light circle, galactose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose.

Chapter 3

Targeted glycoproteomic analysis reveals that kappa-5 is a major, uniquely glycosylated component of *Schistosoma mansoni* egg antigens

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Abstract

Glycans present on glycoproteins from the eggs of the parasite *Schistosoma mansoni* are mediators of various immune responses of the human host, including T-cell modulation and granuloma formation, and they are the target of glycan-specific antibodies. Here we have analyzed the glycosylation of kappa-5, a major glycoprotein antigen from *S. mansoni* eggs using a targeted approach of lectin purification followed by mass spectrometry of glycopeptides as well as released glycans. We demonstrate that kappa-5 has four fully occupied N-glycosylation sites carrying unique triantennary glycans composed of a difucosylated and xylosylated core region, and immunogenic GalNAcβ1-4GlcNAc (LDN) termini. Furthermore, we show that the kappa-5 specific IgE antibodies in sera of *S. mansoni*-infected individuals are directed against the core region of the kappa-5 glycans. Whereas two previously analyzed immunomodulatory egg glycoproteins, IPSE/α1 and omega-1, both express diantennary N-glycans with a difucosylated core and one or two Galβ1-4(Fucα1-3)GlcNAc (LeX) antennae, the kappa-5 glycosylation appears unique among the major soluble egg antigens of *S. mansoni*. The distinct structural and antigenic properties of kappa-5 glycans suggest a specific role for kappa-5 in schistosome egg immunogenicity.

Introduction

Schistosomiasis, a debilitating disease caused by infection with *Schistosoma* parasites, forms a major public health burden in many areas in the tropics and sub-tropics. The main pathological symptoms of *Schistosoma mansoni* infection are caused by the deposition of parasite eggs into the organs of the host and the subsequent immunological consequences, which include the formation of perioval granulomas associated with a pronounced T helper 2 (Th2) response [1].

Many aspects of these egg-induced immune processes are thought to be at least partly mediated by protein glycosylation [2-5]. The role of glycans during granuloma formation has been explored *in vivo* by hepatic implantation of antigen-coated Sepharose beads as artificial eggs into mice [3]. In this model, beads that carry soluble egg antigens (SEA) of *S. mansoni* give rise to granulomas very similar to granulomas around actual schistosome eggs. In contrast, granulomas were not formed around beads coated with SEA of which the glycans had been destroyed by periodate treatment. Intact glycosylation has also been reported to be crucial for Th2-polarizing properties of SEA in a murine model of intranasal sensitization giving rise to antigen-specific IgE production and induction of IL-4 and IL-10 [2].

The structure of SEA glycans has been studied extensively, mainly by mass spectrometric (MS) analysis of released N- and O-glycans [6-8]. However, to obtain knowledge about the contribution of glycosylation to the functional properties of individual glycoproteins, protein-specific glycosylation analyses, rather than studies on glycans released from glycoprotein mixtures, are essential. Up to now, only for two schistosome egg glycoproteins, omega-1 [9] and IPSE/α1 [10], a protein-specific in-depth glycosylation analysis has been carried out [11;12], and both these glycoproteins were found to carry the immunogenic Galβ1-4(Fucα1-3)GlcNAc (Lewis X. LeX) motif [13-17]. The similar glycosylation patterns of these proteins do not account for the variety of antigenic glycan elements found in schistosome eggs, emphasizing the need for detailed studies on the glycosylation of other subsets of the egg glycoproteome. Recently, kappa-5, an immunogenic egg glycoprotein from *S. mansoni* putatively involved in host-parasite interactions, has been identified [18]. Schramm et al. showed that kappa-5 is the target of a pronounced IgE response in the human host [18]. Recombinant kappa-5 expressed in human embryonic kidney (HEK) cells did 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. In addition, kappa-5 was shown to be the primary *S. mansoni* SEA constituent that binds to soybean agglutinin (SBA), a lectin that is specific for terminal αβ-D-*N*-acetylgalactosamine (GalNAc) [19]. Terminal GalNAc is a common monosaccharide in several *S. mansoni* life stages [6, 20, 21] including eggs [6] as part of GalNAcβ1-4GlcNAc (LacdiNAc,

LDN), a structure to which several immunogenic properties have been attributed [4, 22-24]. The selective binding of SBA to kappa-5 suggests that kappa-5 carries such GalNAc-containing glycans.

The significance of schistosome glycans with respect to host-schistosome interactions and the peculiar properties of kappa-5 glycosylation prompted us to perform a detailed analysis of kappa-5 using nanoscale LC-MS(/MS) and MALDI-TOF(/TOF)-MS measurements of released glycans as well as tryptic glycopeptides, in combination with exoglycosidase treatments. We here show that each glycosylation site of kappa-5 to a large degree carries a unique type of core-difucosylated, core-xylosylated triantennary glycan with three terminal LDN motifs, setting it apart from the other members of the egg glycoproteome. Furthermore, we show that IgE reactivity of kappa-5 is attributable to its glycans. These observations underscore the antigenic properties of kappa-5 glycans and emphasize the need for further unraveling the role of glycosylation in the interaction of individual SEA components with the immune system of the host.

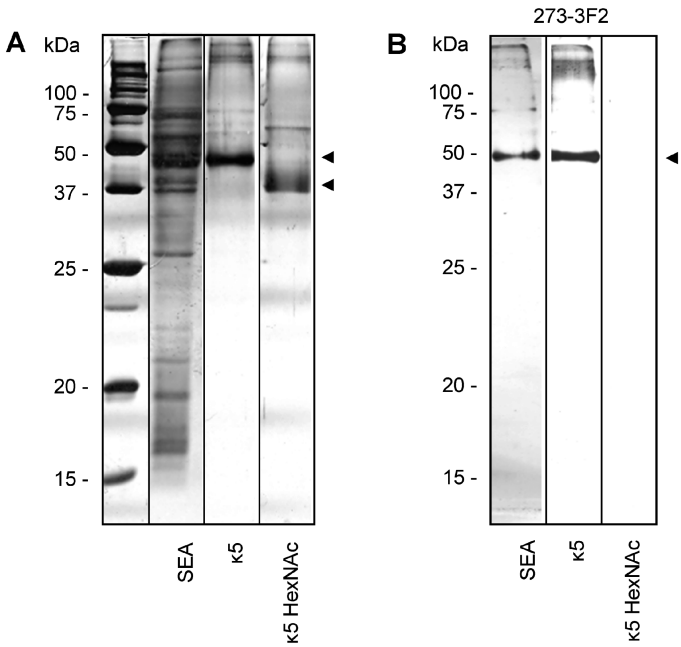

Figure 1. Visualization and characterization of kappa-5. SEA, kappa-5 (κ5) and β-*N*-acetylhexosaminidase-treated kappa-5 (κ5 HexNAc) were separated under reducing conditions by SDS-PAGE and silver stained (A). For testing the presence of terminal LDN, the antigens were subjected to Western blot using a monoclonal antibody against LDN (273-3F2) (B). Kappa-5 is indicated by arrowheads.

Results

Kappa-5 is the major LDN-containing glycoprotein in S. mansoni soluble egg antigens (SEA)

Kappa-5 was purified from SEA by SBA-affinity chromatography and subjected to SDS-PAGE under reducing conditions, giving rise to a prominent kappa-5 band at ~ 50 kDa (Figure 1A), as previously shown [18]. The peptide mass fingerprint analysis of the tryptic digest matched to the secretory glycoprotein k5 precursor of *Schistosoma mansoni* (kappa-5), accession AAX83117 (NCBInr) with a Mowse score of 198, a sequence coverage of 58%, and 27 of 59 mass values searched matched. Mass spectrometric analysis demonstrated that the 50 kDa kappa-5 isomer contains four glycosylation sites N116, N143, N174 and N251 (Figure 2). In a previous study, an additional protein band at a lower apparent molecular weight was found, containing an isomer of kappa-5 with only three glycosylation sites due to a single nucleotide mutation [18]. Mass spectrometric analysis of glycopeptides isolated from this protein band (data not shown) indicated that the glycans carried by the kappa-5 isomer with three glycosylation sites were identical to those of the kappa-5 carrying four glycans.

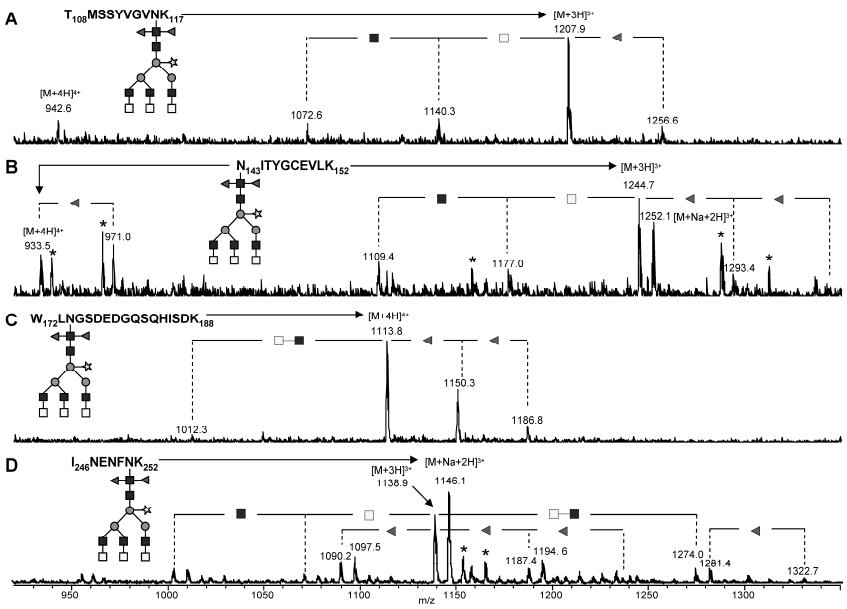

Figure 2. Heterogeneity of the glycan moieties on the four glycosylation sites of kappa-5. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides were analyzed by LC-ion trap-MS. Mass spectra cover the four glycosylation sites N116 (A), N143 (B), N174 (C), and N251 (D). Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Non-glycopeptide signals are marked with asterisks (*). Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.

SBA binds terminal GalNAc [19], a monosaccharide which abundantly occurs in *S. mansoni* glycoconjugates in the context of the disaccharide element LDN [6;20;21]. To investigate whether kappa-5 carries LDN motifs, SBA-purified kappa-5 was subjected to Western blot analysis. The LDN-reactive mAb 273-3F2 was found to bind to kappa-5 (Figure 1B), demonstrating the presence of LDN. Treatment of kappa-5 with β-*N*-acetylhexosaminidase (κ5 HexNAc) to remove terminal β-linked HexNAc residues and thereby LDN completely abrogated binding of the antibody, verifying the presence of a terminal LDN motif on kappa-5. Moreover, on SDS-PAGE the molecular weight of κ5 HexNAc decreased by > 5kD upon β-*N*-acetylhexosaminidase treatment (Figure 1A), which implies the presence of multiple LDN termini on kappa-5. Western blot analysis of SEA with mAb 273-3F2 showed one reactive protein band at the same molecular weight as isolated kappa-5 (Figure 1B). Together, these data demonstrate that kappa-5 is the major glycoprotein in SEA that carries terminal, unsubstituted LDN.

Kappa-5-reactive IgE antibodies in human infection sera are directed against glycans

To investigate the hypothesis that glycans are the target of kappa-5-reactive IgE in *S. mansoni* infection sera [18], SBA-purified kappa-5 was subjected to SDS-PAGE under reducing conditions, blotted onto a nitrocellulose membrane and treated with periodate to destroy its glycan structures. After treatment, SBA-reactivity of kappa-5 was lost, while protein staining using silver stain and reactivity to a mAb against the kappa-5 protein were unaffected (Figure 3A), indicating that by periodate treatment of kappa-5 on a blotting membrane the integrity of the glycans is destroyed, while the protein remains intact. Untreated kappa-5 is bound by IgE antibodies in human *S. mansoni* infection sera (Figure 3B), as previously shown by Schramm *et al.* [18]. Upon treatment with periodate, IgE reactivity was lost or strongly diminished (Figure 3B), confirming that kappa-5 glycans are responsible for the binding of IgE antibodies in human infection sera to kappa-5.

Next, to investigate whether the reactivity of IgE antibodies from *S. mansoni* infection sera is targeted against the LDN antennae of kappa-5 or not, we performed an ELISA to measure IgE binding to kappa-5 variants generated by β-*N*-acetylhexosaminidase and fucosidase treatments. To ensure success of removal of HexNAc and fucose residues, glycopeptides generated after tryptic digestion of an aliquot of the exoglycosidase-treated kappa-5 variants were analyzed by LC-MS (data not shown), similarly as demonstrated for the exoglycosidase-treated glycopeptides (see Figure 4 and 5). Figure 3C shows that β-*N*-acetylhexosaminidase-treated kappa-5 exhibits similar IgE reactivity as untreated kappa-5, indicating that LDN on kappa-5 is not the primary target for serum IgE. Similarly, kappa-5 of which antenna fucoses

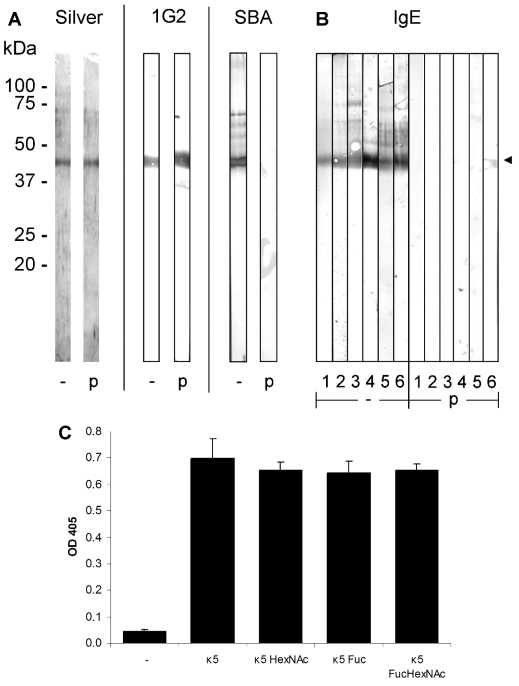

Figure 3. Reaction of IgE in human infection sera with kappa-5. (A,B) Blots containing SBA-purified kappa-5 were treated (p) with metaperiodate to destroy glycan structures, or without (-). Kappa-5 is indicated by the arrowhead. (A) Treatment efficiency was analyzed with a protein stain (Silver), a monoclonal antibody against kappa-5 (1G2) and SBA reactivity (SBA). (B) Sera from six human subjects known to be infected with *S. mansoni* were used to probe the kappa-5 blots. IgE antibody reactivity was visualized by a secondary antibody specific for IgE. (C) Kappa-5 was treated with β-*N*-acetylhexosaminidase (κ5 HexNAc), α-fucosidase from *X. manihotis* (κ5 Fuc) or a combination of the enzymes (κ5 FucHexNAc), and the kappa-5 variants were tested in an ELISA for their reactivity with IgE from two human infection sera. One out of two representative experiments is shown.

were removed by treatment with α1-(3,4)-fucosidase from *X. manihotis*, and a variant treated sequentially with the fucosidase and β-*N*-acetylhexosaminidase, respectively, also display IgE reactivity comparable to untreated kappa-5 (κ5 Fuc and κ5 FucHexNAc, respectively, in Figure 3C). Together, these observations indicate that the core structure rather than the antenna of kappa-5 glycans are associated with the IgE-reactivity of kappa-5.

The structural details of the antigenic kappa-5 glycans were further analyzed using various MS-based techniques and glycosidase treatments.

Analysis of PNGase A-released N-glycans from kappa-5

To obtain a general overview of N-glycans on kappa-5, tryptic kappa-5 glycopeptides were treated with PNGase A, which releases all N-glycans including those with the core α3-linked fucose modification frequently occurring in schistosome glycoproteins. The glycan pool was analyzed by MALDI-TOF-MS in the positive reflectron mode (Figure 6). Single charged sodiated ions were detected (indicated by single-headed arrows in Figure 6), accompanied by a signal with lower intensity corresponding to a potassium adduct. The major glycan species in the MALDI-TOF-MS spectrum (~ 46% of total glycan pool) is observed at *m*/z 2575.9 $[M+Na]^+$ and *m/z* 2591.9 $[M+K]^+$ (Figure 6), which corresponds to a reducing glycan with a mass of 2552.9 Da and a composition of $H_3N_8F_2P$ (H, hexose; N, *N*-acetylhexosamine; F, deoxyhexose, fucose; P,

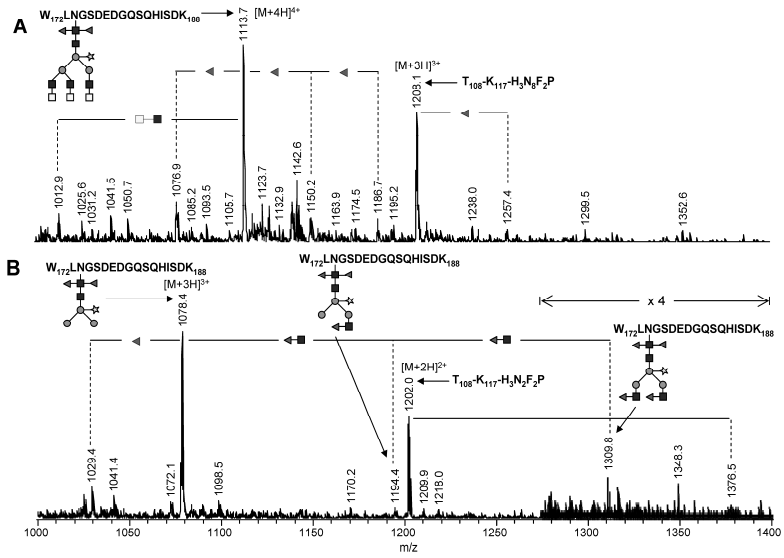

Figure 4. β-*N*-Acetylhexosaminidase treatment of kappa-5 glycopeptides. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides W172-K188 and T108-K117 were analyzed by LC-MS before (A) and after (B) treatment with β-*N*-acetylhexosaminidase. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.

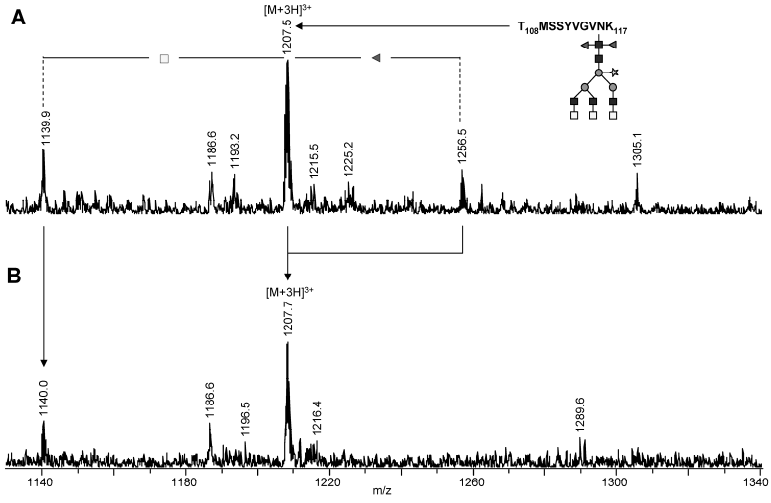

Figure 5. α-Fucosidase treatment of kappa-5 glycopeptides. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptide T_{108}-K_{117} was analyzed by LC-MS before (A) and after (B) treatment with α-fucosidase from *X. manihotis*. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.

pentose, xylose). To further assess the glycan structure, the sodiated species at *m/z* 2575.9 was subjected to MALDI-TOF/TOF-MS fragmentation analysis (Figure 7). Aided by the fragmentation pattern, we propose that $H_3N_8F_2P$ consists of a trimannosyl N-glycan core containing two fucoses at the reducing *N*-acetylglucosamine, a xylose residue linked to the β-linked mannose and three terminal LDN branches. The occurrence of LDN termini was indicated by the signal at *m/z* 429.5 (Figure 7), which corresponds to sodiated $HexNAc_2$ fragments. Based on the strong reactivity of kappa-5 with an LDN antibody (Figure 3B), we interpret these fragments as terminal LDN motifs. This interpretation was supported by the presence of signals at *m/z* 2170.9 and 2374.1 $[M+Na]^+$, corresponding to the loss of respectively one and two terminal HexNAc residues from the parent ion. The positioning of the fucose residues was revealed by a H_3N_7P fragment at *m/z* 2064.7 $[M+Na-H_2O]^+$ in Figure 7, indicative of a chitobiose cleavage product which has lost the reducing end GlcNAc with one α3- and one α6-linked fucose. Furthermore, the signal at *m/z* 1048.3, corresponding to a sodiated H_3N_2P fragment indicates that the xylose residue is associated with the trimannosyl core, as previously described for *S. mansoni* egg [6] and cercarial [33] N-glycans.

The remaining PNGase A-released glycans visualised in Figure 6 were assigned on the basis of mass differences compared to the above described major glycan signal at *m/z* 2575.9 $[M+Na]^+$. Evidently, heterogeneity of the glycan moieties on kappa-5 is caused by variation in the number of HexNAc and fucose residues, as reflected by corresponding mass differences (203 and 146 Da, respectively) between the registered glycans. The second major glycan (at *m/z* 2722.0 $[M+Na]^+$ and 2738.0 $[M+K]^+$, Figure 3) accounts for ~17 % of glycans on kappa-5 and contains an additional fucose residue compared to the major signal, which may imply the presence of a fucose residue linked to one of the LDN antennae. Other glycan variations include structures containing two or four LDN motifs and a mono-fucosylated core (Figure 6).

Identification and characterization of N-glycosylation sites of kappa-5

To obtain in-depth, site-specific glycosylation information, kappa-5 was subjected to reduction, alkylation and trypsinization, after which resulting glycopeptides were analyzed by reversed phase LC-ion trap-MS. The most abundant glycopeptide species for all four glycosylation sites was found to be pep-$H_3N_8F_2P$ (*m/z* 1207.9 $[M+3H]^{3+}$ for site N116, 1244.7 $[M+3H]^{3+}$ and 1252.1 $[M+Na+2H]^{3+}$ for site N143, 1113.8 $[M+4H]^{4+}$ for site N174 and 1138.9 $[M+3H]^{3+}$ and 1146.1 $[M+Na+2H]^{3+}$ for site N251 in Figs. 2A, 2B, 2C and 2D, respectively), which is in accordance with the major glycan species found among the PNGase A-released glycans (Figure 3). Moreover, the same heterogeneity of the number of HexNAc and fucose moieties observed in the pool of released glycans (Figure 3) is observed at the glycopeptide level (Figure 2).

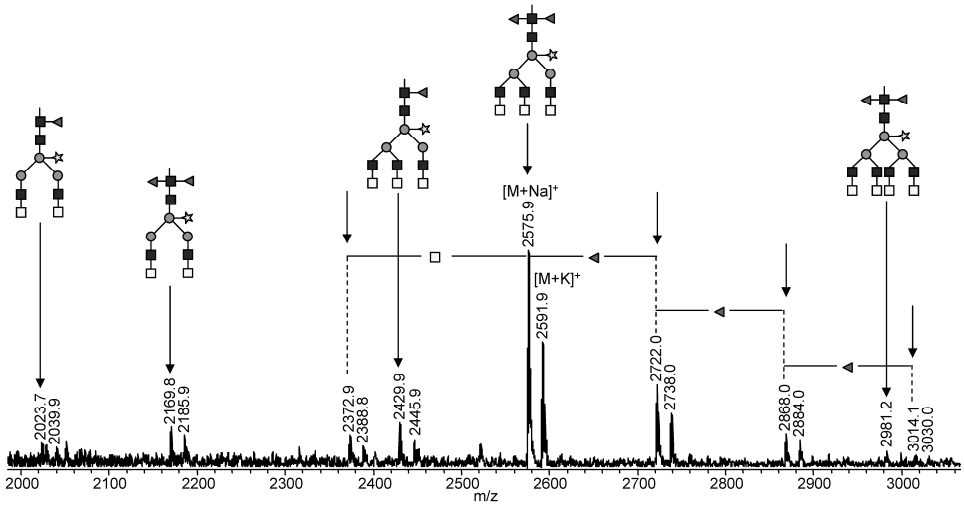

Figure 6. MALDI-TOF-MS of PNGase A-released glycans from kappa-5. SBA- purified kappa-5 was digested with trypsin followed by PNGase A treatment. Released carbohydrates were subsequently purified using carbon adsorption chromatography and measured by MALDI-TOF-MS in the positive-ion reflectron mode. Signals are labeled with monoisotopic masses. *m/z* Values indicated with an arrow correspond to sodiated species. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.

To further substantiate the tentative assignments based on the analysis of the released glycans and to verify the linkage position of HexNAc and fucose residues, kappa-5 glycopeptides were rerun on the RP-LC-MS system, including glycopeptides treated with exoglycosidases. In addition, glycopeptides were subjected to fragmentation using LC-MS/MS. Nano-LC-MS/MS of glycan $H_3N_8F_2P$ on peptide W172-K188 (*m/z* 1113 $[M+4H]^{4+}$; Figure 8A) verifies the presence of LDN antennae by intense signals at *m/z* 407.2 $[HexNAc_2+H]^+$, *m/z* 1349.0 $[M-2HexNAc+3H]^{3+}$ and 2022.9 $[M-2HexNAc+2H]^{2+}$ (loss of one LDN from the parent ion), and *m/z* 1819.9 $[M-4HexNAc+2H]^{2+}$ (loss of two LDN). Furthermore, the absence of signals at *m/z* 610.3 $[HexNAc_3+H]^+$, 553.2 $[HexNAc_2Fuc_1+H]^+$ and 813.4 $[HexNAc_4+H]^+$ confirms the absence of $HexNAc_3$, fucosylated LDN or dimeric LDN antennae, which is a further indication that W172-K188-$H_3N_8F_2P$ contains three, non-fucosylated terminal LDN branches. Additionally, data from β-*N*-acetylhexosaminidase treatment underline this assumption (Figure 4). The signal of untreated W172-K188-$H_3N_8F_2P$ (*m*/z 1113.7 $[M+4H]^{4+}$ in Figure 4A) completely disappears upon treatment, while a similarly intense signal at *m/z* 1078.4 $[M+3H]^{3+}$ (Figure 4B) appears, corresponding to the loss of six HexNAc residues (three LDN antennae). To obtain detailed information on the core structure, a MALDI-TOF/TOF-MS of W172-K188-$H_3N_8F_2P$ was

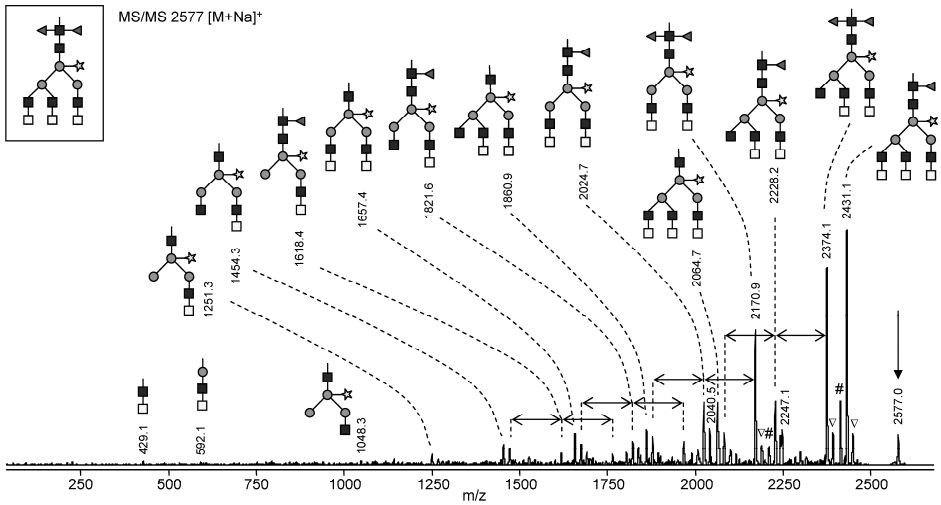

Figure 7. MS/MS of the major PNGase A-released glycan from kappa-5. The major PNGase A-released carbohydrate (*m/z* 2577 $[M+Na]^+$) in Figure 3 was analyzed by MALDI-TOF/TOF-MS. The parent ion is indicated by an arrow. Differences in fucose content are indicated by double-headed arrows. The given glycan structures are examples. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose; #, loss of water; ∇, presumably potassium adduct co-isolated during precursor selection.

recorded (Figure 8B). A core cleavage pattern with an intense signal at *m/z* 2411.7 $[peptide+HexNAc_1Fuc_2+H]^+$ was observed, which verifies that the two fucoses in $H_3N_8F_2P$ are linked to the core GlcNAc residue. In this respect, it must be noted that the lower intensity signals at *m/z* 2265.3 $[peptide+HexNAc_1Fuc_1+H]^+$ and 2119.6 $[peptide+HexNAc_1+H]^+$ are interpreted as the result of a chitobiose cleavage in combination with loss of core fucoses. For glycan $H_3N_8F_2P$ on the other three glycosylation sites, similar results were obtained using fragmentation and exoglycosidase derived data (Figure 4 and Supplementary Figures 1-4), leading to the conclusion that the N-glycan $H_3N_8F_2P$ is structurally identical for all sites and contains three terminal LDN branches, two fucoses linked to the innermost GlcNAc and a xylose linked to the β-linked mannose.

The composition of a substitution present on a minor portion of each glycosylation site, $H_3N_8F_3P$ (Figure 2), points to an N-glycan with two core fucoses, one core xylose and three LDN antennae of which one is fucosylated, as verified by LC-MS/MS (Supplementary Figure 5). The position of the antenna fucose was determined by enzymatic degradation using a set of three exoglycosidases. First, the specificities of α1-(3,4)-fucosidase from *X. manihotis*, α-L-fucosidase from bovine kidney and β-*N*-acetylhexosaminidase from *C. ensiformis* were tested using two synthetic glycoconjugates containing the terminal GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F-tag) and Fucα1-3GalNAcβ1-4GlcNAc (F-LDN-tag) sequences [25, 26]. Treatment with α-fucosidase

from *X. manihotis* resulted in the partial loss of one fucose from LDN-F-tag (*m/z* 936.5 $[M+H]^+$ in Figure 8A shifts to *m/z* 790.5 $[M+H]^+$ in Figure 9B), while F-LDN-tag was not affected by the enzyme (*m/z* 936.5 $[M+H]^+$ in Figure 8E does not shift in Fig 9F). Interestingly, α-fucosidase from bovine kidney has the opposite specificity. Under the conditions applied, it was able to partially cleave the fucose of F-LDN-tag (Figure 9G) but not of LDN-F-tag (Figure 9C). Upon treatment with β-*N*-acetylhexosaminidase, LDN-F-tag loses one HexNAc (*m/z* 936.5 $[M+Na]^+$ in Figure 9A shifts to *m/z* 733.4 $[M+Na]^+$ in Figure 9D), while the enzyme has no effect on F-LDN-tag (Figure 9H). The tryptic glycopeptides of kappa-5 were treated with the same glycosidases. We used glycopeptide T108-K117 containing glycosylation site N116 to monitor the effect of exoglycosidase treatment on glycan $H_3N_8F_3P$ by LC-MS. Treatment of kappa-5 glycopeptides with α-fucosidase from *X. manihotis* results in removal of one fucose from T108-K117-$H_3N_8F_3P$

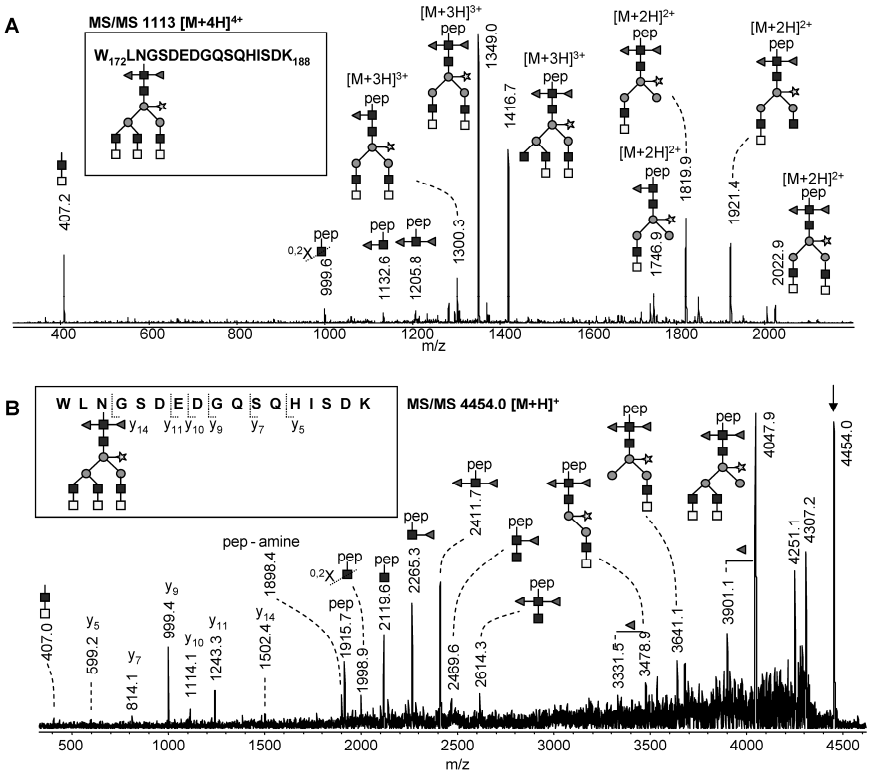

Figure 8. MS/MS of peptide W172-K188 carrying $H_3N_8F_2P$ N-glycans. The MS/MS spectra were acquired by LC-ion trap-MS/MS (A) and MALDI-TOF/TOF-MS/MS (B) from a tryptic digest of kappa-5. Glycopeptides are singly positively charged, unless specified otherwise. The given glycan structures are examples. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose; pep, peptide. 0,2X, ring fragmentation of the N-linked *N*-acetylglucosamine.

as the signal at *m/z* 1256.5 $[M+3H]^{3+}$ in Figure 5A completely shifts to the signal at *m/z* 1207.5 $[M+3H]^{3+}$ in Figure 5B, while the α-fucosidase derived from bovine kidney has no effect on the glycopeptide (data not shown). In addition, treatment with β-*N*-acetylhexosaminidase results in the disappearance of the signal at *m/z* 1257.4 $[M+3H]^{3+}$ in Figure 4A and the appearance of a signal at *m/z* 1376.5 $[M+2H]^{2+}$ in Figure 4B, representing the loss of the two (non-fucosylated) LDN structures and one HexNAc from the fucosylated LDN antenna, as confirmed by fragmentation analysis (Supplementary Figure 6). Together, these data demonstrate the presence of the LDN-F but not the F-LDN motif on glycopeptide T108-K117-$H_3N_8F_3P$.

Fragmentation spectra and exoglycosidase treatment data were also obtained for the other glycans on kappa-5, as summarized in Table 1. Interestingly, the glycosylation patterns were found to be remarkably similar for the four glycosylation sites, although some differences in relative abundances as well as presence or absence of low abundant glycans existed.

Discussion

Our data show that the *S. mansoni* egg antigen kappa-5 carries a unique set of glycans with three LDN antennae on an α3/α6-difucosylated, xylosylated N-glycan core. These glycans appear to be exclusively expressed on kappa-5. No other SEA components were detected that are bound by an LDN-reactive mAb or SBA, the GalNAc-binding lectin used to purify kappa-5 from *S. mansoni* egg protein extracts. So far, two other abundant *S. mansoni* egg glycoproteins have been characterized in terms of glycosylation, IPSE/α1 [34] and omega-1 [9]. The glycan profiles of IPSE/α1 and omega-1 are highly similar to each other and are characterized by the presence of diantennary glycans with core difucosylation and LeX antennae [11;12], whereas in contrast to kappa-5 core xylosylation is absent and LDN-motifs were observed only in minor amounts in these two proteins.

The striking differences between the glycosylation of kappa-5 on the one hand and IPSE/α1 and omega-1 on the other, emphasize the value of protein-specific MS-based glycosylation analyses. Although glycomics studies of glycans released from protein mixtures derived from cercariae, worms or eggs provide useful information on the glycan repertoire of a cell or organism, such studies usually lack the structural information required to address glycan-related properties of individual glycoproteins.

Schistosome-derived glycoproteins play an important role in immunomodulatory mechanisms associated with schistosomiasis via their action on a range of immune cells including dendritic cells (DCs) and T-cells [35]. Glycosylated SEA components from *S. mansoni* are internalized by immature monocyte-derived DCs primarily via the C-type lectin receptors

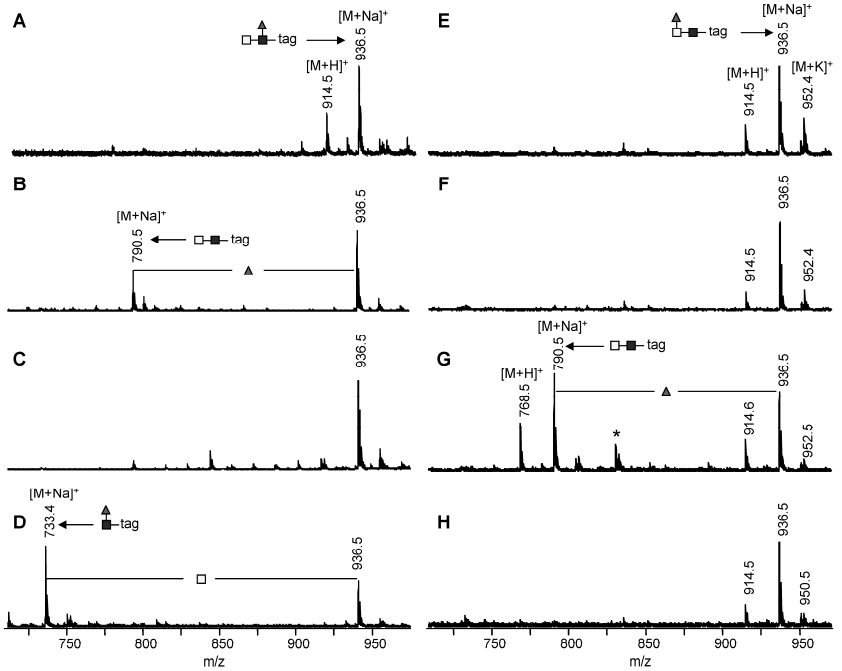

Figure 9. MALDI-TOF-MS of exoglycosidase treated glycoconjugates. To test the specificity of exoglycosidases, GalNAcβ1-4(Fucα1-3)GlcNAcβ1-3Galα1-$(CH_2)_5$-squarate (A-D) and Fucα1-3GalNAcβ1-4GlcNAcβ1-3Galα1-$(CH_2)_5$-squarate (E–H) were either not treated (A and E) or treated with α1-(3,4)-fucosidase from *Xanthomonas manihotis* (B and F), α-L-fucosidase from bovine kidney (C and G) or β-*N*-acetyl-hexosaminidase from *Canavalia ensiformis* (D and H) and analyzed by MALDI-TOF-MS. Non-glycoconjugate signals are marked with asterisks (*).Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine.

(CLRs) Mannose receptor (MR), macrophage galactose-type lectin (MGL) and DC-SIGN, leading to suppression of Toll like receptor-induced DC activation [36]. It is currently unknown which individual glycoproteins in SEA bind to these different CLRs. Binding of pathogen-derived glycans to DC-SIGN and MR can trigger intracellular pathways associated with immunomodulation [37;38], but MGL-mediated signaling pathways involving pathogen glycans have to our knowledge not yet been described. The structural elucidation of kappa-5 glycans, and the previously reported studies on the glycosylation of IPSE/α1 [12], a basophil modulator [39] and of omega-1 [11], an SEA component recently described to condition DC to drive Th2 responses [40], provide a starting point to further detail the contribution of these abundant SEA glycoproteins to CLR-mediated immunomodulatory processes. Guided by the reported binding characteristics of MR, MGL and DC-SIGN, the LeX elements present on both IPSE/α1 and omega-1 are potential ligands for DC-SIGN [41], and they may also confer MR binding [42;43]. In contrast, kappa-5 is a candidate ligand for MGL that binds to α and β-linked GalNAc residues [44].

Table 1. Kappa-5 glycopeptide species detected after trypsin digestion

Glycan composition	*Glycopeptide signal (m/z)*	Structural characteristics
Peptide T106-K117 with glycosylation site N116		
$H_3N_6F_2P$	1072.6 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN
$H_3N_7F_2P$	1140.3 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN, one HexNAc
$H_3N_8F_2P$	906.1 $[M+H]^{4+}$ 1207.9 $[M+3H]^{3+}$	^{3,6}F, Xyl, three LDN
$H_3N_8F_3P$	942.6 $[M+4H]^{4+}$ 1256.6 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN, one LDN-F
Peptide N143-K152 with glycosylation site N143		
$H_3N_6F_2P$	1109.4 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN
$H_3N_7F_2P$	1177.0 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN, one HexNAc
$H_3N_8F_2P$	933.5 $[M+4H]^{4+}$ 1244.7 $[M+3H]^{3+}$ 1252.1 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, three LDN
$H_3N_8F_3P$	971.0 $[M+4H]^{4+}$ 1293.4 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN, one LDN-F
$H_3N_8F_4P$	1342.4 $[M+3H]^{3+}$	^{3,6}F, Xyl, one LDN, two LDN-F
Peptide W172-K188 with glycosylation site N174		
$H_3N_6F_2P$	1012.3 $[M+4H]^{4+}$	^{3,6}F, Xyl, two LDN
$H_3N_8F_2P$	1113.8 $[M+4H]^{4+}$	^{3,6}F, Xyl, three LDN
$H_3N_8F_3P$	1150.3 $[M+4H]^{4+}$	^{3,6}F, Xyl, two LDN, one LDN-F
$H_3N_8F_4P$	1186.8 $[M+4H]^{4+}$	^{3,6}F, Xyl, one LDN, two LDN-F
Peptide I246-K252 with glycosylation site N251		
$H_3N_6F_2P$	1003.7 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN
$H_3N_7F_2P$	1071.3 $[M+3H]^{3+}$	^{3,6}F, Xyl, two LDN, one HexNAc
$H_3N_8F_1P$	1090.2 $[M+3H]^{3+}$ 1097.5 $[M+Na+2H]^{3+}$	Monofucosylated core, Xyl, three LDN
$H_3N_8F_2P$	1138.9 $[M+3H]^{3+}$ 1146.1 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, three LDN
$H_3N_8F_3P$	1187.4 $[M+3H]^{3+}$ 1194.6 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, two LDN, one LDN-F
$H_3N_8F_4P$	1235.7 $[M+3H]^{3+}$ 1243.7 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, one LDN, two LDN-F
$H_3N_{10}F_2P$	1274.0 $[M+3H]^{3+}$ 1281.4 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, four LDN
$H_3N_{10}F_3P$	1322.7 $[M+Na+2H]^{3+}$	^{3,6}F, Xyl, three LDN, one LDN-F

^{3,6}F, α3⁄α6-di-fucosylation of the innermost GlcNAc.

Kappa-5 was subjected to reduction, alkylation and subsequent trypsinization. Resulting glycopeptides were analyzed by LC-ESI-MS. Glycan compositions are given in terms of hexose (H), *N*-acetylhexosamine (N), fucose (F) and pentose/xylose (P). Monoisotopic masses of glycopeptide precursors are given throughout. Proposed glycan structures are deduced from glycopeptide MS after enzyme treatments and fragmentation data. Another special property of kappa-5 is that it is recognized by IgE as well as IgG antibodies in sera from *S. mansoni* infected individuals but not from uninfected individuals, whereas no other SEA components are as strongly reactive with IgE [18]. In serum from *S. mansoni*-infected mice IgE has been detected that is specific for the N-glycan core modifications α3-fucose and possibly β2-xylose [45], which can both be present on *S. mansoni* egg glycans [6;8]. Interestingly, recombinant kappa-5 from HEK-cells, a mammalian cell type not capable of producing core α3-fucosylated and/or β2-xylosylated glycans, did not reveal IgE reactivity but had ample IgG reactivity in human sera, suggesting that glycans are the IgE targets [18]. Our data confirm that kappa-5 glycans constitute the IgE epitopes (Figure 3) and moreover demonstrate that the antenna structures on kappa-5 glycans, including the LDN epitopes, are not involved in IgE binding of kappa-5 to human infection sera (Figure 3C). As the N-glycan core of kappa-5 glycans contains two fucoses linked to the innermost GlcNAc and a xylose (Table 1), our data together with the previously published data on the antigenic properties of the α3-core fucose and β2-xylose [45], strongly suggest that the IgE antibodies in *S. mansoni* infection sera are targeted against the core decorations of kappa-5. Notably, asparagine-linked glycans belong to the most abundant environmental immune determinants and form the basis of the so-called cross-reactive carbohydrate determinants (CCDs) [46]. About 20% or more of allergic patients generate specific anti-glycan IgE, often accompanied by IgG. Despite some structural variation, the two main motifs in these CCDs are the xylose and the core α3-linked fucose, which form the essential part of two independent epitopes. With respect to kappa-5, we believe that in particular the xylose residue forms part of the minimal glycan IgE epitope, since omega-1 and IPSE/α1, which also contain core di-fucosylated glycans but lack the xylose residue, are not bound by IgE in schistosomiasis infection sera [18].

In a global glycomic comparison of glycans from *S. mansoni* and *S. japonicum* eggs, N-glycans with a α3-, β6-difucosylated, xylosylated core were found exclusively in the *S. japonicum* egg-derived glycan fraction [6]. We here show that also *S. mansoni* is capable of expressing this highly unusual combination of core decorations. The specific three LDN antennae-containing glycan of kappa-5 was not observed by Khoo *et al.* [6]. It is possible that within the total PNGase A-released glycan pool of *S mansoni* eggs, the major kappa-5-derived glycan $H_3N_8F_2P$ forms a minor fraction that could not be detected because it was obscured by other more abundant

glycans. However, in a recent MALDI-TOF MS based profiling study of released glycans from several life stages of *S. mansoni*, the PNGase A-sensitive $H_3N_8F_2P$ glycan species was observed in eggs as well as miracidia [7]. Also, kappa-5 was detected in eggs as well as miracidia, both at the protein and mRNA level [18], which is in line with the current data indicating that the $H_3N_8F_2P$ glycan is associated with kappa-5. Interestingly, the core-difucosylated LeX-containing glycans observed on IPSE/α1 and omega-1 [11;12] were detected in the PNGase A-released fraction of eggs, but not of miracidia [7], which is in line with the observation that IPSE/α1 and omega-1 are expressed in the sub shell area of the egg [9;10]. Kappa-5 has been located to the same area, but as yet it is not clear whether kappa-5 is glycosylated differently from IPSE/α1 and omega-1 because it is produced in the context of a different cellular glycosylation machinery, or because protein-specific effects play a role.

The observation that kappa-5 is the major LDN-expressing glycoprotein from schistosome eggs is additionally relevant in view of a previous report that a synthetic LDN-glycoconjugate can induce granulomas in a mouse model based on the implantation of coated Sepharose beads as artificial eggs [4]. Liver granulomas with high similarity in terms of cellular constituency and temporal regulation compared to granulomas induced by schistosome eggs in a natural infection were induced by SEA- and LDN-coated beads, but not by a range of fucosylated conjugates including LeX [4]. The potential role of kappa-5 as an authentic granuloma-inducing component of schistosome eggs should be investigated.

MS techniques are at the basis of structural analysis of glycans, and MS is an indispensible tool to obtain sensitive and detailed structural information on scarce and complex material such as pathogen-derived glycoproteins. It remains particularly difficult, however, to determine the specific positions and linkages of antenna elements in branched glycans. Therefore, validation of the structural assignments by glycosidase treatments and/or anti-glycan antibodies is critical for a detailed and solid interpretation of MS data and the determination of glycan structures. To generate a tool to discriminate between the immunogenic LDN-F and F-LDN elements, we investigated the specificity of two α-fucosidases and one β-*N*-acetylhexosaminidase using synthetic LDN-F- and F-LDN-containing glycoconjugates. Interestingly, under the conditions applied, α1(-3,4)-fucosidase derived from *X. manihotis* was found to specifically cleave the fucose from LDN-F, whereas α-L-fucosidase from bovine kidney could cleave off the fucose of F-LDN but not LDN-F. In addition, β-*N*-acetylhexosaminidase from *C. ensiformis* provided structural information by the cleavage of unsubstituted HexNAc from LDN-F. Using this knowledge, we were able to demonstrate that a minor subset of kappa-5 glycans carries terminal LDN-F motifs, whilst no F-LDN motifs were observed on kappa-5. These specific glycosidases will be useful tools to distinguish F-LDN and LDN-F in a variety of

settings, as these isomers form antigenically different motifs. For instance, in schistosome infections, high IgG antibody responses are found against F-LDN, but not against LDN-F [25;47].

With the completion of the glycosylation analysis of kappa-5, three major *S. mansoni* egg antigens [48;49] have now been identified in terms of protein and glycan sequence. This information will contribute to our understanding of the immunological mechanisms induced by these proteins and/or the glycans that they carry. The collective glycans detected on these three major egg antigens seem to make up the majority of N-glycans detected in *S. mansoni* ES glycoproteins [8] and in the specific PNGase A-sensitive fraction of egg N-glycans [7].

Materials and Methods

Antigens, glycoconjugates and sera

S. mansoni SEA was prepared as described previously [10]. Kappa-5 was isolated by soybean agglutinin (SBA; Sigma, Zwijndrecht, the Netherlands) affinity chromatography as described previously [18] or via a slightly adapted method. For the adapted method, two mg of SBA was coupled to one ml N-hydroxysuccinimide-activated Sepharose beads (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. SEA in PBS, pH 7.4, was applied to a column containing the SBA beads and the bound material was eluted with 0.1 M galactose in PBS, pH 7.4. To maximize extraction efficiency, the effluent was reapplied once to the column. Effluent and eluate fractions were analyzed by SDS-PAGE with silver staining and Western blotting for the presence and purity of kappa-5. Eluate fractions containing purified kappa-5 were pooled, concentrated by ICON concentrators (9K MWCO, Thermo scientific, Rockford, IL, USA), dialyzed against PBS using Slide-a-lyzer dialysis cassettes (3.5 MWCO, Thermo scientific) and stored at -20°C.

For testing the specificity of exoglycosidases used in this study, the synthetic glycoconjugates Fucα1-3GalNAcβ1-4GlcNAcβ1-3Galα1-$(CH_2)_5$-squarate (F-LDN-tag) and GalNAcβ1-4(Fucα1-3)GlcNAcβ1-3Galα1-$(CH_2)_5$-squarate (LDN-F-tag), recovered after derivatization with diethylsquarate during a protein coupling procedure [25;26] were used.

Approval for this study has been granted by the Medical Ethics Committee of the Leiden University Medical Center. The serum samples used to determine the IgE-reactive properties of kappa-5 were provided from an immunoepidemiological study that was previously carried out in the village of Ndombo, Senegal. The study design, epidemiology, sample collection and ethical procedures have been described in detail [27;28]. In short, consent forms were developed in the local language. The purpose and contents of the study were explained in detail to the community in the local language. Informed consent was obtained from individual adult

participants but for children, the parents or guardians consented on their behalf. Each individual signed a consent form before commencement of any activity. All information obtained from participants was kept confidential.

Venous blood samples were collected, allowed to stand at room temperature for 1 hour, and centrifuged at 1500 rpm. The serum was carefully removed and stored frozen at -15°C. The serum samples were transported on dry ice to the Netherlands, aliquoted in 1.5 ml tubes (Eppendorf, Hamburg, Germany) and stored at -80°C until use.

SDS-PAGE and Western blotting

Kappa-5 was separated on a 12% polyacrylamide gel by SDS-PAGE under reducing conditions using the Mini-Protean Cell system (Bio-rad, Veenendaal, the Netherlands). Proteins were detected with silver stain, as previously described [29]. For Western blotting, the proteins were transferred onto a nitrocellulose membrane in a Bio-rad Criterion Blotter system according to manufacturer's instructions. For detection of LDN glycosylation, protein blots were stained as described previously [30]. In short, blots were blocked with BSA and incubated with monoclonal antibody (mAb) 273-3F2, which binds to LDN [21]. Blots were subsequently incubated with alkaline phosphatase-labeled goat-anti-mouse IgG,A,M (Caltag; Invitrogen, Breda, the Netherlands) and stained with nitro blue tetrazolium (NBT) / 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

To test the glycan-dependent binding of IgE antibodies to kappa-5, nitrocellulose membranes carrying kappa-5 were blocked with PBS/0.1% Tween and treated with meta-periodate in acetate buffer or only acetate buffer as a control. Then, membranes were incubated with human infection sera diluted 1:100 and subsequently with alkaline phosphatase-labeled mouse anti-human IgE (Sigma). Antibody binding was visualized by NBT/BCIP. To determine the efficacy of meta-periodate treatment, meta-periodate- and mock-treated membranes were stained with SBA-HRP and 3,3'-Diaminobenzidine (DAB). As a control, membranes were stained with silver stain as described before [31] and the anti-kappa-5 antibody 1G2 ([18] and unpublished results).

IgE binding ELISA

Maxisorp immuno plates (NUNC, Roskilde, Denmark) were coated with 5µg/ml of the indicated kappa-5 variants and blocked with PBS/0.05% Tween. Then, plates were incubated with human infection sera diluted 1:100 and subsequently with alkaline phosphatase-labeled mouse anti-human IgE (Sigma). Binding was visualized with para-nitrophenylphosphate (pNPP).

Sample preparation for mass spectrometry

Tryptic digestions were either performed in solution or in gel after SDS-PAGE. For the in-solution approach, to ~ 5 µg of kappa-5 in 50 µl 50 mM ammonium bicarbonate, 0.1 µg of trypsin was added (Promega, Leiden, the Netherlands) followed by overnight incubation at 37°C with or without prior reduction and alkylation. For reduction and alkylation, the sample was mixed with 0.05 volumes of 200 mM dithiothreitol, incubated for 30 min at 56°C, after which 0.2 volumes of 200 mM iodoacetamide were added and the sample was incubated for 30 min in the dark at room temperature. After digestion, the sample was stored at −20°C and used for nano-LC-MS and PNGase A treatment.

In-gel digestion was performed as described previously [32]. Individual protein bands of interest were excised from the gel. After tryptic digestion, (glyco)peptides were collected using two rounds of extraction with 20 µl of 0.1% trifluoroacetic acid (TFA) and stored at -20°C.

Exo-glycosidase treatments

Glycoproteins, tryptic glycopeptides or glycoconjugates were treated with β-*N*-acetylhexosaminidase from *Canavalia ensiformis* (62.5 mU; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 24 h at 37°C. α-Fucosidase treatment on these samples was performed with α1-(3,4)-fucosidase from *Xanthomonas manihotis* (0.5 mU; Sigma) or α-L-fucosidase from bovine kidney (15 mU; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 24 h at 37°C. Untreated and treated samples were subjected to nano-LC-MS(/MS), in case of glycoproteins after trypsin digestion.

PNGase A release

A tryptic digest of kappa-5 was adjusted to pH 5.0 by addition of TFA and incubated with PNGase A (10 mU; Roche Diagnostics) overnight at 37°C. The sample was applied to a Zip-Tip_{C18} (Millipore, Billerica, MA, USA), washed 5 times with 20 µl 0.1% TFA and eluted with 50% acetonitrile (ACN), 0.1% TFA. The flow-through containing released glycans was applied to a self-packed (20 µl) porous graphitized carbon column (Carbograph; Alltech, Deerfield, IL, U.S.A.). The column was first equilibrated with a standard of 10 pmol maltopentaose to prevent irreversible binding of kappa-5-released carbohydrates. The maltopentaose was eluted from the column with 70% aqueous ACN and after washing the carbon column with water, the kappa-5-released carbohydrates were loaded on this column. The glycans were eluted with 50% aqueous ACN.

MALDI-TOF(/TOF)-MS

PNGase A-released and purified glycans of kappa-5 were spotted directly on the MALDI target plate and mixed with one µl of matrix solution, 2,5-dihydroxybenzoic acid (DHB; Bruker Daltonics, Bremen, Germany), 10 mg/ml 50% ACN/0.1% TFA. Sample spots were dried under a stream of warm air. MALDI-TOF(/TOF)-MS data were obtained using an Ultraflex II time-of-flight mass spectrometer (Bruker Daltonics) equipped with a LIFT-MS/MS facility. Spectra were acquired in the positive-ion reflectron mode. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated with 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. For confirmation of the identity of the isolated kappa-5, peptides extracted after in-gel digestion were purified by Zip-Tip$_{C18}$, eluted with 1 µl 50% ACN, 0.1% TFA onto the sample target plate, and mixed with 1 µl of matrix solution (10 mg/ml DHB in 50% ACN, 0.1% TFA). The peptide mass fingerprint (PMF) spectrum acquired was processed with FlexAnalysis version 3.3. The baseline subtraction algorithm Median was used, and peaks were detected using the Snap algorithm with following settings: S/N threshold >7, relative intensity threshold 10%, minimum intensity threshold 50, maximal number of peaks 100, quality factor threshold 90, Snap average composition averagine. The PMF data were searched with Mascot version 2.2.06 (Matrix Science, London, UK) against all entries (12,852,469) in the NCBInr database (release 31 Jan 2011), using Biotools version 3.2 as interface. Mascot parameter settings were trypsin digestion, up to 2 missed cleavages allowed, fixed carbamidomethyl modification of cysteine and variable methionine modification. The maximum mass tolerance was set at 0.1 Da. A protein score greater than 85 was significant ($p > 0.05$), with the scores for hits to the kappa-5 variants in the NCBInr protein database ranging from 162 to 198.

Nano-HPLC-ESI-ion trap-MS(/MS)

(Glyco)peptide samples were applied to a reverse-phase column (PepMap, 3 µm, 75 µm × 100 mm; Dionex, Amsterdam, the Netherlands) using a Ultimate 3000 nano-LC system (Dionex). The column was equilibrated with eluent A (0.1% formic acid in water) at a flow rate of 300 nL·min^{-1}. After injection of the sample, a linear gradient was applied to 25% B (95% ACN, 0.1% formic acid) in 15 min, followed by a gradient to 70% B in 10 min and subsequent isocratic elution of 5 min. The eluate was monitored by absorption at 215 nm.

The LC column was coupled to an Esquire HCT Ultra ESI-IT-MS (Bruker Daltonics) equipped with an online nanospray source operating in the positive-ion mode. Electropolished, stainless steel LC-MS emitters (150 µm OD, 30 µm ID) from Proxeon A/S (Odense, Denmark)

were used for electrospray (1100-1250 V). The solvent was evaporated at 170°C employing a nitrogen stream of 6 L min^{-1} and ions from *m/z* 400 to *m/z* 1800 were registered in the MS mode. When operated in the auto MS/MS mode, registering ions from *m/z* 140 to 2200, each MS scan was followed by the acquisition of MS/MS spectra of up to five of the most abundant ions in the MS spectrum.

Acknowledgements

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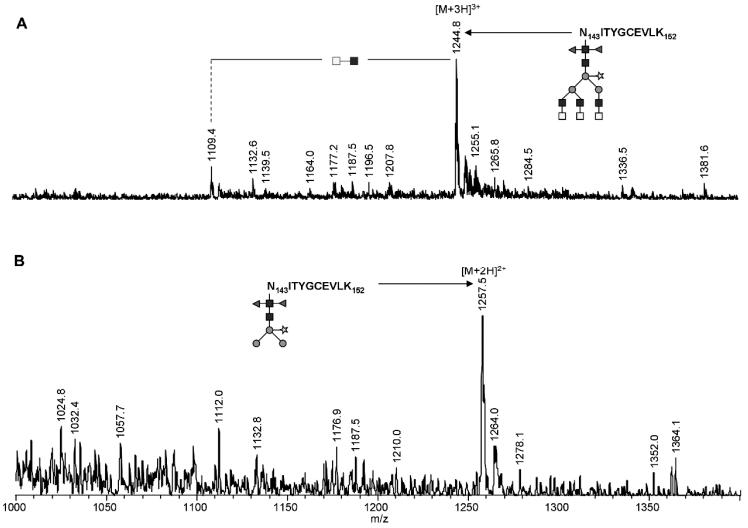
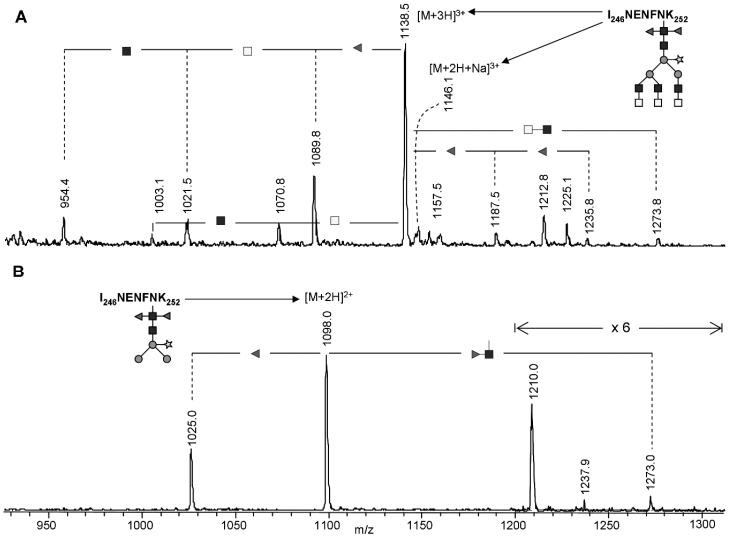
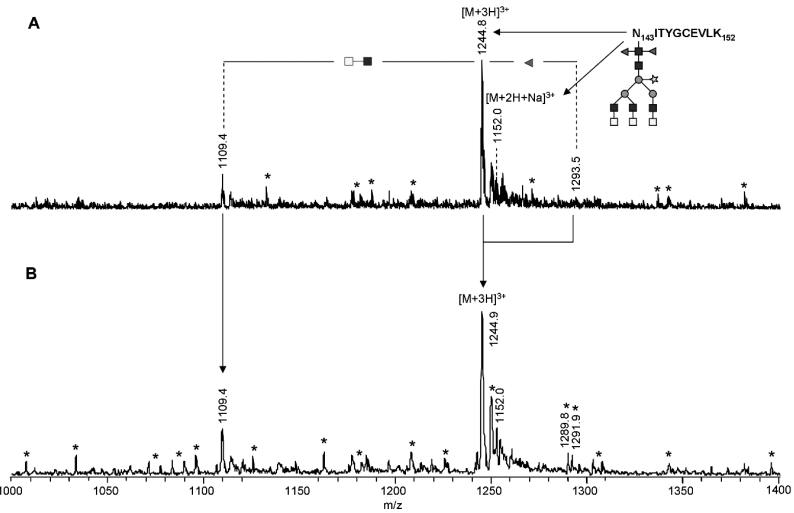
Supplementary Figures



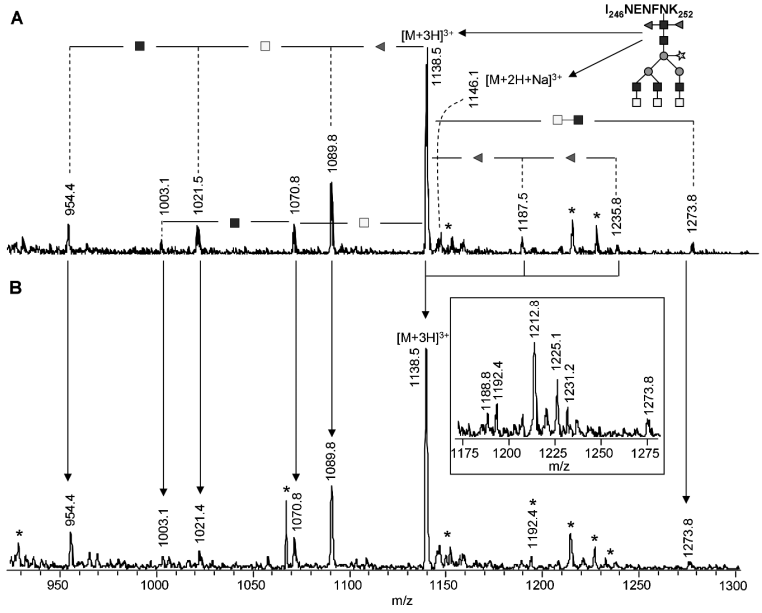
Supplementary figure 1. β-*N*-acetylhexosaminidase treatment of glycopeptide N143-K152. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides were analyzed by LC-MS before (A) and after (B) treatment with β-*N*-acetylhexosaminidase. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.



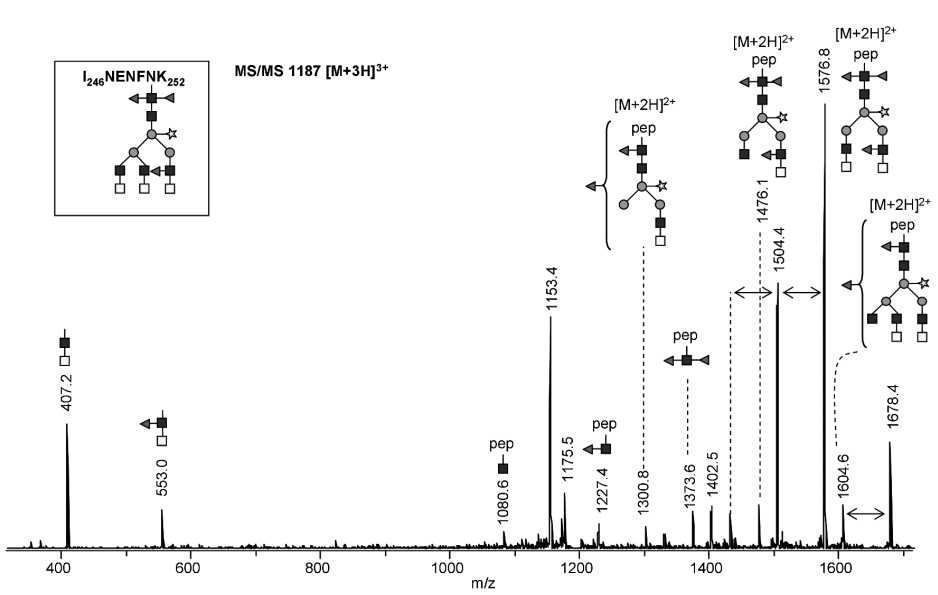
Supplementary figure 2. β-*N*-acetylhexosaminidase treatment of glycopeptide I246-K252. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides were analyzed by LC-MS before (A) and after (B) treatment with β-*N*-acetylhexosaminidase. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.



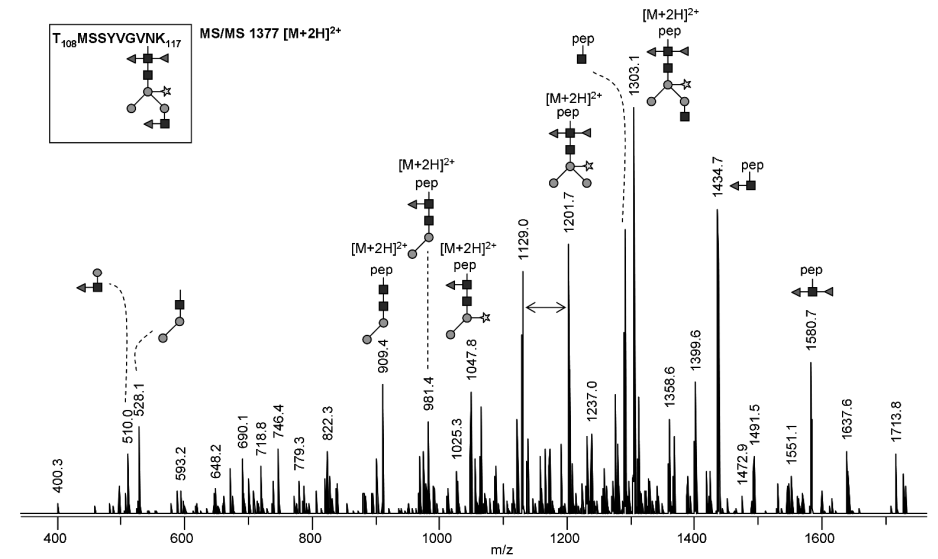
Supplementary figure 3. α-Fucosidase treatment of glycopeptide N143-K152. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides were analyzed by LC-MS before (A) and after (B) treatment with α-fucosidase from *X. manihotis*. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.



Supplementary figure 4. α-Fucosidase treatment of glycopeptide I246-K252. Kappa-5 was subjected to reduction and alkylation, digested with trypsin and the resulting glycopeptides were analyzed by LC-MS before (A) and after (B) treatment with α-fucosidase from *X. manihotis*. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose.



Supplementary figure 5. MS/MS of peptide I246-K262 carrying $H_3N_8F_3P$ N-glycans. The MS/MS spectrum was acquired by LC-ion trap-MS/MS of a tryptic digest of kappa-5. Glycopeptides are singly positively charged, unless specified otherwise. The given glycan structures are examples. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose; pep, peptide.



Supplementary figure 6. MS/MS of peptide T108-K117 carrying $H_3N_3F_3P$ N-glycans. The MS/MS spectrum was acquired by LC-ion trap-MS/MS of a tryptic digest of kappa-5 after treatment with β-*N*-acetylhexosaminidase. Glycopeptides are singly positively charged, unless specified otherwise. The given glycan structures are examples. Differences in fucose content are indicated by double-headed arrows. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; open star, xylose; pep, peptide.

Chapter 4

Specific glycan elements determine differential binding of individual egg glycoproteins of the human parasite *Schistosoma mansoni* by host C-type lectin receptors

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Abstract

During infection with the blood fluke *Schistosoma mansoni*, glycan motifs present on glycoproteins of the parasite's eggs mediate immunomodulatory effects on the host. The recognition of these glycan motifs is primarily mediated by C-type lectin receptors (CLR) on dendritic cells (DC) and other cells of the immune system. So far, it is unknown however which individual glycoproteins interact with the different CLR, and which structural components are involved. Here we investigated the structural basis of the binding of two abundant egg antigens, kappa-5 and IPSE/α1, by the CLR DC-specific ICAM3-grabbing non-integrin (DC-SIGN), macrophage galactose-type lectin (MGL) and mannose receptor (MR). In the natural soluble form, the secretory egg glycoprotein IPSE/α1 interacts with DC mainly via MR. Surprisingly, in plate-based assays MR preferentially bound to mannose conjugates, while in cell-based assays, IPSE/α1 is bound via the fucosylated Galβ1-4(Fucα1-3)GlcNAc (LeX) motif on diantennary N-glycans. Kappa-5, in contrast, is bound by DC via all three CLR studied and for a minor part also via other, non-CLR receptors. Kappa-5 interacts with MGL via the GalNAcβ1-4GlcNAc (LDN) antenna present on its triantennary N-glycans, as well as the GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) antennae present on a minor N-glycan subset. DC-SIGN binding of kappa-5 was mediated via the LDN-F antennae, whereas binding of MR may involve either LDN-F antennae or the fucosylated and xylosylated chitobiose core. This study provides a molecular and structural basis for future studies of the interaction between CLR and other soluble egg antigen (SEA) glycoproteins and their effects on the host immune response.

Introduction

Schistosomes are parasitic helminths that infect over 200 million people world-wide. The main pathological symptoms of schistosomiasis are caused by the deposition of parasite eggs by adult worms into the organs of its human host and the subsequent immunological consequences, which include the formation of perioval granulomas associated with a pronounced Th2-type immune response [1]. Many aspects of these egg-induced immune processes are thought to be at least partly mediated by protein glycosylation of the soluble egg antigens (SEA) [2]. This requires binding of the glycans to host receptors on antigen presenting cells (APCs) such as macrophages and dendritic cells (DC), leading to internalisation of the glycoprotein and/or activation of signaling pathways.

Previously, Van Liempt et al. demonstrated that the complex *Schistosoma mansoni* SEA mixture when coated on beads, is recognised by monocyte-derived human immature DC (iDCs) primarily through three C-type lectin receptors (CLR): dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), macrophage galactose-type lectin (MGL) and mannose receptor (MR) [3]. The binding of DC-SIGN to the SEA-coated beads could be partly inhibited using monoclonal antibodies (mAbs) directed against the glycans Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), suggesting that these two glycan structures on SEA glycoproteins contribute to the interaction between DC-SIGN and SEA [4]. In contrast, MGL appears to recognise terminal GalNAc moieties in (fucosylated) GalNAcβ1-4GlcNAc (LDN) ligands on solid phase coupled SEA glycoproteins, as concluded from studies using anti-LDN and anti-LDN-F blocking antibodies [5]. MR ligands in SEA have not been investigated yet, but it is known that MR can bind fucose- and mannose-containing glycans [6].

While these studies provide valuable indications for the involvement of specific glycan elements in SEA-CLR interactions, the role of individual glycoprotein components of the complex SEA mixture in these interactions has not yet been assessed. Moreover, binding and uptake of SEA by the CLRs has only been studied using SEA coupled to a solid phase as in coated to beads or to ELISA-plates [3;4]. In the natural *S. mansoni* egg context, however, protein and glycoprotein constituents of SEA are presented to the host as soluble molecules rather than bound to a particle, cell or whole organism. Some SEA components are excreted by the schistosome egg through pores in the egg shell, forming the so-called excretory/secretory fraction (ES) while others come into contact with the host after eggs die and release their soluble contents into the tissue surrounding the egg. Therefore, the biological context may be better reflected by studying the binding between the natural soluble egg components and cell-associated CLR.

Previously, three major soluble *S. mansoni* egg glycoproteins displaying immunogenic activities have been identified: omega-1 [7], which is able to drive DC-mediated Th2 responses both *in vitro* and *in vivo* [8]; IPSE/α1 [9], an inducer of IgE-dependent IL-4-release in human and murine basophils [10;11]; and kappa-5 [12], which has been shown to be a target of IgE in human *S. mansoni* infection sera. Detailed glycosylation analysis showed that the glycans expressed by these glycoproteins make up a major subset of glycans found in total SEA [13;14]. Omega-1 and IPSE/α1 are glycosylated almost identically, both carrying diantennary N-glycans with a difucosylated core and LeX antennae (Figure 1 and [15;16]). Kappa-5, on the other hand, expresses a completely different set of SEA glycans, characterised by the presence of triantennary N-glycans with terminal LDN and, to a lesser extent, LDN-F motifs (Figure 1 and [17]).

In this study, we have examined the interaction of IPSE/α1 and kappa-5 with DC-SIGN, MGL and MR as expressed by DC and single CLR-cell lines. Using exo-glycosidases, we were able to create specific glycoprotein variants and could precisely define the structural elements involved in the binding of the egg glycoproteins by individual cellular CLR. To our knowledge, this is the first report that assesses the binding of individual, native S. mansoni egg glycoproteins to CLR in a cellular context. These data provide a molecular basis for the CLR-mediated interaction of DC with soluble glycoproteins secreted/excreted by schistosome eggs and thereby contribute to the understanding of how individual components of SEA induce CLR-dependent immune responses.

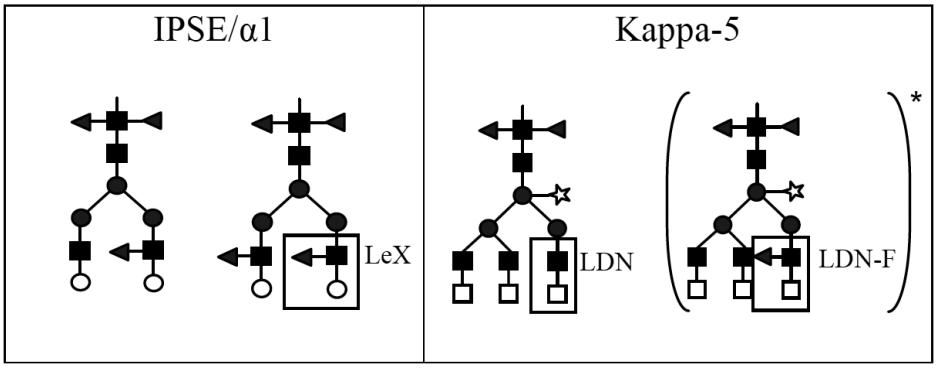

Figure 1. Major glycan structures of IPSE/α1 and kappa-5. Triangle, fucose; open square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; open circle, galactose; dark circle, mannose; open star, xylose; LeX, Galβ1-4(Fucα1-3)GlcNAc; LDN, GalNAcβ1-4GlcNAc; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAc. *Structure is present on a small subset of kappa-5 glycoproteins.

Results

Binding of CLR-Fc constructs to SEA-associated glycoconjugates

SEA is a largely uncharacterised mixture of proteins and glycoproteins that interacts with DC-SIGN, MR and MGL on cells of the innate immune system [3]. We have previously studied the glycosylation of two proteins abundantly present in SEA; IPSE/α1 carries diantennary glycans with one or two LeX antennae ([15] and Figure 1), while kappa-5 glycosylation is characterised by the presence of triantennary N-glycans with terminal LDN motifs with a minor subset of kappa-5 glycans carrying terminal LDN-F motifs ([17] and Figure 1). To test and confirm that DC-SIGN, MR and MGL can bind to glycan elements present on kappa-5 and IPSE/α1, binding of Fc constructs of these CLR to a glycoconjugate microarray containing several schistosome-related synthetic glycoconjugates was analysed (Figure 2).

The majority of fucose-containing conjugates on the array, including LeX and LDN-F, were bound by DC-SIGN-Fc in a concentration-dependent manner (Figure 2A). This is in line with previous data showing that DC-SIGN has a broad specificity for α1-3 and α1-4 fucosylated motifs [18;19]. Relatively weak binding of DC-SIGN was observed to conjugates terminating with

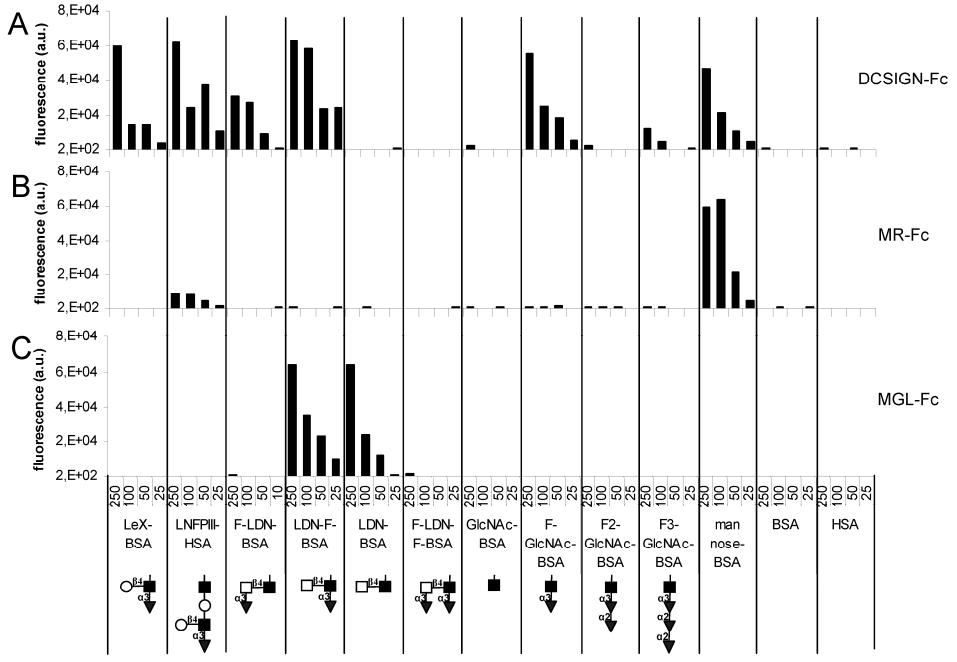

Figure 2. Binding of Fc-constructs of DC-SIGN, MGL and MR to an extensive set of schistosome-related, synthetic glycoconjugates. Glycan-binding specificities of human DC-SIGN (A), murine MR (B) and human MGL (C) were tested by glycan array screening. The glycan array was probed with 2 μg ml^{-1} DC-SIGN-Fc or 5 μg ml^{-1} MGL-Fc and MR-Fc. Results of one out of two independent experiments are shown. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose.

Fucα1-2Fucα1- (see F2-GlcNAc and F3-GlcNAc in Figure 1A), a sequence that has so far only been found in schistosomes [13;20]. F-LDN-F is not bound by DC-SIGN-Fc (Figure 2A), even though it contains α1-3-linked fucoses, which may be explained by spatial interference due to close proximity of the two fucoses. MR, like DC-SIGN, is considered to be a mannose- and fucose-recognising receptor [6;18]. On the glycoconjugate array, MR-Fc showed a relatively high affinity only for mannose-BSA (Figure 2B), while binding of DC-SIGN-Fc to mannose-BSA was similar to the binding to fucosylated ligands such as LeX (Figure 2A). MGL-Fc selectively bound to LDN and LDN-F on our array (Figure 2C), which is in accordance with the reported affinity of MGL to terminal, unsubstituted GalNAc residues [5].

Conventional ELISA confirms that DC-SIGN-Fc binds to LeX-expressing IPSE/α1 (Figure 3A). Surprisingly, DC-SIGN-Fc bound kappa-5 to a similar extent as IPSE/α1 (Figure 3A), which is remarkable as the only known DC-SIGN-ligand present on kappa-5, LDN-F (Figure 2A and [18]), is present on only a very minor subset of kappa-5 glycoproteins ([17] and Figure

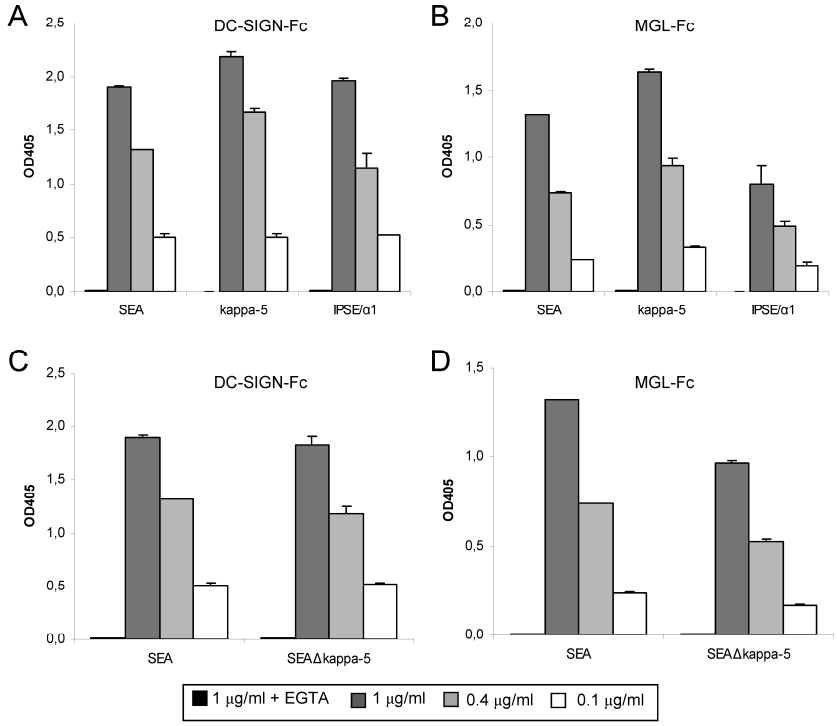

Figure 3. Interaction of IPSE/α1 and kappa-5 with DC-SIGN-Fc and MGL-Fc constructs. Binding of DC-SIGN-Fc (A, C) and MGL-Fc (B, D) to IPSE/α1 and kappa-5 (A,B) and SEA depleted of kappa-5 (SEAΔkappa-5) (C,D) was determined by ELISA in the presence (black bars) or absence (grey and white bars) of EGTA. Various concentrations for each Fc construct were used. Results are representative for three independent experiments.

1). In ELISA, MGL-Fc binds relatively intensely to kappa-5 as compared to IPSE/α1 (Figure 3B), likely because LDN-motifs are abundantly expressed on kappa-5 ([17] and Figure 1). Kappa-5 has been demonstrated to be the major LDN-containing glycoprotein in SEA [17]. In line with this observation, MGL-Fc binding to SEA depleted of kappa-5 (SEAΔkappa-5) is decreased compared to that of total SEA (Figure 3D), but significant other MGL ligands are still present. The affinity of DC-SIGN-Fc for SEA upon kappa-5 depletion remained high (Figure 3C).

Human iDCs bind the differentially glycosylated kappa-5 and IPSE/α1 through different CLR

The array and ELISA data using Fc-constructs of CLRs indicate that MGL and DC-SIGN are candidate receptors for kappa-5 and IPSE/α1. As a more accurate reflection of the natural situation in which soluble egg glycoproteins are bound by cell surface expressed CLRs, we next incubated fluorescently labeled kappa-5 and IPSE/α1 *in vitro* with human iDCs. The iDCs expressed each of the CLR DC-SIGN, MGL and MR (Supplementary Figure 1). The contribution of each single CLR in binding of kappa-5 and IPSE by DCs was assessed by addition of specific CLR blocking agents (Figure 4).

The observation that binding of IPSE/α1 to iDCs was strongly inhibited by addition of the calcium chelator EGTA indicates that this binding is CLR mediated (Figure 4A). Moreover, mannan, a natural ligand that competes for binding to DC-SIGN and MR, significantly reduced IPSE/α1-binding by DCs. More specifically, preincubation with blocking antibodies against DC-SIGN, MGL and MR showed that MR is the major receptor for IPSE/α1 on iDCs, while MGL and DC-SIGN both appeared to play only a minor role (Figure 4A).

In contrast, for kappa-5, preincubation with the specific CLR antibodies as well as with EGTA and mannan suggested the involvement of all three CLRs in kappa-5 binding by iDCs without one or the other in a dominant role (Figure 4B). Inhibition induced by mannan evidently exceeded the inhibition by the specific antibodies against DC-SIGN or MR, strongly supporting a role for both MR and DC-SIGN in the binding of kappa-5 by iDCs. Mannan inhibited the binding of kappa-5 by iDCs to a lesser extent as compared to IPSE/α1-iDC binding, in line with the larger role of MR for the binding of IPSE/α1. In addition, after preincubation of the iDCs with EGTA, still 24% of the initial kappa-5 binding could be detected (Figure 4B), indicating a role for other, Ca^{2+}-independent receptors in the interaction of kappa-5 with DCs.

In summary, iDCs bound IPSE/α1 preferentially via MR, while kappa-5 was recognised through multiple CLRs including DC-SIGN, MGL and MR, as well as via other, Ca^{2+}-independent receptor(s).

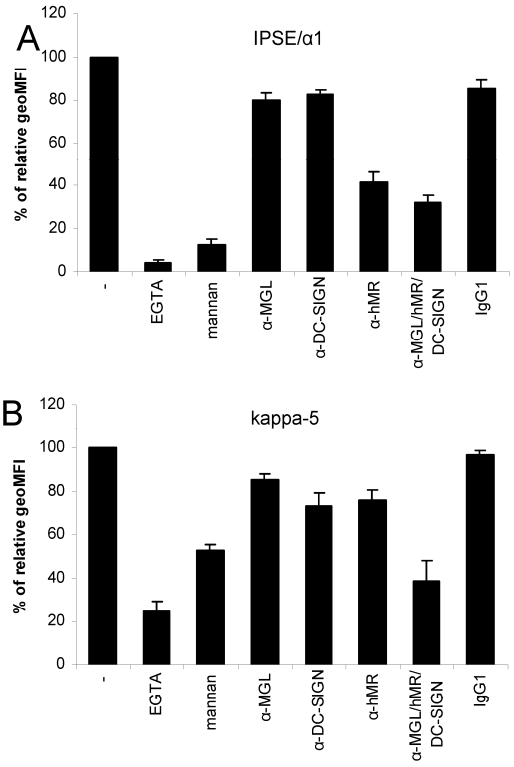

Figure 4. Interaction of IPSE/α1 and kappa-5 with CLR on immature DCs. Binding of fluorescently-labeled IPSE/α1 (A) and kappa-5 (B) to monocyte-derived human iDCs was assessed in the absence or presence of EGTA (blocking all CLR), mannan (blocking DC-SIGN and MR) or blocking mAbs directed against DC-SIGN, MGL, MR or a control antibody (IgG1). Unblocked conditions (-) were set to 100 %. The presented results represent three combined, independent experiments. Error bars represent standard error of the mean. * $p < 0.05$, ** $p<0.01$, for significant differences compared to the unblocked conditions (-), based on a two-sided paired *t*-test. # n=2, no statistical analysis was performed. MFI; mean fluorescence intensity.

Antenna fucoses on kappa-5 and IPSE/a1 are involved in binding to DC-SIGN and MR, while terminal GalNAc residues confer binding of kappa-5 to MGL

Next, we set out to identify the precise glycan elements which mediate kappa-5 and IPSE/α1 recognition by the individual CLRs in a cellular context. Kappa-5 and IPSE/α1 were treated with β-*N*-acetylhexosaminidase (hexnac) and/or α-fucosidase (fuc) to remove specific monosaccharides that form potential CLR ligands. Of note, α-fucosidase from *X. manihotis* was used which specifically removes α1-3-linked antenna fucoses of N-glycopeptides while being unable to cleave off the core-linked fucoses [17]. α-Fucosidase treatment of IPSE/α1 generated a glycoprotein variant mainly carrying diantennary glycans with unsubstituted Galβ1-4GlcNAc (LN) antennae (major set), as well as some LDN antennae (minor fraction) on an N-glycan core

which is substituted with α3- and α6-linked fucoses (IPSE/α1fuc, Supplementary Figure 2 for site N80). The effect of this enzyme was previously established in a mass spectrometric structural study on the egg glycoprotein omega-1, which carries nearly identical glycosylation [16]. Treatment of kappa-5 with β-*N*-acetylhexosaminidase resulted in the complete removal of unsubstituted GlcNAc and GalNAc residues in LDN and LDN-F, as verified by nano-LC-MS analysis of site N_{251} (κ5hexnac, Supplementary Figure 3). In addition, a kappa-5 variant was generated of which fucoses were removed from LDN-F by treatment with α-fucosidase (κ5fuc, Supplementary Figure 3), as well as a variant treated sequentially with α-fucosidase and β-*N*-acetylhexosaminidase (κ5fuc/hexnac, data not shown) leaving intact only the trimannosyl core structure with α3/α6-fucose and β2-xylose core-modifications.

Subsequently, binding of the native and modified glycoprotein variants by cell lines expressing only one of the three human CLR, DC-SIGN, MR or MGL, was tested (Figure 5). Incubation of IPSE/α1 with the cell lines showed that all three CLRs had affinity for IPSE/α1 in a Ca^{2+}-dependent manner (Figures 5A, B and C). Interestingly, while MR proved to be the dominant receptor for IPSE/α1 on iDC which express all three CLRs (Figure 4A), IPSE/α1 was bound by the DC-SIGN cell line similarly well as the MR cell line (Figures 5A and B, respectively). Binding of soluble IPSE/α1 by the MGL-expressing cell line appeared minor as compared to binding by the MR- and DC-SIGN cell lines (Figure 5C). In contrast, in the corresponding ELISA (Figure 3B), soluble MGL-Fc showed clear Ca^{2+}-dependent binding to plate-bound IPSE/α1, which indicates that significantly different affinities or specificities are observed in these two binding assay types. Furthermore, α-fucosidase treatment of IPSE/α1 revealed the involvement of the antenna fucose, as present in the major antenna motif LeX, in the interaction with both DC-SIGN and MR (Figures 5A and B). As expected, the fucoses did not have any role in the interaction between MGL and IPSE/α1 (Figure 5C).

Incubation of kappa-5 with the cell lines showed strong interactions between kappa-5 and cellular DC-SIGN (Figure 5D) as well as MGL (Figure 5F). Exo-glycosidase treatment of kappa-5 revealed that the terminal GalNAc in LDN(-F) is required for MGL-binding (Figure 5F), while the fucoses of LDN-F appeared essential for the binding of DC-SIGN for kappa-5 (Figure 5D). The latter finding is striking, as an estimated less than 5% of kappa-5 LDN antennae are fucosylated [17], which may indicate a relatively high affinity of DC-SIGN for LDN-F. Binding of kappa-5 by the MR cell line seemed only minor as compared to binding observed for the DC-SIGN and MGL cell lines, and α-fucosidase treatment did reduce kappa-5-MR binding only slightly (Figure 5E). Indeed, the glycoconjugate array data indicated that MR has little to no affinity for LDN-F (Figure 2B). Possibly MR displays affinity for the core-fucoses which remain unaffected by the α-fucosidase treatment (Supplementary Figure 3). Interestingly,

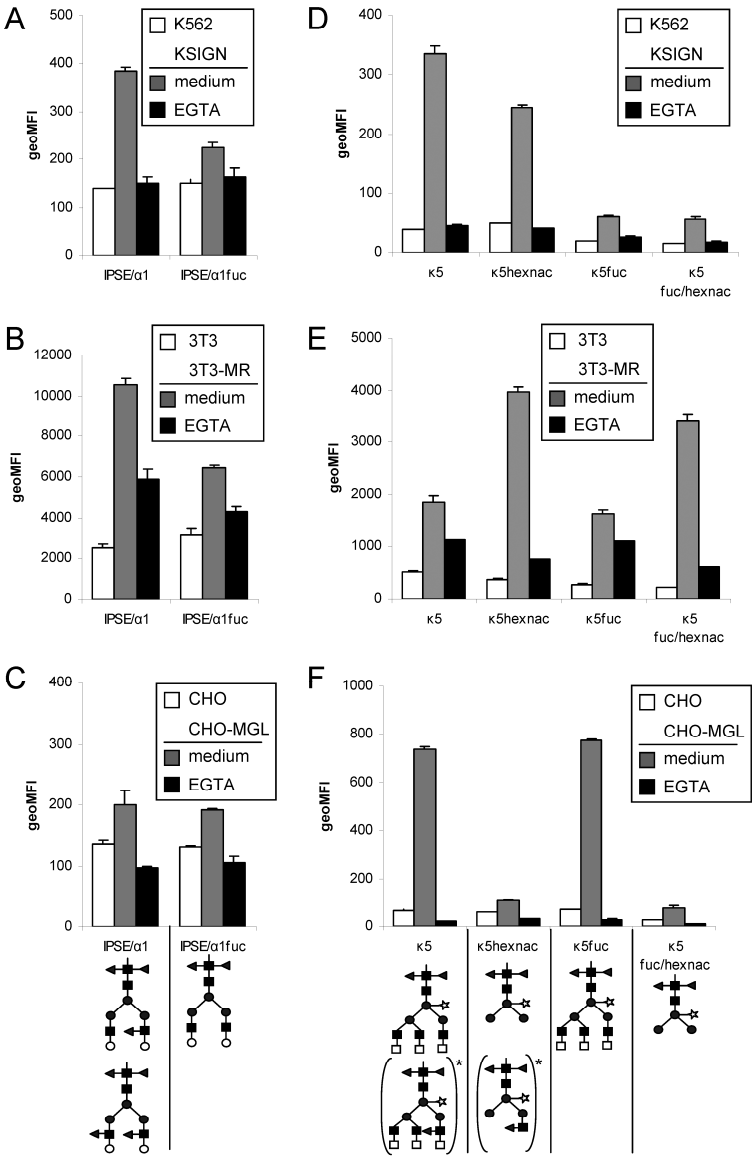

Figure 5. Structural characteristics of the glycan epitopes on IPSE/α1 and kappa-5 involved in CLR binding. Binding of untreated and exo-glycosidase-treated fluorescently labeled IPSE/α1 (A,B,C) and kappa-5 (D,E,F) to a DC-SIGN-expressing cell line (A,D), a MR-expressing cell line (B,E) and a MGL-expressing cell line (C,F), was assessed in the absence (grey bars) or presence (black bars) of EGTA. Untransfected or mock-transfected cell lines were used as control (white bars). The given glycan structures represent the major glycans present on the (treated) glycoproteins, while the structures marked with an asterisk (*) are present on a small subset of kappa-5 glycoproteins. The experiments were performed in duplicate and one representative experiment out of two is shown. Error bars represent standard deviation. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; open star, xylose; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase; κ5fuc/hexnac, kappa-5 treated sequentially treated with α-fucosidase and β-*N*-acetylhexosaminidase; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase. MFI; mean fluorescence intensity.

β-*N*-acetylhexosaminidase treatment of kappa-5 increased binding of kappa-5 by MR (Figure 5B), which is probably due to exposure of the xylosylated trimannoside, a structure also frequently observed on native glycoproteins of various *S. mansoni* life stages including eggs [14]. This effect induced by β-*N*-acetylhexosaminidase treatment was not observed in relation to kappa-5-binding by DC-SIGN (Figure 5D). On the contrary, β-*N*-acetylhexosaminidase treatment of kappa-5 reduced the interaction with DC-SIGN, indicating a higher affinity of DC-SIGN for intact LDN-F as compared to the Fucα1-3GlcNAc elements left after treatment. Furthermore, in contrast to MR, DC-SIGN seems not able to bind the xylosylated and fucosylated trimannosyl core (Figure 5D; κ5fuc/hexnac). Finally, ELISA format experiments revealed a recognition pattern of the exo-glycosidase treated glycoproteins by DC-SIGN and MGL (Supplementary Figure 4) analogous to the cell based binding assays. In conclusion, the three CLRs DC-SIGN, MGL, and MR, display differential affinity for the individual glycoproteins which is determined by specific glycan elements.

Discussion

During *S. mansoni* infection, immunogenic SEA glycoproteins are released by eggs and bound and internalised by DCs and other APCs patrolling the host tissues. We investigated the differential interaction of DC-SIGN, MR and MGL with two abundant soluble *S. mansoni* egg glycoproteins IPSE/α1 and kappa-5 each representing a differently glycosylated group of SEA glycoproteins. A first surprising observation was that on iDCs, MR is the main receptor for IPSE/α1 (Figure 4A) via its LeX glycans, while in the glycan array format LeX-glycoconjugates form relatively weak ligands for MR-Fc (Figure 2 and [21]). Secondly, although we hypothesised that MGL would play a dominant role in kappa-5-iDC interaction due to its capacity to bind to the LDN motifs on kappa-5 [5], all three CLR studied appeared to be involved in the interaction between iDCs and kappa-5 (Figure 4B). In particular the presence of LDN-F motifs on a minor subset of kappa-5 glycans contributed significantly to cellular binding via DC-SIGN.

van Liempt *et al.* previously indicated the importance of the DC-SIGN, MR and MGL, in the interaction of iDCs with SEA [3]. A mixture of blocking antibodies against these CLRs could abrogate binding of iDCs to SEA-coated 1 μm-beads by 90%, which is to the same level as reached with EGTA, a Ca^{2+}-chelator which blocks the binding of all CLRs. Also studies using synthetic glycoconjugates have suggested that DC-SIGN and MGL can bind glycan motifs that have been detected in schistosomes [4;5]. However, SEA is a complex mixture of (glyco)proteins [22], and it is yet unknown how many and which SEA glycoproteins actually carry glycan motifs that can be bound by CLRs, possibly leading to modulation of DC function. We believe that the

cellular binding studies using single well-characterised glycoproteins and enzymatically modified glycoforms thereof presented here are required to more precisely define the molecular basis of SEA-DC interactions.

Both the DC-SIGN- and the MR-expressing cell line bound IPSE/α1 in a Ca^{2+}-dependent way (Figures 5A and B). While LeX-glycoconjugates form relatively weak ligands for MR but strong ligands for DC-SIGN in the glycan array format (Figure 2 and [18;21]), on iDCs, the LeX-expressing IPSE/α1 is predominately bound by MR (Figure 4A). To be able to bind IPSE/α1, both DC-SIGN and MR require the presence of the antenna fucoses present mainly in the form of LeX (Figures 5A and B). Studies using bead-coupled glycans [4] or glycan arrays (Figure 1A and [4;18;19]) suggest that DC-SIGN has a strong preference for LeX motifs presented on pathogens, particles or a solid surface in a multivalent fashion. It can be hypothesised from our observations that MR plays a dominant role over DC-SIGN in the binding of LeX-containing glycoproteins if presented as soluble ligands. This is in line with a previous report showing a role for MR in the uptake of soluble antigens, but not in that of cell-associated antigens [23]. For DC-SIGN molecules, which bear only one CRD domain, it has been shown that the extracellular domains can aggregate *in vitro* to form DC-SIGN tetramers, which have been suggested to possess enhanced DC-SIGN capacity to bind multivalent ligands [24]. In addition, clustering of DC-SIGN in lipid rafts on the surface of iDCs has been shown to enhance binding and internalisation of viral particles [25].

Although the main glycans present on kappa-5 carry multiple termini of the MGL-ligand LDN [17], MGL did not display a dominant function in kappa-5-iDC interaction (Figure 4B). Clearly, multiple glycan elements present on kappa-5 contribute to the binding to different CLRs. Removal of the terminal GalNAc and GlcNAc units of LDN in kappa-5 by β-*N*-acetylhexosaminidase treatment abolishes MGL binding (Figure 5F), whereas removal of LDN-F associated fucose leads to the loss of DC-SIGN binding (Figure 5D) and the partial loss of MR binding (Figure 5E). These observations suggest that exposure of CLRs or CLR-expressing cells to single glycoproteins can still include multiple putative ligands due to glycan microheterogeneity, the natural variation in protein glycosylation leading to different so-called glycoforms of a single glycoprotein. This underlines that a thorough investigation of all major and minor glycan structures on a specific glycoprotein is imperative for the identification of glycan motifs putatively involved in CLR binding. Moreover, the observation that the LDN-F unit, which is present in only a very minor subset of kappa-5 glycoforms [17], dictates its binding by DC-SIGN (Figure 5D) indicates that small variations in glycosylation may have important biological effects. Consistent with this observation, LDN-F has been shown to be a strong ligand for DC-SIGN by glycan array studies ([18] and Fig. 2).

The current study, together with the structural data of the glycans of kappa-5 [17] and IPSE/α1 [15], as well as a third SEA glycoprotein omega-1 [16], provides structural background knowledge to several recent functional studies. First, kappa-5 is the major glycoprotein in SEA that carries the granulomogenic LDN motif [17;26], raising the question whether the kappa-5 associated LDN receptor MGL (Figure 5F) plays a role in the induction of the granulomatous immune responses around *S. mansoni* eggs. Second, the observations that MR rather than DC-SIGN is the most relevant CLR involved in the binding of the LeX-containing IPSE to iDCs might indicate an important function for MR during natural infection, as the secreted glycoproteins are generally believed to be the major immunoregulatory molecules of *S. mansoni* eggs [7;8;27-29]. IPSE/α1 is postulated to have a Th2-regulating role during *S. mansoni* infection via the induction of IL-4 production by basophils [10;11], as well as an anti-inflammatory role by reducing cellular infiltration into granulomas [30]. Both immunomodulatory properties are not related to its glycosylation, and thus CLR recognition will not be involved in this process. However, recently it was demonstrated that IPSE/α1 contains a functional C-terminal nuclear localisation signal via which it is transported to the nucleus in monocyte-derived human DCs [31]. Interestingly, this was a Ca^{2+}-dependent process, implicating that CLRs are involved in the initial uptake into the cell. Together with our results, it can be hypothesised that MR, which we found to be the primary CLR binding to IPSE/α1 via its antenna fucoses (Figure 4B), can mediate uptake of IPSE/α1 into cells, after which it is translocated to the nucleus to exert a currently unknown function. Interestingly, another major *S. mansoni* egg glycoprotein omega-1, which has RNase activity and Th2-inducing properties [8;32] expresses LeX-carrying glycans similar to IPSE/α1 [16]. In the mouse, a synthetic LeX conjugate has been shown to drive Th2 responses, involving the production of antigen-specific IgE and induction of IL-4 and IL-10 following intranasal immunization [32;33]. However, the SEA glycoprotein associated LeX epitopes alone are not sufficient to confer the Th2-inducing properties initiated by *S. mansoni* eggs, as IPSE/α1 in contrast to omega-1 is not able to induce the strong DC-mediated type 2 responses [8]. Since our current study shows that DCs recognise IPSE/α1 via MR by the same glycans as present on omega-1, it is conceivable that the latter glycoprotein is also bound by MR on DCs. Furthermore, experiments in mice as well as with bone marrow derived macrophages deficient for MR show the involvement of MR in the uptake of cercarial secretions and the downregulation of IFN-γ production in response to *S. mansoni* cercariae [34]. These results demonstrate that soluble schistosome glycoconjugates indeed may depend on recognition by MR on APCs for modulation of the immune response.

In summary, while SEA is bound and internalised by iDCs via three CLRs, DC-SIGN, MGL, and MR, we found that single components in SEA are differentially bound. We

demonstrated that while in plate-or array based assays DC-SIGN but not MR binds relatively strongly to LeX conjugates including the LeX-containing glycoprotein IPSE/α1, MR is the major receptor for IPSE/α1 on iDCs. Furthermore, exo-glycosidase treatment of the two differentially glycosylated egg proteins, kappa-5 and IPSE/α1, revealed the preference of the three CLRs for specific elements in/of the glycan structures: antenna fucoses in LeX for DC-SIGN and MR, antenna fucoses in LDN-F for DC-SIGN, and the GalNAc-termini in LDN(-F) for MGL. This study provides a molecular and structural basis for future studies of the interaction between CLRs and other SEA glycoproteins, and their effects on the host immune response.

Material and Methods

Antigens and exo-glycosidase treatments

SEA and IPSE/α1 were obtained as described previously [10]. Kappa-5 was isolated by soybean agglutinin (SBA) affinity chromatography as described previously [17]. Kappa-5-depleted SEA is defined as the non SBA-binding fraction in the kappa-5 isolation procedure. Kappa-5 and IPSE/α1 were fluorescently labeled with PF-647 using the Promofluor labeling kit according to the manufacturer's recommendations (Promokine, Heidelberg, Germany). Either native or fluorescently labeled kappa-5 was treated with β-*N*-acetylhexosaminidase from *Canavalia ensiformis* (62.5 mU; Sigma, Zwijndrecht, the Netherlands) in 100 mM sodium phosphate buffer, pH 5.0, for 48 h at 37°C. α-Fucosidase treatment of (labeled) kappa-5 and IPSE/α1 was performed with α1-(3,4)-fucosidase from *Xanthomonas manihotis* (1 mU and 0.5 mU, respectively; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 48 h at 37°C.

Glycoconjugate array

BSA-conjugates were prepared as described previously [35;36]. Lacto-N-fucopentaose III (LNFPIII)-HSA was obtained from Glycotech (Gaithersburg, MD, USA) and mannose-BSA from Sigma. The glycoconjugates were printed and immobilized on epoxide-modified glass slides (Slide E, Schott Nexterion, Jena, Germany) as described previously [37]. Each glycoconjugate microarray was blocked for 60 min with TSM (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) containing 1% (w/v) BSA (Sigma) and 50 mM ethanolamine. After washing, arrays were incubated with either 2 μg mL^{-1} DC-SIGN-Fc (R&D Systems, Minneapolis, MN, USA), 5 μg mL^{-1} MGL-Fc [5], or 5 μg mL^{-1} MR-Fc (a kind gift from L. Martinez-Pomares [38]) in TSM/1% BSA/0.01% (v/v) Tween-20 for one hour. Slides were flushed and subsequently incubated with anti-human-IgG(Fc)-Cy3 (Sigma) for 30 min. After thorough washing and drying, the slides were scanned using the Agilent DNA Microarray Scanner. Data was analysed using GenePix Pro

6.1 (Molecular Devices). Mean fluorescence intensities (MFI) of triplicates were calculated on basis of median spot intensity using circular alignment and local background subtraction [39].

DC-SIGN- and MGL-Fc ELISA

96-well Maxi-Sorp plates (NUNC, Roskilde, Denmark) were coated overnight with antigens (1µg ml^{-1}) in 0.1M $NaHCO_3$ at 4°C in duplicate. Plates were washed three times with TSM, followed by incubation with TSM/1% BSA for 30 min. After three washes with TSM, various concentrations of DC-SIGN-Fc or MGL-Fc in TSM were added for 2 h, with or without a prior incubation for 15 min with EGTA (10 mM). The plates were washed five times with TSM/0.05% Tween and bound DC-SIGN-Fc or MGL-Fc was detected with alkaline phosphatase-labeled goat anti-human Fc (1:25 000; Sigma) in TSM/0.05% Tween.

Cells

DCs were generated from monocytes, isolated from venous blood of healthy volunteers by density centrifugation on Ficoll followed by a Percoll gradient as previously described [8;40]. Monocytes were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (80 ng ml^{-1}, Biosource-Invitrogen, Breda, The Netherlands) and human rIL-4 (250 units ml^{-1}, R&D Systems).

The K562 cell line stably expressing DC-SIGN and a mock transfected control (a kind gift from C. Figdor [41]), were maintained in mixed medium consisting of 25% IMDM (Gibco) medium + 5% FCS and 75% RPMI (Gibco) medium + 10% FCS. The CHO cell line stably expressing MGL [41] and an untransfected control were maintained in RPMI (Gibco) containing 10% FCS. NIH3T3 cell lines expressing human MR (3T3.hMR) or the control pFB vector were a kind gift from Joanna Miller and Gordon Brown [38]. 3T3 cell lines were cultured in DMEM (BioWitthaker) medium + 10% FCS. All media were supplemented with penicillin and streptomycin and transfected cell lines were continuously kept under selection of 0.5 mg ml^{-1} geneticin (Gibco).

Cellular adhesion assay

Fluorescently labeled kappa-5 and IPSE/α1 were treated or not with various exo-glycosidases, as described in the section "Antigens and exo-glycosidase treatments". Cells (50.000/well) were seeded in a V-bottom 96 well plate. Where indicated cells were pre-incubated with 10mM EGTA, 100 ug ml^{-1} mannan (Sigma-Aldrich), 20 µg ml^{-1} anti-DC-SIGN (clone AZN-D1, azide free, Beckman Coulter, Woerden, the Netherlands), 20 µg ml^{-1} anti-MR (clone 15.2, Biolegend, San Diego, CA, USA) or a combination of two anti-MGL antibodies (20 µg ml^{-1} clone

125A10.03, Dendritics, and 20 μg ml^{-1} 1G6.6 [42]) for 45 min at 37 °C. Subsequently, cells were incubated with 2 μg ml^{-1} PF-647 labeled glycoprotein variants at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry (FACSCalibur, Becton Dickinson).

Mass spectrometry

Trypsin (Promega, Leiden, The Netherlands) was added to a sample of IPSE/α1 or kappa-5 variants at a 1:100 trypsin/antigen ratio and incubated overnight at 37 °C. Resulting IPSE/α1 and kappa-5 (glyco)peptides were analysed as described previously using respectively MALDI-TOF-MS [15] and nano-HPLC-ESI-ion trap-MS [16].

Acknowledgements

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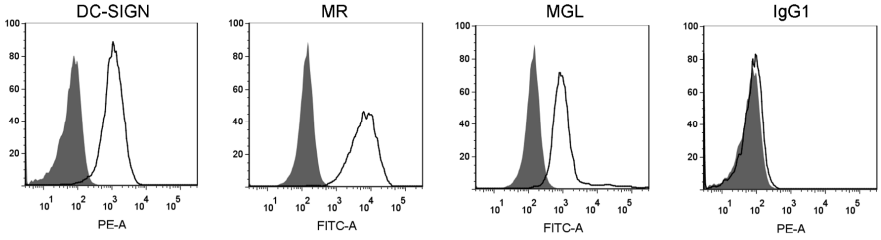
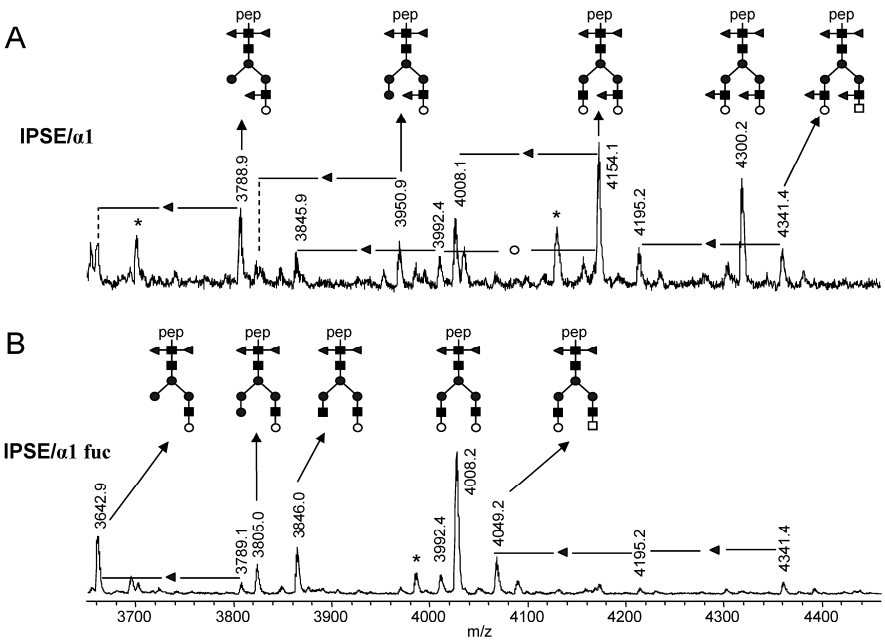
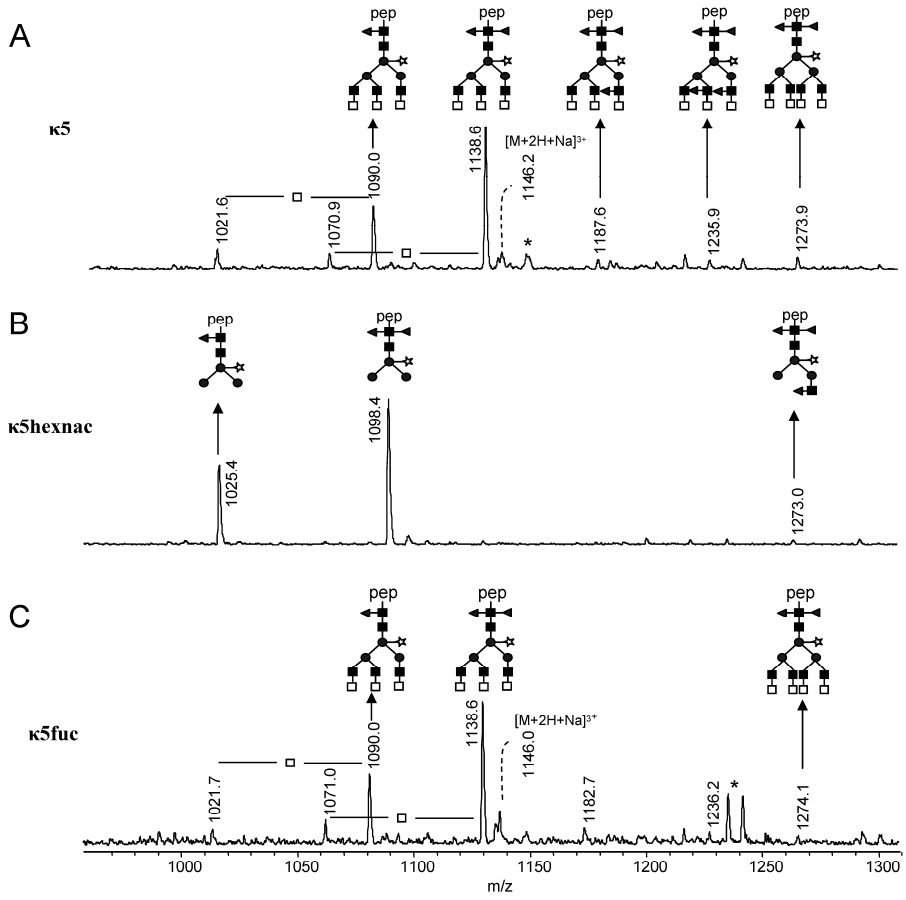
Supplementary Figures



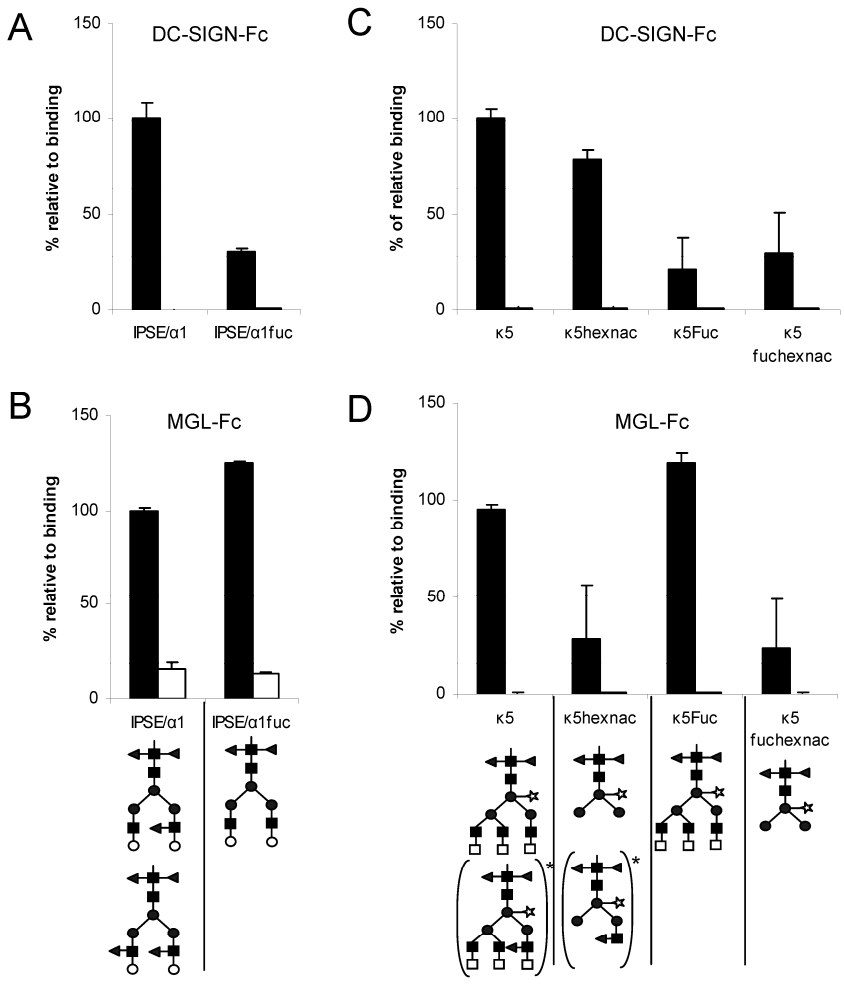
Supplementary figure 1. Expression of DC-SIGN, MR, MGL and isotype control (IgG1) on monocyte-derived immature DC. Open histograms represent the mAb staining, filled histrograms represent unstained cells. The presented results are representative of one out of three experiments.



Supplementary figure 2. Exo-glycosidase treatment of IPSE/α1. IPSE/α1, either untreated (A) or treated with α-fucosidase (B), was digested with trypsin and the resulting glycopeptides were analyzed by MALDI. Analysis of glycans on glycosylation site N_{80} in peptide E_{69}RPYWYLFDNVNYTGR$_{84}$ is given. Signals are labeled with monoisotopic masses and single charged. Non-glycopeptide signals are marked with asterisks (*).Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; pep, peptide; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase.



Supplementary Figure 3. Exo-glycosidase treatment of kappa-5. Kappa-5, either untreated (A) or treated with β-*N*-acetylhexosaminidase (B) and α-fucosidase (C), was digested and the resulting glycopeptides were analyzed by nano-LC-MS. Analysis of glycans on glycosylation site N_{251} in peptide I_{246}NENFNK$_{252}$ is given. Signals are labeled with monoisotopic masses. Signals are triple charged (A and C) or double charged (B), unless indicated otherwise. Non-glycopeptide signals are marked with asterisks (*).Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, mannose; open star, xylose; pep, peptide; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase.



Supplementary figure 4. Interactions of exo-glycosidase treated IPSE/α1 and kappa-5 with DC-SIGN-Fc and MGL-Fc. Binding of 1 μg ml^{-1} of DC-SIGN-Fc (A, C) and MGL-Fc (B, D) to exo-glycosidase-treated IPSE/α1 (A,B) and kappa-5 (C,D) was determined by ELISA in the absence (black bars) or presence (white bars) of EGTA. The given glycan structures represent the major glycans present on the (treated) glycoproteins, while the structures marked with an asterisk (*) are present on a small subset of kappa-5 glycoproteins. Binding of Fc-constructs to untreated kappa-5 or IPSE/α1 was set to 100%. The presented results represent two combined, independent experiments. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; open star, xylose; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase; κ5fuc/hexnac, kappa-5 treated sequentially treated with α-fucosidase and β-*N*-acetylhexosaminidase; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase.

Chapter 5

Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by mannose receptor

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Manuscript in preparation for publication.

Abstract

Omega-1, a glycosylated ribonuclease secreted by *Schistosoma mansoni* eggs and abundantly present in soluble egg antigen (SEA), has recently been shown to condition dendritic cells (DCs) to prime Th2 responses. However, the molecular mechanisms underlying this effect remain unknown. We show here by site-directed mutagenesis of omega-1 that both the glycosylation and the RNase activity are essential to condition DCs for Th2 polarization. Mechanistically, we demonstrate that omega-1 is bound and internalized via its glycans by the mannose receptor (MR) and subsequently impairs protein synthesis by degrading ribosomal RNA. These studies reveal an unrecognized pathway, involving MR and interference with protein synthesis that conditions DCs for Th2 priming.

Introduction

Dendritic cells (DCs) play a central role in the development and maintenance of immune responses during infection, as they govern both the activation and polarization of adaptive T helper (Th) cells. Classically, upon recognition of invading pathogens, resting DCs undergo a process of activation, so-called maturation, that involves stable presentation of peptides in the context of major histocompatibility complex (MHC)-II, up-regulation of co-stimulatory molecules, and production of polarizing cytokines, that collectively enable DCs to potently activate and direct $CD4^+$ T cell responses [1].

This paradigm is largely based on observations of responses towards pathogens, like bacteria, viruses and fungi. These pathogens harbour pathogen associated molecular patterns (PAMPs) that lead to classic DC activation by engaging several classes of innate pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs). Binding of PAMPs to these receptors initiates signalling cascades that generally result in the conditioning of DCs for priming of Th1- or Th17-biased responses which are instrumental in combating prokaryotic and single cell eukaryotic pathogens [2]. In contrast to this classical view of DC activation, components derived from parasitic helminths fail to induce all traditional signs of DC maturation. However, although overt maturation is not observed, unlike immature DCs, helminth antigen-treated DCs are altered such that they prime Th2-polarized immune responses [3].

Despite this consistent picture, the pathways through which helminth antigens manipulate DC function and drive Th2 responses are still poorly understood [4]. The majority of the studies have been conducted with a complex mixture of soluble egg antigens (SEA) from the trematode *Schistosoma mansoni*. SEA is regarded as one of the most potent helminth-derived antigenic extracts that instruct DCs to drive Th2 polarization [3;5]. So far these studies have mainly suggested that carbohydrate structures play a role in DC modulation by SEA, given that chemical modification of glycans on proteins present in SEA is known to abolish their capacity to induce Th2 polarization [6]. In this respect, another class of PRRs expressed by DCs, the carbohydrate-binding C-type lectin receptors (CLRs), have been suggested to play a role in modulation of DC function by SEA [7]. For instance, SEA contains carbohydrate structures, such as Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX), that can be recognized by DC-SIGN [8-10]. Engagement of this receptor by components from pathogens such as *Helicobacter pylori* has been shown to suppress IL-12 production and modulate TLR-induced DC activation and T cell polarization [8;11]. In addition, more recently it has been shown that SEA can modulate cytokine responses through another CLR, Dectin-2 [12]. Finally, a number of studies have raised the possibility that TLRs are involved in SEA-mediated Th2 induction [13;14]. However, direct evidence

for involvement of specific receptors or downstream pathways in SEA-driven Th2 polarization has been missing.

The recent identification of a defined glycoprotein with RNase activity, omega-1, as the major component in schistosome eggs that is responsible for conditioning DCs for Th2 polarization [15;16], has allowed us to dissect the involved molecular pathways in a precise manner. Through site-directed mutagenesis we show that the RNase activity as well as the glycosylation of omega-1 are essential for programming of DCs for Th2 induction. Furthermore, we provide evidence that mannose receptor (MR) is critical for omega-1-driven Th2 responses and that internalization via this receptor is needed for biological activity of omega-1, as it allows omega-1 to interfere with ribosomal function and thereby to condition these cells to prime Th2 responses.

Results

Omega-1 requires both its glycosylation and RNase activity to condition DCs for priming of Th2 responses

A role for the RNase activity of omega-1 has been proposed in the conditioning of DCs to prime Th2 responses [16]. However, this was based on a chemical inactivation of the RNase activity by DEPC-treatment, which may also alter the function or structure of the whole protein. Therefore, we addressed the role of RNase activity in a more stringent and specific manner by creating a mutant of recombinant wildtype (WT) omega-1 lacking RNase activity by site-directed mutagenesis. Specifically, a histidine residue in its catalytic domain, known from other T2 RNases to be essential for the enzymatic activity, was replaced by phenylalanine (omega-1 H58F) [17] (Supplementary figure 1A). Apart from RNase activity, glycosylation of omega-1 may also be important for its Th2-priming capacity, since chemical modification of glycans on proteins present in SEA is known to abolish the ability of SEA to induce Th2 polarization [6]. Moreover, potentially Th2-polarizing LeX glycan motifs have recently been described to be present in glycans on omega-1 [18]. To address the role of glycosylation in Th2 priming by omega-1, a glycosylation mutant was generated by a single amino-acid replacement at each of the two putative N-linked glycosylation sites (omega-1-N71/176Q) (Supplementary figure 1A) [19]. An RNase assay showed that RNase mutant did not have any RNase activity, while that the RNase activity of the glycosylation mutant was unaffected (Supplementary figure 1B). In addition, using a silver-stained SDS PAGE as well as an anti-omega-1 Western blot, we confirmed the absence of glycosylation on the glycosylation mutant. On the other hand, the mass shift of the RNase mutant on a silver-stained SDS PAGE was identical to recombinant WT omega-1, suggesting the glycosylation on the RNase mutant was still intact (Supplementary figure 1C). With regard to

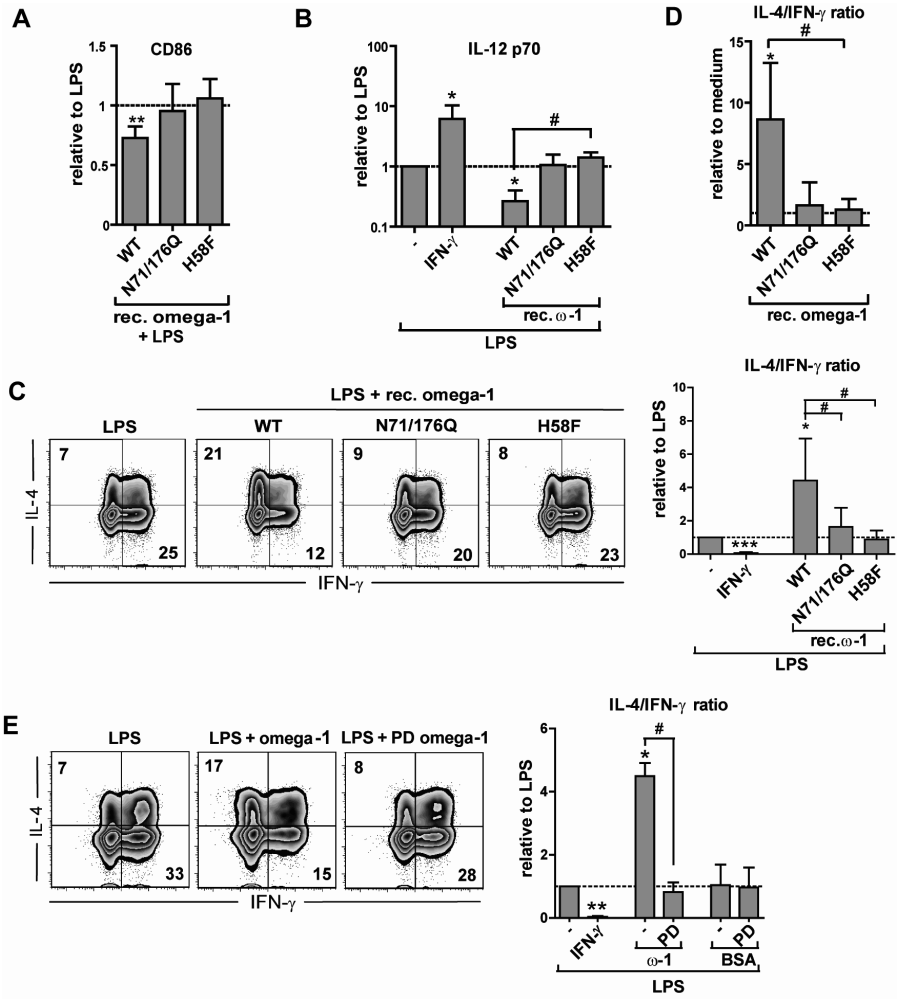

Figure 1. The glycosylation and RNase activity of omega-1 are essential for conditioning DCs to prime Th2 responses (A) Monocyte-derived DCs were pulsed for 48 h with the different variants of recombinant omega-1 (2 μg/ml) in combination with LPS (100 ng/ml) as a maturation factor and surface expression of CD86 was determined by FACS analysis. The expression levels, based on geometric mean fluorescence, are shown relative to the DCs stimulated with LPS alone, which is set to 100%. (B) Conditioned DCs were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1. (C+D) Conditioned DCs were cultured with allogeneic naive $CD4^+$ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2. Intracellular cytokine production was assayed by FACS 6 h after the stimulation of primed T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The frequencies of each population are indicated as percentages in the plot. One representative result from 3 independent experiments is shown. Based on intracellular cytokine staining, the ratio of T cells single-positive for either IL-4 or IFN-γ was calculated relative to the control condition. (E) DCs were pulsed for 40 h with mock- or periodate-treated (PD) natural omega-1 (500 ng/ml) in combination with LPS (100 ng/ml). Conditioned DCs were cultured with allogeneic naive $CD4^+$ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and analyzed as described in (D). Data are representative of 3 independent experiments. Bars represent mean ± SD. *,# $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (two-sided paired *t*-test). ω-1, omega-1.

the glycans present on recombinant WT omega-1 and the RNase mutant, mass spectrometric analysis of tryptic glycopeptides showed the presence of N-glycans on Asn176 with the monosaccharide composition $Hex_3HexNAc_6Fuc_{2/3}$ (Supplementary figure 2). This is indicative of the presence of fucosylated LDN antennae (LDN-F), a glycan element previously found on a protein from HEK293 cells, the cell type in which recombinant omega-1 is expressed [20]. LDN-F is thought to have similar immunological characteristics as the LeX motifs present on natural omega-1 [9;10;18].

To assess the role of glycosylation and RNase activity in omega-1-driven Th2 polarization, a well established *in vitro* culture system of human monocyte-derived DCs and naïve $CD4^+$ T cells was used, which mimics *in vivo* DC-mediated Th cell polarization [1]. Similar to natural omega-1 [15], recombinant WT omega-1 consistently and significantly suppressed the LPS-induced upregulation of the costimulatory molecule CD86 (Figure 1A), as well as the production of IL-12 (Figure 1B), which is an important characteristic of Th2-priming DCs [3]. However, both the glycosylation as well as the RNase mutant failed to alter LPS-induced CD86 expression or IL-12 production of DCs. Importantly, in contrast to DCs primed with recombinant WT omega-1, those conditioned with the glycosylation mutant or the RNase

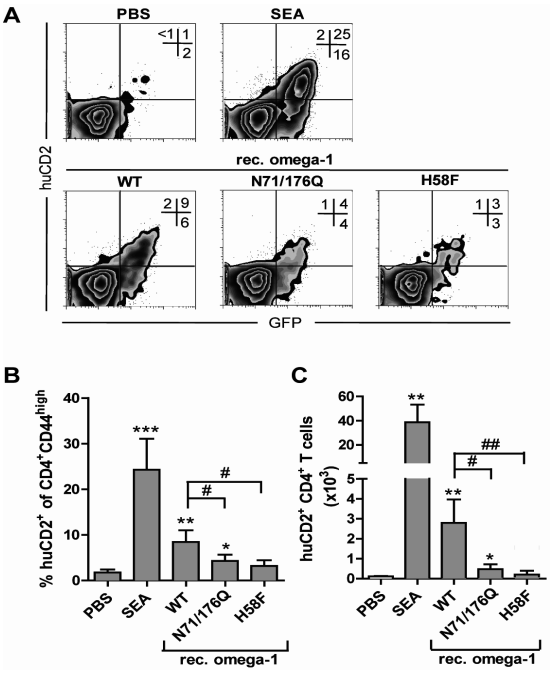

Figure 2. Glycosylation and RNase activity are essential for omega-1 to prime Th2 responses *in vivo* 4get/KN2 IL-4 dual reporter mice were injected s.c. with SEA (20 µg) or WT recombinant omega-1 and the two mutants (3 µg) into the footpad. After 7 days the frequency of GFP^+ and $huCD2^+$ within the $CD4^+CD44^{high}$ effector T cell population was determined by flow cytometry in the draining popliteal lymph nodes. Depicted are (A) concatenated FACS plots, (B) frequencies of $huCD2^+$ within the $CD4^+CD44^{high}$ population and (C) total $huCD2^+$ T cell numbers in draining lymph nodes of combined data of 4 mice per group. (A) The frequencies of each population are indicated as percentages in the plots. One of 3 independent experiments is shown. Bars represent mean ± SD. *,# $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for values significantly different from the PBS control (*) or between test conditions (#) based on two-sided *t*-test.

mutant did not prime a Th2 response (Figure 1C). Similar results were obtained with cultures in which DCs were conditioned by the omega-1 variants in the absence of LPS (Figure 1D). The importance of omega-1 glycosylation for polarizing towards Th2 was further substantiated by experiments performed with natural omega-1 on which the glycans were disrupted by periodate treatment (Figure 1E). These data show that the RNase activity and the glycosylation of omega-1 are both essential, but as single property not sufficient, for the induction of Th2 responses via DCs.

Omega-1 requires both its glycosylation and RNase activity to prime Th2 responses in vivo

To test whether the *in vivo* Th2-priming capacity of omega-1 is dependent on glycosylation and RNase activity, recombinant WT omega-1 or its mutants were administered to 4get/KN2 IL-4 dual-reporter mice [21]. In these mice IL-4-competent cells are GFP^+ and IL-4-producing cells additionally express huCD2, allowing the direct visualization of Th2 differentiation and IL-4 production. Following the s.c. injection of the antigens into the footpad, the draining popliteal lymph nodes (LNs) were harvested on day 7 and $CD4^+CD44^{high}$ effector T cells were analyzed for the expression of GFP and huCD2 directly ex vivo. Injection of SEA resulted in a significant increase of GFP^+ and $huCD2^+$ cells, reflecting the induction of Th2 differentiation and acute IL-4 production *in vivo* (Figure 2). Importantly, while recombinant WT omega-1 alone also induced a marked Th2 response and the production of IL-4, both mutants were significantly impaired to prime this response as evidenced by lower frequencies (Figures 2A and 2B) as well as total numbers of $huCD2^+$ T cells (Figure 2C) in the draining LN. Taken together, these data show that the glycosylation and the RNase activity of omega-1 play a crucial role in Th2 polarization induced by omega-1 *in vivo*.

Omega-1 is internalized by DCs via mannose receptor (MR)

To get a better understanding of how glycosylation is involved in omega-1-driven Th2 polarization, we tested whether recognition of omega-1 by DCs was dependent on glycans from omega-1. To this end an assay was used that quantifies binding and uptake of fluorescently-labelled antigens by DCs (Supplementary figure 3). While DCs were capable of binding and internalizing recombinant WT omega-1 or the RNase mutant, DCs failed to bind and internalize the glycosylation mutant, demonstrating that glycans present on omega-1 are essential for recognition by DCs (Figure 3A). Given the importance of glycosylation of omega-1 for binding to DCs, we explored the involvement of carbohydrate-binding CLRs in the recognition of omega-1. When DCs were pre-incubated with calcium-chelator EGTA, which blocks CLR function, binding and internalization of natural omega-1 was totally prevented (Figure 3B),

suggesting that DCs recognize omega-1 via CLRs. SEA has been reported to be recognized and endocytosed by human DCs via the CLRs DC-SIGN and MR [7], that have the capacity to bind fucose-residues such as ones found in LeX [9;10;22], a glycan motif present on natural omega-1 [18]. To determine whether MR and DC-SIGN are involved in recognition and internalization of natural omega-1, DCs were pre-incubated with mannan (a natural ligand that competes for binding to DC-SIGN and MR), or DC-SIGN- and MR-specific blocking antibodies, followed by an 1h incubation with fluorescently-labelled SEA or natural omega-1. Uptake of SEA, as previously published [7], could be reduced by mannan and either DC-SIGN or MR blocking antibodies in an additive manner. With regard to omega-1, pre-treatment with mannan could almost completely block binding and uptake of the molecule. Interestingly, binding and uptake of natural omega-1 were significantly reduced by MR but not by DC-SIGN blocking antibodies (Figure 3C).

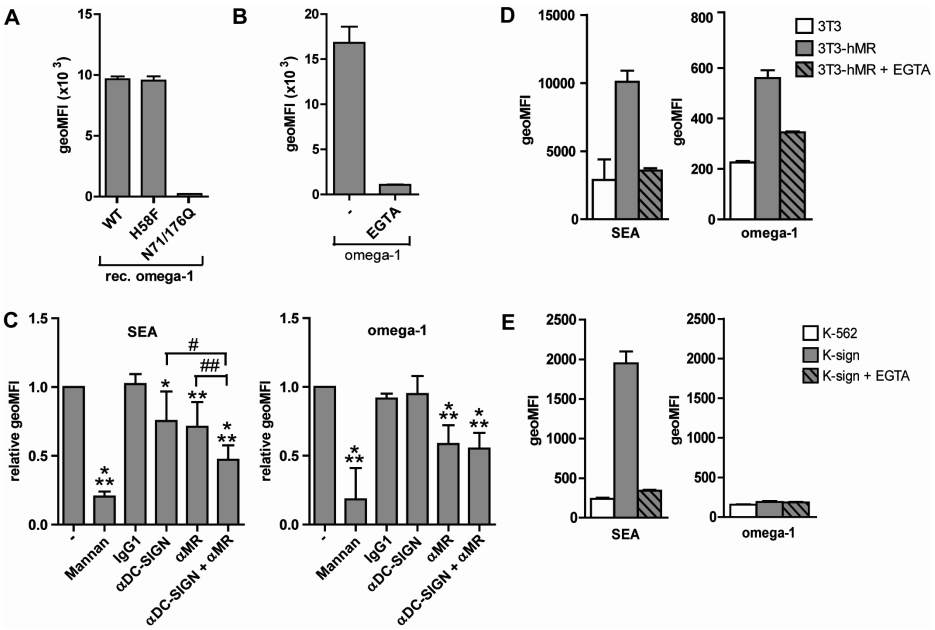

Figure 3. Mannose Receptor (MR) is the principal receptor through which omega-1 is recognized and internalized by DCs (A) Human monocyte-derived DCs were incubated for 1 h with PF-647-labeled recombinant WT omega-1, the glycosylation mutant or the RNase mutant and analyzed for uptake of antigens by FACS analysis. One representative experiment with duplicate samples out of 2 experiments is shown. (B) A binding and internalization assay on immature monocyte-derived DCs was performed as described in (A) following preincubation with EGTA. One representative experiment based on duplicate samples out of 5 is shown. Data are shown as mean ± S.D. (C) A binding and internalization assay on immature monocyte-derived DCs was performed as described in (A) following preincubation with indicated reagents. Binding and internalization are shown relative to mock pre-treatment. Data are representative of 5 experiments. (D) 3T3 cell-line expressing MR and (E) K-SIGN expressing DC-SIGN or parental control cell lines (3T3 and K-562) were incubated with PF-647-labeled omega-1 and SEA in the presence or absence of EGTA to determine specificity. One representative experiment based on duplicate samples out of 2 is shown. Bars represent mean ± SD. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for significant differences compared to the control (two-sided *t*-test).

Pre-incubation with the combination of both blocking antibodies did not have any additional effect on the uptake of omega-1 as compared to pre-incubation with anti-MR antibody alone. Of note, we found that recombinant omega-1 was recognized and internalized by DCs in similar MR-dependent fashion as natural omega-1 (data not shown). To further investigate the observations of selective recognition and uptake of omega-1 by MR, we made use of the K562 and 3T3 cell lines selectively expressing human DC-SIGN and MR, respectively. Fluorescently-labelled SEA was readily bound by both the DC-SIGN- and MR-expressing cells, which was not observed upon pre-incubation with EGTA or in parent control cell lines lacking CLR expression. In line with the DC-binding and uptake data, omega-1 binding could be observed in the cell line expressing MR (Figure 3D), but not in the cell line expressing DC-SIGN (Figure 3E). It should be noted, that not only in DC uptake experiments but also in the omega-1 binding experiments with the cell line selectively expressing MR, blocking with anti-MR antibody was not complete (Figure 3C and data not shown), suggesting that a relatively low affinity of the anti-MR antibody accounts for this finding rather than that other receptors are involved. Taken together, our data suggest that recognition and internalization of omega-1 by human DCs is dependent on its glycosylation and that MR is the primary CLR involved in this process.

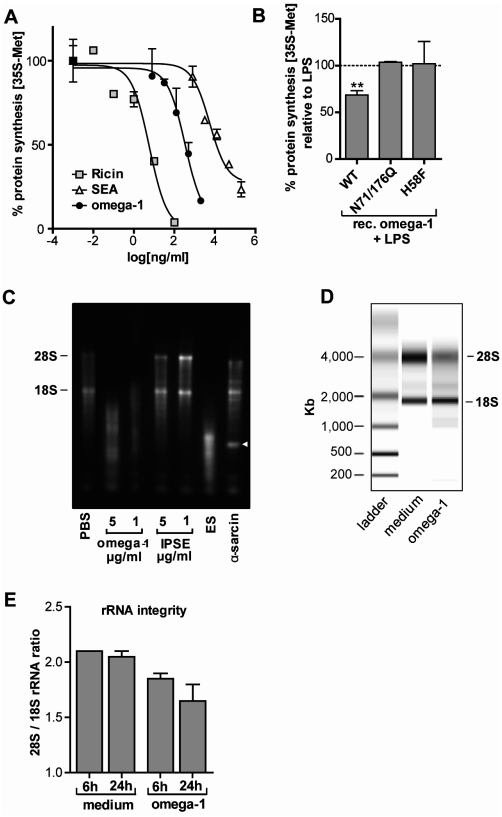

Figure 4. Omega-1 suppresses protein synthesis through interference with ribosomal function (A) Following 16 h incubation of DCs with a concentration range of indicated reagents in the presence of LPS (100 ng/ml), protein synthesis was assessed after a 2 h pulse with radioactive labelled methionine. Ricin, as potent inhibitor of protein synthesis, was taken along as positive control [23]. (B) Protein synthesis by DCs following exposure to the recombinant omega-1 variants (2 µg/ml) was assessed as described in (A). (C) After rabbit reticulocyte lysate containing functional ribosomes was incubated for 1 h with omega-1 or IPSE/α1, as a negative control, isolated ribosomal RNA was analyzed for breakdown on a 2% agarose gel. The RNase α-sarcin was taken along as positive control as it should give a single rRNA cleavage product when incubated with functional ribosomes (white arrowhead) [35]. (D) rRNA isolated from 24h omega-1-stimulated DCs and was visualized by running a lab-on-a-chip picogel and (E) 28S/18S rRNA ratio was determined from these samples as a measure for rRNA integrity. (A-E) One representative experiment from 3 independent experiments is shown. Bars represent mean ± SD.

Omega-1 suppresses DC function by interfering with protein synthesis

Next we examined the molecular mechanism through which the RNase activity of omega-1 exerts its modulatory effects on DCs. We noted that omega-1-stimulated DCs in response to CD40 ligation were not only impaired in their capacity to produce IL-12 p70, as reported previously [15], but also fail to express a large panel of other cytokines (Supplementary figure 4). This indicates that the suppression may not be gene specific, but could be the result of inhibition of protein synthesis globally. Indeed, following exposure of DCs to omega-1 or SEA, a dose-dependent reduction of protein synthesis could be observed, similar to what is found in DCs exposed to ricin, a well known protein synthesis inhibitor [23] (Figure 4A). The capacity to inhibit protein synthesis was dependent on its RNase activity and uptake via its glycans, since the RNase as well as the glycosylation mutant failed to interfere with protein synthesis (Figure 4B). As several fungal ribonucleolytic proteins, so-called ribotoxins, have been described to inhibit protein synthesis through cleavage of ribosomal RNA (rRNA) [24], we tested whether omega-1 could cleave rRNA in the context of functional ribosomes in a cell free assay. Omega-1 was able to break down rRNA, while IPSE/α1, another *S. mansoni* egg-derived protein that lacks RNase activity but has identical glycans as omega-1 [25], did not induce any rRNA digestion (Figure 4C), indicating that omega-1 is able to interfere with ribosomal function by cleavage of rRNA. Finally analysis of the integrity of rRNA isolated from omega-1-exposed DCs revealed a selective breakdown of 28S rRNA (Figure 4D), resulting in time dependent decrease in 28S/18S ratio (Figure 4E). Taken together, these data support the notion that the RNase activity enables omega-1 to modulate DC function by interfering with protein synthesis through cleavage of rRNA.

MR mediates omega-1-induced protein synthesis inhibition, DC modulation and Th2 polarization

To address the role of omega-1 binding by MR in mediating RNase-dependent DC modulation and Th2 priming by omega-1, we used blocking antibodies directed against MR or DC-SIGN. Blocking of MR during the stimulation of DCs with omega-1 significantly prevented the inhibition of protein synthesis (Figure 5A), while blocking of DC-SIGN had no effect, showing that the interference with protein synthesis by omega-1 is dependent on MR. In line with these observations, blocking of MR significantly reduced the capacity of omega-1 to suppress LPS-induced CD86 expression (Figure 5B) and IL-12 production following CD40 ligation (Figure 5C) or to condition DCs to induce a Th2 response (Figure 5D). As a last step, murine MR-/- splenic DCs were tested for their Th2-priming capacity in response to omega-1. While OVA peptide-pulsed WT splenic DCs, when conditioned with omega-1, primed a Th2-skewed OVA peptide-specific T cell response *in vitro*, MR-/- splenic DCs failed to do so (Figure 5E).

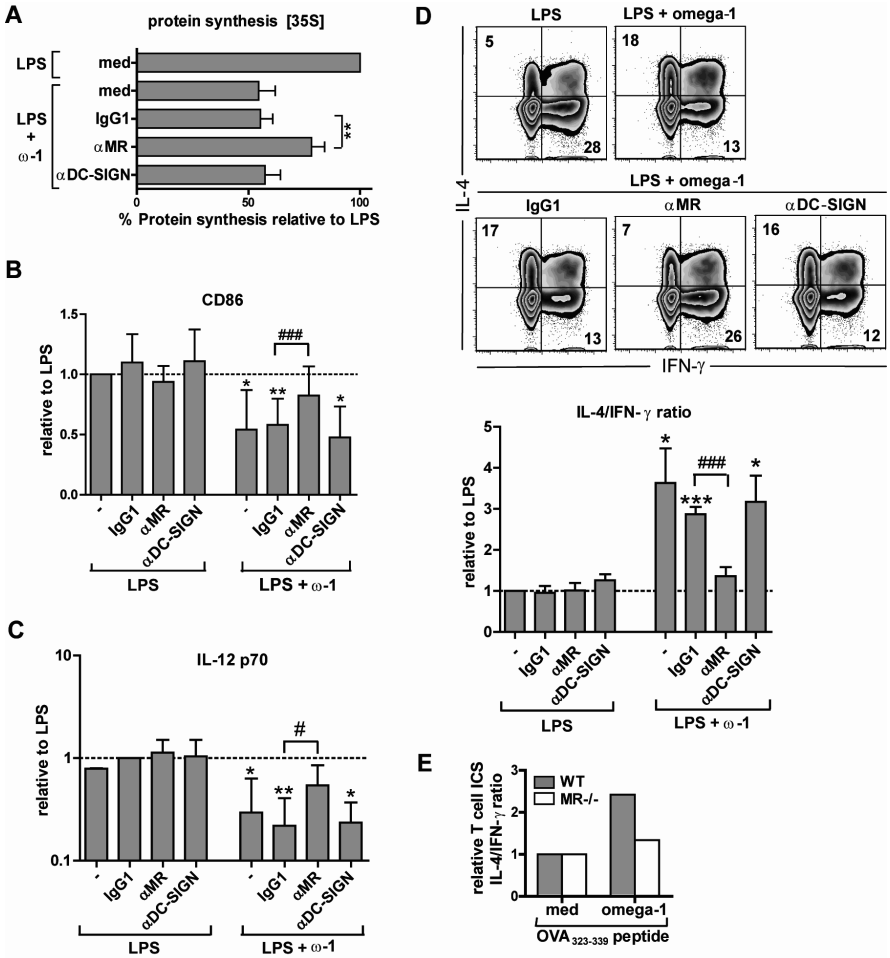

Figure 5. MR mediates omega-1-induced DC modulation and Th2 polarization *in vitro*. Following 1 h pre-incubation with blocking antibodies against MR, DC-SIGN or an isotype control (20 μg/ml), monocyte-derived DCs were pulsed for 16 h (A) or 48 h (B-D) with natural omega-1 (500 ng/ml) in combination with LPS (100 ng/ml). (A) Protein synthesis was assessed as described in Fig. 4 A. One representative experiment based on duplicate samples out of 3 experiments is shown. (B) The expression levels of CD86 on DCs were assessed by FACS and are shown, based on geometric mean fluorescence, relative to the DCs stimulated with LPS alone, which is set to 100% (dashed line). Data are based on 3 independent experiments (C) Conditioned DCs were co-cultured for 24 h with a CD40-L expressing cell line, to mimic the interaction with T cells. IL-12p70 cytokine expression levels are shown relative to the DCs stimulated with LPS alone, which is set to 1 (dashed line). Data are based on 3 independent experiments. (D) Conditioned DCs were cultured with allogeneic naive $CD4^+$ T cells for 12 days in the presence of staphylococcal enterotoxin B and IL-2 and T cell polarization was analyzed as described in figure 1. Data are based on 6 independent experiments. (E) Splenic WT or MR-/- DCs were co-cultured with OVA-specific OT-II cells for 4 days in the presence of indicated antigens. At day 4 T cells were restimulated with PMA and ionomycin and analyzed for intracellular IL-4/IFN-γ cytokine ratio as described in Fig. 1 D. 1 representative experiment out of 2 experiments is shown. Bars represent mean ± SD. *,# $p < 0.05$, **,## $p<0.01$, *** $p<0.001$ for significant differences with the LPS control (*) or between test conditions (#) based on paired analysis (two-sided paired *t*-test). ω-1, omega-1.

These data establish that MR is essential for the omega-1-driven Th2 polarization via DCs *in vitro*.

Omega-1 requires MR to prime Th2 responses in vivo

Finally, to investigate the role of MR in Th2-priming by omega-1 *in vivo,* natural omega-1 or PBS were injected subcutaneously into the footpad of WT and MR-/- mice. After 7 days the draining popliteal LNs were harvested and restimulated *in vitro* with PBS, omega-1 or a polyclonal stimulus PHA and analyzed for cytokine production. Antigen specific restimulation of omega-1-primed LNs from WT mice resulted in a Th2-polarized response as evidenced by elevated levels of Th2-associated cytokine IL-5 but not of Th1-associated cytokine IFN-γ, which was absent in LN cells derived from MR-/- (Figure 6A). Furthermore, intracellular staining for IFN-γ and IL-4 following antigen-specific restimulation of $CD4^+$ T cells from omega-1-primed LNs, showed a significant increase in the ratio between IL-4- and IFN-γ-producing T cells from WT but not MR-/- mice (Figure 6B). The failure of MR-/- mice to prime a Th2-polarized response in response to omega-1 was not due to a general failure of MR-/- cells to produce these cytokines as the responses to PHA were comparable in WT and MR-/- mice (Figures 6A and 6B). Taken together, these data show that MR is essential for priming of Th2 responses by omega-1 *in vivo*.

Discussion

Utilizing omega-1, a single molecule derived from *Schistosoma mansoni*, we studied the molecular mechanisms involved in conditioning DCs to induce Th2 responses. By generating mutant proteins we could show that both the glycosylation and RNase activity of omega-1 are essential for its potent Th2-inducing activity both *in vitro* and *in vivo*. The glycan structures on omega-1 suggested that CLRs might play a role in its interaction with DCs. Although both MR and DC-SIGN have been shown to mediate binding and uptake of fucosylated antigens by DCs and omega-1 harbours fucose-containing LeX moieties [18], we observed that omega-1 only significantly bound to a MR-, and not DC-SIGN-expressing cell-line and was internalized by DCs in a MR-, and not DC-SIGN-dependent manner. Lack of potent binding and uptake of omega-1 by DC-SIGN might be explained by the fact that in most DC-SIGN binding studies polyvalent LeX-containing beads or conjugates have been used, which may be bound by DC-SIGN with a higher affinity than soluble glycoproteins, such as omega-1 that presents LeX at a low valency [27]. In line with this observation, DC-SIGN blocking experiments suggest that interactions with DC-SIGN do not play a major role in omega-1-driven Th2 polarization via

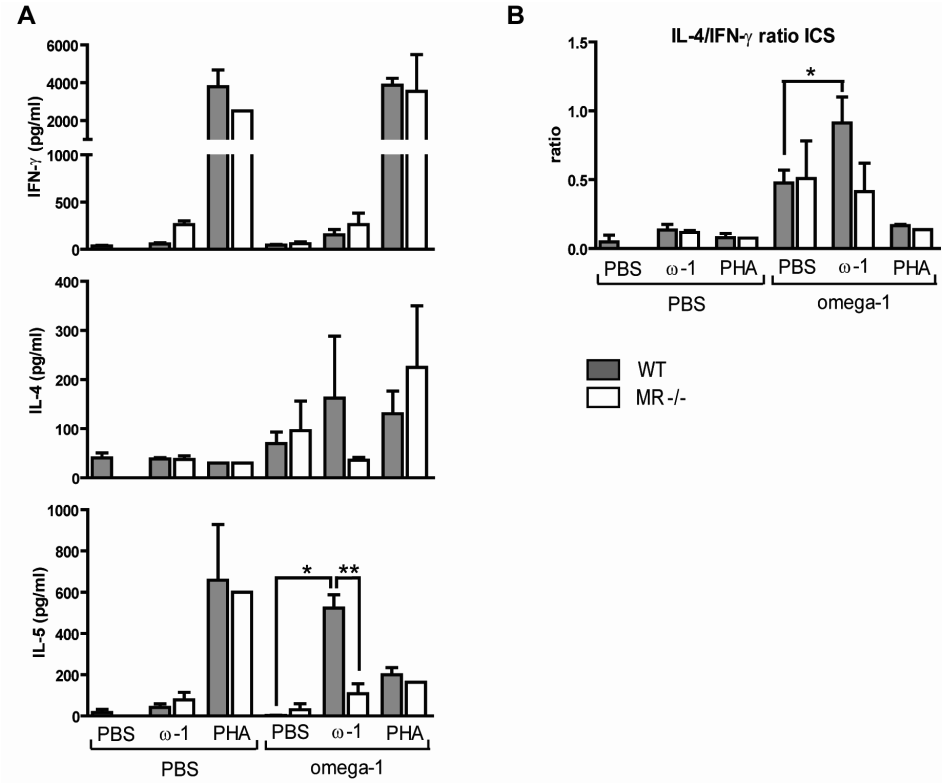

Figure 6. MR is essential for omega-1-driven Th2 polarization *in vivo* MR-/- and WT Bl/6 mice were injected s.c. with omega-1 (2 µg in 30 µl PBS) or PBS into the footpad. (A) After 7 days the cells from the draining lymph node (LNs) were restimulated *in vitro* for 4 days with PBS, omega-1 (2 µg/ml) or PHA (10 µg/ml), as polyclonal stimulus, after which cytokine production was determined by ELISA. (B) Intracellular cytokine production of the $CD3^+/CD4^+$ T cells from these LNs was assayed by FACS after an additional 6 h restimulation with PMA and ionomycin. The percentage of T cells single-positive for either IL-4 or IFN-γ is shown. One experiment of 2 independent experiments is shown. Data are means ± s.e.m. of 4 mice per group based on pooled triplicate wells for each mouse. * $p < 0.05$, ** $p<0.01$ for significant differences based on paired analysis (two-sided paired *t*-test). ω-1, omega-1.

DCs. On the other hand, the importance of MR in recognition and uptake of omega-1 was substantiated by the finding that inhibition of protein synthesis and conditioning of DCs for Th2 polarization by omega-1 was significantly impaired by blocking of MR, but not DC-SIGN. We confirmed and extended the importance of MR in Th2 polarization by omega-1 by showing *in vivo* that an antigen-specific Th2 response induced in MR-deficient mice following footpad injection of omega-1 was strongly reduced compared to the response elicited in WT mice. In this respect it is important to note that human and murine MR have a similar carbohydrate binding specificity [28]. Thus, this establishes that omega-1 relies on MR to drive Th2 polarization. This is consistent with earlier observations that crosslinking of MR with antibodies [29] or engagement by mannosylated antigens [30;31] can drive an anti-inflammatory cytokine program in DCs away from

a Th1-promoting profile [29] and that allergen-driven Th2 polarization by DCs is in part dependent on MR [32;33]. These studies point towards a direct role for MR in Th2 induction, potentially via signalling events. However, our data demonstrate that MR binding alone is not sufficient for Th2 polarization by omega-1, since glycans present on omega-1, in absence of RNase activity, are not sufficient to program DCs to induce Th2 responses. This is in line with the observation that IPSE/α1, another major glycoprotein secreted by *S. mansoni* eggs with identical glycosylation as omega-1 [25], which can bind a cell line expressing MR (chapter 4 of this thesis) but lacking RNase activity, fails to prime Th2 responses [15].

Furthermore, we specifically confirmed that apart from glycosylation, omega-1 requires its RNase activity to induce a Th2 response via modulation of DCs by using an RNase mutant. It was observed that omega-1 could cleave rRNA in the context of ribosomes in a cell free assay, and could induce 28S rRNA breakdown in DCs. The finding that the integrity and yield of mRNA isolated from omega-1-stimulated DCs was not different from unpulsed control DCs (data not shown), suggests that interference with translation via selective cleavage of rRNA is the mode of action through which the RNase activity enables omega-1 to condition DCs for priming of Th2 responses. Some RNases have been linked to Th2 polarization before. For instance, the major birch pollen allergen, Bet v 1 [34], was identified as an RNase. Furthermore, some fungal RNAses that selectively cleave rRNA, such as mitogillin and Aspf-1, are known to be allergens [35]. Interestingly, for Aspf-1 it was found that its allergenicity was lost when its capacity to interfere with ribosomal function was abolished [36]. In addition, a report has linked an endogenous RNase, the eosinophil-derived neurotoxin, to DC-mediated Th2 polarization [37]. Although these studies have not specifically addressed the role of RNase activity in direct priming of Th2 responses, they do highlight the possibility that Th2 priming through interference with ribosomal function may not be a unique feature of *S. mansoni*-derived omega-1, but may be shared by other RNases as well. However, such an RNase needs to be recognized by DCs and routed in a specific manner to reach the ribosomes and exert its enzymatic activity which in turn would result in suppression of protein synthesis, yet without shutting down DC function altogether or inducing cell death before T cell priming has occurred. One would predict that only certain RNases or ribosome-inactivating proteins will meet those requirements, and as such would be able to drive Th2 responses.

It remains to be established how omega-1 would be able to reach the ribosomes present in the cytosol. Some ribosome-inactivating proteins have been shown to translocate from the ER into the cytosol after retrograde transport or by direct escape from endosomes into the cytosol [38]. In this respect, since omega-1 is internalized via MR, it is interesting to note that cross-presentation of OVA by DCs, a process that requires translocation of the antigen from

endosomes into the cytosol, has been shown to be dependent on MR [39;40]. Mechanistically, it was demonstrated that binding of the MR to OVA leads to poly-ubiquitination of MR, resulting in the recruitment of the ATPase p97, a member of the ER-associated degradation machinery, towards the endosomal membrane. p97 in turn was found to provide the energy to pull out the MR ligand into the cytoplasm [41]. This suggests that the MR itself can regulate the transport of its ligand, into the cytoplasm and provides a mechanism through which omega-1 could be translocated into the cytosol of DCs.

Apart from inhibition of protein synthesis, we observed that omega-1 could promote apoptosis in DCs in a RNase dependent manner (unpublished data), reminiscent of ribotoxins that are known to induce cytotoxicity by interfering with ribosomal function [24]. It is important to note that in line with the protective effects of maturation on DC death [42], omega-1-driven cytotoxicity was not observed in LPS-matured DCs, excluding the possibility that the results obtained from our DC-T cell cultures regarding T cell polarization were influenced by potential immunomodulatory effects of apoptotic cells. Furthermore, our observations that omega-1 relies on MR to be internalized by DCs may explain the documented hepatotoxic effects of *S. mansoni* egg-derived protein fractions containing omega-1 [43;44], given that apart from DCs, sinusoidal endothelial cells and Kupfer cells express MR [45;46], which would render them sensitive to omega-1-induced cytotoxicity.

The suppression of protein synthesis in DCs by omega-1, would be in line with the documented inhibitory effects of omega-1 as well as SEA on DC activation and TLR-induced expression of co-stimulatory molecules and cytokines [15;16]. In addition, this mode of action would also provide an explanation for the finding that omega-1 alters DC morphology as a result of cytoskeletal changes [16], since halting of translation and concomitant stress responses can affect actin rearrangements and thereby cell morphology [47]. Importantly, during interactions with naïve T cells, omega-1-conditioned DCs will, in contrast to unconditioned DCs, be largely refractory to respond to CD40 ligation by T cells, as their protein synthesis machinery is impaired. As a consequence, T cells are primed in the absence of IL-12 and in the context of low antigen presentation and/or co-stimulation, a situation that is known to favour the induction of Th2 responses [48;49]. This mechanism would be different from the initially proposed 'default hypothesis' for Th2 induction [50], as it represents a dominant and active suppression of signals during DC-T cell interactions. Such a model of active suppression of DC signals for Th2 polarization would be in line with recent data showing that SEA-pulsed DCs, although still capable of processing antigen to present it on MHC-II, are impaired in their upregulation of surface expression of MHC-II and CD86 or IL-12 in response to CD40 ligation [15;51], as well as the observations that omega-1-primed DCs have a reduced capacity to form T cell-DC conjugates [16].

Taken together, based on our data we propose a model in which the glycans present on omega-1 do not play a dominant role in functional modulation of DC function for induction of Th2 responses, but instead are essential for efficient recognition and internalization by DCs via MR. Subsequently, following translocation into the cytosol omega-1 programs DCs to drive Th2 polarization in an RNase dependent manner by interfering with ribosomal function and protein synthesis. These studies have uncovered a novel mechanism through which DCs can be programmed to drive Th2 responses. It will be of great interest to study whether targeting of MR and the protein synthesis machinery to condition DCs for priming Th2 responses is unique to schistosome-driven Th2 polarization, or a mechanism that is also involved in the initiation of other Th2-polarized immune responses, found during other helminth infections or allergies. In addition, the insight may help the design of Th2-polarizing molecules, that could be used in the development of immune-driven manipulation of metabolic disorders such as insulin resistance, vaccines against parasitic worm infections or approaches to counterbalance unwanted Th1 responses [52;53].

Materials and Methods

Preparation and purification of S. mansoni egg-derived antigens

SEA, omega-1 and IPSE/α1 were prepared and isolated as described previously [15;54]. The purity of the preparations was controlled by SDS-PAGE and silverstaining. Protein concentrations were tested using the Bradford or BCA procedure.

Periodate treatment of antigens

Antigens were treated with sodium periodate as previously described [55;56]. In short, sodium acetate buffer (pH 4.5) was added to the antigens to a final concentration of 0.1M prior to exposure to periodate. Antigens were incubated in 20 mM sodium periodate overnight in the dark at 4ºC. The incubation was stopped by addition of an equal volume of 50 mM sodium borohydride for 30 minutes. To remove the sodium periodate, sample buffer was exchanged to phosphate buffered saline using protein desalting spin columns according to manufacturer's recommendations (Pierce, Rockford, IL, USA).

MALDI-TOF-MS of omega-1 glycopeptides

Glycopeptides of recombinant omega-1 were generated by trypsin treatment of reduced and alkylated omega-1, either in solution or in excised gel bands [18]. Mass spectra were recorded using

an Ultraflex II time-of-flight mass spectrometer (Bruker-Daltonics, Bremen, Germany) as described [25].

Generation and production of WT, glycosylation mutant and RNase mutant forms of recombinant omega-1

Site directed mutagenesis was used to generate a glycosylation and RNase mutant by mutating the two putative N-linked glycosylation sites (N71/176Q) or by targeting a conserved amino-acid residue (H58F) that is known to be critical for enzymatic activity in other homologous RNases [20;85], respectively (see supplemental Figure 1). H58F and N71/176Q mutants were created by polymerase chain reaction (PCR) using mutagenic primers on a DH5α/pProExHtb-plasmid containing the WT omega-1 sequence (Invitrogen). Introduction of the right mutations was confirmed by DNA sequencing. Subsequently, using restriction enzymes HindIII and ApaI the templates for WT and omega-1 mutant were subcloned into a pSecTag2-plasmid (Invitrogen) for stable transfection into HEK cells [15]. Secreted recombinant omega-1 forms were sequentially purified from the HEK cell culture medium by immobilized metal affinity chromatography and size exclusion chromatography as described previously [15].

Human DC culture, stimulation and analysis.

Monocytes were isolated from venous blood of healthy volunteers using Institutional Review Board-approved protocols by density centrifugation on ficoll followed by a Percoll gradient as described [15] and were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (500 units/ml, Invitrogen) and human rIL-4 (250 units/ml) (R&D Systems). On day 3, culture medium including the supplements was replaced and on day 6 immature DCs were stimulated with the indicated reagents in the presence of ultrapure LPS (100 ng/ml) (E. coli 0111 B4 strain, InvivoGen). For CLR blocking indicated cells were pre-incubated with 20μg/ml anti-DC-SIGN (clone AZN-D1, Beckman Coulter) or 20μg/ml anti-MR (clone 15.2, Biolegend) for 60 min at 37 °C. As a Th1 control DCs were also pulsed with IFN-γ (1000 U/ml). After 48 h, DCs were harvested for co-culture with naïve T cells. In addition, $1x10^4$ matured DCs were co-cultured with $1x10^4$ CD40L-expressing J558 cells for 24 h to determine cytokine production by the DCs following activation by CD40L. IL-12p70 concentrations were determined by ELISA using mouse anti-human IL-12, clone 20C2 as capture antibody and biotinylated mouse-anti-human IL-12, clone C8.6 as detection antibody (both Becton Dickinson). Concentrations of IL-10, TNF-α, MIP-1β and RANTES were determined by a multiplex LUMINEX assay according to the manufacturer's instruction (InvivoGen). The expression of CD86-PE pulsed DCs was determined by FACS (FACSCanto) through staining with CD86-FITC (Becton Dickinson).

Murine DC and T cell polarization assay

Splenic $CD11c^+MHCII^+$ DCs and OT-II $CD4^+$ T cells were isolated by sorting from naive splenocytes. 5×10^5 Tg $CD4^+$ lymphocytes/ml were co-cultured with 2.5×10^5 syngeneic splenic DCs/ml and stimulated interchangeably with 100 μg/ml OVA. At d4, $CD3^+/CD4^+$ T cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 2 μg/ml ionomycin for 6 h. 10 μg/ml brefeldin A was added during the last 2 h (all Sigma). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies (BD). and analyzed for intracellular IL-4/IFN-γ cytokine ratio.

Human T cell culture and determination of T cell polarization

To determine T cell polarization, 5 x 10^3 48 h-pulsed DCs were co-cultured with 2 x 10^4 naïve T cells that were purified using a human $CD4^+/CD45RO^-$ column kit (R&D, Minneapolis, MN) in the presence of staphylococcal enterotoxin B (10 pg/ml; Sigma) in 96-well flat-bottom plates (Corning). On day 5, rhuIL-2 (10 U/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded for another 7 days. For intracellular cytokine production, the primed $CD4^+$ T cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 2 μg/ml ionomycin for 6 h. 10 μg/ml brefeldin A was added during the last 2 h (all Sigma). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies (BD).

TLR-transfected HEK cell activation

HEK-293-CD14, HEK-293-CD14/TLR2 and HEK-293-CD14/TLR4 cells were maintained in DMEM culture medium, supplemented with 10% FCS, 10 μg/ml ciprofloxacin and 5 μg/ml puromycin. For stimulation experiments, cells were seeded at 3.5 x 10^4 cells/well in 96-well flatbottom plates and were stimulated the next day. For stimulation of HEK-293-CD14/TLR4 cells, 12.5% supernatant of MD-2 transfected cells was added. IL-8 production was measured in supernatants after 22 hours using a commercial kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

DC-SIGN- and MR-expressing cell line

K562 cell line stably expressing DC-SIGN (a kind gift from K. Figdor [57]) or 3T3 cellline stably expressing human MR (a kind gift from G. Brown [58]) and their respective parental control cell lines were seeded overnight in a 96 well plate at 10.000 cells/well. Where indicated, cells were pre-incubated with 10mM EGTA for 30 min at 37 °C. Subsequently, cells were incubated with 2μg/ml PF-647 labeled SEA or 500ng/ml PF-647 labeled omega-1 at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry.

Protein synthesis inhibition

Immature DC were seeded overnight in 96 well flatbottom plates before stimulation with indicated reagents in the presence of LPS. 16 h after stimulation protein synthesis was determined by a 2 h pulse at 37 °C with 3µCi /0,05 ml [^{35}S]-methionine (EasyTag Express Protein labeling mix, Perkin Elmer) in serum- and and L-methionine free RPMI1640. After a double washing step in PBS, cells were lysed for 5 min in AV-lysis buffer (20mM Tris HCl, pH7.6, 150 mM NaCl, 0.5% DOC, 1.0% NP40, 0.1% SDS) in the presence of protease inhibitors Leupeptin and Aprotinin 200ug/ml. Lysates were transferred on a filter (Perkin Elmer) and dried. After radioactive labeled proteins were precipitated on the filter with trichloroacetic acid, filters were washed with 96% ethanol and dried. The radioactivity present on the filters was measured in a β-counter by a liquid scintillation cocktail for aqueous solution.

RNase activity assay

RNA was extracted from PBMC using the RNeasy kit (Qiagen). RNA was incubated for 1 h at 37 ˚C with indicated antigens 0.01M Tris 0.02% Cu. Subsequently, RNA breakdown was visualized by running the samples on a 2% agarose gel containing ethidium bromide.

Ribosomal RNA breakdown

Rabbit Reticulocyte Lysate (Promega) was incubated with antigens as described by others [35]. Briefly, following 1 h incubation at 37 °C in Tris-HCl (15 mM NaCl, 50 mM KCl, 2,5 mM EDTA), the reaction was stopped with 10% SDS and RNA was extracted from the ribosomes with phenol/chlorophorm. Next, isolated ribosomal RNA was denatured at 95°C and visualized by running the samples on a 2% agarose gel containing ethidium bromide.

Analysis of ribosomal RNA integrity in human DCs

mRNA was isolated from DCs conditioned by omega-1 for indicated time points using RNeasy mini Kit (Qiagen) according to the manufacturers recommendations. Integrity of rRNA was visualized and quantified using Agilent RNA 6000 Pico Kit in a 2100 Bioanalyzer (Agilent) according to the manufacturers recommendations.

Antigen uptake by DCs

SEA and omega-1 were fluorescently labeled with PF-647 using the Promofluor labeling kit (Promokine and according to the manufacturers recommendations). 10.000 immature DC/well were seeded in a 96 well plate. Where indicated cells were pre-incubated with 10mM EGTA, 100ug/ml Mannan (Sigma Aldrich), 20µg/ml anti-DC-SIGN (clone AZN-D1, Beckman Coulter)

or 20μg/ml anti-MR (clone 15.2, Biolegend) for 60 min at 37 °C. Subsequently, cells were incubated with 2μg/ml PF-647 labeled SEA or 500ng/ml PF-647 labeled omega-1 at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry.

In vivo experiments

4get/KN2 [59] mice were bred and housed in the animal facility of the Trudeau Institute and used at 8-12 weeks of age. MR-/- mice on a C57BL/6 background were provided by Dr. M. C. Nussenzweig (Rockefeller University, New York, NY) and were bred and housed in the animal facility of the Institutes of Molecular Medicine and Experimental Immunology at the University Hospital, Bonn. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Mice were immunized s.c. into one hind footpad with SEA (20μg), omega-1 (3μg), in a volume of 50 ul and the draining popliteal lymph nodes were analyzed one week later.

In vitro restimulation of lymph node cells

1.5×10^6 popliteal LN cells/ml from individual animals were restimulated with 10 μg/ml SEA or 2 μg/ml omega-1. IL-5, IL-4 and IFN-γ were measured by ELISA in day 4 supernatants according to the manufacturer's recommendations (R&D). Following removal of the supernatants, cells were restimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 2 μg/ml ionomycin for 6 h. 10 μg/ml brefeldin A was added during the last 2 h (all Sigma). The cells were stained with a combination of IL-4-PE and IFN-γ-FITC antibodies (BD).

Statistical analysis

Data were analyzed for statistical significance using a two-sided paired Student's *t*-test or where indicated a two-sided unpaired Student's *t*-test. All p-values < 0.05 were considered significant.

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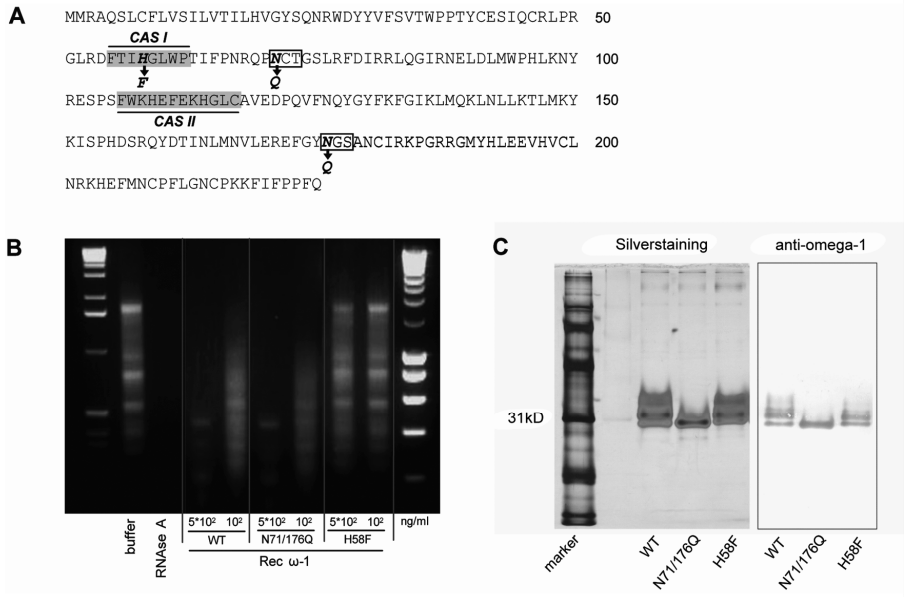
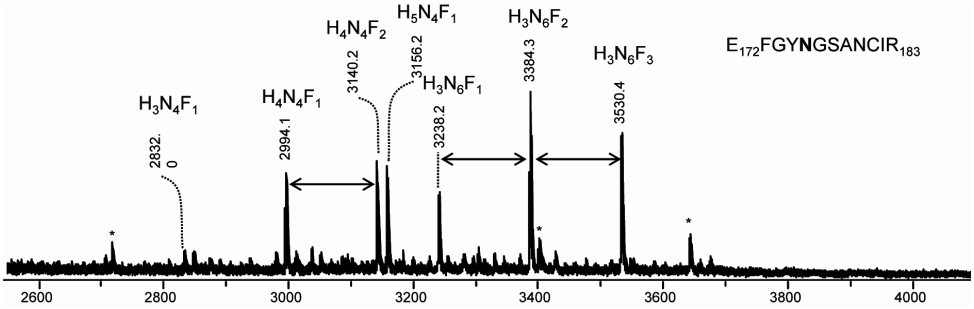
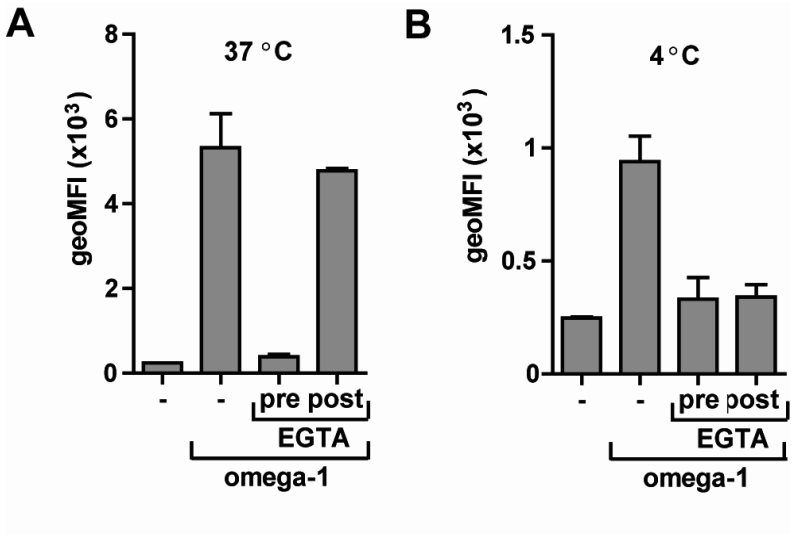
Supplementary figures



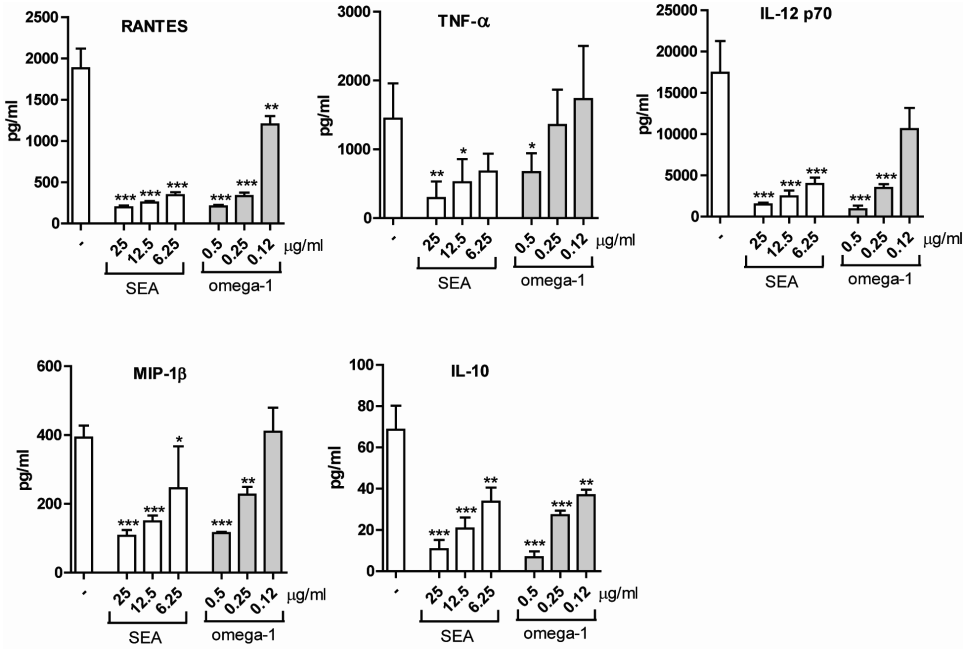
Supplementary figure 1. Generation and evaluation of glycosylation and RNase mutants of recombinant omega-1 (A) The amino acid sequence of omega-1 is shown in which the mutation sites are depicted. The two conserved amino acid sequence (CAS)-domains essential for catalytical activity are marked in grey and the two N-linked glycosylation sites are depicted in white boxes. (B) RNA from PBMCs was incubated for 1 h with the different omega-1 variants and analyzed on a 2% agarosegel for breakdown. The RNase mutant, in contrast to the glycosylation mutant and WT recombinant omega-1, fails to degrade RNA (C) The omega-1 variants were run under non-reducing conditions by SDS-PAGE and silver stained. A Western Blot by staining with a specific anti-omega-1 monoclonal antibody confirmed native conformation of the different omega-1 mutants as well as the absence of glycosylation only on the omega-1 glycosylation mutant, as evidenced by a single band instead of the 3 glycosylation forms of recombinant omega-1 normally secreted by HEK293 cells.



Supplementatry figure 2. Glycosylation of recombinant omega-1 MALDI-TOF mass spectrum of glycopeptides from a tryptic digest of recombinant omega-1, covering the glycosylation site N176. Recombinant omega-1 was subjected to SDS-PAGE under reducing conditions and stained with Colloidal blue. Stained bands were excised, subjected to reduction and alkylation and digested with trypsin. The MALDI-TOF-MS spectrum derived from the upper band in the SDS-PAGE pattern is depicted. Signals ($[M+H^+]$) are labeled with monoisotopic masses. Composition of the glycan moieties are given in terms of hexose (H), *N*-acetylhexosamine (N) and fucose (F). Differences in fucose content are indicated by double-headed arrows. Signals that cannot be assigned to glycopeptides are marked with asteriks (*). Based on the presence of the common N-glycan core structure (H_3N_2), the monosaccharide composition of the glycan species $H_3N_6F_2$ and $H_3N_6F_3$ indicates that these contain fucosylated antennae.



Supplementary figure 3. Validation of an assay for binding and uptake of antigens by DCs (A) DCs were incubated for 1 h with PF-647-labeled omega-1 at 37 °C and, where indicated, either preincubated with EGTA to prevent binding to CLRs or treated afterwards with EGTA to remove any potential antigen bound to CLRs on the cell surface. After 1h incubation at 37 °C, EGTA treatment afterwards did not remove any fluorescently-labeled omega-1 from the surface, given that the fluorescent intensity of the DCs was not affected, suggesting that all omega-1 is internalized by DCs, (B) As a control, the same assay was performed at 4 °C, a temperature that precludes receptor-mediated uptake, to show that EGTA treatment afterwards, can abolish surface binding of omega-1.



Supplementary figure 4. Omega-1 dose dependently suppresses expression of multiple cytokines After DCs had been pulsed for 40 h with the different antigen preparations in combination with LPS (100 ng/ml), the cells were co-cultured for 24 h with the J558 cell-line, expressing CD40-L, to mimic the interaction with T cells. Concentrations of the stimuli are given in μg/ml. Bars represent mean ± SD of triplicate wells. * $p < 0.05$, ** $p<0.01$, *** $p<0.001$ for values significantly different from the LPS control.

Chapter 6

The major GalNAcβ1-4GlcNAc-containing glycoprotein of *Schistosoma mansoni* eggs, kappa-5, induces type 2-polarized granulomas in a pulmonary mouse model

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Abstract

The main pathology of schistosomiasis is caused by a granulomatous response which develops around eggs that get trapped in host tissue. Previously, granuloma formation has been explored in pulmonary and hepatic mouse models using antigen-coated Sepharose beads as artificial eggs. Using the hepatic model, it has been shown that the glycan portion of soluble egg antigens (SEA) can induce granuloma formation similar to SEA. Moreover, from a group of synthetic schistosome-related glycoconjugates, only those terminating in GalNAcβ1-4GlcNAc (LDN) and Galβ1-3/4GlcNAc (LN) were able to induce the same granulomatous responses. So far, the native *S. mansoni* egg glycoproteins expressing these granuloma-inducing glycans have remained elusive. Recently, we have identified kappa-5 as the major glycoprotein in SEA that expresses LDN-motifs. In this report, we show in a pulmonary mouse model for granuloma formation that kappa-5 coated to Sepharose beads is able to induce type 2-polarized granulomatous responses. Furthermore, we show that the capacity of kappa-5 to induce granulomas involves its LDN motifs, as selective removal of LDN significantly diminished granuloma formation.

Introduction

Schistosomes are parasitic helminths that infect over 200 million people in (sub-) tropical areas around the world. The main pathology of *S. mansoni* infection is initiated by the large proportion of parasite eggs that instead of being excreted with the feces, get trapped in various organs such as the liver. Here, egg antigens induce a type 2 inflammatory response, leading to the formation of periovular granulomas composed of mainly CD4+ T cells, macrophages and eosinophils, and collagen fibers [1]. The development of the egg granuloma is shown to be highly dependent on Th2-polarized CD4+ T cells [2] and alternatively activated macrophages [3].

The nature of *S. mansoni* egg molecules involved in the induction and modulation of granulomas has been explored in established experimental mouse models by injection of antigen-coated Sepharose beads as artificial eggs into the liver [4-6] and lungs [7-9]. In the liver model, beads coated with *S. mansoni* soluble egg antigens (SEA) give rise to granulomas comparable to those around schistosome eggs in terms of cellular composition and expression of adhesion molecules and extracellular matrix components [4;6]. Destruction of the integrity of SEA glycans by meta-periodate abolished the inflammatory properties of the SEA beads, demonstrating a major role for glycosylation in the initiation of the granulomatous reaction [6]. To explore the type of glycans involved, beads were injected coated with a selected set of synthetic model glycoconjugates containing glycan elements representative for schistosome eggs, such as GalNAcβ1-4GlcNAc (LacdiNAc, LDN), Galβ1-4(Fucα1-3)GlcNAc (Lewis X), Fucα1-2Fucα1-3GlcNAc (DF-Gn) and Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) [10]. Interestingly, from the tested set, only beads coated with a synthetic LDN-conjugate and beads coated with asialofetuin, a glycoprotein with terminal Galβ1-3/4GlcNAc (LacNAc, LN) groups, were able to induce granulomas in this model. All other glycoconjugates tested elicited only a monolayer of macrophages, similar to uncoated and albumin-coated beads.

Recently, we have described kappa-5 as one of the major antigens in *S. mansoni* SEA [11;12]. Glycosylation analysis demonstrated that kappa-5 contains four N-glycosylation sites which for a large part carry triantennary LDN motifs. Moreover, kappa-5 was found to be the major LDN-containing glycoprotein in SEA [11], in terms of abundance as well as reactivity with the GalNAc-specific lectin soybean agglutinin and an LDN-binding antibody. These characteristics prompted us to test the immunological properties of kappa-5 in the pulmonary mouse model. Although kappa-5 is presumably not present during the early stages of egg development, it is definitely presented to the immune system later on, as it gives rise to antibody responses in infected hosts [13]. Therefore we set out to investigate whether kappa-5 might have a role in granuloma formation and/or modulation. Using the pulmonary model, we demonstrate that

kappa-5 coated beads are able to induce granulomas of a similar nature as egg- and SEA-induced granuloma. Furthermore, beads containing kappa-5 of which the LDN motifs were enzymatically removed induce significantly less and smaller sized granulomas opposed to untreated kappa-5 coated beads. Conclusively, here we describe the first native *S. mansoni* egg glycoprotein with glycan-dependent granuloma-inducing properties.

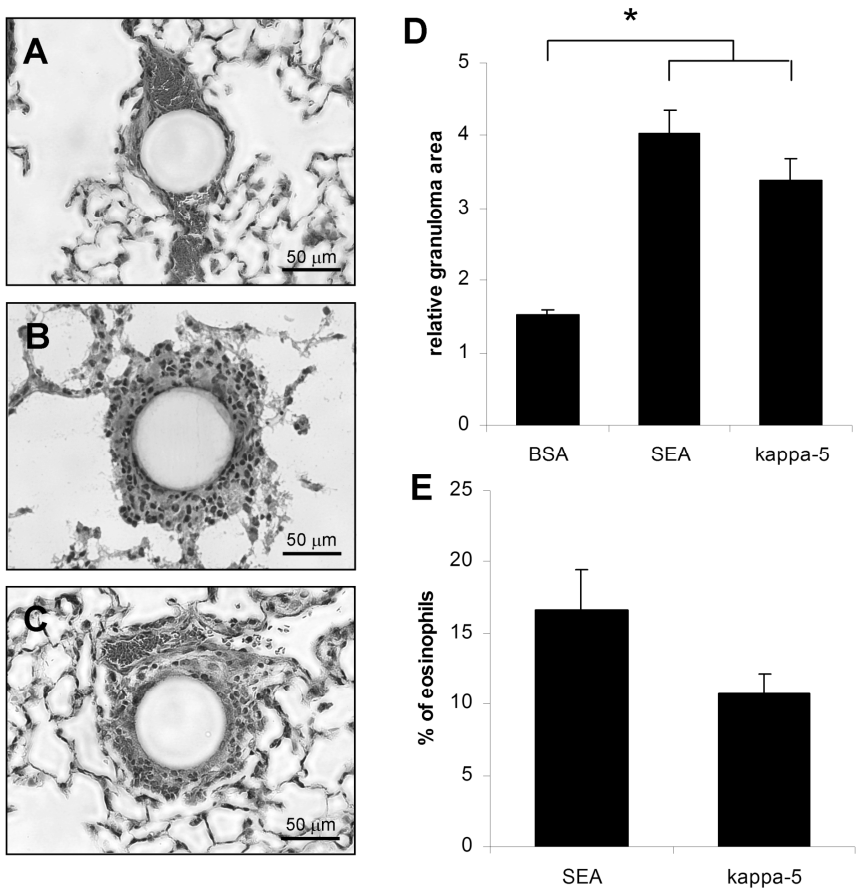

Figure 1. Induction of pulmonary granuloma formation by kappa-5 in an experimental mouse model. Antigen-coated beads were injected into the lungs of mice and at 14 days post-injection, granuloma formation was assessed. (A-C) Representative examples of BSA- (A), SEA- (B) and kappa-5- (C) induced granuloma are shown for every experimental group. Lung slides are stained with H&E. (D) The relative granuloma area (granuloma area divided by bead area) was assessed for each experimental group. (E) The percentage of eosinophils was calculated for SEA- and kappa-5-induced granulomas Graphs show mean values with SEM (Mann-Whitney: * indicates $p < 0.001$).

Results

The S. mansoni egg glycoprotein kappa-5 induces Th2-polarized granuloma formation

Sepharose beads were coated with SEA or the SEA component kappa-5, and tested in an established mouse model for pulmonary granuloma formation [7]. Fourteen days after bead injection, control beads coated with bovine serum albumin (BSA) showed no cellular reaction except for a monolayer of cells (Figure 1A), while granuloma formation was observed around beads coated with SEA (Figure 1B). Importantly, the majority of beads (93 %) coated with isolated, native kappa-5 were able to induce granulomatous responses (Figure 1C). As the size of the commercially obtained beads varied significantly, the granuloma area relative to the bead area was used as a measure for the granuloma size (relative granuloma area; Figure 1D). We found that there was no significant difference between the granuloma sizes induced by SEA- and kappa-5-coated beads.

To investigate the nature of the granulomas induced by the antigen-coated beads, granulomas within H&E-stained lung slides were screened for the presence of eosinophils, which is a hallmark for type 2 granulomas [3;6]. 16.6 ± 2.9 % of the cells within the SEA-induced granulomas were eosinophils (Figure 1E). For the kappa-5-induced granulomas, the majority of inspected granulomas also contained eosinophils (10.7 ± 1.4 %; Figure 1E).

The granuloma-inducing properties of kappa-5 are partly mediated by its LDN motifs

To investigate whether the LDN motifs on kappa-5 are involved in the granuloma-inducing properties of this glycoprotein, kappa-5-coated beads were treated with β-*N*-acetylhexosaminidase to remove LDN motifs (kappa-5Δhexnac beads). Nano-LC-MS analysis verified the nearly complete removal of unsubstituted GalNAc and GlcNAc residues from kappa-5, as shown for site N251 in Supplementary Figure 1. When tested in the pulmonary mouse model, the overall relative granuloma area of kappa-5Δhexnac beads was significantly lower as compared to that of untreated kappa-5 beads (Figure 2A). To further explore this difference, the potency of coated beads to induce granulomas, as well as the distribution of the different sizes of the granulomas induced, was studied. Interestingly, 33 % of the kappa-5Δhexnac beads was no longer able to induce granulomas but instead induced monolayer reactions similar to the BSA control, compared to only 7 % of kappa-5 coated beads (Figures 2B and 2C). Moreover, when evaluating granuloma formation around individual beads, it became evident that the kappa5Δhexnac bead population contained more small-sized and less large-sized granulomas as compared to untreated beads (Figure 2D and Supplementary Figure 2). The reduction in granuloma size upon LDN removal was due to a decrease in cell numbers, as a

strong correlation was found between granuloma area and cell numbers (r=0.468, p<0.001). To summarize, removal of LDN from kappa-5-coated beads resulted in a higher percentage of beads unable to induce granulomas and a reduction in granuloma cell numbers around the remaining, granuloma-inducing beads.

Notably, kappa-5Δhexnac beads initiated similar, Th2-type granulomas as untreated kappa-5 beads, as was demonstrated by a comparable percentage of eosinophils within kappa-5Δhexnac- and kappa-5-induced granulomas (10.3 ± 0.9 % and 10.7 ± 1.4 %, respectively).

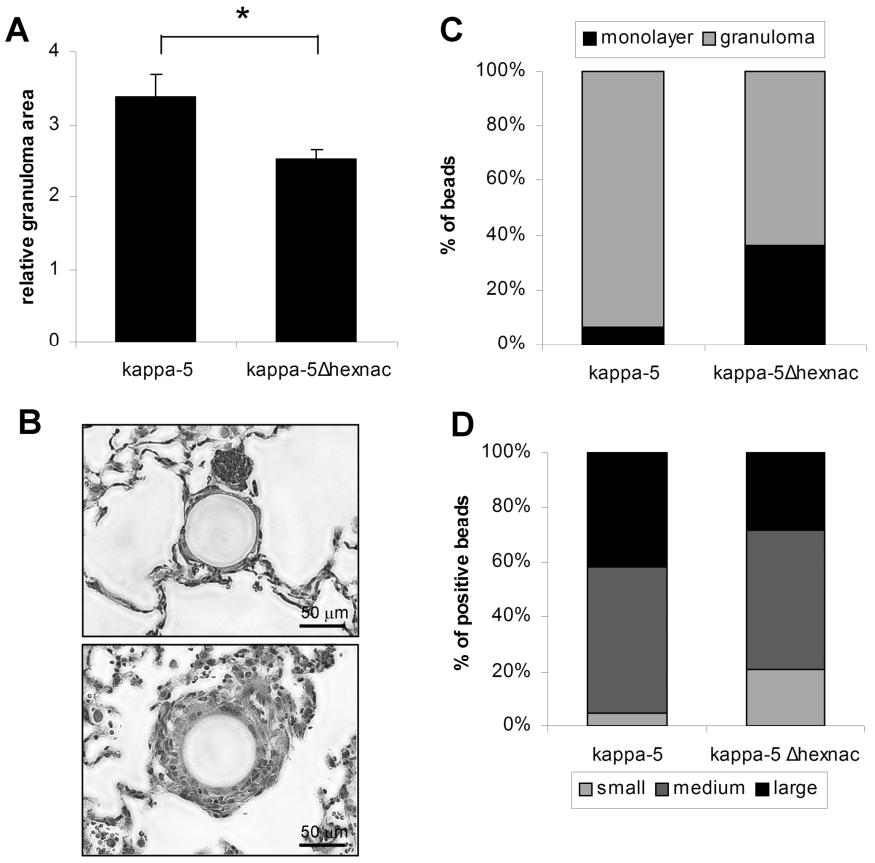

Figure 2. Enzymatic removal of LDN from kappa-5 leads to less and smaller sized granulomas. β-*N*-hexosaminidase-treated and untreated kappa-5 beads were injected into the lungs of mice and at 14 days post-injection, granuloma formation was assessed. (A) The relative granuloma area (granuloma area divided by bead area) was assessed for untreated and treated kappa-5 beads. Graphs show mean values with SEM (Mann-Whitney: * indicates $p < 0.05$). (B) Representative example of kappa-5 beads treated with β-*N*-acetylhexosaminidase inducing either a monolayer of cells or a multilayer granuloma. Lung slides are stained with H&E. (C) Percentage of beads surrounded by a monolayer of cells (monolayer) and beads surrounded by a granulomatous response (granuloma) for treated and untreated kappa-5 beads was calculated. (D) Untreated and treated kappa-5 beads surrounded by a granulomatous response (positive beads) were grouped on the basis of their relative granuloma area; small (<2), medium (2-4) and large (>4).

Discussion

S. mansoni egg glycoconjugates induce strong Th2 type inflammatory responses in the host, which include the formation of granulomas around tissue-lodged eggs. Previous reports using a hepatic mouse model have demonstrated that glycans from *S. mansoni* SEA play a major role in the initiation of this inflammatory response [6]. Also, in the same mouse model, LDN in the context of a synthetic glycoconjugate coated to beads was demonstrated to have similar granuloma-inducing properties [10]. However, the native *S. mansoni* glycoproteins that carry these immunogenic glycans have remained undetermined. In this report, we present for the first time a single, native *S. mansoni* egg glycoprotein, the SEA-component kappa-5, with granuloma-inducing properties. Moreover, we have found that the glycan motif LDN significantly contributes to this immunogenic effect.

The type 2 granulomatous response induced by *S. mansoni* eggs and SEA-coated beads is characterized by an influx of Th2-polarized CD4+ cells, alternatively activated macrophages as well as eosinophils [3;6]. As confirmed by the presence of eosinophils, we here show that granulomas induced by beads coated with kappa-5 and kappa-5Δhexnac share this type 2-polarized nature. This indicates that the granulomatous properties of LDN and other, yet unknown components of kappa-5, could be of relevance during natural *S. mansoni* infection. Notably, the percentage of eosinophils in our SEA-induced granulomas is comparable with granulomas elicited by SEA beads in a similar model of pulmonary granuloma induction [9]. Granulomas surrounding kappa-5 (Figure 1E) and kappa-5Δhexnac (data not shown) beads appear to contain lower percentages of eosinophils, although experiments need to be repeated to achieve statistical relevance. This might indicate that SEA contains other (glycosylated) proteins that have a positive effect on eosinophil infiltration.

The mechanisms through which kappa-5 exerts the immunomodulatory effects described in this report, still need to be elucidated. However, the involvement of the glycan motif LDN indicates that recognition by pattern recognition receptors (PRRs) on antigen-presenting cells (APCs) might play an important role. PRRs which were previously shown to recognize LDN and/or GalNAc are galectin-3, MGL and Dectin-2. MGL is a C-type lectin with a narrow specificity for unsubstituted, terminal α- and β-linked *N*-acetylgalactosamine [14]. Recognition of SEA by MGL has been demonstrated to be at least partially conferred by the GalNAc-containing LDN motifs [14]. Moreover, *in vitro* recognition of kappa-5 by dendritic cells (DCs) is in part dependent on MGL binding (Chapter 4 of this thesis). However, MGL does not seem to recognize LN [14], a motif structurally related to LDN which has been shown to exhibit comparable granuloma-inducing properties, suggesting that other receptors recognizing both LDN as well as

LN are more likely to mediate their immunomodulatory effects. Galectin-3 recognizes both LDN and LN motifs and has previously been proposed to be a key molecule in *S. mansoni* granuloma formation, as it is upregulated in egg granulomas of *S. mansoni*-infected hamsters and mice and colocalizes with LDN-glycans on eggshells [10;15]. However, galectin-3 $^{-/-}$ mice infected with *S. mansoni* only showed minor phenotypic changes compared to normal mice in terms of perioval granuloma formation [16-18]. Therefore, this receptor does not seem to play an important role in the initiation or modulation of granulomas during natural infection. Recently, Dectin-2 has been shown to bind terminal galactose and GalNAc residues [19], and thus could be a potential receptor for both LN and LDN motifs. SEA has been demonstrated to be recognized by and signal via Dectin-2, and mice deficient for ASC and Nlrp2, signal molecules involved in SEA-induced Dectin-2 signaling, developed smaller granulomas around *S. mansoni* eggs [20]. Hence, Dectin-2 signaling might pose a potential mechanism for kappa-5 to modulate perioval granuloma formation.

While we show that the LDN motifs on kappa-5 are involved in the granuloma-inducing properties of kappa-5, the glycoprotein must contain other granulomagenic features, as β-*N*-acetylhexosaminidase-treated kappa-5 still contains some activity (Figure 2). Upon LDN removal, the major fraction of kappa-5 glycans consist of an N-glycan core containing two core fucose and a xylose, while a small fraction of glycans additionally carry an α3-fucosylated GlcNAc (Supplementary Figure 1). The latter structural element was however previously shown to lack granuloma-inducing properties in the hepatic mouse model [10]. Also all other fucosylated structures tested were inactive in this model [10], suggesting that the di-fucosylated core is also not involved. A possible role for the core xylose residues on kappa-5 glycans in granuloma formation has so far not been investigated. In addition, involvement of the protein part of kappa-5 can also not be excluded, although the amino acid sequence of kappa-5 shares no similarities with any known functional motifs [11].

Granuloma formation is initiated when eggs lodge into the organs of the host. The egg-derived molecules involved in activation and maintenance of the granulomatous response may include soluble excretory/secretory (ES) glycoproteins as well as molecules associated with the egg shell. As kappa-5 is not considered to be secreted by eggs at the time point when granuloma formation sets in [11], it is unlikely that kappa-5 plays a dominant role in this stage of granulomagenesis. However, kappa-5 is effectively presented to the immune system as evident by the high antibody titers against kappa-5 that have been detected in *S. mansoni*-infected individuals [13]. Moreover, it is believed that kappa-5 is a constituent of hatching fluid [11], to which the host presumably is exposed when eggs die in the tissues. While kappa-5 is capable of inducing granulomas in our model, in the biological context it might therefore be involved in the

maintenance or modulation of granulomas. Well-studied immunomodulatory molecules that are probably presented to the immune system at the earlier stages of granuloma development are the major ES glycoproteins IPSE/α1 and omega-1. The LeX-dominated glycosylation of these two ES glycoproteins is unlikely to be directly involved in the initiation of granuloma formation, as LeX motifs on a synthetic glycoconjugate did not show any granuloma-inducing properties in a hepatic mouse model [10;21]. IPSE/α1 has instead been reported to play a role in the down modulation of granuloma formation, as *S. mansoni*-infected mice that were treated with antibodies to block IPSE/α1 activity, developed significantly larger perioval granulomas compared to untreated infected mice [22]. Besides a possible role for other secreted egg glycoproteins, also the egg shell may have glycan-dependent granuloma-inducing properties. It has been recently demonstrated that the shell of *S. mansoni* eggs is glycosylated [23], but clear structural information is lacking so far.

To conclude, our data suggest that kappa-5 plays a role in the induction and modulation of periovular granulomas during schistosome infection, and that this property is partly dependent on the terminal LDN structures on the kappa-5 glycans. These data provide new insights in the glycan-dependent immunomodulatory properties of *S. mansoni* egg molecules.

Material and Methods

Animals

Six week old female BALB/c mice were purchased from Harlan. Mice were housed under SPF conditions and all animal experiments were approved by the animal experimental committee of the University of Leiden, The Netherlands.

Preparation of antigens and antigen-coated beads

S. mansoni soluble egg antigens (SEA) was prepared and isolated as described previously [24]. Kappa-5 was isolated by SBA affinity chromatography as described previously [12]. For bead coating, 1 mg of glycoprotein was coupled to 1 ml Cyanogen bromide-activated Sepharose 4B heads (Sigma, St. Louis, MO), ranging in diameter from 45 to 165 μm, as previously described [7]. 50.000 kappa-5 coated beads were treated with 62.5 mU β-*N*-acetylhexosaminidase from *Canavalia ensiformis* (Sigma) in 100 mM sodium phosphate buffer, pH 5.0 for 24 h at 37°C, after which the same amount of enzyme was added for another 24 h.

Induction of pulmonary granulomas in mice

BALB/c mice received 5000 antigen-coated beads suspended in sterile PBS i.v. in the lateral tail vein. Four mice were used for every set of beads. The animals were sacrificed 14 days after injection and lungs were removed.

Light microscopy and immunohistochemistry

To evaluate the granulomatous response, 4 μm-thick formalin-fixed paraffin-embedded lung sections as well as acetone-fixed cryostat sections were stained with H&E. Granuloma as well as bead areas were quantified using Bersoft Image Measurement Software v4.03 (Bersoft Inc., Puerto Plata, Dominican Republic) and the relative granuloma area was calculated, representing the average of the granuloma area divided by the average of the bead area. Only granulomas with visible central beads and bead area above 1600 μm^2 were included. Also, granuloma sizes were grouped in small, medium and large-sized granulomas, based on relative granuloma areas of under 2 (small), between 2 to 4 (medium) and over 4 (large). For each experimental group, at least 50 granulomas were assessed and the variables were compared and statistically analyzed with a Mann-Whitney test. P values of less than 0.05 were considered significant. In addition, at least 20 granulomas per experimental group were visually inspected for the total number of cells and number of eosinophils.

Mass spectrometry

Trypsin (Promega, Leiden, The Netherlands) was added to a sample of the β-*N*-acetylhexosaminidase and untreated kappa-5 beads at a 1:100 trypsin/antigen ratio and incubated overnight at 37 °C. Resulting (glyco)peptides were analyzed using nano-HPLC-ESI-ion trap-MS as described previously [21].

Acknowledgements

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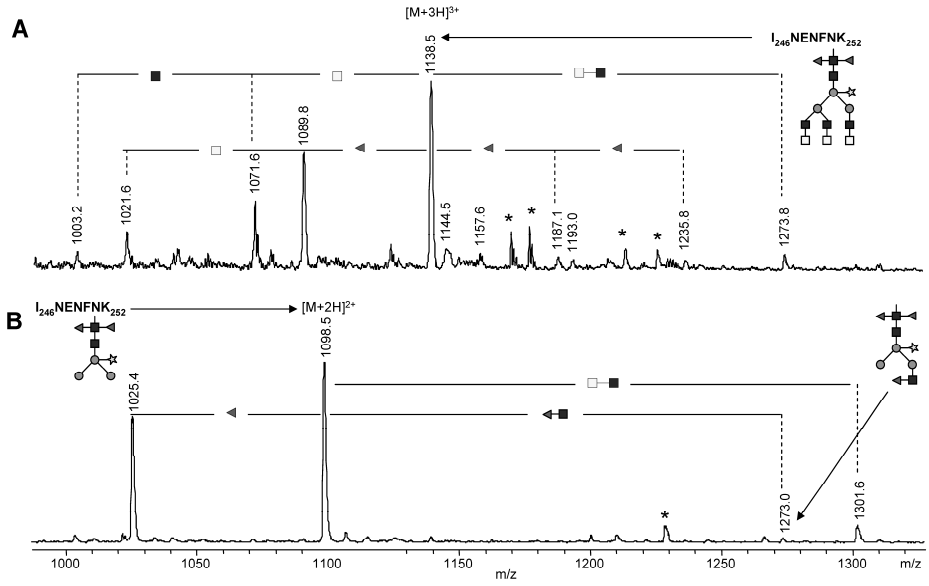
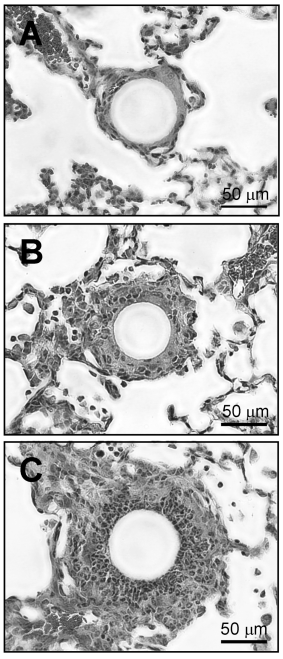
Supplementary Figures



Supplementary figure 1. β-*N*-acetylhexosaminidase treatment of kappa-5 beads. Beads coated with kappa-5 were either left untreated (A) or treated with β-*N*-acetylhexosaminidase (B) and a sample of beads was digested with trypsin. Resulting kappa-5 glycopeptides were analyzed by nano-LC-MS. The accumulative mass spectrum representing all glycoforms of the tryptic glycopeptide containing glycosylation site N251 is shown. Signals are labeled with monoisotopic masses. Differences in fucose, *N*-acetylglucosamine and *N*-acetylgalactosamine content of the glycan moiety are indicated. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; dark circle, mannose; light star, xylose.



Supplementary figure 2. Representative examples of small (A), medium (B) and large (C) sized granulomas. Granulomas were grouped on the ba sis of their relative granuloma area; small (<2), medium (2-4) and large (>4).

Chapter 7

General Discussion

Soluble egg glycoproteins of *Schistosoma mansoni* exhibit potent immunomodulatory properties including those that lead to T-helper 2 (Th2) polarization and granuloma formation [1]. The glycans carried by these proteins are thought to be responsible for a large part of these properties by directing interactions with host lectins. Until now, most experiments supporting this line of thought have been performed with soluble egg antigen (SEA) preparations and/or synthetic, schistosome-related glycoconjugates [2-6]. However, SEA comprises a very complex mixture of soluble glycoproteins [7] and therefore it is difficult to determine which individual glycoproteins or specific structural characteristics of SEA are involved. Moreover, when using synthetic glycoconjugates as a basis for immunological studies, certain aspects of native glycoproteins are not addressed, such as the spatial presentation of glycans and the influence of the underlying protein structure. Comprehensive analysis of single, native *S. mansoni* glycoproteins in terms of glycan and protein structure, lectin binding behavior as well as functional capacities is essential to achieve a more profound understanding of immunological properties of schistosome eggs. In this thesis, the structural features of the glycans expressed on three major egg glycoproteins and the role that these glycans play in various aspects of host-parasite interplay have been studied.

One of these egg glycoproteins is the major ES component omega-1 [8]. Recently, omega-1 has been shown to harbor potent Th2 polarizing activities via modulation of dendritic cells (DCs), *in vitro* using human blood-derived cells and *in vivo* in mouse models [9;10]. We show in **chapter 2** that omega-1 carries core-difucosylated diantennary N-glycans with one or more Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX) motifs in the antennae. These structural observations are particularly interesting because a) the same glycans are present on IPSE/α1, a second major ES glycoprotein with immunomodulatory properties [11;12]; b) LeX as part of model glycoconjugates can modulate DC activation via DC-SIGN signaling, resulting in a immunological phenotype that is thought to be capable of inducing Th2 polarization [3;4;13-15]. It is reasonable to speculate that omega-1 uses this mechanism to condition DCs for Th2 priming. However, in **chapter 5** we show that DC-SIGN is dispensable for omega-1-induced Th2 polarization. Moreover, IPSE/α1, despite having similar glycans and C-type lectin receptor (CLR)-binding characteristics as omega-1 ([12] and **chapter 4**), does not exhibit the same Th2-polarizing properties observed for omega-1 [10]. Interestingly, the reported immunomodulatory effects of IPSE/α1, i.e. induction of IL-4 release in human and murine basophils [16;17] and reduction of cellular infiltration into granulomas [18], do not require intact glycosylation [16-18]. While literature suggests that respectively IgE binding- and chemotactic activities of IPSE/α1 are responsible for the immunological properties of this glycoprotein [11;16-18], we show in **chapter 5** that the mechanism through which

native omega-1 induces DC-mediated Th2 polarization is based on internalization of omega-1 via LeX-MR interactions, allowing its RNase activity to functionally modulate DCs.

In accordance with a major role for MR in the immunomodulatory effects of omega-1, we demonstrate that MR is the dominant receptor on monocyte-derived immature DCs (iDCs) that recognizes the LeX-dominated glycosylation on soluble IPSE/α1 and omega-1, while DC-SIGN interactions seem to be of only minor importance (**chapter 4 and 5**). In contrast to a dominant role for MR over DC-SIGN for these *soluble* LeX-expressing glycoproteins, MR binds only marginally to *plate-coated* LeX glycoconjugates (**chapter 4** and [19]), while DC-SIGN displays a strong affinity for these coated glycoconjugates as well as *plate- and particle-coated* SEA (**chapter 4** and [5;20;21]). These findings imply that the specificity of these CLR is at least partially dependent on the molecular presentation of the ligand.

Several studies describing the CLR-dependent molecular mechanisms of immunomodulatory molecules from other pathogens in addition imply that the multivalency characteristics of a ligand can influence the mechanism of action. Ligation of MR on DC by specific mannosylated molecules, including the mannose-capped lipoarabinomannans (ManLAM) from *Mycobacterium species*, has been demonstrated to promote anti-inflammatory responses via the induction of anti-inflammatory cytokines and interference with pro-inflammatory TLR signalling [15;22;23]. Other mannosylated ligands for MR, including mannan and thyroglobulin, are not able to induce these signalling pathways [22]. Cross-linking of MR by anti-MR antibodies leads to similar effects as ManLAM, indicating that the polyvalent nature of ManLAM is involved in its MR-mediated effects [22;23]. For DC-SIGN, it has been shown that pathogens expressing mannosylated (e.g. *Mycobacterium tuberculosis* and human immunodeficiency virus type 1; HIV-1) as well as fucosylated (*Helicobacter pylori*) glycan structures on a pathogenic surface can interfere with TLR-induced signaling via modulation of a signaling complex associated with DC-SIGN [15;24]. The type of glycan moiety recognized by DC-SIGN is critically important for the recruitment or dissociation of signaling molecules to the signaling complex. More importantly, the glycan moiety thereby affects the consequential modulation of cytokine responses. Mannose-mediated DC-SIGN signaling leads to upregulation of IL-10, IL-12 and IL-6 transcript while fucose-mediated signaling induces a more anti-inflammatory profile with enhanced IL-10 levels and decreased IL-12 and IL-6 levels [15].

These findings, combined with ours, indicate the presence of at least two separate mechanisms via which interplay of pathogen-associated or -derived glycoconjugates with CLRs can condition DCs for immune modulation of T cells: a) CLR-mediated internalization of bioactive molecules; and b) CLR-induced interference with TLR signalling. Which mechanism is exploited by pathogen-derived molecules may depend on the glycan motifs expressed, their

spatial presentation, and co-stimulatory factors provided by the targeted molecule itself and/or surrounding pathogenic molecules. Interestingly, the fucosylated and mannosylated pathogeni-derived molecules that are thought to induce functional DC signaling, are expressed on the surface of pathogens. Such a multivalent presentation of glycan ligands might be a prerequisite for functional signalling in DCs leading to Th polarization, and could explain why omega-1 and IPSE/α1, being secreted glycoproteins, are unable to use this specific type of mechanism (**chapter 5**).

For kappa-5, the third major egg glycoprotein studied, no immunomodulatory function has been described in literature. We show in **chapter 3** that kappa-5 mainly carries core-difucosylated and -xylosylated triantennary N-glycans that are substituted with GalNAcβ1-4GlcNAc (LacdiNAc, LDN) antennae, a minority of which carry an additional α3-linked fucose on the GlcNAc residue (LDN-F). These glycans mediate interaction of kappa-5 with DC-SIGN, MGL and MR (**chapter 4**), and at the same time are a target for IgE-binding (**chapter 3**), suggesting multiple roles for these glycans in the host-pathogen interaction. In **chapter 6**, we demonstrate in a pulmonary mouse model that native kappa-5 coated to Sepharose beads can induce type 2-polarized granulomatous responses, and that the LDN motifs on kappa-5 are involved in this process. These results indicate a dual role for kappa-5 as a granulomagenic as well as a Th2-modulating agent.

The mechanism by which LDN induces granulomatous responses remains to be elucidated, but its recognition by CLRs on DCs suggests that CLR-expressing antigen-presenting cells (APCs) are likely to be involved. Also, preliminary data indicate a role for the chemokines CCL17 and CCL22, as mRNA expression of these glycoproteins could be induced in DCs upon kappa-5 stimulation (data not shown). CCL17 and CCL22 have been previously shown to be upregulated in presensitized mice upon tail vein injections of *S. mansoni* eggs [25], Moreover, these chemokines can functionally act via CCR4 which has been shown to be expressed on a wide array of immune cells including Th2 cells, eosinophils and macrophages, cells that are major constituents of the *S. mansoni* egg-induced granuloma [26-29]. Neutralization of either of these chemokines in mice resulted in reduced egg granuloma size and eosinophil numbers as well as increased cytokine levels [26]. Given these and our findings, we hypothesize that recognition of LDN motifs by CLRs on APCs leads to the release of CCL17 and CCL22 and therefore the attraction of CCR4-expressing immune cells. This may provide one mechanism of action via which eggs can induce and/or modulate type 2 granuloma formation.

Unpublished findings indicate that kappa-5 is not the only granulomagenic component in SEA or ES, as depletion of kappa-5 does not completely abrogate the capacity of these mixtures to

induce granuloma formation in a pulmonary mouse model (data not shown). Likewise, SEA depleted of omega-1 is still able to induce Th2 polarization in a mouse model [10], indicating that other soluble egg (glyco)proteins are in addition involved in these immunomodulatory processes. Considerable data is now available on the proteome and glycome of *S. mansoni* eggs [7;30;31]. However, apart from the glycoproteins described in this thesis, it is generally unknown which proteins express which glycans, complicating the search for new immunogenic molecules. Still, the structural information of SEA and ES glycans provides interesting clues. Omega-1 and IPSE/α1 have been shown to be the main carriers of LeX motifs within the ES glycome [31], while kappa-5 is the major LDN-expressing glycoprotein within SEA (**chapter 3**). Therefore, we suggest that the remaining immunoactive soluble egg glycoproteins can modulate immune responses via other, yet undetermined, glycans and/or protein motifs. Major glycan motifs on these glycoproteins include α3-fucose and β2-xylose core modifications, fucosylated LDN motifs, and Fucα1-2Fucα1-3-HexNAc elements [30;31].

For the fucosylated LDN motifs present on *S. mansoni* egg glycoproteins (see Table 2 in **chapter 1**), we and others have shown that DC-SIGN recognizes the majority of these structures (**chapter 4** and [20,21]). Interestingly, LDN-based structures are expressed by many other helminth parasites that induce Th2-polarized responses, including *Haemonchus contortus* [32], *Fasciola hepatica* [33] and *Trichinella spiralis* [34;35]. This is in contrast to LN/LeX motifs, that have only been found on schistosomes and the nematode *Dictyocaulus viviparus* [31;33]. Thus, the fucosylated LDN motifs form common helminth ligands for DC-SIGN, although the significance of these motifs remains unknown.

Fucα1-2Fucα1-3 (DF) elements are frequently occurring on LDN structures of egg O-glycans, while only a minority of N-glycans seem to display this element [31;36]. DF has so far not been reported in any species other than schistosomes, and therefore could potentially be very interesting in terms of immune recognition. Indeed, DF elements elicit high antibody responses during *S. mansoni* infection in humans and chimpanzees [37-39]. Moreover, LDN-DF glycoconjugates induce a pronounced production of pro- and anti-inflammatory cytokines in peripheral-blood mononuclear cells (PBMCs) from non-exposed humans, to a level far higher than the production induced by other glycoconjugates including those terminating in LeX and LDN-F [40]. The latter observation may indicate that DF elements may be involved in innate recognition of eggs by APCs. Our glycoconjugate array data show that DC-SIGN, MGL and MR do not or only slightly bind the DF-element on a single GlcNAc residue (**chapter 4**), and it was previously demonstrated that DC-SIGN is unable to bind LDN-DF [20], indicating that DC-SIGN, MGL and MR do not play a role in the recognition of DF elements by the immune system. To date, no other receptors have been found that display affinity for this element.

In addition to the described properties of soluble egg glycoproteins, insoluble components of the *S. mansoni* egg shell as well as glycolipids may also contain (glycosylated) molecules with immunomodulatory properties. Egg glycolipids have been shown to express LDN, LDN-F and LeX, as well as the DF-element in the form of repetitive Fucα1-2Fucα1-3-HexNAc stretches [36,41], and have been linked to innate immunity [40]. Recently, it has been shown that the egg shell also appears to be glycosylated [42]. Moreover, we found that egg shell N-glycans are dominated by Galβ1-4GlcNAc (LacNAc, LN) motifs (unpublished finding), which as a part of asialofetuin on Sepharose beads have been shown to harbor granulomagenic properties [43].

Concluding remarks

Pathogens are recognized by the innate immune system via a multitude of conserved molecules that are bound by pattern recognition receptors (PRR) such as CLRs, leading to activation of the adaptive immune system. However, pathogens have evolved to modulate immune responses via the same group of molecules to ensure survival within the host. Schistosomes are particularly persistent, as clearance of these parasites usually does not occur without treatment. Immunogenic glycan motifs of egg molecules are suggested to play a major role in the immunomodulation leading to long term survival.

While affecting CLR-induced signaling in DCs seems to be a common mechanism for pathogens to polarize Th-cell responses, we have shown that the *S. mansoni* egg glycoprotein omega-1 uses LeX-CLR interplay for its internalization into DCs, which is an essential step for the Th2 priming effect of this glycoprotein (**chapter 2 and 5**). In the case of IPSE/α1, intact glycosylation is dispensable for its anti-inflammatory and Th2 regulatory effects, although its glycans are efficiently recognized by CLRs (**chapter 4**). For kappa-5, we have shown that LDN glycosylation is involved in its granulomagenic properties (**chapter 3 and 6**). The exact mechanisms via which the immune system mediates this effect remain unclear, although CLR recognition of kappa-5 glycans might play a role (**chapter 4**). The approach used in this thesis, in which studies on primary structural features, binding properties, as well as functional effects of a single glycoprotein are combined, has helped to gain insight in the mechanisms of action of three *S. mansoni* egg glycoproteins and could also be useful for the identification of other glycosylated molecules involved in helminth-induced immune responses.

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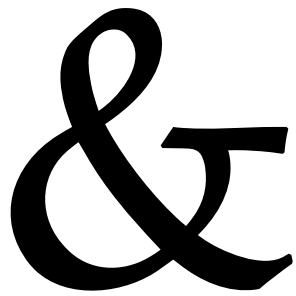
Addendum



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Summary

Schistosomes are parasitic helminths that cause chronical infections in over 200 million people in tropical and sub-tropical areas around the world. In the case of *Schistosoma mansoni*, one of the three major schistosome species infectious to humans, infection starts when cercariae penetrate the skin, after which the developing *S. mansoni* larvae travel to the mesenteric venules near the liver where they further mature. Adult male and female worms form pairs and start to produce eggs, which either leave the body to continue the life cycle, or get trapped in the liver and other organs. The host reacts to eggs and egg products by inducing Th2-mediated granulomatous immune responses which can lead to pathological tissue remodeling, fibrosis, severe organ damage and potentially death.

The majority of molecular and immunological *S. mansoni* egg-related studies have been performed using SEA (soluble egg antigens), an experimental preparation consisting of the soluble egg proteins and glycoproteins. Part of the immunological activity associated with *S. mansoni* eggs is mediated by protein glycosylation. An extensive overview of the immunological capacities of SEA, as well as the current knowledge on immunogenic SEA glycans and glycoproteins, is given in **chapter 1**. This chapter in addition introduces common mass spectrometry-based methods and their application to identify glycan structures on egg glycoproteins.

Three major, immunogenic SEA glycoproteins have been identified: omega-1, IPSE/α1 and kappa-5. The studies described in this thesis focus on the structural and molecular details of these three glycoproteins and their interactions with the human and murine immune systems. In **chapter 2 and 3**, the glycosylation of omega-1 and kappa-5 were studied by a mass spectrometry (MS)-based approach. Glycoproteins were digested with trypsine and analysed by nanoscale LC-MS and MALDI-TOF-MS to generate site-specific glycosylation information. Structural details were obtained by tandem mass analysis, exo-glycosidase treatments and anti-glycan antibodies We found that omega-1 contains two occupied glycosylation sites which for primarily carry diantennary N-glycans of the complex-type (**chapter 2**). The antennae are predominantly composed of Lewis X and *N*-acetyllactosamine structures, with a minority composed of (fucosylated) LDN structures. The innermost core *N*-acetylglucosamine is typically decorated with α3/α6 difucosylation. Interestingly, the glycosylation of IPSE/α1, which has previously been analyzed using a similar MS-based approach, is highly identical to that of omega-1. Kappa-5 on the other hand was found to express a completely different set of glycans (**chapter 3**). It mainly carries triantennary N-glycans that are substituted with LDN antennae, a minority of which carry an additional α3-fucose on the GlcNAc (LDN-F). Like omega-1 and IPSE/α1, most kappa-

5 glycans are α3/α6 core fucosylated, however kappa-5 glycans are in addition modified with a xylose attached to the β4-linked mannose. Notably, we found that kappa-5 is the major LDN-expressing glycoprotein within SEA.

SEA glycan motifs are primarily recognized by C type lectin receptors (CLRs) that are expressed by antigen-presenting cells, a type of innate immune cells that include the dendritic cells (DCs). SEA glycoproteins induce responses in DCs that lead to a characteristic Th2 skewing of the host immune system.The recognition and uptake of SEA glycoproteins by DCs is largely mediated through three CLRs; DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), mannose receptor (MR) and macrophage galactose-type lectin (MGL). **Chapter 4** investigates the binding of these receptors to native IPSE/α1 and kappa-5, in a plate-based assay as well as in a cellular context. To define the structural elements involved in binding to the three receptors, glycoprotein variants were created using exo-glycosidase digestions. In **Chapter 5**, a similar strategy was used to investigate binding of omega-1 to DC-SIGN and MR. We found that IPSE/α1 and omega-1 are primarily recognized by DCs via the interaction of LeX on the egg glycoproteins and MR on DCs. DC-SIGN and MGL are in addition involved in binding of DCs to IPSE/α1, albeit to a much lesser extent. Kappa-5 is recognized by DC-SIGN, MR and MGL without one of these being the dominant receptor, as well as via other, Ca^{2+}-independent receptor(s). Kappa-5 interacts with DC-SIGN via its LDN-F antennae, whereas binding of MR may involve either LDN-F antennae or the fucosylated and xylosylated chitobiose core. MGL binding of kappa-5 was mediated via LDN as well as LDN-F antennae. The binding studies in **chapter 4 and 5** provide a molecular basis for the CLR-mediated interaction of DCs with individual, native glycoproteins of schistosome eggs.

It has long been established that the SEA mixture has Th2 polarizing properties;, but the SEA molecules responsible for this effect have remained unknown. Recently, omega-1 was reported to be a major Th2-inducing component of SEA through functional modulation of DCs. In **Chapter 5**, we show using site-directed mutagenesis that two features of omega-1, its glycosylation and RNase activity, are involved in this process. Omega-1 glycosylation is necessary for the internalization of omega-1 by DCs. We show that this process is mediated via the interaction between LeX on omega-1 and MR on DCs. Within the cell, the RNase activity of omega-1 can then block protein production via cleavage of ribosomal RNA, leading to the conditioning of DCs for Th2 polarization. The immunological data as described in **chapter 5** provide new insights in the molecular processes that direct Th2 induction.

To study *S. mansoni* perioval granuloma formation, mouse models involving the injection of Sepharose beads into liver or lungs are commonly used tools. The beads can be coated with various schistosome egg preparations or schistosome-related molecules, thereby

enabling the identification of native granuloma-inducing and/or modulating molecules. Using such a model, SEA-induced granuloma formation has previously been demonstrated to be dependent on glycosylation. Interestingly, beads coated with LDN and *N*-acetyllactosamine-terminating glycoconjugates were able to induce granulomatous responses similar to SEA. As we found in **chapter 4** that kappa-5 is the major LDN-expressing glycoprotein in SEA, we hypothesized that kappa-5 might be an important granuloma-modulating molecule within eggs. Indeed, we showed in **chapter 6** that kappa-5 coated beads are able to induce granulomatous reactions in a pulmonary mouse model. Kappa-5-induced granulomas contain eosinophils, indicating a Th2-polarized nature that is also observed in egg- and SEA-induced granulomas. LDN is partly responsible for this effect, as enzymatic removal of LDN from kappa-5 coated beads results in significantly less and smaller granulomas as opposed to untreated kappa-5. **Chapter 6** describes kappa-5 as the first, native *S. mansoni* egg molecule with glycan-dependent, granuloma-inducing properties.

Various mechanisms exist via which interplay of pathogen-derived glycoconjugates with CLR on innate immune cells can mediate immunomodulation. In **Chapter 7,** we discuss the CLR-dependent mechanisms exploited by omega-1, IPSE/α1 and kappa-5, as well as related pathogenic molecules, in the light of the results presented in this thesis and previously published data. We conclude that pathogens make use of at least two separate CLR-dependent mechanisms to condition DCs for immune modulation of T cells: CLR-mediated internalization of bioactive molecules; and CLR-induced interference with TLR signalling. In **chapter 7** we additionally review the knowledge on other egg glycoconjugates and glycan motifs that might also be involved in the immunomodulatory activities of *S. mansoni* eggs.

Nederlandse samenvatting

Schistosomen zijn parasitaire wormen die in veel tropische en sub-tropische landen voor grote medische en sociaal-economische problemen zorgen. In het geval van *Schistosoma mansoni*, een van de drie belangrijkste schistosoom soorten die mensen infecteren, start de infectie als cercariën de huid binnendringen. Eenmaal in het lichaam verplaatsen ze zich naar de lever, terwijl ze zich ontwikkelen tot volwassen wormen. Een volwassen vrouwelijke en mannelijke worm vormen een paartje en beginnen eieren te produceren. Een substantieel deel van deze eieren verlaat het menselijk lichaam om de levenscyclus te vervolgen, maar een ander deel loopt vast in de lever en andere organen. De menselijke gastheer reageert op deze eieren door middel van een Th2-gemedieerde granulomateuze immuunrespons, die kan leiden tot pathologische weefselveranderingen, fibrose, ernstige orgaanschade en in sommige gevallen zelfs tot de dood.

De meerderheid van de moleculaire en immunologische *S. mansoni* ei-gerelateerde studies is uitgevoerd met behulp van SEA (oplosbare -Soluble- Ei Antigenen). SEA is een experimenteel mengsel van oplosbare ei proteïnen en glycoproteïnen. Een deel van de immunologische activiteit geassocieerd met *S. mansoni* eieren wordt gemedieerd door de glycosylering op de SEA glycoproteïnen. **Hoofdstuk 1** geeft een uitgebreid overzicht van de immunologische capaciteiten van SEA en van de huidige kennis over immunogene SEA glycanen en glycoproteïnen. Dit hoofdstuk beschrijft daarnaast de meest gangbare massa spectrometrie (MS) methoden die gebruikt worden om glycaan structuren op ei glycoproteïnen te identificeren.

Er zijn drie belangrijke, immunogene glycoproteïnen in SEA geïdentificeerd; omega-1, IPSE/α1 and kappa-5. De studies die in dit proefschrift worden beschreven richten zich op de structurele en moleculaire details van deze drie glycoproteïnen en hun interacties met de immuunsystemen van de mens en de muis. In **hoofdstuk 2 en 3** hebben we met behulp van MS de glycosylering van omega-1 en kappa-5 bestudeerd. Om site-specifieke glycosylerings informatie te verkrijgen, zijn de glycoproteïnen behandeld met trypsine en geanalyseerd met nano-LC-MS en MALDI-TOF-MS. Daarnaast is gebruik gemaakt van tandem mass analyse, exo-glycosidase behandelingen en anti-glycaan antilichamen om de structurele details te ontrafelen. Het blijkt dat omega-1 twee glycosylerings sites bevat die voor het grootste deel bezet zijn met diantennaire N-glycanen van het complexe type (**hoofdstuk 2**). De antennes bestaan voornamelijk uit Lewis X en N-acetyllactosamine structuren, en voor een kleiner gedeelte uit (gefucosyleerde) LDN structuren. De core van de meeste omega-1 glycanen is bezet een α3- en een α6-gebonden fucose. Interessant is dat de glycosylering van IPSE/α1, die al eerder is geanalyseerd met behulp van een vergelijkbare methode, erg lijkt op die van omega-1. Kappa-5

bevat daarentegen een compleet verschillende set glycanen (**hoofdstuk 3**). Dit glycoproteïne draagt voornamelijk triantennaire N-glycanen met LDN antennes, waarvan een klein gedeelte nog een extra α3-fucose op de GlcNAc heeft (LDN-F). Net als omega-1 en IPSE/α1 hebben de meeste kappa-5 glycanen twee α3/α6-gebonden fucoses op de core, maar kappa-5 glycanen dragen nog een extra xylose op de β4-gebonden mannose. We laten in hoofdstuk 3 ook zien dat kappa-5 het belangrijkste, LDN-bevattende glycoproteïne in SEA is.

SEA glycaan motieven worden voornamelijk herkend door C-type lectine receptoren (CLRs), die zich bevinden op het oppervlak van antigeen-presenterende cellen (APCs). APCs zijn een type immuuncellen waaronder onder meer de dendritische cellen (DCs) vallen. SEA glycoproteïnen induceren responsen in DCs die leiden tot een karakteristieke Th2 polarisatie van het immuunsysteem van de gastheer. De herkenning en opname van SEA glycoproteïnen door DCs wordt voor een groot deel bepaald door drie CLRs; DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), mannose receptor (MR) and macrophage galactose-type lectin (MGL). In **hoofdstuk 4** hebben we de binding van deze drie receptoren aan natuurlijk IPSE/α1 and kappa-5 onderzocht. Om de structurele elementen te definiëren die betrokken zijn bij de binding aan de drie receptoren, zijn varianten van IPSE/α1 en kappa-5 gemaakt met behulp van exo-glycosidase digesties. In **hoofdstuk 5** hebben we een soortgelijke strategie gebruikt om de binding van omega-1 aan DC-SIGN en MR te onderzoeken. Het blijkt dat IPSE/α1 en omega-1 voornamelijk door DCs worden herkend via de interactie van LeX op deze ei glycoproteïnen met de MR op DCs. Bij de binding van DCs aan IPSE/α1 zijn daarnaast DC-SIGN en MGL betrokken, alhoewel in veel mindere mate. Kappa-5 wordt in vergelijkbare mate herkend door zowel DC-SIGN, MR en MGL op DCs. Daarnaast zijn nog andere, Ca^{2+}-onafhankelijke, receptoren betrokken bij de herkenning van kappa-5 door DCs. De binding tussen kappa-5 en DC-SIGN wordt gemedieerd door LDN-F, terwijl bij de kappa-5-MR binding mogelijk de LDN-F danwel de fucoses en/of xylose op de chitobiose core betrokken zijn. De binding van MGL aan kappa-5 gaat via de LDN en LDN-F motieven. De binding studies van **hoofdstuk 4 en 5** geven een moleculaire basis voor de CLR-gemedieerde interactie tussen DCs en individuele schistosoom ei glycoproteïnen.

Het is al eerder vastgesteld dat het SEA mengsel een Th2-polariserend effect heeft. Echter, de SEA moleculen die verantwoordelijk zijn voor dit effect waren nog onbekend. Recent is gebleken dat omega-1 een belangrijke component in SEA is die Th2 responsen kan induceren via functionele modulatie van DCs. In **hoofdstuk 5** beschrijven we twee eigenschappen van omega-1 die betrokken zijn bij dit proces: glycosylering en RNase activiteit. De glycosylering van omega-1, en meer specifiek de interactie tussen LeX op omega-1 en MR op DCs, is nodig voor de internalisatie van omega-1 in DCs. Eenmaal in de cel, blokkeert de RNase activiteit van

omega-1 de proteïne productie via de afbraak van ribosomaal RNA. Dit leidt uiteindelijk tot de conditionering van DCs voor Th2 polarisatie. De immunologische data die in **hoofdstuk 5** worden beschreven bevatten nieuwe inzichten in de moleculaire processen die leiden tot de inductie van Th2 responsen.

Bij de bestudering van granuloomvorming rondom *S. mansoni* eieren wordt vaak gebruik gemaakt van muis modellen waarbij Sepharose partikels in the lever of longen worden gespoten. Aan de partikels kunnen verscheidene schistosoom ei moleculen worden gekoppeld, waardoor identificatie van natuurlijke granuloma-inducerende en -modulerende moleculen mogelijk wordt. Met behulp van een dergelijk model is eerder aangetoond dat SEA granulomen kan induceren, en dat dit afhankelijk is van glycosylering. Daarnaast is ook gebleken dat glycoconjugaten met LDN en *N*-acetyllactosamine een granulomateuze respons kunnen induceren die lijkt op de respons geïnduceerd door SEA. Omdat we in **hoofdstuk 4** lieten zien dat kappa-5 het belangrijkste LDN-bevattende glycoproteïne in SEA is, vermoedden wij dat kappa-5 wellicht een belangrijk granulomogeen molecuul in eieren is. We laten in **hoofdstuk 6** zien dat kappa-5 gekoppeld aan partikels inderdaad granulomen kan induceren in een long model in muizen. We laten verder zien dat deze granulomen eosinofielen bevatten, wat aangeeft dat ze, net als de granulomen rondom eieren en SEA, Th2 gepolariseerd zijn. LDN is deels verantwoordelijk voor het granulomogene effect, aangezien kappa-5 partikels waarvan LDN op enzymatische wijze verwijderd is, minder en kleinere granulomen induceren in vergelijking met onbehandelde kappa-5 partikels. **Hoofdstuk 6** beschrijft kappa-5 als het eerste, natuurlijke *S. mansoni* ei molecuul met glycaan-afhankelijke granulomogene eigenschappen.

Er bestaan verscheidene mechanismen waarop de interacties tussen glycoconjugaten van pathogenen en CLR op immuuncellen kunnen leiden tot immuunmodulatie, zoals de inductie van signaleringsroutes of de internalisatie van bioactieve moleculen. In **hoofdstuk 7** bespreken we, in het licht van de resultaten uit dit proefschrift, de CLR-afhankelijke mechanismen betrokken bij de immunologische effecten van omega-1, IPSE/α1, kappa-5 en gerelateerde pathogene moleculen. We beschrijven ook andere ei glycoconjugaten en glycaan motieven die mogelijk betrokken kunnen zijn bij de immunomodulatoire activiteiten van *S. mansoni* eieren.

Dankwoord

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Moniek

Curriculum vitae

Moniek Hubertina Joanna Meevissen was born on the 31th of May, 1983 in Heythuysen. After passing her VWO exams at the St Ursula College in Horn in 2001, she started her scientific carreer at the University of Utrecht, studying Biomedical Sciences. She proceeded with the Master "Infection and Immunity" at the same university. During this period she performed her first internship at the department of Dermatology and Allergology under the supervision of Dr. E.F. Knol and Dr. D.J. Hijnen. This intership focused on the role of the chemokines TARC and CTACK in the homing of T cells from the blood into the skin. Her second internship was part of a communication & education profile and took place at the Astmafonds in Leusden. Under supervision of N. Knaan and C. Alders, she developed, together with a fellow student, a decision aid for people with astma and/or COPD to help them assess whether or not they are a candidate for lungrevalidation. In 2006, she started her PhD project at the department of Parasitology of the LUMC under the supervision of Dr. C.H. Hokke, Prof. Dr. A.M. Deelder and Prof. Dr. M. Yazdanbakhsh. Her work on *Schistosoma mansoni* egg glycoproteins and their interactions with the human immune system has resulted in this thesis.

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MHJ Meevissen, M Wuhrer, MJ Doenhoff, G Schramm, H Haas, AM Deelder, CH Hokke. Structural characterization of glycans on omega-1, a major *Schistosoma mansoni* egg glycoprotein that drives Th2 responses. *J Proteome Res* 2010 May 7;9(5):2630-42.

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