Interactions of glycoconjugates from the parasitic trematode *Schistosoma mansoni* with C-type lectins

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Tag der mündlichen Prüfung:

„Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will."

Galileo Galilei (1564 – 1642)

Publications resulting from this work

Sandra Meyer, Boris Tefsen, Anne Imberty, Rudolf Geyer and Irma van Die (2007)
The C-type lectin L-SIGN differentially recognizes glycan antigens on egg glycosphingolipids and soluble egg glycoproteins from *Schistosoma mansoni.* Glycobiology, in press, doi:10.1093/glycob/cwm073:

CH. Grün, SJ. van Vliet, WECM. Schiphorst, CMC. Bank, S. Meyer, I. van Die and Y. van Kooyk (2006)
One-step biotinylation procedure for carbohydrates to study carbohydrate–protein interactions.
Analytical Biochemistry, 354, 1, 54-63

Meyer, S., van Liempt, E., Imberty, A., van Kooyk, Y., Geyer, H., Geyer, R., van Die I. (2005)
DC-SIGN mediates binding of dendritic cells to authentic pseudo-Lewis^{Y} glycolipids of *Schistosoma mansoni* cercariae, the first parasite-specific ligand of DC-SIGN. Journal of Biological Chemistry (JBC), 280, 37349-37359

Contributions to conferences (oral presentations)

Sandra Meyer, Ellis van Liempt, Anne Imberty, Yvette van Kooyk, Hildegard Geyer, Rudolf Geyer and Irma van Die.
Interaction of Lewis^{X} and pseudo-Lewis^{Y} glycolipids from *Schistosoma mansoni* with the C-type lectin DC-SIGN.
16th Joint Meeting of the „Studiengruppe Glykobiologie der Gesellschaft für Biochemie und Molekularbiologie“, the „Netherlands Society for Glycobiology“ and the „Groupe Lillois de Glycobiologie“ and the “Belgian Working Group for Glycosciences” 27.-29th October 2005, Hannover, Germany.

Sandra Meyer and Rudolf Geyer.
Characterization of glycoconjugates from *Schistosoma mansoni* interacting with DC-SIGN.
1st Course for Young Parasitologists of the “Deutschen Gesellschaft für Parasitologie”, 15.-17th March 2004, Würzburg, Germany.

Contributions to conferences (posters)

Meyer, S., van Die, I., Imberty, A., and Geyer, R.
The human C-type lectins DC-SIGN and L-SIGN differentially recognize glycolipids of the helminth *Schistosoma mansoni*
8th Jenner Glycobiology and medicine symposium of the Royal Society of Medicine, 21.-23rd October 2007, Dublin, Ireland.

Sandra Meyer, Boris Tefsen, Lynn Meurs, Caroline M.W. van Stijn, Yvette van Kooyk, Rudolf Geyer and Irma van Die.
The C-type lectin L-SIGN differentially recognizes glycan antigens on egg glycosphingolipids and soluble egg antigens from *Schistosoma mansoni*
17th Joint Meeting of the "Studiengruppe Glykobiologie der Gesellschaft für Biochemie und Molekularbiologie", the "Netherlands Society for Glycobiology" and the "Groupe Lillois de Glycobiologie" and the "Belgian Working Group for Glycosciences", 5.-7th November 2006, Brugge, Belgium.

Sandra Meyer, Ellis van Liempt, Anne Imberty, Yvette van Kooyk, Hildegard Geyer, Rudolf Geyer, and Irma van Die.
DC-SIGN mediates binding of dendritic cells to authentic pseudo-Lewis^{y} glycolipids of *Schistosoma mansoni* cercariae - the first parasite-specific ligand of DC-SIGN. Conference of the American Society for Glycobiology, 9.-12th November 2005, Boston, USA.

T. Lehr, S. Meyer, H. Geyer, M. Wuhrer, I. van Die, MJ Doenhoff and R. Geyer
Developmental expression of carbohydrate epitopes contributes to molecular mimicry during the life-cycle of *Schistosoma mansoni*.
Gordon Research Conference "Glycobiology", 6.-11th March 2005, Ventura, USA.

Meyer Sandra, van Liempt E, Geyer H, van Die I, Geyer R.
Interaction of glycolipids from *Schistosoma mansoni* cercariae with DC SIGN. 15th Joint Meeting of the "Studiengruppe Glykobiologie der Gesellschaft für Biochemie und Molekularbiologie", the "Netherlands Society for Glycobiology" and the "Groupe Lillois de Glycobiologie", 28.-30th November, 2004, Wageningen, The Netherlands. *Glycoconjugate J: 21, 410.*

List of Abbreviations

0-3 hRP	Products released by schistosome larvae 0-3 hours after transformation
Ag	Antigen
AKT	Protein kinase B
APCs	Antigen presenting cells
CAA	Circulating anodic antigen
CCA	Circulating cathodic antigen
CD	Cluster of differentiation
CLR	C-type lectin receptors
CRD	Carbohydrate recognition domain
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
dHex	Deoxyhexose
E	Amino acid glutamic acid
ELISA	Enzyme-linked immunosorbent assay
Endo H	Endoglycosidase H
ERK	Extracellular-signal regulated kinase
ES products	Excretory/secretory products
Fuc	L-Fucose
Gal	D-Galactose
GalNAc	2-*N*-Acetamido-2-deoxy-D-galactose (*N*-Acetylgalactosamin)
Glc	D-Glucose
GlcA	Glucuronic acid
GlcNAc	2-*N*-Acetamido-2-deoxy-D-glucose (*N*-Acetylglucosamin)
Hex	Hexose
HexNAc	*N*-Acetylhexosamin
HPLC	High-performance liquid chromatography
ICAM	Intercellular adhesion molecule
IL	Interleukin
INF	Interferon
JNK	C-Jun N-terminal kinase
KLH	Keyhole Limpet-Hemocyanine of *Megathura crenulata*
Le^A	Lewis A
Le^B	Lewis B
Le^X	Lewis X

Le^Y	Lewis Y
LN	LacNAc
LNFP III	Lacto-N-fucopentaose III
LPS	Lipopolysaccharide
L-SIGN	Liver/Lymph node-specific ICAM-3 grabbing nonintegrin
LSEC	Liver sinusoidal endothelial cells
Lyso-PS	Lyso-phosphatidyl-serine
mAb	Monoclonal antibody
MAP	Mitogen-activated protein
MHC	Major Histocompatibility Complex
MS	Mass spectrometry
NKT	Natural killer T-cells
PLCγ	Phospholipase C gamma
PAMPs	Pathogen-Associated molecule patterns
PBMCs	Peripheral-blood mononuclear cells
PRR	Pathogen-recognition receptor
SEA	Soluble egg antigens
TLR	Toll-like receptors
TNF α	Tumor-necrosis-factor alpha
Treg	Regulatory T-cells
Val	Valine
YKSL	Amino acids tyrosine, lysine, serine and leucine

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Outline of this thesis

The experiments described in this thesis have been performed in order to gain more information about the recognition of *Schistosoma mansoni* glycans of glycosphingolipids or glycoproteins by the C-type lectins DC-SIGN and L-SIGN.

In **chapter 1** an introduction in the topic concerning schistosomes, their manifold glycoconjugates, expressed as glycoproteins and glycosphingolipids, as well as the immunobiology of schistosome infection is presented. Moreover, the C-type lectins DC-SIGN and L-SIGN are introduced and the multiple roles of carbohydrates during the immune response to schistosome infection are addressed.

In **chapter 2** the original publications resulting from this work are displayed. In the first publication (Meyer *et al*. JBC 2005) we demonstrated that besides the Lewis X epitope also the pseudo-Lewis Y motif, both of which are expressed on cercarial glycosphingolipids of schistosomes, are ligands of DC-SIGN. The pseudo-Lewis Y moiety is the first parasite-specific ligand described for this lectin.

In the second publication, a quick method for biotinylation of oligosaccharides is presented allowing a fast analysis of carbohydrate-lectin interactions.

In the third publication (Meyer *et al.* Glycobiology 2007) we demonstrated that the C-type lectin L-SIGN has a broad ligand specificity recognizing high-mannose structures on SEA as well as fucosylated glycolipids of schistosome eggs carrying the F-LDN-F epitope.

In **chapter 3** the results presented in this thesis are integrated in a general discussion including a future outlook concerning this topic of research.

Finally, in **chapter 4 and 5** a summary of this work is given in English and German.

1 Introduction and literature overview

1.1 The parasite *Schistosoma mansoni*

1.1.1 Zoological classification of schistosomes

Within the helminths, i.e. parasitic metazoans classified in the clades plathelminthes, nematodes and acanthocephala, schistosomes belong to the first named clade and to the class of trematodes. They are summarized to the family of schistosomatidae which consists of 12 genuses, all of which live parasitically in the blood vessels of vertebrates (121). Humans are mainly infected by *S. mansoni*, *S. haematobium, S. japonicum, S. mekongi* and *S. intercalatum*.

1.1.2 Life cycle of *Schistosoma mansoni*

The life cycle of schistosomes alternates between an asexual phase in the invertebrate host, a freshwater snail, and a sexual generation in the vertebrate host. Due to these switches between hosts and the way of reproduction, the life cycle of schistosomes belongs to the most complex ones in the whole animality. An overview of the life cycle of *S. mansoni* is depicted in Figure 1. A schistosome egg hatches in fresh water, releasing a motile larva, the miracidium. Within eight to twelve hours the miracidium has to find its intermediate host, in the case of *S. mansoni* a freshwater snail of the genus *Biomphalaria*. After invasion of the snail the miracidium develops into the mother sporocyst and, via the open circulation system, daughter sporocysts reach the mid-gut-gland and continue the further development resulting in the release of cercariae. Four to six weeks after snail infection, the cercariae hatch in response to light attraction. These human pathogenic larvae, being equipped with a bifurcated tail, actively search for their definitive host, penetrate his skin, release their tails and transform to schistosomula. This transformation is accompanied by profound changes in their metabolism and antigenic structures at their surface (59; 60; 86). Schistosomula dwell in the human skin for two to three days and migrate afterwards in the blood vessels und reach the lungs. After several days they continue their way via the blood stream to the mesenterial veins and into the liver. After maturation to adult worms, male and female worms pair and migrate against the blood stream into the intestinal venules of the portal drainage, where they permanently settle using their oral sucker. Four to five weeks post infection female worms start with egg production (135). Schistosomal couples produce up to three

hundreds of eggs per day and their intended fate is to pass from their intravascular site of deposition to the intestinal lumen, from where they can leave the body. The eggs reaching fresh water release miracidia, which can find and invade the aquatic snail host (Figure 1). An alternative dead-end street for the eggs is that they are caught by the bloodstream and brought to distal tissue sites in which they become trapped. The tissue in which half or even more of the eggs of *S. mansoni* are captured is the liver, as the sinusoids of which are of insufficient diameter to allow the eggs to pass. Eggs lodged in the liver, as well as in the intestine, induce strong granuloma formation (114).

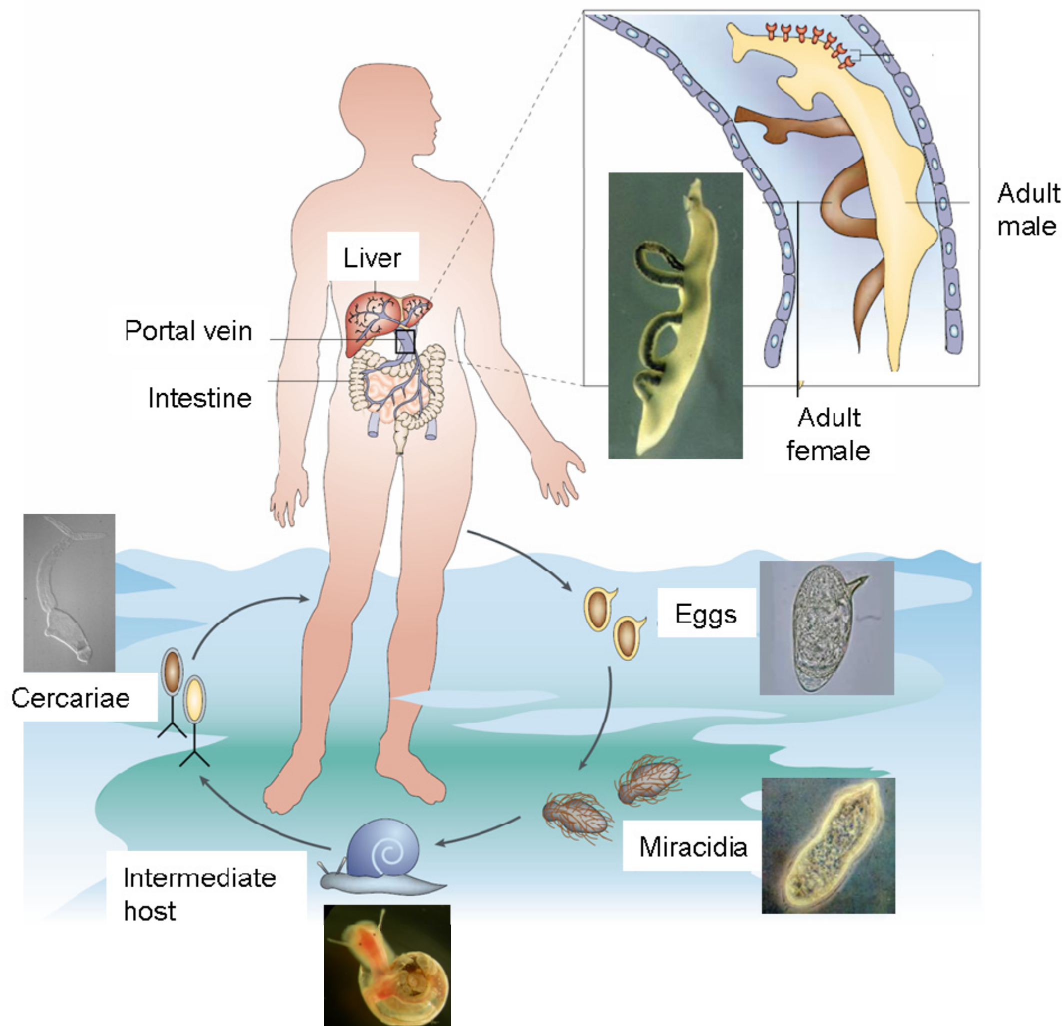


Figure 1: The life cycle of *Schistosoma mansoni* (modified after (35)).

Infection of humans is initiated by cercariae which penetrate the skin, transform into schistosomula, enter the vasculature and migrate to the portal system, where they mature to adult worms. The eggs produced are released within the vasculature, cross the endothelium of the veins and transverse to the epithelium of the intestine. After the eggs have reached fresh water, free swimming larvae, called miracidia hatch, which actively seek their intermediate host Biomphalaria glabrata. Asexual reproduction steps lead to production of infective cercariae.

1.2 The disease Schistosomiasis

Many publications on schistosome research begin with the phrase "Schistosomiasis is a parasitic disease affecting over 200 million people in 74 countries of the developing world". Perhaps the frequent use of this quotation has made us thoughtless about the fact, that among human parasitic diseases, schistosomiasis ranks second behind malaria in terms of socio-economic and public health importance in tropical and subtropical areas. Of the 200 million people infected, 20 million suffer from severe consequences of the disease. An estimated number of 500 to 600 million people worldwide are at risk to get infected. Nowadays, the treatment of schistosomiasis depends primarily on chemotherapy using praziquantel ((RS)-2-(Cyclohexylcarbonyl)-2,3,4,6,7,11b-hexahydro-1H-pyrazino[2,1] isochino-lin-4-on), an isoquinoline-pyrazine derivative with a wide antiparasitic effect. The exact mechanism of action of praziquantel is still not known, but it leads to an opening of calcium channels at the surface of the adult worm, resulting in a paralysis of the parasites. Unfortunately, there is increasing evidence for an emerging drug resistance. Due to rather unspecific symptoms, the diagnosis of schistosomiasis is still not easy. Therefore, direct and indirect methods have to be employed. One easy way is the identification of species-specific parasitic eggs in the stool. A further direct diagnostic tool is the detection of circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) (see paragraph 1.3 for details) (1; 6; 29-31). Indirect methods to diagnose schistosomiasis are, e.g., the detection of hemoglobin in the urine (26) or the verification of schistosome-specific antibodies in the serum (169).

The main pathology of a schistosome infection resides in a pronounced hepatosplenomegaly (enlargement of spleen and liver), caused by schistosome eggs. The female worms deposit several hundreds of eggs per day for up to 30 years (50). As outlined above, about up to two third of the eggs fail to adhere to the endothelium and become trapped in the hepatic capillary bed. Embolized eggs die within 20 days, initiate a local immunpathological reaction and lead to granuloma formation. The dieing eggs secrete large amounts of proteins, glycoproteins and glycolipids, which are highly antigenic and stimulate the immune response. Before reviewing the immunology of schistosomes in more detail (see paragraph 1.4), the major glycoconjugates of schistosomes will be described.

1.3 Glycoconjugates of schistosomes

Schistosome glycoconjugates play an eminent role in the parasite's biology, in particular, in the interaction with and the survival within the host. It has been shown that carbohydrate determinants are major targets of the immune response (38). Furthermore, surface-expressed glycans provide the basis for direct diagnostic methods (29). An enormous amount of structural data regarding the glycosylation of different schistosomal life stages has been collected in the last decades. It became evident that the parasites express a great variety of carbohydrate structures, including both host-like structural elements and highly antigenic, parasite-specific determinants.

1.3.1 N-glycans of *S. mansoni*

Schistosomal glycoproteins contain complex glycan moieties linked via N- or O-glycosidic linkage to the polypeptide backbone (32; 166). The major structural features of these glycoprotein-glycans can be summarized as follows: Glycoproteins of *S. mansoni* **adult** worms contain typical N-linked oligomannose structures also occurring in many eukaryotes (111). In addition, diantennary complex-type N-glycans from adult worms carry terminal GalNAcβ1-4GlcNAc (LDN) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) elements (112; 129). Moreover, adult worm glycoproteins may carry repeating units of Galβ1-4(Fucα1-3)GlcNAc (Lewis X, Le^X) on multi-antennary N-glycans. Finally, a large proportion of N-glycans of *S. mansoni* adult worms has been shown to represent non-fucosylated, or core-(α1-6)-fucosylated paucimannosidic structures (79; 110). In particular complex-type glycans have been ascribed a vast structural variety (77). Complex-type species may contain two LDN antennae, both of which can be fucosylated (129). Alternatively, N-glycans may consist of 2-4 *N*-acetyllactosamine antennae with additional oligomeric Lewis X epitopes (128).

Glycoproteins from *S. mansoni* **eggs** contain high-mannose type glycans similar to those from adult worms and complex type glycans with Galβ1-4GlcNAc (LN) as well as LDN elements. Egg glycoproteins further express truncated N-glycan core structures carrying β2-linked xylose and α3-linked fucose residues, which are also described for other helminths, insects and even plants (62; 130). In addition, terminal $(HexNAc)_3$ epitopes were found in the egg N-glycans with oligofucosyl-units attached to the terminal HexNAc residue (78).

While the glycosylation of soluble egg antigens (SEA) is thought to play an important role in schistosome immunology, data regarding the precise structures of the respective glycoprotein-glycans expressed are still incomplete (43; 166). Until now three glycoproteins were purified from SEA, named α-1, Ω-1 and κ-5 (34; 36). Ω-1 is a secreted, cytotoxic ribonuclease (46), whereas α-1 is identical to a highly immunogenic, secreted egg glycoprotein termed IPSE (=interleukin-4-inducing principle from *S. mansoni* eggs) (122). IPSE is a general activator of human basophiles triggering the release of IL-4, IL-13 and histamine. Monomeric IPSE carries core-difucosylated, diantennary N-glycans with one or more Lewis X motifs (161).

N-glycans of *S. mansoni* **cercariae** are mainly hybrid-type or diantennary structures with Lewis X trisaccharide units in terminal positions (166). They are core-(α1-6)-fucosylated throughout and carry often a (β1-2)-core bound xylose (79).

An overview of typical carbohydrate epitopes present on N-glycans of *S. mansoni* glycoproteins of eggs, cercariae and adult worms is given in Figure 2.

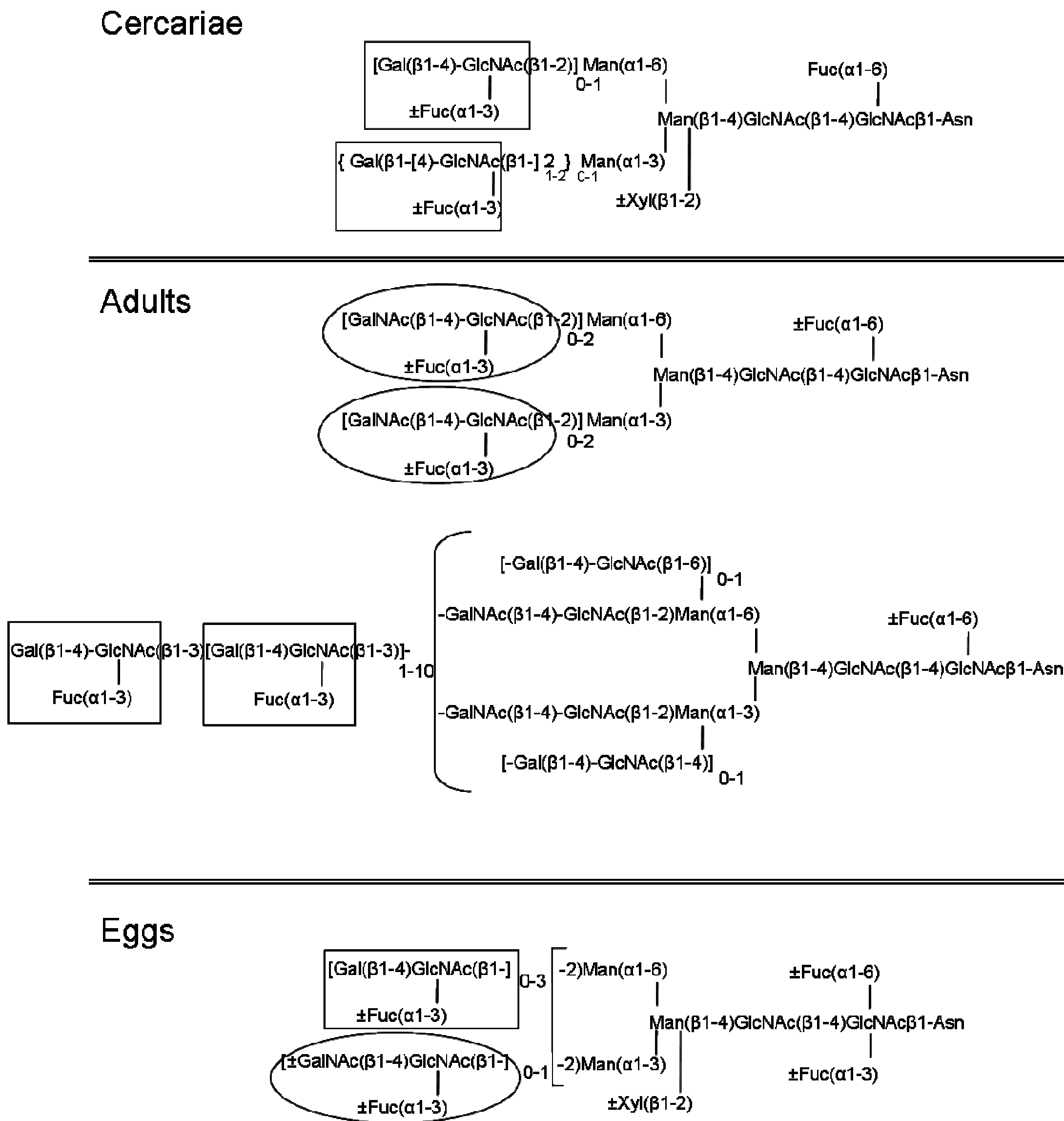


Figure 2. Glycoprotein-N-glycans of *S. mansoni* cercariae, adults and eggs.

In the egg stage the pentasaccharid-core can be up to twofold fucosylated and can be modified with β1-2-linked Xylose. Terminal Lewis X and LDN-F units are boxed and circled, respectively.

1.3.2 O-glycans of *S. mansoni*

Schistosome O-glycans range from single O-linked GlcNAc residues or short Galβ1-3GalNAcα mucin-type disaccharides on glycoproteins from schistosomula or adult worms (111) to complex multiply fucosylated glycans from the highly immunogenic **cercarial** glycocalyx (80). These complex O-glycans contain specific epitopes of repeating GalNAcß1-4GlcNAcß1-3Galα1-3 units carrying oligofucosyl chains $(Fuc\alpha1\text{-}2)_{0/1}$Fucα1-2Fucα1-3 motifs linked to the internal GlcNAc, as well as terminal $(Fuc\alpha1\text{-}2)_{0\text{-}1}$Fucα1-3GalNAc structures (Figure 3). These multimeric glycans have been shown to be based on conventional type 1 and 2 core structures (66; 80). A second group of cercarial O-glycans is based on a novel core-structure comprising an O-linked GalNAc with β-linked Gal residues in both 3- and 6-positions (67). Studies on O-glycosylation in **adult** worms revealed the presence of O-linked Tn-antigens (GalNAcα1-Ser/Thr), T-antigen (Galβ1-3GalNAcα1-Ser/Thr), and O-linked GlcNAc (23; 109). So far, only a few publications describe carbohydrate structures of purified glycoproteins from adult worms. Well-characterized examples are the two major gut-associated excreted antigens CCA (circulating cathodic antigen) and CAA (circulating anodic antigen) (12; 140). CCA carries mainly long, linear multimers of Lewis X trisaccharides and the highly negatively charged O-glycans of CAA comprise a polymeric (β1-6)-linked GalNAc backbone which is substituted by (β1-3)-linked glucuronic acid (GlcA) residues (140). The structures of *S. mansoni* O-glycans from CAA and CCA are given in Figure 3.

An overview of characteristic glycan epitopes of schistosome glycoproteins and their stage-specific occurrence is listed in Table 1.

O-glycans of CAA (adults)

[GalNAc(β1-6)] – core glycan –Ser/Thr
~30
GlcA(β1-3)

O-glycans of CCA (adults)

[Gal(β1-4)GlcNAc(β1-3)]–
Fuc(α1-3)
~25
[– Gal(β1-4)GlcNAc(β1-6)]
0-1
GalNAc –Ser/Thr
– Gal(β1-3)

O-glycans of cercariae

Gal(β1-4)GlcNAc(β1-3)Gal(β1-6)
±Fuc(α1-3)
GalNAc-Ser/Thr
±Gal(β1-6)
Gal(β1-4)GlcNAc(β1-3)Gal(β1-3)
±Fuc(α1-3)

±Fuc(α1-2)Fuc(α1-[3)GalNAc(β1-4)GlcNAc(β1-3)Gal(α1-]3)GalNAc(β1-4)GlcNAc(β1-6)
n
±Fuc(α1-2)Fuc(α1-2)Fuc(α1-3) ±Fuc(α1-2)Fuc(α1-3) GalNAc-Ser/Thr

±Fuc(α1-2)Fuc(α1-[3)GalNAc(β1-4)GlcNAc(β1-3)Gal(α1-]3)GalNAc(β1-4)GlcNAc(β1-3)Gal(β1-3)
m
±Fuc(α1-2)Fuc(α1-2)Fuc(α1-3) ±Fuc(α1-2)Fuc(α1-3) n + m = 0,1,2,3 oder 4

Figure 3. O-glycans of CAA and CCA from *S. mansoni* adult worms and O-glycans of cercariae.

O-glycans of CAA express poly-Lewis X motifs and the O-glycans of CAA carry (β1-3)-bound glucuronic acid (GlcA). Present Lewis X motifs are boxed.

Table 1. Common glycan antigens of *S. mansoni* and their distribution in the different life-cycle stages cercariae (C), adults (A) and eggs (E). Glycan epitopes occurring also in humans (H) are marked for comparison. Structure plots were generated in the notation of the Consortium for Functional Glycomics (http://www.functionalglycomics.org) using the visual editor of "GlycoWorkbench". This software application is developed and available as part of the EUROCarbDB project (http://www.eurocarbdb.org/applications/ms-tools). Symbols used are: light square, GalNAc; dark square, GlcNAc; triangle, Fuc; light circle, Gal.

Carbohydrate epitope	Structure	Structure plot	C	A	E	H
LacdiNAc (LDN)	GalNAcβ1-4GlcNAc		+	+	+	+
Terminally fucosylated LDN (F-LDN)	Fucα1-3GalNAcβ1-4GlcNAc		+	+	+	-
Fucosylated LDN (LDN-F)	GalNAcβ1-4(Fucα1-3)GlcNAc		+	+	+	+
Fucosylated LDN-F (F-LDN-F)	Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc		+	+	+	-
Difucosylated LDN (LDN-DF)	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc		+		+	-
Fucosylated LDN-DF (F-LDN-DF)	Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc		+	+	+	-
Lewis ^{X} (Le^{X})	Galβ1-4(Fucα1-3)GlcNAc		+	+	+	+
Pseudo-Lewis ^{Y} (pseudo-Le^{Y})	Fucα1-3Galβ1-4(Fucα1-3)GlcNAc		+	-	-	-

1.3.3 Glycosphingolipids of *S. mansoni*

While mammalian glycosphingolipids are based on lactosylceramide (Galβ1-4Glcβ1-1-ceramide) schistosomal glycolipids carry the so-called "schisto-core" (GalNAcβ1-4Glc-ceramide) (Figure 4) (98; 165).

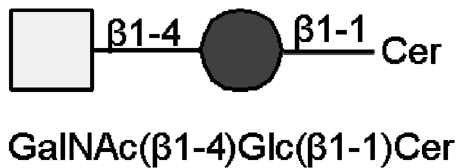


Figure 4. The "schisto-core".

Graphic representation of sugar moieties with symbols as in Table 1.

Besides simple glucosyl- and galactosylceramides, this schisto-specific glycosphingolipid seems to be expressed in all life-cycle stages, nevertheless exhibiting strong variations in the ceramide part (164). Elongation of this schisto-core leads to very complex glycans including either structural elements occurring also in N- or O-glycans, or specific modifications which are found so far only on glycolipids. In the context of these more complex *S. mansoni* glycolipids, epitope-typing, using mAb and infection sera, revealed a marked antigenicity and a stage-specific expression of individual classes of glycolipid species (160). A summary of *S. mansoni* glycolipids and their occurrence in different life-cycle stages is depicted in Figure 5.

Glycolipid	Ad	Ce	Egg
Gal(β1-1)Cer	Ad	Ce	Egg
Glc(β1-1)Cer	Ad	Ce	Egg
GalNAc(β1-4)Glc(β1-1)Cer	Ad	Ce	Egg
GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer		Ce	Egg
Gal(β1-4)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer		Ce	
Gal(β1-4)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with Fuc(α1-3) on GlcNAc		Ce	Egg
Gal(β1-4)GlcNAc(β1-3)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with Fuc(α1-3) on GlcNAc		Ce	
Gal(β1-4)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with Fuc(α1-3) on Gal and Fuc(α1-3) on GlcNAc		Ce	
GalNAc(β1-4)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with Fuc(α1-3) on GalNAc and ±Fuc(α1-3) on GlcNAc			Egg
GalNAc(β1-4)GlcNAc(β1-3)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with ±Fuc(α1-3) on GalNAc, Fuc(α1-3) ±Fuc(α1-2) ±Fuc(α1-2) on GlcNAc			Egg
GalNAc(β1-[4)GlcNAc(β1-]1-4 4)GlcNAc(β1-3)GalNAc(β1-4)Glc(β1-1)Cer with Fuc(α1-3) ±Fuc(α1-2) on GalNAc, Fuc(α1-3) Fuc(α1-2) on repeating GlcNAc, ±Fuc(α1-3) ±Fuc(α1-2) on GlcNAc			Egg

Figure 5. Summary of *S. mansoni* glycolipids and their reported stage-specific occurrence (modified after (166)).

The three fucose-containing carbohydrate-motifs Lewis X (box), Pseudo-Lewis Y (dotted box) and M2D3H (dashed box) are indicated. The occurrence in each life-cycle stage is listed. Ad, adult worms; Ce, cercariae; Egg, eggs.

Cercarial glycolipids were shown to be based on the schisto-core GalNAcβ1-4Glc-ceramide and to carry mainly terminal Lewis X trisaccharide units (165). Some of these glycolipids carry an additional fucose residue which is α1-3-linked to galactose thereby forming the so-called "pseudo-Lewis Y" motif Fucα1-3Galβ1-4[Fucα3]GlcNAcβ- (165). The pseudo-Lewis Y motif has been, so far, neither detected in the adult nor in egg stages (165). Both glycan motifs are presented in Figure 6. In addition to the aforementioned glycolipids, cercariae express also glycolipids species that are highly antigenic and share antigenic motifs with the large glycolipids of the egg stage (160; 162). The role of Lewis X for the immune response to and the survival of the parasite is described in paragraph 1.3.4.

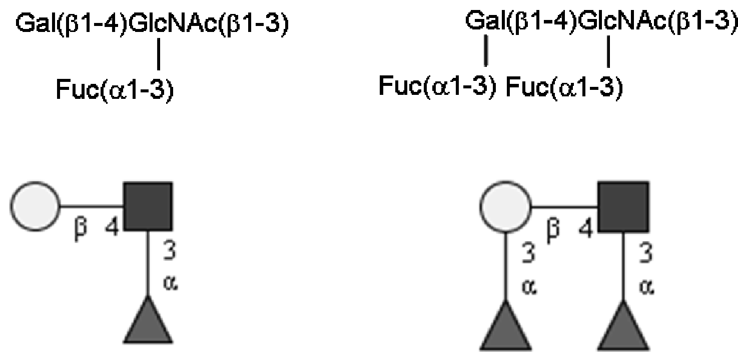


Figure 6. The Lewis X and pseudo-Lewis Y motifs.

Graphic representation of sugar moieties as in Table 1.

Size, complexity and antigenicity are increased in the **egg** glycolipids, being characterized by a backbone of *N*-acetylhexosamine residues decorated with oligofucosyl side chains, like Fucα1-3GalNAcβ1-4[±Fucα1-2±Fucα1-2Fucα1-3]GlcNAcβ-. Analyses of egg glycosphingolipids demonstrated the preponderant presence of the repeating unit -4(Fucα1-2Fucα1-3)GlcNAcβ1- terminating in the case of *S. mansoni* in (Fucα1-2)$_{0/1}$Fucα1-3GalNAcβ1- at the non-reducing end. In this context a panel of distinct carbohydrate epitopes can be defined: GalNAcβ1-4GlcNAc- (LDN), Fucα1-3GalNAcβ1-4GlcNAc (F-LDN), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) (cf. Table 1). The major antigenic determinant found in egg glycosphingolipids was Fucα1-3GalNAcβ- as recognized by the monoclonal antibody (mAb) M2D3H (167). *S. mansoni* egg glycolipids exhibit strong biological activities as they were shown to induce cytokine production, thus leading to elevated interleukin-10 (IL-10), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) levels (146).

Stage-specific expression pattern have not only been described for the glycan elements, but also for the ceramide part of schistosome mono- or dihexosides (164). In addition, the structural basis for the serological cross-reactivity between schistosome infection sera, schistosome glycolipid glycans and keyhole limpet hemocyanine (KLH) can be explained in terms of the N-linked glycan epitope Fucα1-3GalNAcβ1-4[Fucα1-3]GlcNAcβ-, i.e., F-LDN-F (55; 56). This cross-reactivity might be of biological relevance, as hemolymph glycoproteins of the *S. mansoni* intermediate host, *Biomphalaria glabrata*, also show cross-reactivity with

parasite glycoproteins and glycolipids (33; 89; 163). This sharing of carbohydrate motifs between parasite and host could be interpreted as an attempt of the parasite to camouflage itself and fits into the concept of "molecular mimicry" (25).

1.3.4 The Lewis X motif in host-parasite relationship

A number of recent studies have indicated a major role for Lewis X-containing glycoconjugates in the interaction of host and parasite. In this context, the most striking feature of this carbohydrate epitope is its shared occurrence in both the parasite and its host, as Lewis X is expressed in a wide range of human tissue (47) including glycolipids and glycoproteins of human granulocytes (49; 127; 132). Secondly, the temporal and spatial expression of Lewis X in schistosomes is of high interest. It has been observed that the cercariae express Lewis X only around gland openings (153), whereas this expression pattern changes completely when the parasite enters the mammalian host. During the transformation from cercariae to schistosomula the highly antigenic glycocalyx is stripped off, the tail is lost, the Lewis X epitope can be now multiply detected and on the entire, newly-formed surface of the schistosomulum by monoclonal antibodies (83; 85; 153). After developing to adult worms Lewis X remains to be constantly exposed at the site of parasite/host interaction, but is weakly expressed in the parasitic tegument. On the other hand, Lewis X-containing gut antigens like CCA are constantly secreted. Likewise, the shell of schistosomal eggs is decorated with Lewis X motifs, thus verifying again the continuous expression of Lewis X by the parasite (153). It could be further demonstrated that the Lewis X epitope is a target of the host´s immune response in schistosomiasis causing autoimmunity (107; 108). These auto-anti-Lewis X antibodies have been shown to induce complement-dependent cytolysis of Lewis X-presenting host cells (108; 141). Furthermore, glycoconjugates carrying Lewis X moieties have an immunomodulatory effect as could be demonstrated by the induction of secretion of interleukin-10 *in vitro* (155; 156). Finally, which is a main topic of this thesis, parasitic Lewis X units can also target the innate immune system through binding to the C-type lectins, such as DC-SIGN, and might thereby be involved in the early cellular immune reaction to schistosomula.

1.4 Immune response to schistosomes

During infection with schistosomes and most other helminths, the dominant immune response can be classified as Th2-like (95). Simplistically, Th responses can be considered to diverse into those dominated by IFN-γ producing Th1 cells, or IL-4/IL-5/IL-13-producing Th2 cells. Two main clinical conditions are distinguished during schistosome infection: acute and chronic schistosomiasis.

1.4.1 Acute schistosomiasis- a Th1 response

During acute illness, i.e., during the first 6 to 8 weeks post infection before eggs are deposited, often unspecific syndromes like fever occur ("Katayama syndrome"). When the parasite enters the host, prostaglandin D_2 (PGD_2), produced by the skin stage of *S. mansoni*, inhibits the migration of Langerhans cells to the draining lymph nodes, thereby delaying the immune response (4). These first weeks of infection represent a classical T helper 1 (Th1) type of response which is characterized by an increase of measurable levels of tumor-necrosis factor (TNF) in the plasma. In addition, peripheral-blood mononuclear cells (PBMCs) produce large amounts of IL-1 and IL-6 (27). The early phase of schistosome infection is less well investigated, but several mechanisms of immune regulation during the migration of schistosome have been described so far and are summarized in Figure 7.

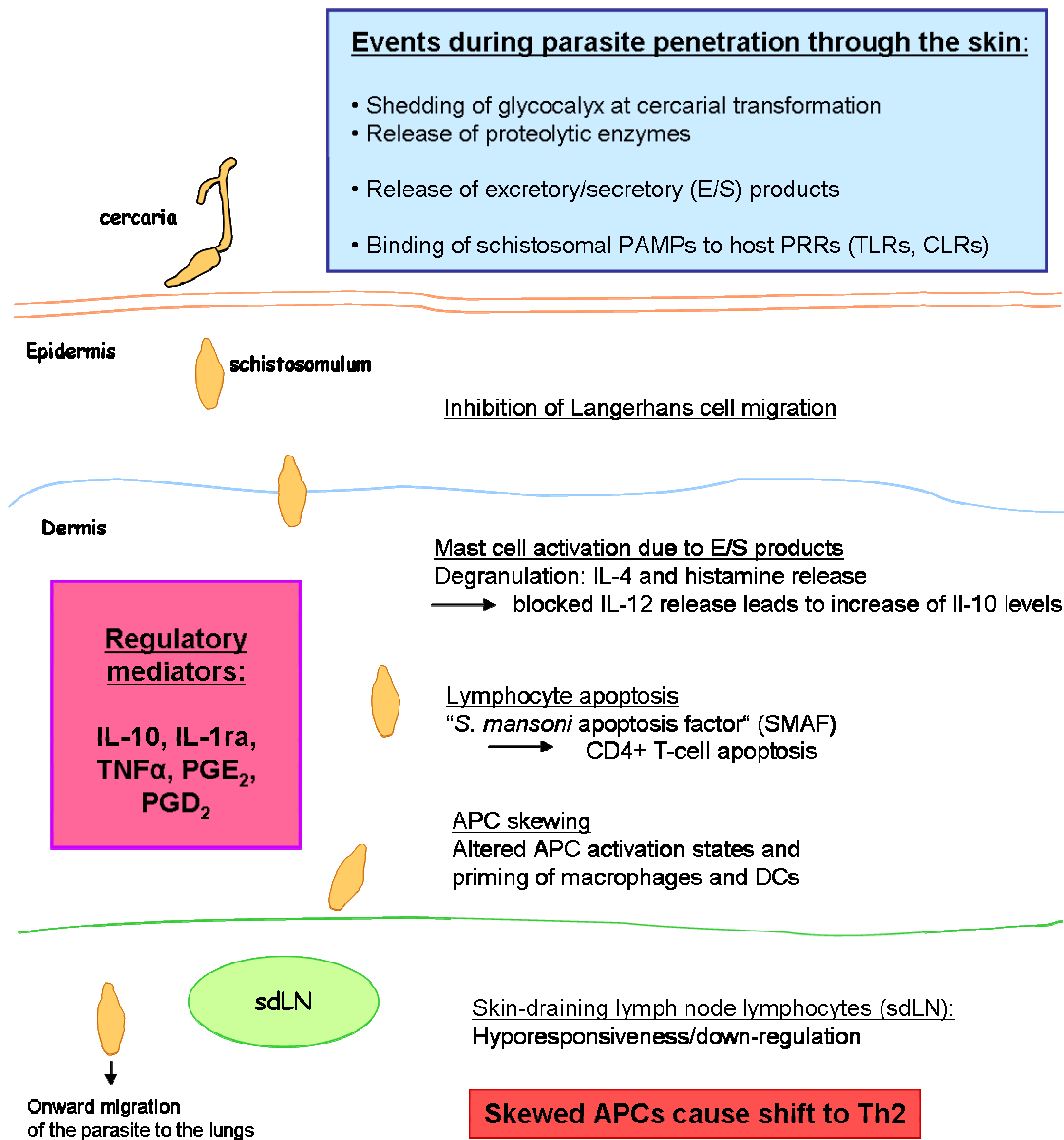


Figure 7. Schematic diagram showing the possible mechanisms of immune regulation during the acute phase of schistosome infection whilst the parasite migrates through the human skin (modified after (70)).

The abbreviations used are: APC, antigen-presenting cell; CLRs, C-type lectin receptors; DCs, dendritic cells; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; PAMPs, pathogen-associated molecular patterns; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PRRs, pattern recognition receptors; sdLN, skin-draining lymph node lymphocyte; SMAF, *S. mansoni* apoptosis factor; TLRs, Toll-like receptors; TNFα, tumor necrosis factor alpha.

1.4.2 Chronic schistosomiasis - a Th2 response

During infection with schistosome species, a chronic disease is the result of the ongoing host response to accumulating eggs trapped in the tissue. In the progression of the disease the egg-driven, developing Th2 response down regulates the production of the aforementioned pro-inflammatory cytokines. The switch from a Th1 to a Th2 response at the point of egg deposition is illustrated in Figure 8.

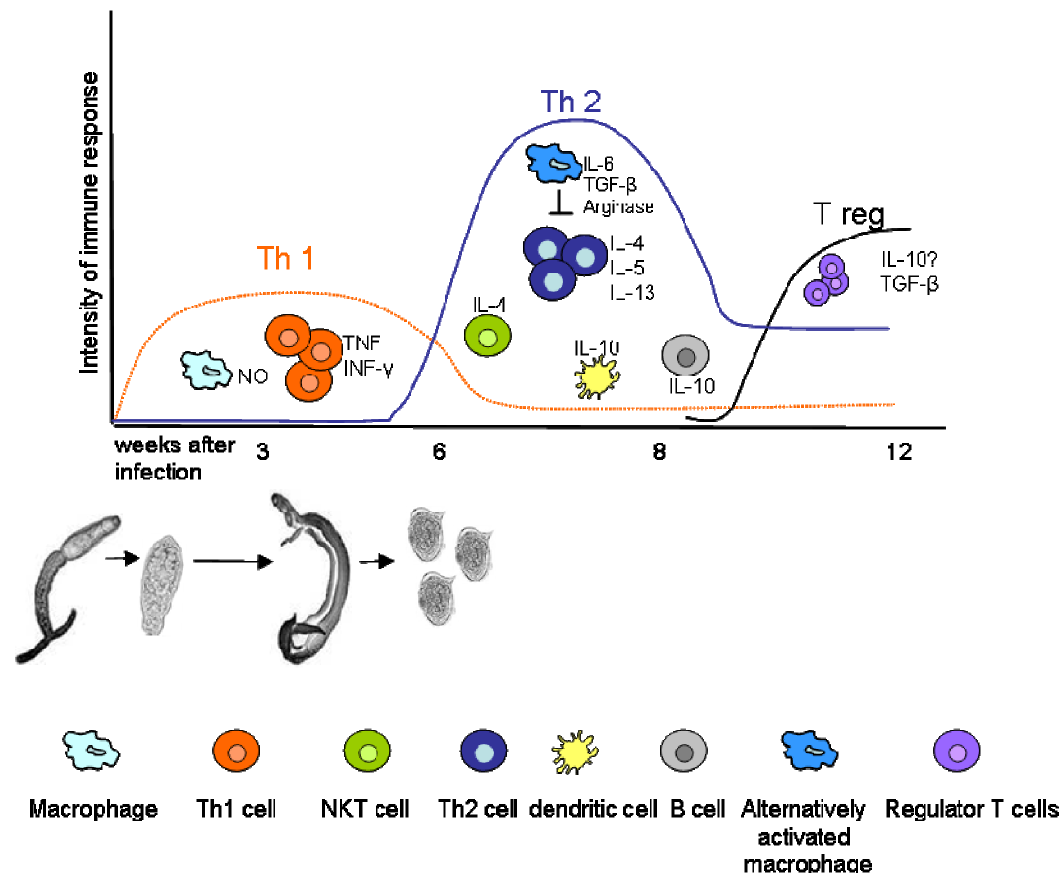


Figure 8. Schistosome eggs induce a switch in the immune response from Th1 to Th2 (modified after (35)).
The intensity of immune response during the time post infection is depicted for Th1 cells (orange line), Th2 cells (blue line) and regulatory T-cells (T reg, black line). After infection with schistosomes the first immune response is a Th1 cell response. As the worms develop and egg production starts, natural killer (NKT) cells are activated, dendritic cells produce more IL-10 and less IL-12, and a Th2 cell response develops. In addition, B cells produce IL-10 in response to egg-derived antigens. Furthermore, populations of alternatively activated macrophages and regulatory T-cells are formed. IFN-γ interferon-γ; NO nitric oxide; TGF-β transforming growth factor beta; TNF tumor necrosis factor.

In *S. mansoni* infection the liver is the mainly affected organ. As the sinusoids of the liver are too small for the eggs, they get stuck and die in the liver tissue. The $CD4^+$ T-cell response that is induced by the trapped eggs orchestrates the development of a strong granuloma formation, composed of collagen fibers, macrophages and

eosinophiles, thus causing severe hepatosplenomegaly and increased liver portal blood pressure, as the liver becomes increasingly fibrotic. The main Th2 cytokine responsible for liver fibrosis is IL-13 (21). The fibrotic role of IL-13, together with IL-4, seems to be based on its ability to induce the expression of arginase in macrophages (64). Mediators that are associated with a Th1 response, like IFN-γ, IL-12, TNF or NO can prevent the IL-13 mediated fibrosis in mice (64). But IL-13 itself is obviously not necessary for the Th2 development *per se* (41).

S. mansoni egg-induced granuloma formation and the composition of a granuloma in the liver are outlined in Figure 9.

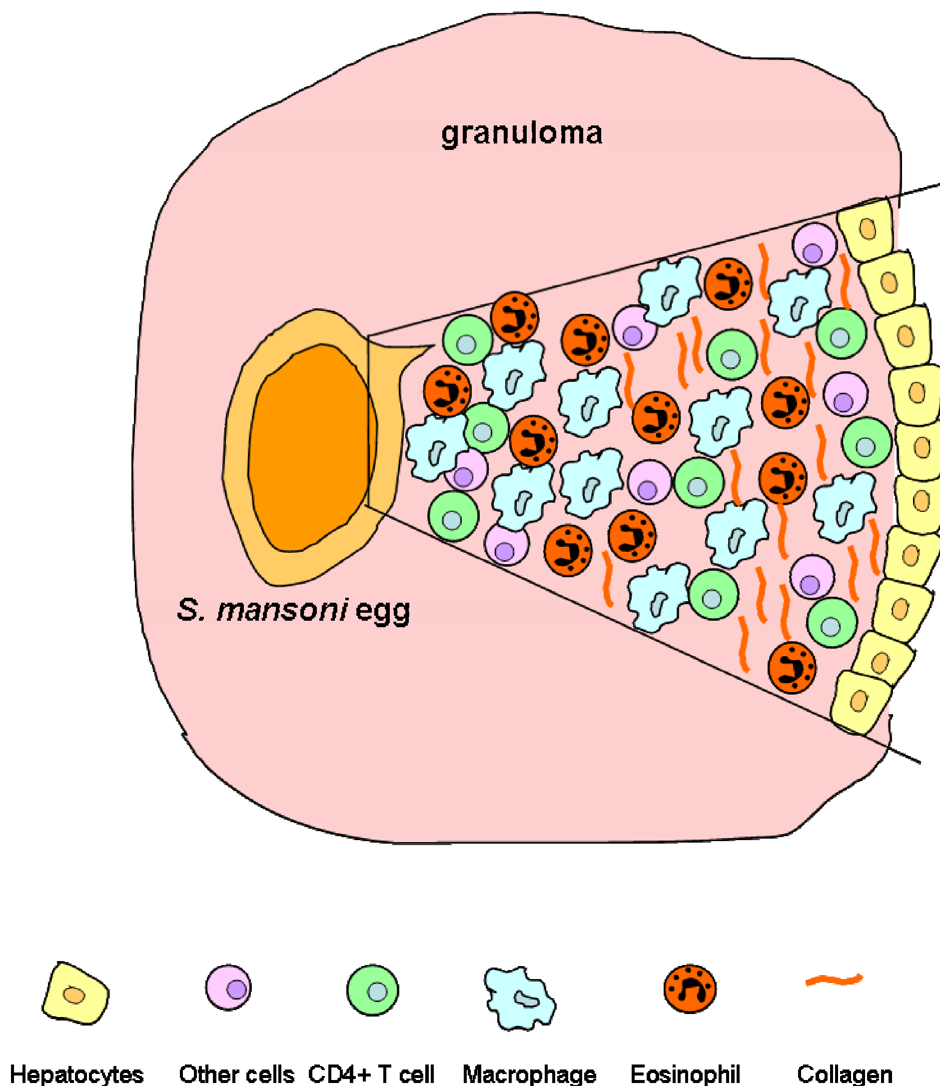


Figure 9. Formation of liver granuloma due to dieing parasitic eggs **(modified after (117)).**

The CD4+ T-cell response that is induced by trapped eggs, mainly in the liver, leads to the development of granulomatous lesions. Granulomes are composed of collagen fibers and different cell types, including macrophages, eosinophiles and CD4+ T-cells.

Several cytokines other than IL-4 have been implicated in the Th2 development, but have been found to be of minimal importance for the expression of this type of immune response (117). IL-6, for example, does not play a main role during Th2 phase, although it might be involved at some level in the regulation of IFN-γ and IL-12 production (87).

The function of B cells during schistosomiasis is still under investigation. One study showed that effector B cells can induce a Th2 response through the production of polarizing cytokines (61). The finding that CD40-CD154 interactions are important for the development of a Th2 type of response during schistosome infection also implicates that B cells might be involved in this process, since B cell responses are markedly impaired in the absence of CD40 signaling (100; 149). In addition, it has been demonstrated that $CD40^{-/-}$ DCs are incapable of inducing egg-antigen-specific Th2 responses (97).

Several studies underline that it is the egg stage of the schistosome that is responsible for the induction of a Th2 response (57; 116). In contrast, the worms themselves seem to be poor inducers of a Th2 answer. The Th2 phase is characterized by increased numbers of eosinophiles, basophiles and mast cells as well as high levels of circulating IgE. Hence, schistosomes, as well as, other parasitic helminths inducing Th2 responses, may be used as model organisms for studying the development and function of this type of immune response.

The induction of a Th2 response by schistosomes is summarized in Figure 10.

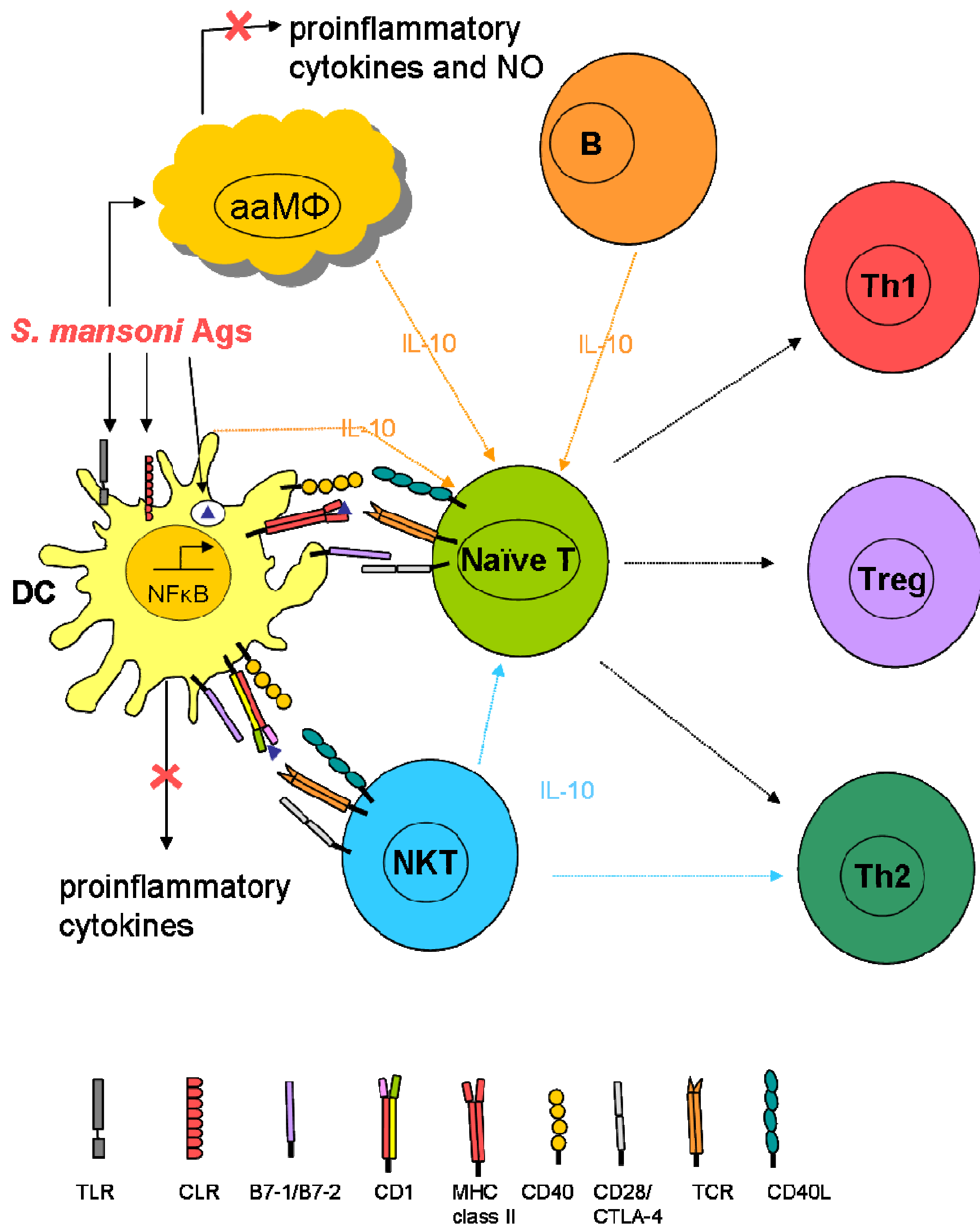


Figure 10. Induction of a Th2 response (modified after (117)).

S. mansoni **alive helminths and excreted/secreted antigens modify cells of the innate immune system by interaction with TLRs and CLRs to stop the production of inflammatory mediators and to elicit the output of immunoregulatory cytokines like IL-10. This results in the generation of suppressive regulatory T-cells (Treg) and a shift towards a Th2 response. Abbreviations used are: aaMΦ, alternatively activated macrophage; B7-1/B7-2, protein on the surface of antigen-presenting cells (2nd signal); CD, cluster of differentiation; CLR, C-type lectin receptor; CD40L, CD40 ligand; CTLA-4, cytotoxic T-lymphocyte antigen 4; DC, dendritic cell; MHC, major histocompatibility complex; NKT, natural killer cell; NFκB, nuclear factor kappa B; NO, nitric oxide;** ***S. mansoni*** **Ags,** ***S. mansoni*** **antigens; TCR, T-cell receptor; TLR, Toll-like receptor.**

1.5 Immunevasion of the pathogen

At least three early findings arouse the interest of immunologists in schistosomiasis: the immune response is clearly involved in the development of many of the pathological changes accompanying infection, the patients can exhibit resistance to superinfection, and schistosomes may survive for years in the host despite a strong immune response (117). To this end parasites have evolved an intricate series of adaptations enabling them to survive for decades in the host without causing host mortality. They manage to escape or suppress the immune response while and simultaneously avoid overwhelming their hosts. In this context, schistosomes have developed an impressive array of immune evasion mechanisms including the ability to coat themselves with host antigens (molecular mimicry), to enzymatically cleave antibodies and to rapidly release distinct surface layers with unique double membranes, using pre-formed membrane vesicles (118). Apart from employing immune evasion strategies, schistosomes also subvert and thereby utilize the host's immune response to facilitate their own development and transmission. The production of eggs by female worms is, for example, dependent on host-derived TNF (3). Adult worms may also use host-derived TGF-β as a regulator of their own growth (10). Furthermore, schistosomes are adapted to and might promote genetic changes in both the intermediate and the definitive human host. So far, only in snails evidence for this conclusion is provided by the fact that snail strains can be selected dependent on their susceptibility towards schistosomal infection (158).

1.6 Glycans and their role in schistosomiasis

A wide range of both parasite- and host-derived glycoconjugates have been investigated for their role in the induction of a Th2 response by schistosomes in order to correlate defined carbohydrate structures with their biological properties in schistosome immunology.

Glycosylated components of schistosomal eggs have been shown to affect the production of cytokines by DCs (171). Furthermore, DCs stimulated by egg-derived antigens *in vitro* are able to promote a Th2 cell response *in vivo* (96). Egg glycoproteins can also bind to macrophage mannose receptor (63). Recent studies have shown that carbohydrates off egg antigens are of integral importance for the induction of a Th2 response. Likewise, related model compounds like Lacto-N-Fucopentaose III (LNFP, containing the Lewis X motif) induce a strong Th2 response (113), whereas the non-fucosylated form lacks this ability. It could be further demonstrated that the activation of TLR4 via LNFP III leads to an activation of the ERK (Extracellular-signal regulated kinase) pathway only, whereas after LPS-induced stimulation 3 different pathways (ERK, P23, JNK) are addressed (138).

Natural killer T (NKT) cells are similarly activated and stimulated by glycolipids of eggs and adult worms (171). NKT-cells as well as eosinophiles and mast cells are potent sources of IL-4; however, the absence of these cell types does not prevent the establishment of Th2 cell responses in infected mice (15; 119). It could be demonstrated that schistosome-specific phosphatidylserine activates TLR2 at the cell surface of DCs. This results in mature DCs (mDCs) with the capacity to promote the development of IL-10 secreting regulatory T-cells (Tregs) (145). Tregs induced by helminths have the potential to suppress the Th1 cell response to helminths-derived antigens, thereby ensuring Th2 cell polarization (102). The described induction of Tregs seems to be an essential control mechanism preventing an over-vigorous immune response during chronic infection.

One of the most fascinating features in parasite immunology is how the host detects the pathogen and gets rid of the invader without destroying self-antigens/-tissues. Recent work underlines that glycoconjugates, that are expressed through all life-cycle stages of the parasite, play an important role in the escape mechanisms of the pathogen. The first cells that come into contact with invading schistosomula are dendritic cells. They recognize pathogens and, via interaction with T-cells, an immune response is activated. The emerging role of carbohydrates in the

immunology of schistosomiasis opens up the possibility that innate patter-recognition receptors, that recognize parasitic glycans, may have a crucial role in the induction of a Th2 response. In the next paragraph pattern recognition receptors, dendritic cells and their role in immune responses to schistosomes will be discussed.

1.7 Dendritic cells

Beside macrophages, dendritic cells (DCs) are the major antigen presenting cells (APCs). In 1868 Paul Langerhans observed a subpopulation of DCs - the so-called Langerhans cells- in the skin, but he misclassified them as nerve cells (88). It took more than 100 years until the function of dendritic cells could be defined, in more detail, as Ralph Steinman and Zanvil Cohn reported in 1973 a new cell type in murine lymphoid organs, which they named dendritic cells in accordance to their tree-like cytoplasmic extensions (131). DCs represent a special type of leukocytes which is able to alert the immune system to the presence of infectious agents and is responsible for activation and control of both innate and adaptive immune responses. DCs are especially distributed in tissues that interface the external environment like the skin, the gut and the lungs. DCs have a unique capacity to sample sites of pathogen entry, respond to pathogenic signals and potently activate naïve T-cells to proliferate and differentiate into Th1, Th2 or Treg cells. Three signals are necessarily delivered by APCs to determine the fate of the naïve T-cells (106). Signal 1 is induced by the T-cell receptor (TCR), when binding an appropriate peptide-MHC complex. Signal 1 alone is thought to promote naïve T-cells into anergy or deletion, thereby promoting tolerance. Co-stimulatory signals in combination with signal 1 induce immunity, mirrored in T-cell clonal expansion and differentiation into effector cells. Signal 2 is the co-stimulatory signal mediated by triggering of CD28 by CD80 and CD86. Signal 3 is the polarizing signal mediated by soluble or membrane bound factors like IL-12 (Th1) or CC-chemokine ligand 2 (CCL2) (Th2) (72).

Following antigen uptake, DCs efficiently process antigens for their presentation in association with major histocompatibility complex (MHC) molecules. As a prerequisite for being able to prime the adaptive immune response, however, DCs have to mature. Maturation of DCs is characterized by a decreased antigen-uptake capacity and an increased cell surface expression of MHC and co-stimulatory molecules (8; 19). In addition, rearrangements of cytoskeleton, adhesion molecules

and cytokine receptors occur during maturation and allow DCs to migrate from peripheral tissues to secondary lymphoid organs (120).

In summary, the interaction with pathogens results in activated DCs which migrate to the T-cell area of the lymph nodes, where antigen-specific cells of the adaptive immune response can be primed. Therefore dendritic cells are often called "sensors of the immune system".

Interest in the function of DCs during schistosomiasis was strongly increased, when MacDonald and coworkers discovered that DCs pulsed *in vitro* with SEA induced a SEA-specific Th2 response, when reinjected in mice (96). Furthermore, SEA could condition human DCs to polarize Th responses in a Th2 direction *in vitro* (28).

The adaptive immune response to a pathogen develops after the initiation of innate immune reactions that requires the recognition of pathogenic antigens. Pathogen-expressed molecules that stimulate the innate immune response are termed pathogen-associated molecular patterns (PAMPs). Prominent examples are lipopolysaccharid (LPS) of gram-negative bacteria and peptidoglycans of gram-positive bacteria, as well as polysaccharides of fungal cell walls (136). The recognition of PAMPs is mediated by pattern-recognition receptors (PRRs) that consist of the large families of toll-like receptors (TLRs) and C-type lectins (CLRs).

1.8 C-type lectins

Recognition of carbohydrates is mediated by a specialized family of host receptors, named lectins, which bind glycans via one or more so- called carbohydrate recognition domains (CRDs). There are different classes of animal lectins, such as siglecs, galectins or the C-type lectin receptors (CLRs), which can be distinguished, on the basis of conserved regions in their amino acid sequence. The CLRs bind carbohydrates in a calcium-dependent manner, and the Ca^{2+}-ions are directly involved in the binding of the ligand as well as in maintaining the structural integrity of the lectin. The high affinity binding of some lectins is achieved through high avidity by multivalency of the ligand, by multiple CRDs within one lectin or via clustering of lectins containing one single CRD. The C-type lectins comprise a prototypic lectin fold, consisting of two anti-parallel β-strands and two α-Helices (159). The CLRs are either transmembrane proteins or secreted as soluble proteins, like members of the collectins family and the mannose-binding protein (MBP) (76; 93). The transmembrane CLRs are divided into two subfamilies, type I or type II C-type lectins depending on their N-terminus pointing outwards or inwards the cytoplasm. Examples of transmembrane C-type lectins are the selectins, the mannose receptor (MMR) family and DC-SIGN (37; 53; 90). A summary of type I and type II C-type lectins that are present on dendritic cells or Langerhans cells is given in Figure 11.

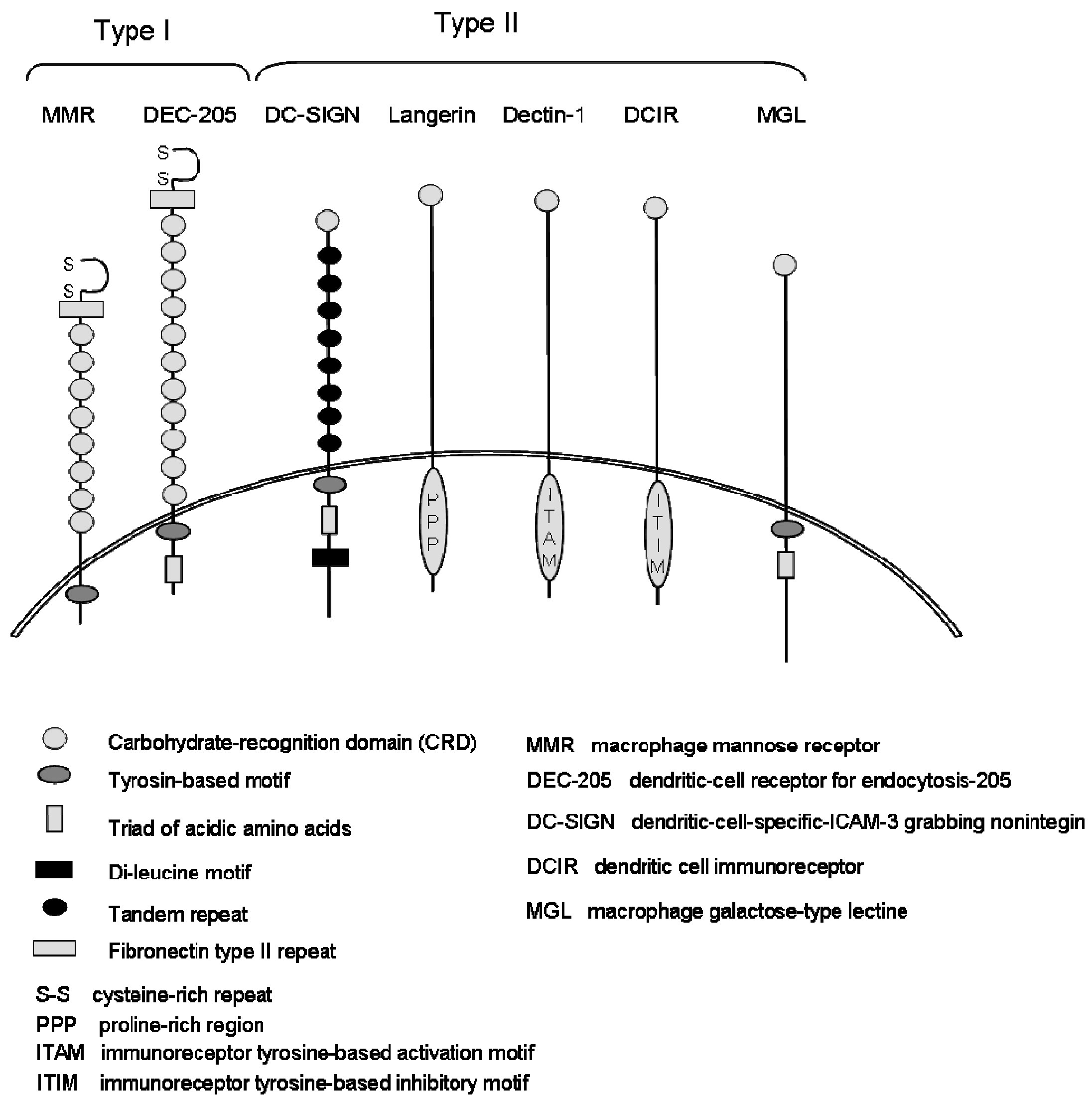


Figure 11. Classification of C-type lectins (modified after (45)).

Type I C-type lectins (MMR and DEC-205) contain an aminoterminal cysteine rich repeat, a fibronectin type II repeat and 8 to 10 carbohydrate recognition domains (CRD). Type II C-type lectins contain only one CRD at their carboxyterminal extracellular domain.

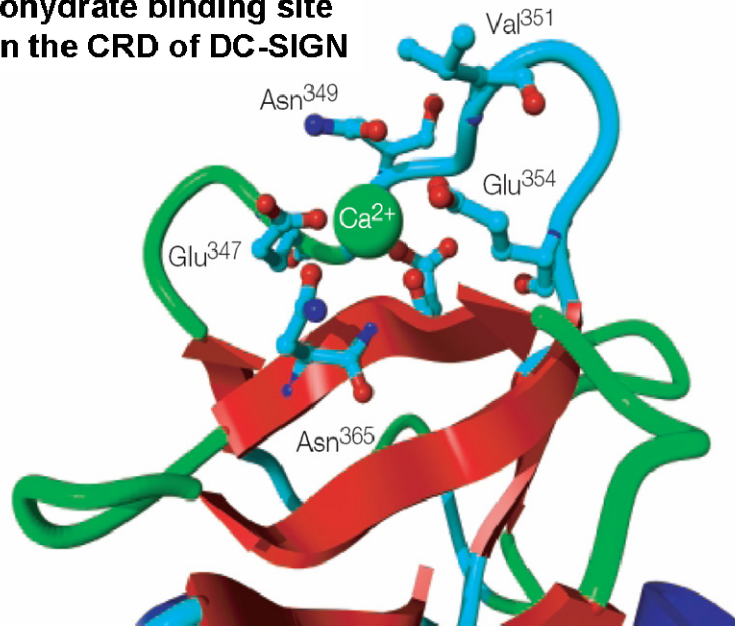
Despite the fact that several CLRs share a common CRD the different assembly architecture and spacing of these domains create unique sets of carbohydrate recognition profiles for each receptor. A good example is the different recognition of Lewis blood-group antigens by completely different CLRs. While DC-SIGN recognizes unsialylated Lewis X and Lewis A units, P- and E-selectin express high affinity towards sialylated Lewis X and Lewis A moieties (5; 48).

The main function of CLRs is the binding and subsequent internalization of antigens for direct elimination by macrophages. At the same time lysosomal degradation produces antigenic fragments which stimulate the adaptive immune system after their presentation by DCs and macrophages via MHC molecules at the cell surface (45).

1.8.1 DC-SIGN

The dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN, CD209) was first characterized as a receptor interacting with intercellular adhesion molecule 3 (ICAM-3) (53). It was subsequently shown to bind also ICAM-2 on vascular endothelial cells, thereby regulating DC migration (51). Both of these interactions are mediated by N-linked high-mannose structures (typically consisting of 5-9 mannose units). Mannose-dependent recognition processes are also responsible for the interaction of DC-SIGN with different pathogens. DC-SIGN was primarily described as HIV-1 receptor that enhances infection of T lymphocytes *in trans* (52). Ever since, increasing evidence has been accumulated that, besides HIV, a multitude of pathogens uses DC-SIGN as entry gate, as already described for severe acute respiratory syndrome coronavirus (SARS) (168), filoviruses (101), Hepatitis C virus (94), the fungus *Candida albicans* (17) as well as the bacterium *Mycobacterium tuberculosis* (133). Biochemical studies have shown that a significant proportion of DC-SIGN is located in detergent-resistant membrane fractions, where lipid rafts can also be found. However, disruption of lipid rafts by cholesterol extraction did not change the integrity of DC-SIGN microdomains, suggesting the existence of additional molecular determinants regulating the association of transmembrane protein, like DC-SIGN, with lipid rafts (16). Nevertheless, the localization of DC-SIGN in these lipid microdomains may create a scaffold that favors pathogen binding and may simultaneously allow the interplay of DC-SIGN with signaling molecules that are located in the same membrane domains.

As outlined above, DC-SIGN is a type II membrane C-type lectin with a short N-terminal cytoplasmic tail and a single C-terminal CRD (53). This CRD recognizes equally well internal branched mannose units as well as terminal di-mannosyl motifs (44; 105). In addition, DC-SIGN binds fucosylated epitopes, preferentially α1-3 and/or α1-4 fucosylated tri- or tetrasaccharides including Lewis antigens and LDNF (5; 148). The cytoplasmic tail contains three internalization motifs, as one main role of C-type lectins is to internalize pathogen specific antigens for processing and antigen presentation, thereby inducing the specific immune response (150). The internalization motifs of DC-SIGN are composed of a di-leucine motif, a tyrosine-based motif with the sequence YKSL, and a triacidic amino acid cluster EEE, the first two of which are involved in antigen internalization (40). A schematic structure of DC-SIGN is given in Figure 12.



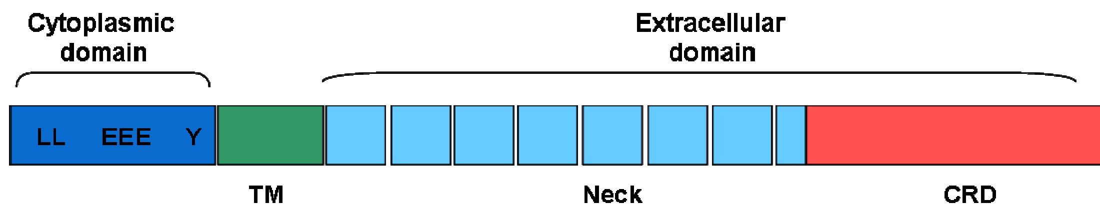


Figure 12. Schematic overview of the structure of DC-SIGN (modified after (150)).
One of the two Ca^{2+} is shown and interacts with four amino acids (Glu347, Asn349, Glu354, Asn365). Val351 has been shown to be involved in the recognition of some ligands. The CRD (red) of DC-SIGN is separated from the transmembrane (TM, green) region by a neck domain that consists of seven complete and one incomplete tandem repeats (light blue). The cytoplasmic tail (dark blue) contains internalization motifs such as the di-leucine (LL) motif and the tri-acidic (EEE) cluster and an incomplete immunoreceptor tyrosine (Y)-based activation motif (ITAM).

DC-SIGN delivers antigens to intracellular vesicles, which are comparable with late endosomes/lysosomes. As shown for HCV, resulting antigen fragments are subsequently presented to T-cells *in vitro* (40; 94). Some pathogens, however, may selectively use the method of DC-SIGN-mediated antigen uptake to escape from the host's immune defence (150).

DC-SIGN interacts with *S. mansoni* SEA. Using anti-glycan antibodies it could be demonstrated that this binding is mediated by high-mannose type glycans as well as Lewis X and LDNF motifs (148). Glycan array studies verified that DC-SIGN recognizes besides glycans with terminal Manα1-2Man units also fucosylated glycans with Fucα1-3GlcNAc and Fucα1-4GlcNAc moieties (58).

In chapter 2 of this thesis we demonstrate that DC-SIGN interacts not only with the Lewis X motif present in cercarial glycolipids, but also with the pseudo-Lewis Y epitope, thus representing the first parasite-specific ligand for DC-SIGN.

1.8.2 L-SIGN

Liver/lymph node specific ICAM-3-grabbing nonintegrin (L-SIGN, CD209L, DC-SIGN-R) is a homologue of DC-SIGN (9; 123). L-SIGN shares 77% amino acid sequence identity with DC-SIGN. L-SIGN is not expressed on human dendritic cells, but occurs on liver sinusoidal endothelial cells (LSECs) (9). These liver-resident cells function as antigen presenting cells and may therefore have the capacity for antigen clearance (81). Moreover, L-SIGN mediates LSEC-leukocyte adhesion (82).
The crystal structures of the CRDs of L-SIGN and DC-SIGN revealed that both receptors recognize high-mannose type glycans (44; 105). Additional binding studies demonstrated that L-SIGN has a higher affinity for mannose than DC-SIGN (58). L-SIGN is able to bind to ICAM-2 and ICAM-3 similar to DC-SIGN and may establish cellular interactions with ICAM-3 expressing T-cells (9). Furthermore, both C-type lectins recognize carbohydrate structures on pathogens, such as ManLAM of *M. tuberculosis* and high-mannose type glycan moieties of viruses like HIV-1, HCV and Ebola (84). The question as to whether L-SIGN is also able to incorporate antigens, is under current discussion. Ludwig *et al.* demonstrated that L-SIGN transfected cells internalize hepatitis C virus particles. The subsequent degradation of these particles, however, is prevented, since they escape into non-lysosomal vesicles (94). In contrast to these findings Guo and co-workers showed that L-SIGN expressed on fibroblasts did not release the ligand, even at low pH and did not mediate endocytosis, which underlines that L-SIGN functions more as adhesion than as internalization receptor (58). Nonetheless, L-SIGN displays considerable homology to DC-SIGN in the cytoplasmic tail and shares potential internalization motifs such as the triacidic cluster (EED) and the di-leucine motif, whereas the tyrosine based motif is lacking (84). The schematic structures of L-SIGN and DC-SIGN are displayed in Figure 13 for comparison.

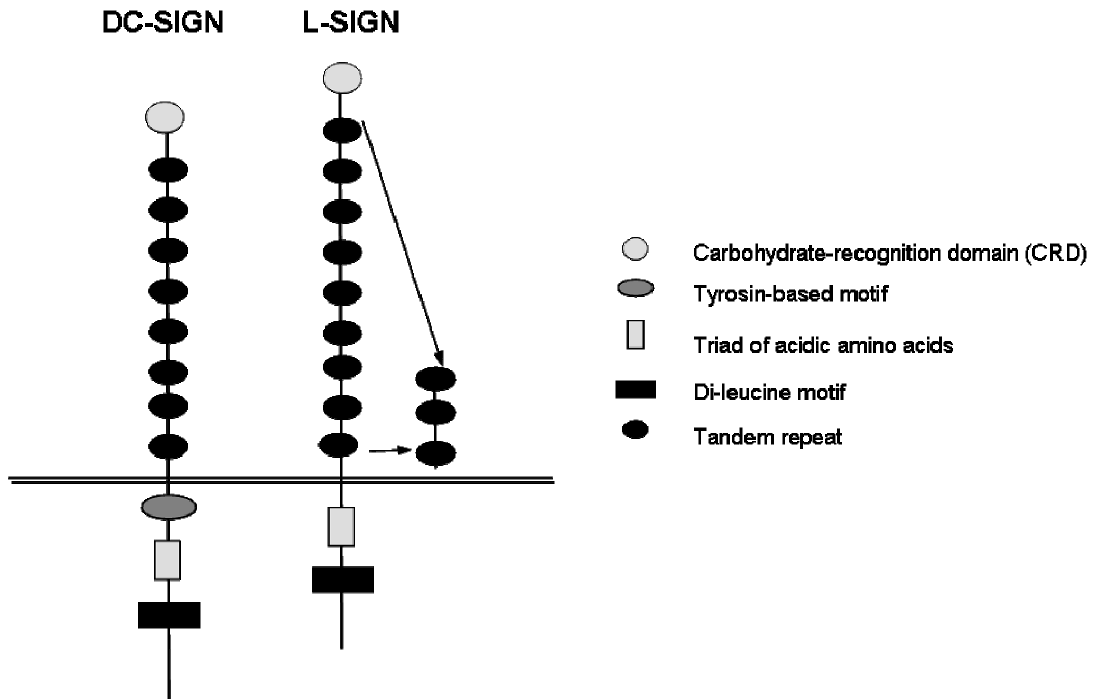


Figure 13. Schematic overview of the structure of DC-SIGN and L-SIGN.

DC-SIGN contains seven complete and one incomplete tandem repeats. The amount of repeats in L-SIGN is variable between three and nine (indicated with arrows). Within the cytoplasmic tails several internalization motifs are present, though L-SIGN lacks the tyrosine-based motif.

As the di-leucine motif has been shown to be essential for internalization in the case of DC-SIGN (125), and as this motif is conserved in L-SIGN, it is still possible that L-SIGN might function as an internalization receptor as well. Like DC-SIGN, L-SIGN binds to several Lewis antigens, except Lewis X (151). It has been also described that L-SIGN binds soluble egg antigens (SEA) from *S. mansoni* (151). A further specification of the ligands involved in these binding processes is presented in the publication Meyer *et al.* (2007) in chapter 2 of this thesis.

1.9 Interaction of C-type lectins with other receptors

In the past few years evidence has been accumulated illustrating the importance of CLRs for proper function of the immune system. From these data, a picture emerged suggesting that the recognition of pathogens is based on a complex network between CLRs and other recognition receptors of the innate immune system. One family of such receptors includes the so-called Toll-like receptors (TLRs) (134; 145). Besides CLRs, TLRs present, in particular, on DCs and macrophages are also involved in the direct recognition of specific PAMPs (134). While the main function of CLRs is to internalize antigens for degradation in order to enhance antigen processing and presentation (45), TLRs recognize foreign macromolecules and trigger intracellular signaling cascades, thus leading to the production of proinflammatory cytokines and T-cell activation (134). The interaction of TLRs and CLRs and their crosstalk is important for the balance between immune tolerance and immune activation. C-type lectin stimulation can either enhance or inhibit TLR signaling. This has been in detail described for *Mycobacterium tuberculosis*, showing that the binding of ManLAM to DC-SIGN inhibits the production of IL-12 and promotes the production of IL-10 elicited by the TLR4 ligand *Escherichia coli* LPS. TLRs control the switch between tolerogenic and activating immune responses. Recently, TLR-2 and TLR-4 have also been shown to be recruited into lipid rafts (126; 139), which favors the communication between neighbored CLRs and TLRs. Hence, pathogen recognition is not the result of an interaction of one compound of a pathogen with a single receptor, but requires a complex network of interacting receptors and ligands. The outcome of this recognition can be totally different, depending on the type of receptors involved and their cellular localization. It can result in the clearance of the pathogen, but may also lead to a strong adaptive immune response or to the induction of tolerance.

Studies on the effect of SEA on DCs revealed that SEA exhibits suppressive effects on the signaling initiated by classical inflammatory TLR ligands. Evidence for this assumption has been provided by co-pulsing DCs with SEA plus *E. coli* LPS or *Toxoplasma gondii* antigen (69; 171). In both cases, the activation by the inflammatory stimuli has been substantially inhibited by SEA. Pearce and coworkers reported, that this reduced responsiveness to TLR-ligands reflects, in part, the fact that SEA augments TLR ligand-induced production of IL-10 (73). However, analyses in IL-10$^{-/-}$ DCs revealed that distinct IL-10-indepedent mechanisms are

responsible for the suppressive effects of SEA. The question, how SEA is able to suppress TLR-initiated signaling, is the focus of ongoing research.

Besides TLRs and C-type lectins, Galectin-3, a well characterized member of the galectin family, recognizing galactose and GalNAc epitopes via its C-terminal CRD has been similarly described to bind to schistosomal glycans (144). Recent data revealed that galectin-3 recognizes LDN epitopes containing terminal β-GalNAc residues and interacts with LDN units in SEA (144). In agreement with this finding, a co-localization of LDN motifs on the parasitic egg shell and galectin-3 during granuloma formation has been observed in liver granuloma of infected hamsters. As galectin-3 is synthesized and secreted by many cell types including dendritic cells, the interaction of C-type lectins and galectins is of high interest in view of the immunology of helminth infection.

The collaborative recognition of, e.g., pathogen-derived glycoconjugates by different types of receptors is crucial for the regulation of an appropriate immune response to schistosome infection. As a prerequisite for further cell biological/immunological studies, however, detailed structural characterization of the ligands bound to the lectin has to be performed. Therefore different methods to define the precise carbohydrate recognition profile have to be applied.

1.10 Methods to study the interaction of lectins and ligands

Therapeutic manipulation of carbohydrate-lectin interactions needs- not only in the case of parasitic infections- detailed knowledge of the specific set of carbohydrates being recognized by the lectin of interest. In recent years different methods have been established to facilitate a systematic and high-throughput analysis of protein-carbohydrate interplay. In chapter 2 of this thesis a collaborative study with C.H. Grün is presented, describing the development of a simple and fast procedure for the biotinylation of carbohydrates based on reductive amination. The method allows complete and stable biotinylation of small quantities of oligosaccharides and includes a rapid and simple procedure to remove excess labeling reagent. Such glycan derivatives can be subsequently used for binding studies. Undoubtedly, however, the use of high-throughput glycan arrays is the method of choice to discriminate between high- and low affinity carbohydrate ligands. This technique could be successfully used in studies demonstrating that L-SIGN and DC-SIGN differ in their carbohydrate-binding profile (58). Release of the glycan moieties from parasitic glycolipids, purification of the glycan epitopes and subsequent attachment to a synthetic lipid-anchor was used as method of choice in the first manuscript presented in chapter 2 of this thesis (Meyer *et al.*; 2005; JBC). Formation of such neoglycolipids could be of general interest not only in the context of released glycolipid-glycans, but also with regard to carbohydrates originating from glycoproteins, proteoglycans and whole organs of the parasite. It could be, therefore, of great interest to combine the neoglycolipid technology with array techniques using different lectins. Two main drawbacks circumvent this goal currently: it is often almost impossible or at least very time-consuming to achieve parasitic material in the required amounts, and isolation of pure compounds to be used in arrays is usually very difficult. Therefore, the use of chemically synthesized oligosaccharides could be a promising strategy to test glycans with known parasitic glycan motifs in lectin binding studies. A further challenging but promising technique is X-ray crystallography which enables the analysis of covalent and non-covalent interactions in three dimensions, thus providing the basis for the understanding of binding of ligands to proteins. Drickamer and coworker were able to crystallize DC-SIGN and DC-SIGNR (= L-SIGN) together with different ligands (44). These crystal structures of the carbohydrate-recognition domains bound to the respective oligosaccharides, in combination with binding studies, revealed that these receptors selectively recognize

endogenous high-mannose type oligosaccharides. The same group was able to crystallize DC-SIGN together with the fucosylated ligand LNFP III showing the affinity of DC-SIGN to fucose-moieties (58). Besides crystallization and subsequent X-ray studies molecular modeling is a further strong technique to gain more insights in ligand-binding. Molecular modeling is a collective term that refers to theoretical methods and computational techniques to model or mimic the behavior of molecules. Thereby the interactions between neighboring atoms may be described and binding modes resulting in the lowest steric energetic state are calculated. A more molecular genetic tool is to induce mutations at specific positions in the CRD to investigate the role of different amino acids in ligand binding. Taken together, all these techniques will draw a clearer picture of ligand/lectin interactions.

2 Publications resulting from this work

DC-SIGN Mediates Binding of Dendritic Cells to Authentic Pseudo-Lewis^Y Glycolipids of *Schistosoma mansoni* Cercariae, the First Parasite-specific Ligand of DC-SIGN*

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One-step biotinylation procedure for carbohydrates to study carbohydrate–protein interactions

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The C-type lectin L-SIGN differentially recognizes glycan antigens on egg glycosphingolipids and soluble egg glycoproteins from *Schistosoma mansoni*

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DC-SIGN Mediates Binding of Dendritic Cells to Authentic Pseudo-Lewis^Y Glycolipids of *Schistosoma mansoni* Cercariae, the First Parasite-specific Ligand of DC-SIGN*

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During schistosomiasis, parasite-derived glycoconjugates play a key role in manipulation of the host immune response, associated with persistence of the parasite. Among the candidate host receptors that are triggered by glycoconjugates are C-type lectins (CLRs) on dendritic cells (DCs), which in concerted action with Toll-like receptors determine the balance in DCs between induction of immunity *versus* tolerance. Here we report that the CLR DC-SIGN mediates adhesion of DCs to authentic glycolipids derived from *Schistosoma mansoni* cercariae and their excretory/secretory products. Structural characterization of the glycolipids, in combination with solid phase and cellular binding studies revealed that DC-SIGN binds to the carbohydrate moieties of both glycosphingolipid species with Galβ1–4(Fucα1–3)GlcNAc (Lewis^X) and Fucα1–3Galβ1–4(Fucα1–3)GlcNAc (pseudo-Lewis^Y) determinants. Importantly, these data indicate that surveying DCs in the skin may encounter schistosome-derived glycolipids immediately after infection. Recent analysis of crystals of the carbohydrate binding domain of DC-SIGN bound to Lewis^X provided insight into the ability of DC-SIGN to bind fucosylated ligands. Using molecular modeling we showed that the observed binding of the schistosome-specific pseudo-Lewis^Y to DC-SIGN is not directly compatible with the model described. To fit pseudo-Lewis^Y into the model, the orientation of the side chain of Phe^{313} in the secondary binding site of DC-SIGN was slightly changed, which results in a perfect stacking of Phe^{313} with the hydrophobic side of the galactose-linked fucose of pseudo-Lewis^Y. We propose that pathogens such as *S. mansoni* may use the observed flexibility in the secondary binding site of DC-SIGN to target DCs, which may contribute to immune escape.

Schistosomiasis is a human parasitic disease caused by helminths of the genus *Schistosoma* that affect more than 200 million people worldwide (1). One of the most striking features of schistosomiasis is that the worms are experts in modulation and evasion of the host immune response, to enable their survival, migration, and development in different host tissues. Schistosomes have a complicated life cycle, requiring both a vertebrate and a snail host. Infection starts when cercariae released by the snail penetrate the host via the skin and transform into schistosomula. Schistosomula migrate to the portal system and develop to mature adult worms that mate and produce eggs. The eggs that become lodged within host tissues are primarily responsible for the development of a strong anti-inflammatory Th2 response that enables parasite survival and induces granuloma formation around the eggs, which is a major cause of pathology (1).

During infection the immune system is continuously challenged with an array of molecules associated with parasite metabolism and reproduction. However, little is known about the molecular mechanism behind this challenging of host immune responses, nor which cellular receptors are involved. Schistosomal glycoconjugates (glycoproteins and glycolipids) are shown to play important roles in host parasite interactions (2), which may include evasion mechanisms exploited by the parasites. These glycoconjugates are often developmentally regulated antigens that are expressed during different life cycle stages. Proteins of different schistosoma life cycle stages carry both *N*- and *O*-glycans (2, 3). In addition, schistosomes synthesize highly immunogenic glycosphingolipids, especially in the egg and cercarial stage (4, 5). The stage-associated synthesis of carbohydrate structures on these glycolipids is paralleled by changes in the ceramide structures during the life cycle (6–8). Schistosome glycosphingolipids have a typical core structure that differs from that in vertebrates. Remarkably, the glucocerebroside is not galactosylated to make lactosylceramide as in vertebrates, but is instead modified by addition of a GalNAc residue to generate GalNAcβ1–4Glcβ1-ceramide, the so-called "schisto-core" (4). Both protein-linked glycans and glycosphingolipids contain a variety of terminal glycan epitopes, many of which are highly fucosylated and include glycan antigens such as GalNAcβ1–4GlcNAc (LacdiNac, LDN),[2] Fucα1–3GalNAcβ1–4GlcNAc (F-LDN), GalNAcβ1–4(Fucα1–3)GlcNAc (LDN-F), GalNAcβ1–4(Fucα1–2Fucα1–3)GlcNAc (LDN-DF), and Galβ1–4(Fucα1–3)GlcNAc (Lewis^X, Le^X) (2, 9–14).

Several findings indicate important roles for Le^X antigens in host-schistosome interactions. Le^X antigens have been found in glycoconju-

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[2] The abbreviations used are: LDN, GalNAcβ1–4GlcNAc; DC, dendritic cell; CLR, C-type lectin; CD, cluster of differentiation; CRD, carbohydrate recognition domain; DC-SIGN, dendritic cell-specific ICAM-3 grabbing nonintegrin; dHex, deoxyhexose; DPPE, 1,2-*sn*-dipalmitoylphosphatidyl-ethanolamine; ES products, excretory/secretory products; ELISA, enzyme-linked immunosorbent assay; Hex, hexose; HexNAc, *N*-acetylhexosamine, ICAM, intercellular adhesion molecule; iDC, immature dendritic cell; LDN-F, GalNAcβ1–4(Fucα1–3)GlcNAc); Le^a or Lewis^a, Galβ1–3(Fucα1–4)GlcNAc; Le^b or Lewis^b, Fucα1–2Galβ1–3(Fucα1–4)GlcNAc; Le^X or Lewis^X, Galβ1–4(Fucα1–3)GlcNAc; Le^Y or Lewis^Y, Fucα1–2Galβ1–4(Fucα1–3)GlcNAc; LNFPIII, lacto-*N*-fucopentaose III; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; pseudo-Lewis^Y or pseudo-Le^Y, Fucα1–3Galβ1–4(Fucα1–3)GlcNAc; SEA, soluble egg antigen; TLR, toll-like receptor; mAb, monoclonal antibody.

gates of all life cycle stages, such as membrane-bound glycoproteins of adult schistosomes, secreted egg and gut glycoproteins (15), and cercarial glycolipids (5). Interestingly, Le^X-containing glycoconjugates are shown to induce proliferation of B-cells from infected animals, which secrete interleukin-10 (IL-10) and prostaglandin E_2 (PGE_2), and to induce the production of IL-10 by peripheral blood mononuclear cells from schistosome-infected individuals (16, 17). In a murine schistosome model, Le^X is an effective adjuvant for induction of a Th2 response (18).

Recognition of an invading pathogen by cells of the immune system is mediated by receptors on antigen-presenting cells. On dendritic cells (DCs) two receptor families are involved in the recognition of pathogens, Toll-like receptors (TLRs) that recognize common pathogen-associated molecular patterns, and C-type lectins (CLRs) that bind to glycan antigens (19). DCs express several TLRs, depending on their developmental stage and lineage (20). Several studies have shown that bacterial products induce maturation of DCs via TLRs (21–23). Recently it was shown that the schistosome-specific phosphatidylserine (PS) activates TLR2 and induces mature DCs to activate IL-10-producing regulatory T cells (24). DCs also express a variety of CLRs that recognize glycan antigens in a Ca^{2+}-dependent manner using highly conserved carbohydrate recognition domains (19, 25). Several CLRs have been implicated to play a role in the recognition of pathogens. An important question still remaining is whether the principal function of CLRs is to capture pathogens, or to recognize self-antigens and suppress immunity (26). Current views are that the balance between triggering TLRs and CLRs may fine tune the immune response toward immune activation or tolerance. Recognition of glycans alone by DC lectins may favor immune suppression, whereas pathogen recognition in a situation of "danger" (when TLRs are triggered) induces immune activation (26, 27).

As a first approach to understand the molecular basis of the role of Le^X and other schistosome glycan antigens in interactions with their host, we set out to investigate the receptors on antigen-presenting cells that recognize the schistosome glycan antigens. Recently we showed that the DC-specific C-type lectin DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin, CD209) binds to *Schistosoma mansoni* soluble egg antigens (SEA) via Le^X, but the actual ligands within SEA have not yet been identified (28). DC-SIGN is a human type II transmembrane CLR that contains only one C-terminal CRD and is abundantly expressed on immature DCs (iDCs). DC-SIGN has affinity for glycoconjugates containing mannose, *N*-acetylglucosamine and fucose and interacts with many pathogens. Multivalent binding of its ligands is thought to be achieved by the formation of tetramers (29, 30). Using site-directed mutagenesis, molecular modeling, and docking of different Lewis antigens in the CRD of DC-SIGN we could demonstrate that the amino acid Val^{351} in DC-SIGN is essential for binding the Fucα1–3/4-GlcNAc moiety of the Lewis antigens Le^X, Le^a, Le^b, and Le^Y (28, 31, 32). In this study we have demonstrated that DC-SIGN strongly binds to authentic cercarial glycosphingolipids of *S. mansoni*, but not to egg glycolipids. Structural characterization of the glycan moieties of the glycosphingolipid species revealed that a pentasaccharide containing Le^X is one of the main ligands recognized by DC-SIGN. Unexpectedly, we found that DC-SIGN also binds to glycosphingolipid species carrying a hexasaccharide terminating with Fucα1–3Gal(β1–4)(Fucα1–3)GlcNAc-R (pseudo-Le^Y), a glycan antigen that so far only has been found within schistosomes.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—iDCs were obtained from human peripheral blood mononuclear cells (PBMCs) by a CD14 magnetic microbeads isolation (MACS; Miltenyibiotec) (33). The obtained CD14$^+$ monocytes were differentiated into iDCs in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (500 and 800 units/ml, respectively; Schering-Plough, Belgium). At day 6, the phenotype of the cultured DCs was confirmed by flow cytometric analysis. The DCs expressed high levels of major histocompatibility complex class I and II, CD11b, CD11c, and ICAM-1 and low levels of CD80 and CD86. Stable transfectants of K562 cells expressing DC-SIGN (34) were kindly provided by Dr. T. Geijtenbeek. The mAb AZN-D1 is a blocking anti-DC-SIGN antibody described previously (35). DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64–404) fused at the C terminus to a human IgG1-Fc fragment into the Sig-pIgG1-Fc vector (32). The peroxidase-labeled goat anti-human IgG-Fc or goat anti-mouse IgM were both from Jackson, West Grove, PA. The goat anti-mouse Alexa Fluor 488 secondary antibody was obtained from Molecular Probes, Inc., Eugene, OR.

Glycolipid Purification—Lyophilized *S. mansoni* cercariae and eggs were kindly provided by Dr. Michael J. Doenhoff (School of Biological Science, University of Wales, Bangor, UK). *S. mansoni* excretory/secretory (ES) products were kindly provided by Dr. M. de Jong-Brink (FALW, VU University, Amsterdam, NL). The cercarial and egg glycolipids were purified by organic solvent extraction, saponification, desalting, and anion-exchange chromatography as described previously (5). Neutral glycolipids were separated by HPLC (Iatrobeads 6RS-8010, 10 μm, 4.6 mm × 500 mm; Macherey and Nagel, Düren, Germany) at a flow rate of 1 ml/min using a binary linear gradient from 100% solvent A (chloroform/methanol/water, 83:16:1, by volume) in 60 min to 60% solvent B (chloroform/methanol/water, 10:70:20, by volume) followed by a 20-min elution step with 100% solvent B.

Release and Purification of Le^X and Pseudo-Le^Y Oligosaccharides from the Ceramide Moieties—Oligosaccharides were released from cercarial and ES glycolipids by treatment with recombinant endoglycoceramidase II (from *Rhodococcus spp.*, Takara Shuzu Co., Otsu, Shiga, Japan). Released oligosaccharides were separated from ceramide moieties by reversed-phase (RP-) chromatography as described previously (5). Le^X and pseudo-Le^Y glycans were fractionated and separated from remaining glycolipid-derived oligosaccharide species by HPLC on a TSK-Amide 80 column (4 mm × 250 mm; Tosoh, Amsterdam, NL) using a linear gradient from 100% solvent A (35% acetic acid, buffered with triethylamine to pH 7.3 and 65% acetonitrile) to 100% solvent B (50% acetic acid, buffered with triethylamine to pH 7.3 and 50% acetonitrile) at a flow rate of 1 ml/min. Fractions (500 μl) were analyzed by MALDI-TOF-MS and MS/MS.

Neoglycolipid Synthesis—Pure glycolipid-derived oligosaccharide fractions containing either Le^X or pseudo-Le^Y glycans (80 μg each) as well as lacto-*N*-fucopentaose III (LNFPIII; 100 μg; Dextra Laboratories, Reading, UK) were used for synthesis of neoglycolipids by coupling to 1,2-*sn*-dipalmitoylphosphatidylethanolamine via reductive amination (36). Resulting products were analyzed by MALDI-TOF-MS.

Enzyme-linked Immunosorbent Assay (ELISA)—Total egg and cercarial glycolipids were diluted in ethanol on NUNC maxisorb plates (Roskilde, Denmark), and incubated for 60 min at 37 °C to coat the glycolipids to the plate. Plates were blocked with 1% ELISA grade bovine serum albumin (fraction V, fatty acid-free; Calbiochem) and incubated with DC-SIGN-Fc (3 μg/ml) (32). Binding was detected using a peroxidase-labeled goat antihuman IgG-Fc (Jackson). Separated glycolipid fractions (8.5 ng) and neoglycolipids were coated on polysorb plates

(Nunc, Wiesbaden, Germany) and similarly analyzed by ELISA for reactivity with DC-SIGN-Fc (3 µg/ml) using peroxidase-conjugated antihuman IgG (4.6 µg/ml; Sigma-Aldrich). EDTA (10 mM, Roth, Karlsruhe, Germany) was added when indicated to investigate whether the binding was calcium-dependent.

MALDI-TOF-MS and MS/MS Analysis—MALDI-TOF-MS analysis was performed on an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonik, Bremen, Germany) equipped with a nitrogen laser and a LIFT-MS/MS facility as described previously.[3] The instrument was operated in the positive-ion reflector mode throughout using 6-*aza*-2-thiothymine (Sigma-Aldrich) as matrix. About 100–500 spectra were summarized in each case.

Cellular Adhesion Assay—96-well plates (NUNC maxisorb) were coated overnight at room temperature with *S. mansoni* cercarial and egg glycolipids, pseudo-Le^Y neoglycolipid, Le^X neoglycolipid, or globotriaosylceramide (Gb3) and blocked with 1% bovine serum albumin. Cells labeled with Calceine AM (Molecular Probes), were added for 1.5 h at 37 °C in the presence or absence of 20 µg/ml mAbs AZN-D1. Non-adherent cells were removed by gently washing. Adherent cells were lysed, and fluorescence was quantified on a Fluostar spectrofluorimeter (BMG Labtech, Offenburg, Germany). Results are expressed as the mean percentage of adhesion of triplicate wells.

Isolation of Schistosoma mansoni Cercarial ES Products—Free cercariae were obtained from *S. mansoni* parasitized *Biomphalaria glabrata* snails by inducing the shedding process basically as described by Sluiters *et al.* (37). The free swimming cercariae obtained were transferred to 60 ml of water. After 5 h, the cercariae/schistosomula were removed, and the remaining water containing the ES products was concentrated.

Molecular Modeling—The coordinates of the crystal structure of human DC-SIGN interacting with the Le^X-containing pentasaccharide LNFPIII (38) (code 1SL5) were taken from the Protein Data Bank (39). The structure was edited using Sybyl software (Tripos Inc., St Louis), to contain only one protein monomer together with calcium ions, the Le^X trisaccharide, and the two water molecules that play an important role in bridging O4 of galactose to the protein surface. Protein hydrogen atoms were added, the peptide atoms partial charges were calculated using the Pullman procedure, and the calcium ions were given a charge of 2. Atom types and charges for oligosaccharides were defined using the PIM parameters developed for carbohydrates (40).

Pseudo-Le^Y was built by adding one fucose on position 3 of the terminal galactose residue. The systematic search procedure of Sybyl was used to vary the two torsion angles at this glycosidic linkage together with the two torsion angles of the Phe^{313} side chain. Only one conformational family was identified. Subsequent energy minimization was performed using the Tripos force field (41) with geometry optimization of the sugar and the side chains of amino acids in the binding sites. A distance-dependent dielectric constant was used in the calculations. Energy minimizations were carried out using the Powell procedure until a gradient deviation of 0.05 $kcal \cdot mol^{-1} \cdot Å^{-1}$ was attained.

RESULTS

Recognition of S. mansoni Cercarial Glycolipids by DC-SIGN—To investigate their binding to DC-SIGN, authentic glycolipids from *S. mansoni* cercariae, and eggs were isolated by organic solvent extraction (5) and assayed by ELISA using soluble DC-SIGN-Fc. In parallel, unrelated glycolipids, such as globotriaosylceramide (Gb3), Forssman

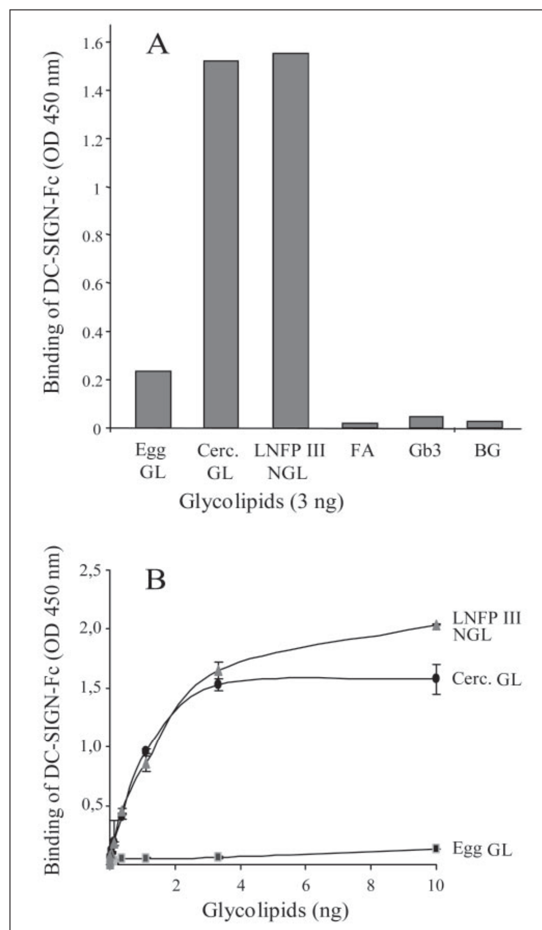

FIGURE 1. **Binding of DC-SIGN to *S. mansoni* cercarial glycolipids and LNFPIII-neoglycolipid.** *A*, ELISA was performed to determine the binding reactivity and specificity of DC-SIGN to egg glycolipids (*Egg GL*), total cercarial glycolipids (*Cerc. GL*), Forssman antigen (*FA*), globotriaosylceramide (*Gb3*), LNFPIII-neoglycolipid (*LNFPIII NGL*), and bovine gangliosides (*BG*). Similar amounts of glycolipids (3 ng/well) were applied in each case. Data represent a typical result out of three experiments performed in duplicate. *B*, titration of egg glycolipids (*Egg GL*), total cercarial glycolipids (*Cerc. GL*), and LNFPIII-neoglycolipid (*LNFPIII NGL*) was performed, starting with 10 ng/well, to determine the binding affinity of DC-SIGN. Results are a typical representative of three independent experiments performed in triplicate.

antigen (FA), and bovine gangliosides (BG) were tested together with a synthetic LNFPIII-neoglycolipid to evaluate the binding specificity of DC-SIGN as well as the potential influence of the structure of the lipid moiety in this assay. The results revealed that DC-SIGN-Fc strongly binds cercarial glycosphingolipids and the LNFPIII-neoglycolipid, whereas a weak binding was observed to egg-derived glycolipids. The remaining types of glycolipids were not recognized at all (Fig. 1, *A* and *B*). Hence, it can be concluded that recognition by DC-SIGN is mediated by the carbohydrate unit and independent of the lipid part of the respective molecules.

Characterization and Fractionation of Total Cercarial Glycolipids—To allow the subsequent analysis of the glycolipid species that bind DC-SIGN, total cercarial glycolipids were analyzed by MALDI-TOF

[3] Geyer, H., Wuhrer, M., Resemann, A., and Geyer, R. (August 31, 2005) *J. Biol. Chem.* 10.1074/jbc.M505985200

Binding of DC-SIGN to Pseudo-Lewis^Y

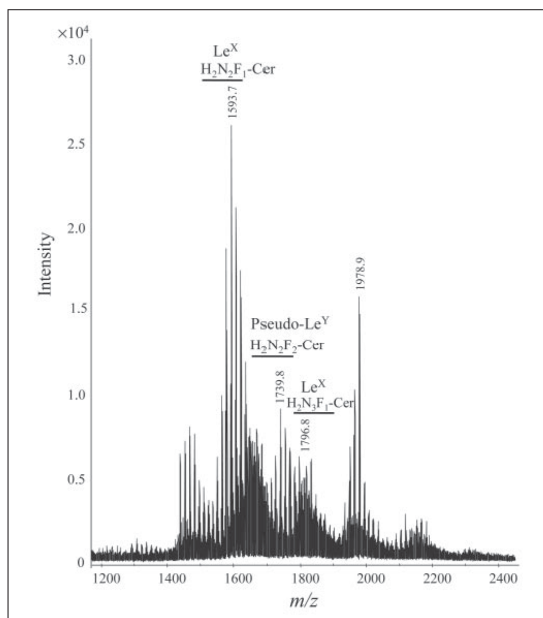

FIGURE 2. **MALDI-TOF-MS analysis of isolated glycolipids from *S. mansoni* cercariae.** Deduced monosaccharide compositions are assigned to major pseudomolecular ions ([M + Na]$^+$) comprising Le^X ($H_2N_2F_1$ at *m/z* 1593.7 or $H_2N_3F_1$ at *m/z* 1796.8) and pseudo-Le^Y epitopes ($H_2N_2F_2$ at *m/z* 1739.8). The cluster of ions culminating in a signal at *m/z* 1978.9 reflects glycolipids with divergent ceramide and carbohydrate moieties including species with monosaccharide compositions of $H_1N_3F_4$ and $H_2N_3F_3$. The complex pattern of registered signals is caused by ceramide heterogeneity. *H*, hexose; *N*, *N*-acetylhexosamine; *F*, deoxyhexose (fucose); *Cer*, ceramide.

MS (Fig. 2). In agreement with previous studies (5), a complex pattern of different glycolipids was registered mainly because of the high heterogeneity of the present ceramide moieties. Prevailing species exhibited monosaccharide compositions of $Hex_2HexNAc_2dHex_1$, $Hex_2HexNAc_3dHex_1$, and $Hex_2HexNAc_2dHex_2$, thus reflecting ceramide pentahexoside and hexahexoside species with the Le^X or pseudo-Le^Y determinants as described (5). In addition, a number of major and minor signals was registered which reflected the presence of additional glycolipids with diverging ceramide and carbohydrate compositions. Based on previous studies on the ceramide composition of cercarial glycolipids (7) the cluster of ions at *m/z* 1978.9 can be concluded to comprise species with monosaccharide compositions of $Hex_1HexNAc_3dHex_4$ and $Hex_2HexNAc_3dHex_3$, which is corroborated by the detection of the respective free oligosaccharides after endoglycoceramidase treatment (TABLE ONE). To obtain individual glycolipid fractions, cercarial glycolipids were subjected to HPLC separation, and the isolated fractions were analyzed by ELISA for their capacity to bind DC-SIGN (Fig. 3*A*). The results revealed that DC-SIGN mainly recognized glycolipids that occurred in HPLC fractions 40–50, whereas species with elongated carbohydrate units did not react. Subsequent analysis of fractions 40–50 by MALDI-TOF-MS demonstrated that each fraction comprised a mixture of glycolipids carrying Le^X or pseudo-Le^Y moieties (Fig. 3, *B–E*). Because of the observed ceramide heterogeneity, a clear separation into fractions containing solely Le^X or pseudo-Le^Y determinants was not possible. To determine which of these glycan moieties are recognized by DC-SIGN, we decided to synthesize neoglycolipids, using purified carbohydrate moieties that were released from the cercarial glycosphingolipids.

Purification of the Glycan Moieties of Cercarial Glycolipids—Glycans were released from total cercarial glycolipids by endoglycoceramidase treatment, separated from remaining (glyco)lipids by reversed-phase chromatography and analyzed by MALDI-TOF-MS (Fig. 4). In agreement with the spectrum obtained in the case of total cercarial glycolipids (Fig. 2), the results confirmed the preponderant occurrence of oligosaccharides with monosaccharide compositions consistent with the presence of a Le^X or a pseudo-Le^Y determinant. In addition, several minor oligosaccharides with divergent compositions have been registered (TABLE ONE). To obtain individual glycan species, the total mixture of oligosaccharides was subjected to HPLC separation using a TSK-amide column. Collected fractions were screened by MALDI-TOF-MS. Fractions containing the Le^X pentasaccharide (*m/z* 917.3 [M + Na]$^+$) plus additional pseudo-Le^Y (*m/z* 1063.6 [M + Na]$^+$) and/or Le^X hexasaccharide species (*m/z* 1120.3 [M + Na]$^+$) were reapplied to HPLC, to reduce peak heterogeneity and to obtain pure compounds as monitored by MALDI-TOF-MS (see *insets* in Fig. 5, *A* and *B*).

Characterization of Le^X and Pseudo-Le^Y Glycans by MALDI-TOF-MS/MS—The identity of the isolated glycans was established by tandem mass spectrometry. MS/MS analysis verified that the parent ion with the mass of *m/z* 917.3 [M + Na]$^+$ consisted of a pentasaccharide with a composition of $Hex_2HexNAc_2dHex_1$ (Fig. 5*A*). In addition to the sequential release of the five monosaccharide units, two characteristic fragment ions, B_2 and C_2 at *m/z* 534.2 and *m/z* 552.2, could be observed in agreement with the presence of a Le^X trisaccharide unit. The linkage of fucose to the subterminal HexNAc residue is confirmed by a $Y_{3\alpha}$ fragment ion at *m/z* 755.5. By the same line of evidence, the glycan with the mass of *m/z* 1063.3 [M + Na]$^+$ (*inset* in Fig. 5*B*) could be shown to comprise a dHex-Hex-(dHex-)HexNAc unit because of the observed B_3 and C_3 fragment ions at *m/z* 680.1 and *m/z* 698.1, respectively (Fig. 5*B*). Hence, the obtained MS/MS spectra displayed all diagnostically relevant fragment ions to be expected for the cercarial glycolipid-derived Le^X pentasaccharide and pseudo-Le^Y hexasaccharide units described previously (5). Furthermore, mass spectrometry revealed a high purity of the Le^X and pseudo-Le^Y glycan fractions obtained.

Binding of DC-SIGN to Le^X and Pseudo-Le^Y Neoglycolipids—Purified Le^X and pseudo-Le^Y glycans were converted into neoglycolipids by coupling to 1,2-*sn*-dipalmitoylphosphatidyl-ethanolamine (DPPE) via reductive amination. Resulting products were analyzed by MALDI-TOF-MS (Fig. 6). Le^X neoglycolipid led to a signal of *m/z* 1615.0 [M-H + 2Na]$^+$ (Fig. 6*A*) in agreement with the calculated mass of the Le^X pentasaccharide (*m/z* 917.3) and the mass increment of DPPE (*m/z* 692), taking into consideration that one oxygen is lost during reductive amination and the acidic proton of DPPE is replaced by a sodium ion. Likewise, pseudo-Le^Y neoglycolipid was registered with masses of *m/z* 1739.4 [M + Na]$^+$ and *m/z* 1761.1 [M-H + 2Na]$^+$ (Fig. 6*B*). Both neoglycolipid samples were quantified by compositional analysis with regard to their carbohydrate content to ensure the application of defined amounts of neoglycolipids in subsequent experiments. The binding of DC-SIGN-Fc to Le^X and pseudo-Le^Y neoglycolipids was studied by ELISA (Fig. 7). The results revealed an almost equivalent recognition of the two neoglycolipids by DC-SIGN-Fc when compared with the LNFPIII-neoglycolipid used as a positive control. This finding is remarkable as the pseudo-Le^Y epitope represents, in contrast to Le^X, a parasite-specific carbohydrate structure. To establish whether natural cell surface-expressed DC-SIGN binds authentic cercarial glycolipids and neoglycolipids, we performed a cellular adhesion assay. K562 cells stably transfected with DC-SIGN express high levels of DC-SIGN on their cell surface as was determined by flow cytometry (Fig. 8*A*). Cercarial glycolipids as well as neoglycolipids containing pseudo-Le^Y or Le^X

TABLE ONE

Compilation of total glycans obtained from glycosphingolipids of *S. mansoni* cercariae and ES products by endoglycoceramidase treatment

Compositions are assigned in terms of hexose (H), *N*-acetylhexosamine (N), and deoxyhexose (fucose; F). Relative occurrence of individual compositional species is roughly estimated from the respective signal intensities registered by MALDI-TOF-MS. Oligosaccharides representing Le^X-pentasaccharides, Le^X-hexasaccharides or pseudo-Le^Y-hexasaccharides are marked in bold type.

Calculated mass $[M+Na]^+$	Observed mass $[M+Na]^+$	Composition	Obtained from[a]	
m/z	*m/z*			
			Glycolipids	E/S-products
917.32	917.3	$\mathbf{H_2N_2F_1}$	+++	+++
933.32	933.2	H_3N_2	++	—
958.35	958.2	$H_1N_3F_1$	+	+
1063.38	1063.6	$\mathbf{H_2N_2F_2}$	++	+
1079.2	1079.2	$H_3N_2F_1$	+	—
1104.41	1104.2	$H_1N_3F_2$	+	+
1120.41	1120.3	$\mathbf{H_2N_3F_1}$	+++	++
1136.39	1136.2	H_3N_3	+	—
1161.43	1161.3	$H_1N_4F_1$	+	—
1241.43	1241.6	$H_4N_2F_1$	—	+
1250.46	1250.3	$H_1N_3F_3$	+	—
1266.46	1266.3	$H_2N_3F_2$	++	+
1282.45	1282.3	$H_3N_3F_1$	+	—
1307.46	1307.3	$H_1N_4F_2$	++	+
1323.48	1323.3	$H_2N_4F_1$	+	—
1396.52	1396.3	$H_1N_3F_4$	++	++
1412.51	1412.3	$H_2N_3F_3$	+	—
1428.51	1428.3	$H_3N_3F_2$	+	—
1453.55	1453.3	$H_1N_4F_3$	+	+
1469.54	1469.3	$H_2N_4F_2$	++	—
1485.54	1485.3	$H_3N_4F_1$	+	—
1510.57	1511.0	$H_1N_5F_2$	—	+
1599.6	1599.4	$H_1N_4F_4$	+	+
1615.59	1615.4	$H_2N_4F_3$	+	—
1631.59	1631.4	$H_3N_4F_2$	+	—
1656.62	1656.4	$H_1N_5F_3$	+	+
1672.62	1672.4	$H_2N_5F_2$	+	—
1713.65	1714.3	$H_1N_6F_2$	—	+
1745.66	1745.4	$H_1N_4F_5$	+	—
1802.68	1803.4	$H_1N_5F_4$	—	+
1818.68	1818.4	$H_2N_5F_3$	+	—
1834.67	1835.4	$H_3N_5F_2$	+	—
1859.70	1860.4	$H_1N_6F_3$	—	++
2005.76	2006.4	$H_1N_6F_4$	—	+++
2062.78	2063.4	$H_1N_7F_3$	—	+
2151.82	2152.4	$H_1N_6F_5$	—	+
2208.84	2210.4	$H_1N_7F_4$	—	++
2354.89	2355.4	$H_1N_7F_5$	—	++
2500.95	2502.3	$H_1N_7F_6$	—	+

[a] Relative amounts were estimated as follows: +, 0–2.2 $\times 10^4$; ++, 2.21–4.6$\times 10^4$; +++, 4.61–6.6$\times 10^4$ intensity counts. —, signal not detected.

showed binding to K562 transfected with DC-SIGN, but not to the parental K562 cell line. There was no binding of cellular DC-SIGN to egg glycolipids and Gb3. The binding could be blocked by AZN-D1, a DC-SIGN blocking antibody, and EGTA (Fig. 8*B*). Human iDCs naturally express DC-SIGN on their cell surface (Fig. 8*A*). Cercarial glycolipids and the neoglycolipids containing Le^X or pseudo-Le^Y are bound by DC-SIGN on iDCs (Fig. 8*C*). Despite the fact that iDCs express multiple CLRs on their cell surface, adhesion is completely inhibited by the Ca^{2+}-chelator EGTA or a DC-SIGN blocking antibody (Fig. 8*C*), indicating that binding of the cells to the glycolipids is mediated via the CRD of DC-SIGN. Hence, these studies demonstrate that DC-SIGN mediates the binding of iDCs to authentic carbohydrate structures uniquely expressed by *S. mansoni* cercarial glycolipids.

Docking of Pseudo-Le^Y Oligosaccharide into DC-SIGN—The docking of pseudo-Le^Y in the DC-SIGN binding site was based on the crystal structure of the DC-SIGN complexed with Le^X-containing oligosaccharide (38). Inclusion of hydrogen atoms (not located by x-ray diffraction) and optimization of the binding site of the DC-SIGN/Le^X complex did not yield any significant change compared with the crystal structure. It allows us to propose the hydrogen bond network displayed in Fig. 9*A*, with involvement of two water molecules that bridge the galactose residue to Ser^{360} and Glu^{358} side chains.

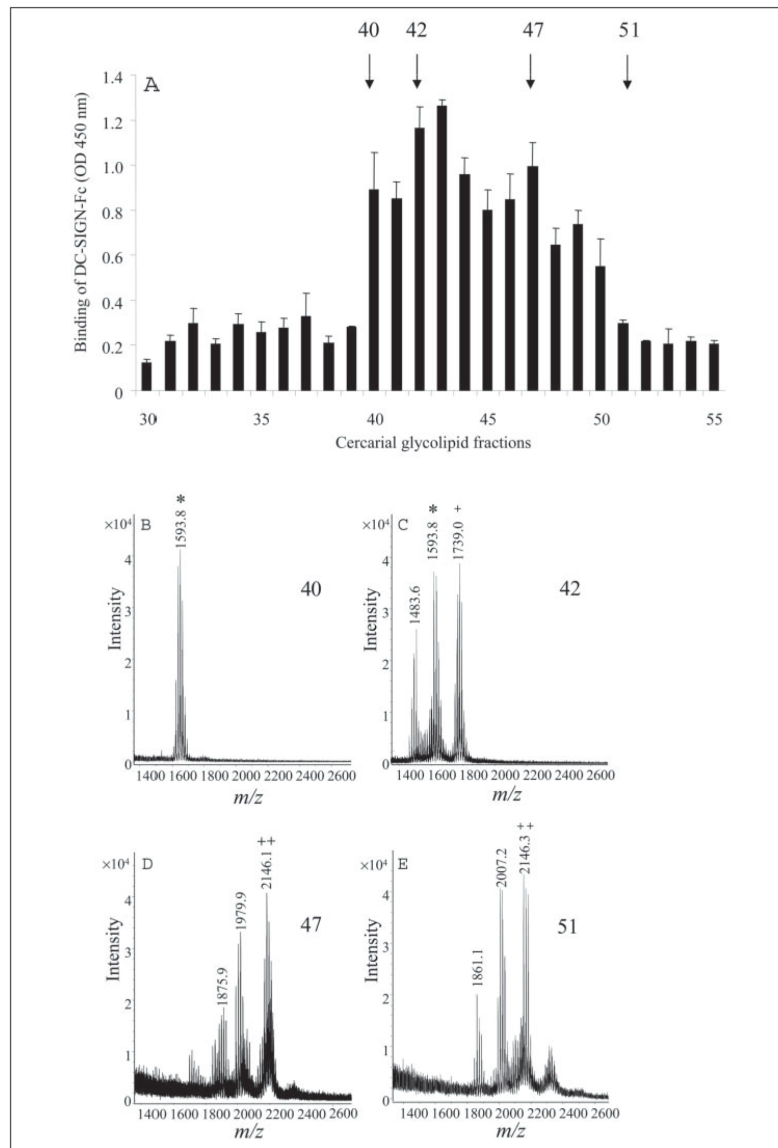

FIGURE 3. **Binding of DC-SIGN to fractionated glycolipids of *S. mansoni* cercariae.** Binding of DC-SIGN to *S. mansoni* cercarial glycolipids was determined by ELISA using soluble DC-SIGN-Fc (*A*). Recognized species occurring in fractions 40–50 are dominated by glycolipids carrying Le^X and pseudo-Le^Y epitopes as confirmed by MALDI-TOF-MS. *B–E*, MALDI-TOF-MS analysis of HPLC-fractions 40, 42, 47, and 51, respectively. Major pseudomolecular ions ([M + Na]$^+$) comprising Le^X-pentasaccharide (*), pseudo-Le^Y-hexasaccharide (+), and pseudo-Le^Y-octasaccharide (++) units are marked.

Pseudo-Le^Y was built from this complex by adding a fucose in position 3 of the galactose. All possible conformations were tested but all of them resulted in a steric conflict with the side chain of Phe^{313}. After a systematic search involving both Phe^{313} side chain and the fucose orientation, one possible mode of interaction was identified. The proposed docking mode is represented in Fig. 9*B*. The Phe^{313} side chain would adopt an orientation different from the one observed in the crystal structure of DC-SIGN complexed with Le^X. The new orientation allows for a strong "stacking" of the aromatic ring of Phe^{313} with the most hydrophobic face of fucose. Such interaction between sugar and planar side chains are commonly observed in protein-carbohydrate interactions.

The reorientation of the Phe^{313} side chain does not cost significant energy. This side chain adopts a different orientation when compared with DC-SIGN complexed with Le^X or with mannose oligosaccharide (30, 38). Furthermore, a recent crystallographic work demonstrated a large conformational change in an arginine residue side chain for stacking to a sugar derivative in a galectin structure (42).

S. mansoni ES Products Comprise Glycolipids with Le^X and Pseudo-Le^Y Epitopes—It remains to be investigated whether the cercarial glycosphingolipids that have been shown to bind to DC-SIGN *in vitro* are in a position that allows an interaction with DCs *in vivo* as well. However, DCs are expected to encounter ES products, a mixture of glycoproteins and glycolipids that is secreted when the cercariae transform to schis-

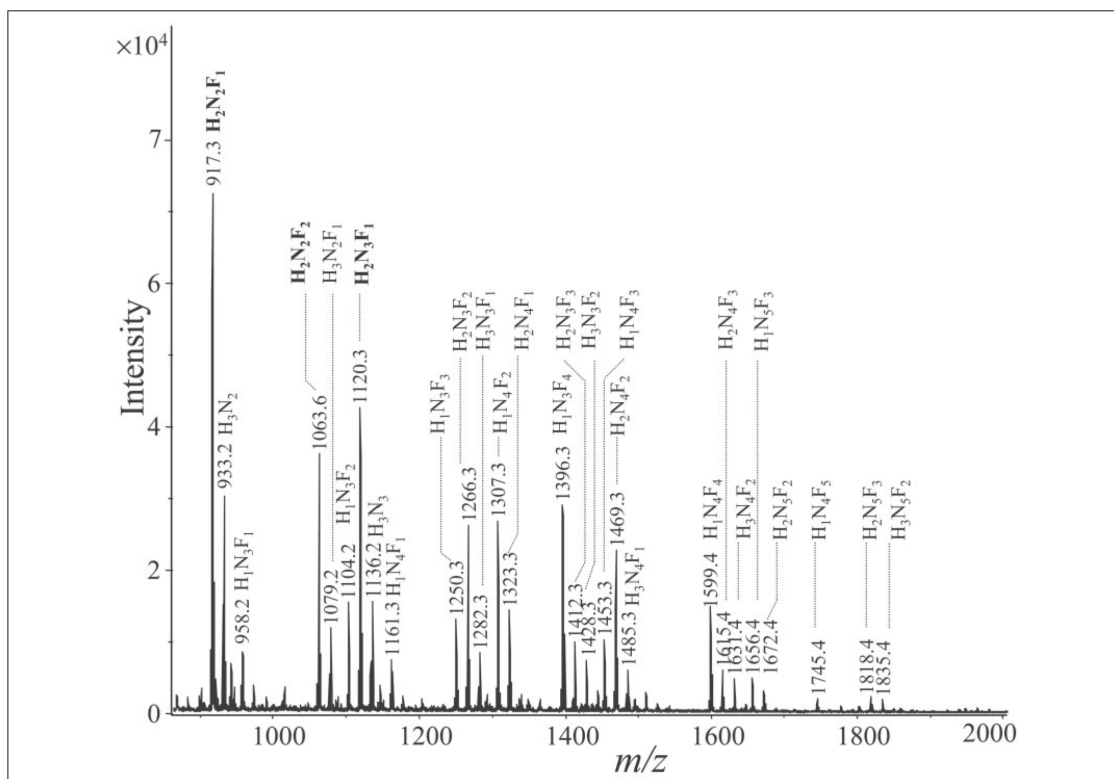

FIGURE 4. **MALDI-TOF-MS analysis of released oligosaccharides.** Oligosaccharides were released from cercarial glycolipids by treatment with endoglycoceramidase and analyzed by MALDI-TOF-MS. Monoisotopic masses of pseudomolecular ions ([M + Na]$^+$) and deduced monosaccharide compositions are assigned. Signals representing free Le^X pentasaccharide (*m/z* 917.3), Le^X hexasaccharide (*m/z* 1120.3) as well as pseudo-Le^Y hexasaccharide (*m/z* 1063.6) are marked in *bold type*. *H*, hexose; *N*, *N*-acetylhexosamine; *F*, deoxyhexose (fucose).

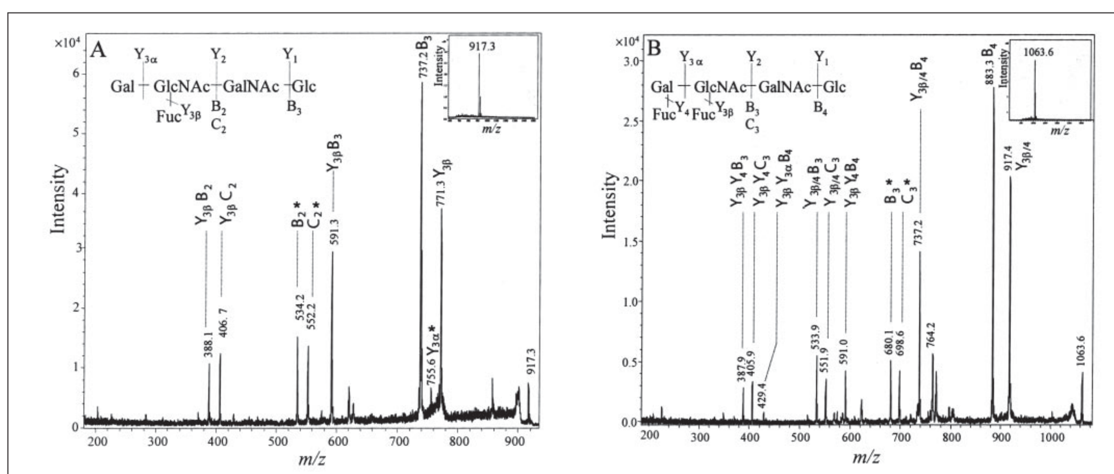

FIGURE 5. **MALDI-TOF-MS and MS/MS analysis of purified glycans with Le^X or pseudo-Le^Y units.** *A*, MALDI-TOF-MS/MS spectrum of the Le^X pentasaccharide (*m/z* 917.3 [M + Na]$^+$). Characteristic Le^X trisaccharide fragment ions (B_2 and C_2) and the diagnostically relevant $Y_{3\alpha}$ fragment are marked by *asterisks* (*). *B*, MALDI-TOF-MS/MS spectrum of the pseudo-Le^Y hexasaccharide (*m/z* 1063.6 [M + Na]$^+$). Characteristic pseudo-Le^Y tetrasaccharide fragment ions (B_3 and C_3) are again marked by *asterisks* (*). The signal at *m/z* 764.2 is assumed to arise from ring fragmentation accompanied by the loss of two water molecules ($^{0,2}A_4$-$2H_2O$). *Insets*, corresponding MS^1 spectra. Assignment of fragment ions is performed according to Domon and Costello (54).

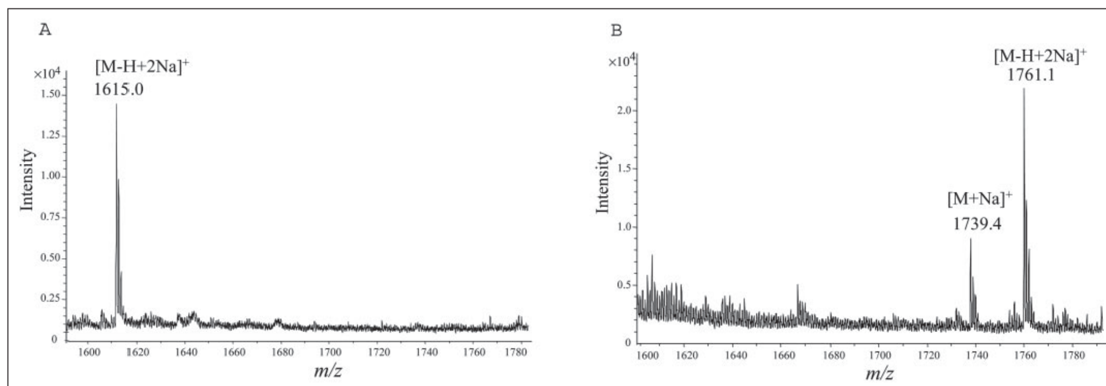

FIGURE 6. **MALDI-TOF-MS analysis of neoglycolipids containing Le^X or pseudo-Le^Y epitopes.** *A* and *B*, MALDI-TOF-MS spectra of Le^X (*m/z* 1615.0 [M-H + 2Na]$^+$) and pseudo-Le^Y (*m/z* 1739.4 [M + Na]$^+$ and 1761.1 [M-H + 2Na]$^+$) neoglycolipids, respectively.

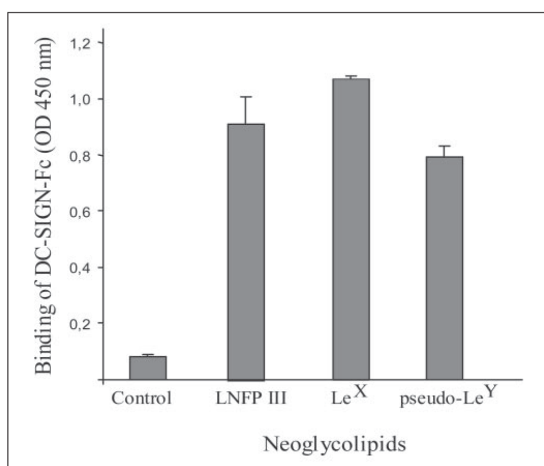

FIGURE 7. **DC-SIGN-Fc binds to Le^X and pseudo-Le^Y neoglycolipids.** Binding of DC-SIGN-Fc to schistosomal neoglycolipids was tested in ELISA. 10 ng of lacto-*N*-fucopentaose III neoglycolipid (*LNFP III*) was coated as positive control. In parallel, 8 ng of Le^X neoglycolipid (*Le^X*) and 8 ng of pseudo-Le^Y neoglycolipid (*pseudo-Le^Y*) were applied to each well. Binding of DC-SIGN-Fc to all neoglycolipids was completely inhibited by addition of EDTA (*Control*, only one example shown). Indicated S.D. are based on nine independent determinations.

tosomula. To determine whether the Le^X and pseudo-Le^Y containing glycosphingolipids are found within ES products, glycolipids were isolated from ES products collected *in vitro* from freshly transformed cercariae. Following treatment with endoglycoceramidase the released oligosaccharides were analyzed by MALDI-TOF-MS. The results revealed that ES product-derived glycolipids comprise species with Le^X and pseudo-Le^Y determinants together with a wide panel of extended oligofucosylated glycan species, many of which were also recovered in the cercarial glycolipid fractions (TABLE ONE).

DISCUSSION

In this study the interaction of DC-SIGN with *S. mansoni* glycolipids was investigated. In contrast to glycosphingolipids derived from eggs, glycosphingolipids of *S. mansoni* cercariae are bound by both recombinant and dendritic cell expressed DC-SIGN. Structural characterization of the glycolipids revealed that DC-SIGN binds two dominant cercarial glycosphingolipids, being Le^X-containing species and pseudo-Le^Y species (5). In contrast to Le^X that is found in both mammals and several pathogens, pseudo-Le^Y is an oligosaccharide determinant that may be unique for schistosomes (5). These are the first natural ligands identified for DC-SIGN in schistosomes, enabling follow-up studies to elucidate the function of the interaction between DC-SIGN and schistosome glycolipids in host immunity. The observation that egg glycolipids interacted poorly with DC-SIGN is in agreement with previous studies demonstrating that species with pseudo-Le^Y determinants are predominantly found in cercarial glycosphingolipids, whereas Le^X containing glycosphingolipids represent only a very small fraction of total egg stage glycosphingolipids (5).

Recently more insight was obtained into the ability of DC-SIGN to bind fucosylated ligands (31, 38). Analysis of crystals of the CRD of DC-SIGN bound to lacto-*N*-fucopentaose III (that comprises the Le^X trisaccharide) showed that the 3- and 4-OH groups of the α1–3-linked fucose form coordination bonds with Ca^{2+} in the primary binding site. In this position the fucose is close to Val^{351}, which forms tight van der Waals contacts with the 2-OH group, whereas the terminal galactose residue contacts the protein via Phe^{313} in a secondary binding site. From the proposed models it appears that Val^{351} in DC-SIGN is close to the fucose binding site and makes a strong hydrophobic contact with CH at position 1 and 2 of fucose (38). By molecular modeling, in combination with binding studies of cell-surface expressed recombinant wild-type and mutant forms of DC-SIGN and its homologue L-SIGN (liver/lymph node-specific ICAM-3 grabbing nonintegrin), we found very similar results for the binding mode of Le^X in DC-SIGN (31). Both models predict that a substituent on the 3-OH group of galactose would give a steric conflict with the side chain of Phe^{313}, which is line with the results of binding studies that showed that 3′-sialylation or sulfation of Le^X abrogates binding (43). However, in the studies described here, we observed binding of soluble DC-SIGN-Fc, as well as cellular expressed DC-SIGN, to pseudo-Le^Y that does carry a fucose α1–3-linked to galactose (5). To fit a fucose on position 3 of galactose into the model, it appeared necessary to slightly change the orientation of the side chain of Phe^{313}, a movement that does not cost significant energy. Furthermore, in this docking mode a perfect stacking with the hydrophobic side of the galactose-linked fucose is created. We propose that the secondary binding site of DC-SIGN is flexible due to the capacity of the side chain of Phe^{313} to change orientation, and that pathogens such as *S. mansoni* may use this property to target DC-SIGN. Recently, a similar change in

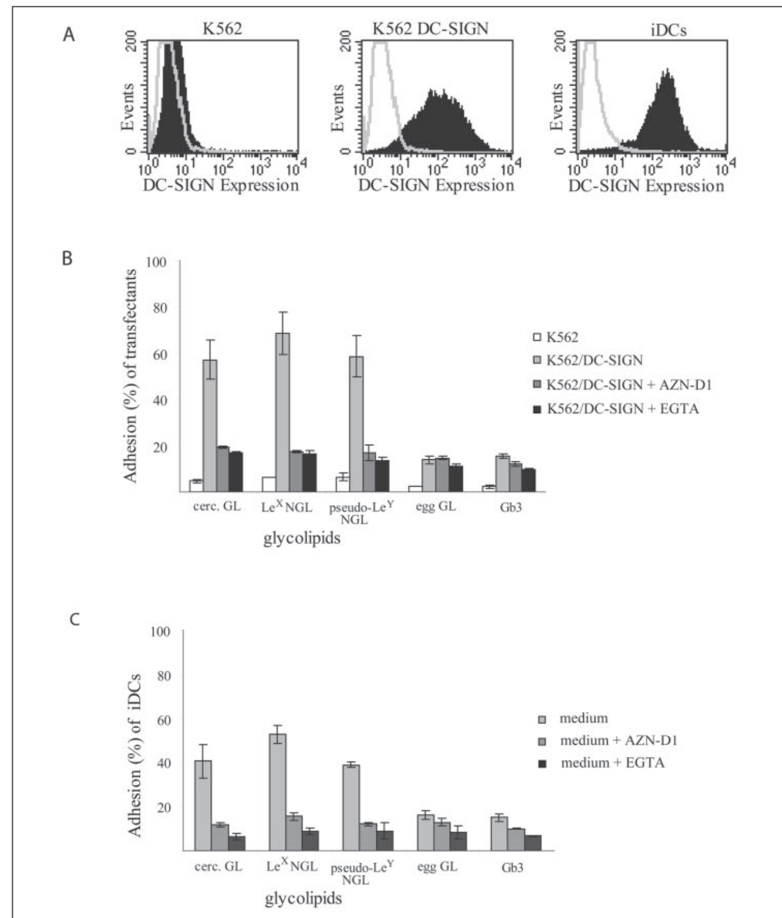

FIGURE 8. **DC-SIGN on human dendritic cells interacts with authentic *S. mansoni* cercarial glycolipids and Le^{X} and pseudo-Le^{Y} neoglycolipids.** The expression of DC-SIGN on transfectants and iDCs was determined by flow cytometry (*A*). Binding of DC-SIGN expressed on K562 transfectants (*B*) or iDCs (*C*) to glycolipids (*GL*) and neoglycolipids (*NGL*) was determined by plate adhesion assay in the presence or absence of EGTA, or a blocking mAb to DC-SIGN (*AZN-D1*). All results are representative of three independent experiments, performed in triplicate.

orientation has been demonstrated for the side chain of Arg^{144} in the CRD of galectin-3 upon ligand binding (42). The high resolution x-ray crystal structures of the CRD of human galectin-3 were solved in complex with *N*-acetyllactosamine (LacNAc) and a high affinity inhibitor. The structures showed that the side chain of Arg^{144} stacks against the aromatic moiety of the inhibitor, which was possible by a reorientation of the side chain relative to that seen in the complex with LacNAc.

Antigen-presenting cells, such as DCs and macrophages are the first immune cells that encounter invading pathogens and are crucially involved in the initiation and control of innate and adaptive immune responses (44). They often recognize pathogens through a wide array of molecules such as (glyco)lipids and acylated proteins or peptides. Interestingly, several studies indicate that glycolipids are capable to modulate the human immune system (45–47). The presence of lipid moieties within pathogen-derived products is essential for activation of specific pattern recognition receptors, in particular TLR2 (48). It was recently shown that schistosomal egg glycolipids induce production of pro- and anti-inflammatory cytokines in monocytes (24). By fractionating and purification of the lipids, the authors showed that mono-acetylated lysophosphatidylserine (lyso-PS) promotes the development of T regulatory cells via interaction with TLR2 on DCs. By contrast, di-acetylated phosphatidylserine promotes maturation of DC into a phenotype, termed DC2, which induces the development of Th2 responses (24).

Here we report that DCs interact with authentic cercarial glycosphingolipids comprising Le^{X} and pseudo-Le^{Y} via the CLR DC-SIGN. This indicates that DCs may likely interact with schistosomes early in infection. Schistosomes enter the human host in the cercarial stage, and these cercariae transform into schistosomula directly after penetration of the skin by shedding their glycocalyx and secretion of ES products. Analysis of the glycolipids derived from ES products showed that they comprise species with Le^{X} and pseudo-Le^{Y} determinants. These ES products that enter the surrounding tissue are good candidate antigens to be encountered by surveying DCs, such as the DC-SIGN positive CD1a negative dermal DCs, which are found mostly in the upper dermis (35, 49, 50).

A remarkable finding is that human DCs recognize Le^{X} and LDN-F glycan antigens within schistosomes (28), which can be considered as "self-glycan" antigens since they are also found on human glycoconjugates. It has been proposed that DC-SIGN, which also interacts with several "self-ligands" such as ICAM-2 and ICAM-3, may principally function in normal homeostasis, rather than being a true pattern recognition receptor (26). Current views are that pathogens target DC-SIGN

Binding of DC-SIGN to Pseudo-Lewis^Y

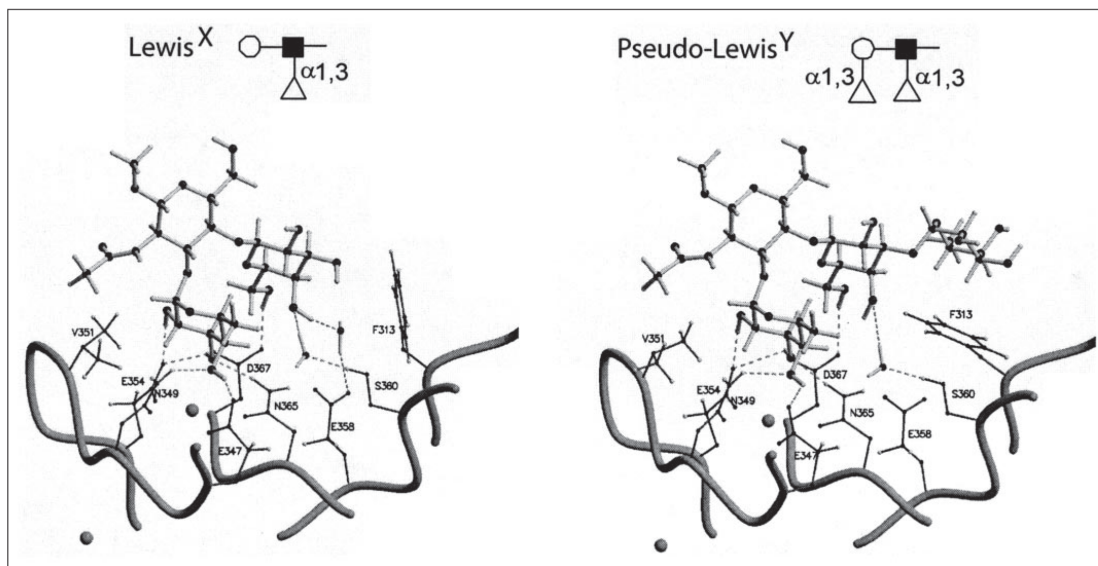

FIGURE 9. **Interaction of DC-SIGN with Le^X and pseudo-Le^Y.** Models of the interaction of DC-SIGN with Le^X trisaccharide (*A*) and pseudo-Le^Y tetrasaccharide (*B*). Calcium ions are represented by *gray spheres*. Only the amino acids interacting directly with the sugars have been displayed.

or other CLRs to promote immune escape (51). For example, *Mycobacterium tuberculosis* secretes glycoconjugates that are recognized by DC-SIGN to down-regulate TLR-induced immune activation (52). Pathogens like HIV-1 have many strategies to evade immune recognition or to modulate immune responses to survive in their hosts. In HIV-1 infection, DC-SIGN plays a role in internalization of the virus into DCs, but instead of being routed to the lysosomal compartment for degradation, part of the infectious virus remains hidden in the DC, to subsequently infect target cells (51). Schistosomes survive for many years in the host despite a pronounced immune response, indicating that these helminths have effective strategies to escape or suppress the host immune system. In a mouse model system, SEA and its major glycan antigen Le^X can induce a Th2-mediated immune response, which is associated with persistence of the pathogen (53). Our data here show that DC-SIGN does not only recognize the self-glycan ligand Le^X within cercarial glycolipids, but also glycolipids carrying pseudo-Le^Y, a non-self structure that so far is only found within schistosome cercarial glycolipids (5) and ES products (this study). Pseudo-Le^Y may be regarded as a glycan antigen that mimics a self-glycan to fit within the CRD of DC-SIGN. The abundant expression of such self-glycans or glycan antigens that mimic self-glycans, may allow schistosomes to mislead the host immune system by down-regulating DC function in all stages of infection. However, DC-SIGN has been shown to internalize schistosome glycoconjugates[3] and could also play a role in processing of these glycoconjugates and antigen presentation. Because currently more than 200 million people have schistosomiasis, it is challenging to understand the central role of DCs in both the strong immune response that is evoked upon infection, as well as in the immune evasion and suppression mechanisms that are exploited by the schistosomes.

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REFERENCES

1. Pearce, E. J., and MacDonald, A. S. (2002) *Nat. Rev. Immunol.* **2,** 499–511
2. Cummings, R. D., and Nyame, A. K. (1999) *Biochim. Biophys. Acta* **1455,** 363–374
3. Hokke, C. H., and Deelder, A. M. (2001) *Glycoconj. J.* **18,** 573–587
4. Makaaru, C. K., Damian, R. T., Smith, D. F., and Cummings, R. D. (1992) *J. Biol. Chem.* **267,** 2251–2257
5. Wuhrer, M., Dennis, R. D., Doenhoff, M. J., Lochnit, G., and Geyer, R. (2000) *Glycobiology* **10,** 89–101
6. Wuhrer, M., Dennis, R. D., Doenhoff, M. J., Bickle, Q., Lochnit, G., and Geyer, R. (1999) *Mol. Biochem. Parasitol.* **103,** 155–169
7. Wuhrer, M., Dennis, R. D., Doenhoff, M. J., and Geyer, R. (2000) *Biochim. Biophys. Acta* **1524,** 155–161
8. Weiss, J. B., Magnani, J. L., and Strand, M. (1986) *J. Immunol.* **136,** 4275–4282
9. Khoo, K. H., Chatterjee, D., Caulfield, J. P., Morris, H. R., and Dell, A. (1997) *Glycobiology* **7,** 653–661
10. Khoo, K. H., Sarda, S., Xu, X., Caulfield, J. P., McNeil, M. R., Homans, S. W., Morris, H. R., and Dell, A. (1995) *J. Biol. Chem.* **270,** 17114–17123
11. Srivatsan, J., Smith, D. F., and Cummings, R. D. (1992) *J. Biol. Chem.* **267,** 20196–20203
12. Nyame, K., Smith, D. F., Damian, R. T., and Cummings, R. D. (1989) *J. Biol. Chem.* **264,** 3235–3243
13. Van Remoortere, A., Hokke, C. H., Van Dam, G. J., Van Die, I., Deelder, A. M., and Van den Eijnden, D. H. (2000) *Glycobiology* **10,** 601–609
14. Wuhrer, M., Kantelhardt, S. R., Dennis, R. D., Doenhoff, M. J., Lochnit, G., and Geyer, R. (2002) *Eur. J. Biochem.* **269,** 481–493
15. Van Dam, G. J., Bergwerff, A. A., Thomas-Oates, J. E., Rotmans, J. P., Kamerling, J. P., Vliegenthart, J. F., and Deelder, A. M. (1994) *Eur. J. Biochem.* **225,** 467–482
16. Velupillai, P., and Harn, D. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 18–22
17. Velupillai, P., dos Reis, E. A., dos Reis, M. G., and Harn, D. A. (2000) *Hum. Immunol.* **61,** 225–232
18. Okano, M., Satoskar, A. R., Nishizaki, K., and Harn, D. A., Jr. (2001) *J. Immunol.* **167,** 442–450
19. Figdor, C. G., Van Kooyk, Y., and Adema, G. J. (2002) *Nat. Rev. Immunol.* **2,** 77–84
20. Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R. W., Kastelein, R. A., Bazan, F., and Liu, Y. J. (2001) *J. Exp. Med.* **194,** 863–869
21. Michelsen, K. S., Aicher, A., Mohaupt, M., Hartung, T., Dimmeler, S., Kirschning, C. J., and Schumann, R. R. (2001) *J. Biol. Chem.* **276,** 25680–25686
22. Visintin, A., Mazzoni, A., Spitzer, J. H., Wyllie, D. H., Dower, S. K., and Segal, D. M. (2001) *J. Immunol.* **166,** 249–255
23. Hertz, C. J., Kiertscher, S. M., Godowski, P. J., Bouis, D. A., Norgard, M. V., Roth, M. D., and Modlin, R. L. (2001) *J. Immunol.* **166,** 2444–2450
24. Van der Kleij, D., Latz, E., Brouwers, J. F., Kruize, Y. C., Schmitz, M., Kurt-Jones, E. A.,

Espevik, T., de Jong, E. C., Kapsenberg, M. L., Golenbock, D. T., Tielens, A. G., and Yazdanbakhsh, M. (2002) *J. Biol. Chem.* **277,** 48122–48129
25. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) *Immunol. Rev.* **163,** 19–34
26. Geijtenbeek, T. B., Van Vliet, S. J., Engering, A., 't Hart, B. A., and Van Kooyk, Y. (2004) *Annu. Rev. Immunol.* **22,** 33–54
27. Van Kooyk, Y., and Geijtenbeek, T. B. (2003) *Nat. Rev. Immunol.* **3,** 697–709
28. Van Die, I., Van Vliet, S. J., Nyame, A. K., Cummings, R. D., Bank, C. M., Appelmelk, B., Geijtenbeek, T. B., and Van Kooyk, Y. (2003) *Glycobiology* **13,** 471–478
29. Feinberg, H., Guo, Y., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2005) *J. Biol. Chem.* **280,** 1327–1335
30. Feinberg, H., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2001) *Science* **294,** 2163–2166
31. Van Liempt, E., Imberty, A., Bank, C. M., Van Vliet, S. J., Van Kooyk, Y., Geijtenbeek, T. B., and Van Die, I. (2004) *J. Biol. Chem.* **279,** 33161–33167
32. Geijtenbeek, T. B., Van Duijnhoven, G. C., Van Vliet, S. J., Krieger, E., Vriend, G., Figdor, C. G., and Van Kooyk, Y. (2002) *J. Biol. Chem.* **277,** 11314–11320
33. Sallusto, F., and Lanzavecchia, A. (1994) *J. Exp. Med.* **179,** 1109–1118
34. Geijtenbeek, T. B., Kwon, D. S., Torensma, R., Van Vliet, S. J., Van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and Van Kooyk, Y. (2000) *Cell* **100,** 587–597
35. Geijtenbeek, T. B., Torensma, R., Van Vliet, S. J., Van Duijnhoven, G. C., Adema, G. J., Van Kooyk, Y., and Figdor, C. G. (2000) *Cell* **100,** 575–585
36. Stoll, M. S., Mizuochi, T., Childs, R. A., and Feizi, T. (1988) *Biochem. J.* **256,** 661–664
37. Sluiters, J. F., Brussaard-Wust, C. M., and Meuleman, E. A. (1980) *Z. Parasitenkd.* **63,** 13–26
38. Guo, Y., Feinberg, H., Conroy, E., Mitchell, D. A., Alvarez, R., Blixt, O., Taylor, M. E., Weis, W. I., and Drickamer, K. (2004) *Nat. Struct. Mol. Biol.* **11,** 591–598
39. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res.* **28,** 235–242
40. Imberty, A., Mikros, E., Koca, J., Mollicone, R., Oriol, R., and Perez, S. (1995) *Glycoconj. J.* **12,** 331–349
41. Clark, M., Cramer, R. D. I., and Van den Opdenbosch, N. J. (1989) *J. Comput. Chem.* **10,** 982–1012
42. Sorme, P., Arnoux, P., Kahl-Knutsson, B., Leffler, H., Rini, J. M., and Nilsson, U. J. (2005) *J. Am. Chem. Soc.* **127,** 1737–1743
43. Appelmelk, B. J., Van Die, I., Van Vliet, S. J., Vandenbroucke-Grauls, C. M., Geijtenbeek, T. B., and Van Kooyk, Y. (2003) *J. Immunol.* **170,** 1635–1639
44. Janeway, C. A., Jr., and Medzhitov, R. (2002) *Annu. Rev. Immunol.* **20,** 197–216
45. Dyatlovitskaya, E. V., and Bergelson, L. D. (1987) *Biochim. Biophys. Acta* **907,** 125–143
46. Ziegler-Heitbrock, H. W., Kafferlein, E., Haas, J. G., Meyer, N., Strobel, M., Weber, C., and Flieger, D. (1992) *J. Immunol.* **148,** 1753–1758
47. Lochnit, G., Dennis, R. D., Ulmer, A. J., and Geyer, R. (1998) *J. Biol. Chem.* **273,** 466–474
48. Lee, H. K., Lee, J., and Tobias, P. S. (2002) *J. Immunol.* **168,** 4012–4017
49. Ebner, S., Ehammer, Z., Holzmann, S., Schwingshackl, P., Forstner, M., Stoitzner, P., Huemer, G. M., Fritsch, P., and Romani, N. (2004) *Int. Immunol.* **16,** 877–887
50. Soilleux, E. J., Morris, L. S., Leslie, G., Chehimi, J., Luo, Q., Levroney, E., Trowsdale, J., Montaner, L. J., Doms, R. W., Weissman, D., Coleman, N., and Lee, B. (2002) *J. Leukoc. Biol.* **71,** 445–457
51. Van Kooyk, Y., Engering, A., Lekkerkerker, A. N., Ludwig, I. S., and Geijtenbeek, T. B. (2004) *Curr. Opin. Immunol.* **16,** 488–493
52. Geijtenbeek T.B., Van Vliet, S. J., Koppel E.A., Sanchez-Hernandez M., Vandenbroucke-Grauls C.M., Appelmelk, B., and Van Kooyk, Y. (2002) *J. Exp. Med.* **197,** 7–17
53. Okano, M., Satoskar, A. R., Nishizaki, K., Abe, M., and Harn, D. A., Jr. (1999) *J. Immunol.* **163,** 6712–6717
54. Domon, B., and Costello, C. E. (1988) *Biochemistry* **27,** 1534–1543

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One-step biotinylation procedure for carbohydrates to study carbohydrate–protein interactions

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Abstract

Protein–carbohydrate interactions play crucial roles in numerous biological processes. To study these interactions, we developed a simple and fast procedure for the biotinylation of carbohydrates based on reductive amination. The method allows complete and stable biotinylation of small quantities of oligosaccharides and includes a rapid and simple procedure to remove excess labeling reagent. After biotinylation, the structural and biological integrity of the glycans was intact as determined by HPLC, mass spectrometry, and a plant lectin assay. By using the human C-type lectin DC–SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin), we demonstrate that the biotinylated glycans can be used in a glycan array to determine binding specificities of lectins. Moreover, we show that fluorescent beads coated with selected biotinylated glycans bind to DC–SIGN-expressing dendritic cells in vitro. Finally, by using biotinylated high-mannose *N*-glycans, we could visualize DC–SIGN-expressing cells in lymph node tissue. The availability of easy biotinylation methods for oligosaccharides such as those described here greatly facilitates the functional analysis of lectins. In addition, the biotinylated glycans will be great tools for investigating functional lectin receptors in situ.
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Keywords: Biotinylated carbohydrates; Protein–carbohydrate interactions; Lectins; Glycan array; DC–SIGN; In situ staining

Lectins are carbohydrate-binding proteins with a principal function in the recognition of carbohydrates present at cell surfaces, attached to circulating proteins, and present in extracellular matrices. By specifically binding to the carbohydrates, lectins mediate biological events such as cell–cell adhesion, host–pathogen recognition, serum glycoprotein turnover, and innate immune responses. Consequently, interest in studying lectin–carbohydrate interactions is increasing, and a wide variety of techniques that can be used to study these interactions are being developed.

Biotin has been widely used for investigating receptor–ligand interactions [1,2]. The use of biotin for labeling biological compounds was first described for localizing membrane proteins by electron microscopy [3], making use of the very high affinity ($10^{15}\,M^{-1}$) of avidin for biotin [4,5]. It has since been used to study the expression and cellular distribution of membrane proteins and glycoproteins, thereby largely replacing the hazardous radioactive iodine labeling. A number of biotinylation procedures in which the label is attached to carbohydrates using oxidative methods have been developed [6,7]. These methods, however, affect the structural integrity of the glycan and therefore may influence the biological function of carbohydrates and their binding properties to lectins. During recent years, a number of nondestructive biotinylation procedures involving the coupling of a biotin group via an amine- or hydrazide-containing linker to the reducing end of purified or synthesized carbohydrates have been described [8–14]. In the first article describing such a method, the label BAP, containing both a biotin and a fluorescent group, was

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coupled via reductive amination to the reducing ends of carbohydrates and permitted detection of carbohydrates during HPLC analysis [8,9]. A drawback of this method, however, is the elaborate preparation of BAP, which is synthesized by coupling biotin–NHS (*N*-hydroxysuccinimide ester)[1] to diaminopyridine (DAP), requiring a preparative chromatography step to separate the formed label from the reactants. Also, the coupling efficiency of BAP to carbohydrates was rather low. Recently, an alternative to this method in which DAP is first coupled to the reducing ends of carbohydrates was presented. Sample purification is obtained by paper chromatography. In a second reaction step, DAP-labeled carbohydrates were coupled to biotin–NHS and purified by HPLC [13]. A much-simplified procedure was developed using the label BNAH, which was coupled to carbohydrates in a one-step reaction, eliminating the elaborate label synthesis. Sample purification again was performed by HPLC [14]. This label showed excellent properties in studying carbohydrate–protein interactions. To our knowledge, however, the BNAH label no longer is commercially available.

Here we describe a one-step procedure for the biotinylation of carbohydrates that differs from previous procedures in its simplicity and efficiency, allowing the method to be used in both chemistry- and biology-oriented laboratories. Using readily available reagents, a variety of carbohydrates are biotinylated and are characterized by anion-exchange chromatography and mass spectrometry and tested in a plant lectin assay. We demonstrate that the biotinylated glycans can be used to determine the binding specificity of isolated lectins in a glycan array as well as to identify lectins expressed on cells using flow cytometry or in human tissue by in situ staining.

Materials and methods

Reagents

Biotinamidocaproyl hydrazide (biotin-LC-hydrazide) and Reacti-Bind streptavidin-coupled microtiter plates were obtained from Pierce Biotechnology (Rockford, IL, USA). Dimethyl sulfoxide over molecular sieves and sodium cyanoborohydride were obtained from Sigma–Aldrich (St. Louis, MO, USA). QM-A quartz microfiber filter was purchased from Whatman International (Maidstone, Kent, UK). 6-Aza-2-thiothymine was purchased from Sigma–Aldrich (Taufkirchen, Germany). Digoxigenin-conjugated lectins (concanavalin A [ConA], *Aleuria aurantia* lectin [AAL], *Sambucus nigra* agglutinin [SNA], and *Datura stramonium* agglutinin [DSA]) were purchased from Boehringer (Mannheim, Germany). Bovine serum albumin (BSA) was obtained from Calbiochem (Merck, Darmstadt, Germany). Peroxidase-labeled goat anti-mouse IgG/IgM and peroxidase-labeled goat anti-human IgG–Fc secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Anti-digoxigenin POD Fab fragments were purchased from Roche (Mannheim, Germany). Biotinylated polyacrylamide-coupled Lewis^x was obtained from Lectinity (Lappeenranta, Finland). Streptavidin-coated fluorescent beads (488/645 nm), streptavidin–Alexa 488, goat anti-mouse–Alexa 594, and Hoechst were purchased from Molecular Probes (Eugene, OR, USA).

Oligosaccharides

Maltohexaose and maltoheptaose were purchased from Sigma–Aldrich (St. Louis, MO, USA). Man9 was purchased from Dextra Laboratories (Reading, UK). 2′Fucosyllactose was obtained from Iso Sep AB (Tullinge, Sweden). $Fuc_2GlcNAc_2Man_3$ was a kind gift from H. Lönn (Biocarb, Lund, Sweden).

Asialo-diantennary, asialo-triantennary, and asialo-tetra-antennary glycans were isolated from α_1-acid glycoprotein (AGP). AGP (1 g) was desialylated in 0.1 M trifluoroacetic acid at 80 °C for 1 h. Desialylated AGP was then dissolved in 50 ml Tris–HCl (50 mM, pH 7.3) containing 50 mM EDTA, 200 mM β-mercaptoethanol, 1 g SDS, 2 g Nonidet NP40, and 80,000 units PNGase F (New England Biolabs, Ipswich, MA, USA) and incubated at 37 °C for 72 h. Released glycans were isolated by precipitating the protein after the addition of trichloroacetic acid. The supernatant was washed with chloroform. Glycans were desalted on a Bio-Gel P-4 gel filtration column (2.5 × 80 cm, Bio-Rad Laboratories, Hercules, CA, USA), and glycoforms were separated by Bio-Gel P-6 gel filtration chromatography (1.6 × 200 cm). In both cases, 50 mM ammonium acetate (50 mM, pH 5.2) was used as a mobile phase. Eluting glycans were detected using the phenol–sulfuric acid assay [15]. Glycan fractions were purified to homogeneity by HPLC on a Spherisorb-NH_2 column (Phase Sep, Clwyd, UK) using a linear gradient of 0.3%/min of potassium phosphate (15 mM, pH 5.2) in acetonitrile starting at 80% acetonitrile. The flow rate was 5.0 ml/min, and the eluent was monitored at 195 nm. Collected fractions were desalted on a Bio-Gel P-2 column (1.5 × 60 cm) using ammonium acetate (50 mM, pH 5.2) as a mobile phase.

Man5 was isolated from the urine of swainsonine-intoxicated sheep as described by Bakker and coworkers [16], and lacto-*N*-fucopentaose I (LND-I) was isolated from human milk according to Neeleman and coworkers [17]. Core

[1] *Abbreviations used:* NHS, *N*-hydroxysuccinimide ester; DAP, diaminopyridine; biotin-LC-hydrazide, biotinamidocaproyl hydrazide; ConA, concanavalin A; AAL, *Aleuria aurantia* lectin; SNA, *Sambucus nigra* agglutinin; DSA, *Datura stramonium* agglutinin; BSA, bovine serum albumin; AGP, α_1-acid glycoprotein; LND-I, lacto-*N*-fucopentaose I; LDN, GalNAcβ1-4GlcNAc; ES–MS, electrospray mass spectrometry; DMSO, dimethyl sulfoxide; HPAEC, high-performance anion-exchange chromatography; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TMB, 3,3′,5,5′-tetramethylbenzidine; DC–SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin; RT, room temperature; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; CID–MS^2, collision-induced dissociation tandem mass spectrometry; DC, dendritic cell.

fucose-containing asialo-diantennary glycan was isolated from human IgG according to Narasimhan and coworkers [18] and was degalactosylated using jack bean β-galactosidase. The resulting core fucose-containing asialo-agalacto-diantennary glycan was separated from its bisecting form using ConA affinity chromatography [19]. Trisialyl-triantennary glycan was prepared by sialylation of asialo-triantennary glycan using bovine colostrum CMP-sialic acid:Galβ 1-4GlcNAc-R α2-6-sialyltransferase according to Joziasse and coworkers [20]. The disaccharide GalNAcβ 1-4GlcNAc (LDN) was prepared enzymatically using GlcNAc as an acceptor and partially purified *Lymnaea stagnalis* UDP-GalNAc:GlcNAcβ-R β1-4*N*-acetylgalactosaminyltransferase as an enzyme source as described previously [21].

All glycans were analyzed by electrospray mass spectrometry (ES–MS). In addition, all isolated glycans were analyzed by ^{1}H NMR spectroscopy.

Coupling of carbohydrates to biotin-LC-hydrazide

Biotin-LC-hydrazide (4.6 mg/12 μmol) was dissolved in dimethyl sulfoxide (DMSO, 70 μl) by vigorous mixing and heating at 65 °C for 1 min. Glacial acetic acid (30 μl) was added, and the mixture was poured onto sodium cyanoborohydride (6.4 mg/100 μmol), which dissolved completely after heating at 65 °C for approximately 1 min. Then 5 to 8 μl of the reaction mixture was added to the dried oligosaccharides (1–100 nmol) to obtain a 10-fold or greater molar excess of label over reducing ends. The reaction was carried out at 65 °C for 2 h, after which the samples were purified immediately. In labeling experiments without reduction, sodium cyanoborohydride was omitted and the samples were allowed to react at 65 °C for 2.5 h.

Sample cleanup

Sample purification cartridges were prepared by cutting two filter discs (~12 mm in diameter) from Whatman QM-A Quartz Microfiber Filter using a hole punch and placing them in a holder (e.g., the housing of a 5-ml syringe). Each cartridge was washed successively with water, 30% acetic acid in water, and acetonitrile before applying the sample. The sample was distributed uniformly on the filter surface and allowed to bind for 15 min before reactants were removed by rinsing with acetonitrile (1 ml) and 4% water in acetonitrile (6 × 1 ml). Labeled products were eluted with water (4 × 0.5 ml), filtered through 0.2-μm syringe filters, and dried in a SpeedVac.

High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex BioLC system equipped with a GS 50 gradient pump and an ED 50 electrochemical detector (Dionex, Sunnyvale, CA, USA). A 2 × 250-mm Carbopac PA-200 column was used for analytical HPAEC using a linear gradient from 0 to 30% B (500 mM NaOAc in 100 mM NaOH) in A (100 mM NaOH) over 20 min. The column was thermostated at 30 °C. A flow rate of 0.5 ml/min was used, and 200-pmol amounts of carbohydrate were injected for analysis.

Mass spectrometry

Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) was performed on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a nitrogen laser and a LIFT–MS/MS facility. The instrument was operated in the positive ion reflectron mode using 6-aza-2-thiothymine as matrix [22]. Approximately 100 to 500 spectra were summarized in each case. Compass 1.1, consisting of FlexControl 2.4 and FlexAnalysis 2.4, was used as instrument control and processing software.

Electrospray ionization

ES–MS experiments were performed on an LCQ DecaXP ion trap mass spectrometer equipped with a nano-ES ionization source (Thermo Finnigan, San Jose, CA, USA). Biotinylated samples were dissolved in methanol/water (1:1) to a concentration of approximately 10 pmol/μl. For each experiment, 2 μl was loaded onto a gold-coated glass capillary nano-ES emitter (Proxeon, Odense, Denmark). The capillary temperature was set to 180 °C. Spectra were taken in the positive ion mode with a spray voltage of 1.5 kV and a capillary voltage of 22.5 V or in the negative ion mode with a spray voltage of 1.0 kV and a capillary voltage of −47.5 V.

Plant lectin assay

Biotinylated carbohydrates were diluted (50 pmol each, 50 μl/well) in 1% BSA in TSM (20 mM Tris–HCl [pH 7.4] containing 150 mM NaCl, 1 mM $CaCl_2$, and 2 mM $MgCl_2$) and coated at 37 °C for 1 h in streptavidin-coupled plates. Plates were blocked with 1% BSA in TSM (100 μl/well), and digoxigenin-conjugated lectins (2 μg/ml, 50 μl/well) were added. The adhesion was performed at 37 °C for 1 h. In between the different incubation steps, plates were washed with TSM containing 0.1% Tween 20. Bound lectins were detected by incubation with 1:2500 diluted anti-digoxigenin peroxidase-labeled Fab fragments (37 °C for 1 h, 50 μl/well). Binding was detected by incubation with 1:2500 diluted peroxidase-labeled anti-digoxigenin Fab fragments (37 °C for 1 h, 50 μl/well) and 3,3′,5,5′-tetramethylbenzidine (TMB) development. The color reaction was stopped by the addition of 0.8 M H_2SO_4, and the emission at 450 nm was recorded.

Glycan array for DC–SIGN binding and bead adhesion assay

Biotinylated carbohydrates were coated (100 pmol/well in 50 μl in TSM) at 37 °C for 1 h in streptavidin-coupled

plates. Biotinylated polyacrylamide-coupled Lewis[x] was used as a positive control (1 µg/ml). In between the different incubation steps, plates were washed with TSM. Coated plates were incubated with dendritic cell-specific ICAM-3-grabbing nonintegrin (DC–SIGN)-Fc (1 µg/ml in TSM, 50 µl/well) in the presence or absence of EGTA (10 mM) or anti-DC–SIGN antibodies (AZN-D1, 20 µg/ml) at room temperature (RT) for 2 h. Binding was detected using an enzyme-linked immunosorbent assay (ELISA)-based assay using peroxidase-labeled anti-human IgG-Fc antibody and TMB development. The color reaction was stopped by the addition of 0.8 M H_2SO_4, and the emission at 450 nm was recorded.

Streptavidin-coated fluorescent beads (100×10^6) were incubated with 500 pmol of the biotinylated carbohydrates or 1 µg of the polyacrylamide-coupled Lewis[x] at 4 °C overnight in 300 µl of phosphate-buffered saline (PBS) containing 0.5% BSA. Fluorescent bead adhesion assay was performed as described previously [23], analyzed on FAC Scalibur, and presented as the percentage of cells that had bound the fluorescent beads.

In situ tissue staining

Nonfixed frozen tissue sections (7 µm thickness) were incubated at 37 °C for 2 h with 500 pmol of the biotinylated carbohydrates in 100 µl of TSM/0.5% BSA per section. Sections were washed gently with warm TSM buffer. All subsequent steps were performed at RT for 30 min, followed by gentle washes with TSM. Binding of the biotinylated carbohydrates was detected using streptavidin–Alexa 488 (1:400 dilution in TSM/0.5% BSA, 100 µl/section). Sections were counterstained with rabbit polyclonal anti-DC–SIGN antibodies (1:250 dilution in TSM/0.5% BSA, 100 µl/section) and goat anti-mouse–Alexa 594 (Molecular Probes, 1:400 dilution in TSM/0.5% BSA, 100 µl/section). Nuclei were visualized using Hoechst (RT for 10 min).

Results

Biotin labeling of carbohydrates

The labeling procedure is based on reductive amination in which a primary amine is coupled to an aldehyde to form an imine or a hydrazone if the primary amine is present as a hydrazide group. This imine (hydrazone) is then reduced to a secondary amine, which stabilizes the formed linkage (Fig. 1). Using this reaction procedure, biotin-LC-hydrazide can be readily coupled to the reducing end of any carbohydrate to form a stable biotin-labeled product. To ensure that all reactants were soluble under reaction conditions, DMSO was chosen as a solvent. Efficient and simple sample purification was obtained by binding the hydrophilic sample to a quartz paper membrane. Excess of hydrophobic biotin label was removed by rinsing the paper with acetonitrile. The efficiency of this procedure was investigated by HPLC, which demonstrated the complete removal of unreacted biotin-LC-hydrazide (data not shown).

Labeling efficiency

To investigate the efficiency for the biotinylation procedure, maltohexaose was biotinylated and was analyzed by HPAEC and MALDI–TOF MS. Biotinylated maltohexaose eluted on HPAEC predominantly as a single peak with

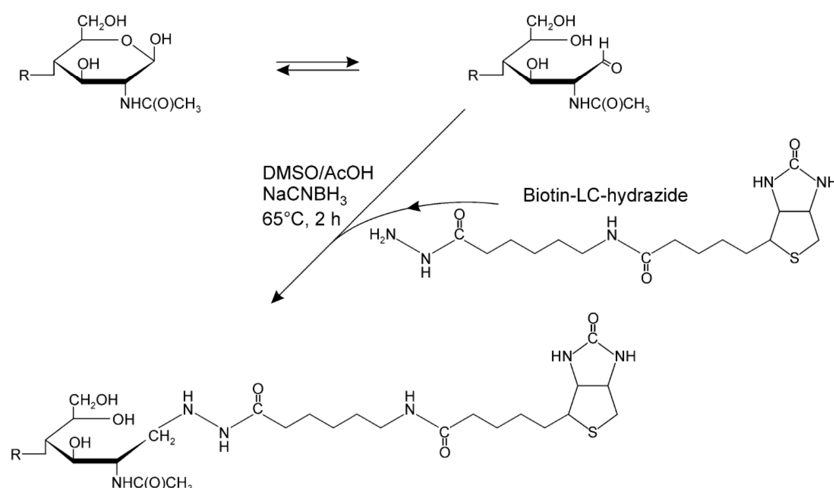

Fig. 1. Coupling of biotin-LC-hydrazide to the reducing ends of carbohydrates. In solution, a semiacetal is formed and reacts with the hydrazide group of biotin-LC-hydrazide to form a hydrazone linkage. In the presence of the reductant $NaCNBH_3$, the hydrazone linkage is reduced to form the stable biotinylated end product.

a retention time different from that of native maltohexaose (Fig. 2A; cf. profile II with profile I). Residual unlabeled maltohexaose was not observed, indicating that quantitative labeling was obtained. Further information on the labeling efficiency of the biotinylation procedure was obtained by MALDI–TOF MS. The MALDI mass spectrum of biotinylated maltohexaose shows a cluster of singly charged pseudomolecular ions corresponding to the proton, sodium, and potassium adducts of the hexasaccharide at *m/z* 1346.1, 1368.1, and 1384.1, respectively (Fig. 2B, lower spectrum). Signals corresponding to unlabeled maltohexaose (Fig. 2B, upper spectrum) were hardly visible, another indication that virtual complete biotinylation could be achieved using the described labeling procedure.

Derivatization and analysis of different types of carbohydrates

To determine whether the biotinylation procedure labels reducing carbohydrates regardless of their chemical composition, a number of standard glycans and glycoprotein-derived glycans, listed in Table 1, were biotinylated and analyzed by ES–MS. Monoisotopic molecular masses were determined by recording high-resolution zoom scans of individual conjugates and corresponded well to theoretical values (Table 1). Ions corresponding to nonreacted glycans were not detected, indicating that the biotinylation was effective for all glycans. To determine the chemical structure of the labeled glycans, collision-induced dissociation tandem mass spectrometry (CID–MS^2) was performed. The CID tandem mass spectrum of one of the compounds, biotinylated asialo-triantennary, is depicted in Fig. 3A, clearly demonstrating the presence of a single biotin label at the former reducing end of the glycan.

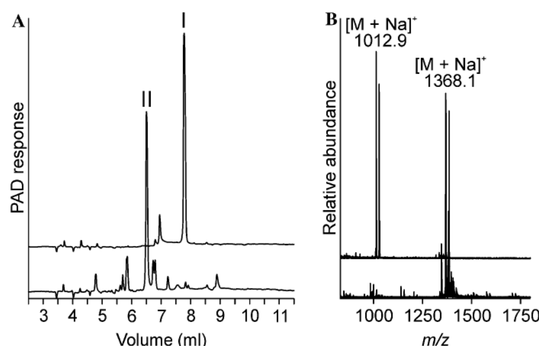

Fig. 2. Labeling efficiency and purity of labeled maltohexaose as determined by HPAEC and MALDI–TOF MS. (A) Maltohexaose (I) and maltohexaose-LC-biotin (II) (200 pmol of each) analyzed by HPAEC eluted at different volumes, indicating that nearly all starting material has been biotinylated. (B) Both native (upper spectrum) and biotinylated (lower spectrum) maltohexaose were analyzed by MALDI–TOF MS. Molecular masses corresponding to sodiated pseudomolecular ions are indicated.

The chemical stability of glycans under reaction conditions was also investigated using mass spectrometry. Sialic acid-containing glycans are especially susceptible to hydrolysis at elevated temperature. Desialylation can be observed by mass spectrometry by the loss of 291 Da from the molecular mass. When analyzing biotinylated trisialylated triantennary glycan in negative ion mode ES–MS, two ions of high abundance were observed at *m/z* 1515 and 1010, corresponding to doubly and triply deprotonated pseudomolecular ions (Fig. 3B). In addition, an ion of low abundance was observed at *m/z* 1370, corresponding to a biotinylated disialylated triantennary glycan, indicating some loss of a single sialic acid residue. Ions corresponding to monosialylated or nonsialylated forms of the biotinylated glycan were not observed in either negative or positive ion mode ES–MS, indicating that desialylation is very limited.

Together, these data suggest that the biotinylation procedure nonselectively labels several types of oligosaccharides and does not degrade the glycan structure significantly.

Binding of biotinylated carbohydrates to plant lectins

To evaluate the recognition by plant lectins, biotinylated glycans were immobilized in streptavidin-coupled microtiter plates and probed with digoxigenin-conjugated lectins. Bound lectins were detected with anti-digoxigenin peroxidase-labeled antibody. The results of this lectin-binding assay are summarized in Table 2.

Concanavalin A

Of the biotinylated carbohydrates tested, high-mannose glycans Man5 and Man9 as well as the asialo-diantennary glycan and the asialo-agalacto diantennary glycan with core fucose showed strong binding to ConA, whereas the asialo-triantennary glycan with only a single 2-substituted mannose residue and the asialo-tetraantennary glycan without monosubstituted mannose residues were not recognized. This is in agreement with previously published results on the binding specificity of ConA [25–29].

Aleuria aurantia lectin

In the binding experiments with AAL, which recognizes terminal fucose residues [29,30], LND-I and the difucosylated $Fuc_2GlcNAc_2Man_3$-biotin hydrazide gave strong binding. AAL further interacts with core fucose, which is present in many *N*-glycan structures. Because this fucose residue is attached to the reducing GlcNAc, we wondered whether biotinylation of the reducing monosaccharide residue influences recognition of the fucose by the lectin; therefore, we tested the binding of biotinylated core fucose-containing asialo-agalacto-diantennary glycan. The measurement showed that the glycan is recognized by AAL, indicating that the ring opening of the core GlcNAc did not abrogate binding. We found,

Table 1
Carbohydrate structures used in this study

Glycan	Structure	Molecular mass (monoisotopic)		
		Native	Biotinylated	
			Calculated	Determined[a]
Maltohexaose	Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc	990.3	1345.8	1346.0
Maltoheptaose	Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glcα1-4Glc	1152.4	1507.9	1507.9
Asialo-diantennary	Galβ1-4GlcNAcβ1-2Manα1 \ 6 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1-2Manα1 / 3	1640.6	1996.1	1996.6
Asialo-triantennary	Galβ1-4GlcNAcβ1-2Manα1 \ 6 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1 \ 4 Manα1 / 3 Galβ1-4GlcNAcβ1 / 2	2006.1	2361.6	2361.8
Asialo-tetraantennary	Galβ1-4GlcNAcβ1 \ 6 Manα1 \ 6 Galβ1-4GlcNAcβ1 / 2 Manβ1-4GlcNAcβ1-4GlcNAc Galβ1-4GlcNAcβ1 \ 4 Manα1 / 3 Galβ1-4GlcNAcβ1 / 2	2370.8	2726.3	2726.0
Man5	Manα1 \ 6 Manα1 \ 6 Manα1 / 3 Manβ1-4GlcNAcβ1-4GlcNAc Manα1 / 3	1234.4	1589.9	1589.5
Man9	Manα1-2Manα1 \ 6 Manα1 \ 6 Manα1-2Manα1 / 3 Manβ1-4GlcNAcβ1-4GlcNAc Manα1-2Manα1-2Manα1 / 3	1882.6	2238.1	2237.8
$Fuc_2GlcNAc_2Man_3$	Fucα1-3GlcNAcβ1-2Manα1 \ 6 Man Fucα1-3GlcNAcβ1-2Manα1 / 3	1202.4	1557.9	1557.6
2′Fucosyllactose	Galβ1-4Glc 2 | Fucα1	488.2	843.7	843.4
LND-I	Galβ1-3GlcNAcβ1-3Galβ1-4Glc 2 4 | | Fucα1 Fucα1	999.4	1354.9	1354.6
Asialo-agalacto-diantennary + core Fuc	Fucα1 | GlcNAcβ1-2Manα1 \ 6 6 Manβ1-4GlcNAcβ1-4GlcNAc GlcNAcβ1-2Manα1 / 3	1462.6	1818.1	1818.2
Trisialyl-triantennary	NeuNAcα2-6Galβ1-4GlcNAcβ1-2Manα1 \ 6 Manβ1-4GlcNAc NeuNAcα2-6Galβ1-4GlcNAcβ1 \ 4 Manα1 / 3 NeuNAcα2-6Galβ1-4GlcNAcβ1 / 2	2675.9	3031.4	3031.5
LDN	GalNAcβ1-4GlcNAc	424.2	779.7	779.4

[a] Monoisotopic molecular masses of biotinylated glycans were determined from zoom scans taken from the pseudomolecular ion.

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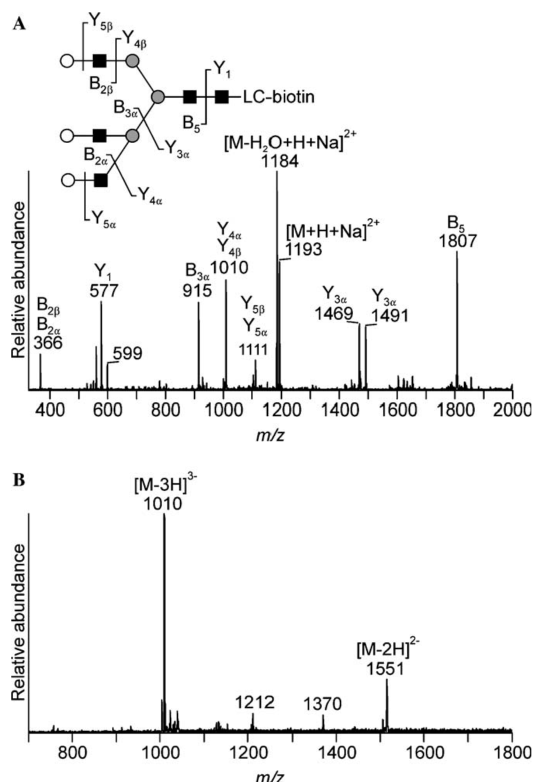

Fig. 3. Structural integrity of biotinylated glycans as determined by mass spectrometry. (A) Positive ion mode nano-ES CID tandem mass spectrum of biotinylated asialo-triantennary glycan demonstrating the coupling of the biotin label to the reducing end of the carbohydrate. The nomenclature used for fragmentation is based on that proposed by Domon and Costello [24]. White circle, galactose; black square, *N*-acetylglucosamine; gray circle, mannose. (B) Negative ion mode nano-ES mass spectrum of biotin-labeled trisialyl-triantennary glycan indicating that the biotinylation procedure does not cause significant desialylation.

however, that when omitting the reduction during the biotinylation reaction, binding to AAL was considerably stronger, indicating that an intact reducing GlcNAc enhanced recognition by the lectin (data not shown). On the other hand, 2′-fucosyllactose biotinylated under non-reducing conditions did not bind AAL, whereas reduction of the compound strongly increased binding (data not shown and Table 2).

Sambucus nigra agglutinin

SNA has a binding specificity for glycans containing the terminal Neu5Acα(2-6)Gal sequence [31]. Consequently, the biotinylated trisialyl-triantennary glycan, which contains three sialic acid residues α(2-6)-linked to galactose, gave strong binding to SNA, a further indication that the biotinylation procedure did not cause large-scale desialylation.

Table 2
Binding of plant lectins to biotinylated carbohydrates

Glycan	Con A	AAL	SNA	DSA
Asialo-diantennary	+[a]			–
Asialo-triantennary	–			+[b]
Asialo-tetraantennary	–			+
Man5	+			
Man9	+			
$Fuc_2GlcNAc_2Man_3$	–	+[b]		
2′-Fucosyllactose	–	+		
LND-I	–	+		
Asialo-agalacto-diantennary + core Fuc	+	+		
Trisialyl-triantennary	–		+	

Note. Abbreviations and monosaccharide specificity: ConA, concanavalin A, Man and Glc; AAL, *Aleuria aurantia* lectin, Fuc; SNA, *Sambucus nigra* agglutinin, NeuNAc; DSA, *Datura stramonium* agglutinin, GlcNAc.

[a] Throughout the table, a plus sign (+) indicates binding, whereas a minus sign (–) indicates negligible interaction between the carbohydrate and the lectin. Blank fields indicate that the interaction was not determined.

[b] Binding was weak but significant.

Datura stramonium agglutinin

Of the glycans tested, only the biotinylated asialo-tetraantennary glycan strongly interacted with DSA, whereas binding of the asialo-triantennary glycan was weak. These results are in agreement with the previously published binding specificity of DSA [32–34].

In conclusion, the results demonstrate that the biotinylated glycans are recognized by plant lectins; hence, their biological function is retained. Therefore, they can be used in biological assays to study lectin binding specificities.

Interaction of the C-type lectin DC–SIGN with biotinylated carbohydrates

To investigate whether our approach of biotinylating carbohydrates is effective in studying the binding specificity of C-type lectins, biotinylated glycans were coated at saturating densities in streptavidin-coupled microtiter plates and incubated with recombinant DC–SIGN-Fc. Bound DC–SIGN was detected using peroxidase-labeled anti-human IgG-Fc antibody. In this assay, the high-mannose-type glycan Man9 and Lewis^b-containing LND-I were recognized by DC–SIGN. In both cases, binding could be blocked completely by the calcium chelator EGTA or the anti-DC–SIGN antibody AZN-D1 (Fig. 4A). No binding could be detected of Man5 or of the non-Lewis-type fucosylated carbohydrates, consistent with previously published data on the binding specificity of DC–SIGN-Fc [35].

We wondered whether the biotinylated carbohydrates could also be used in a bead adhesion assay. To explore this, we incubated streptavidin-coated fluorescent beads with biotinylated carbohydrates and measured the adhesion to DC–SIGN-expressing dendritic cells (DCs) by flow cytometry. Both Man9- and LND-I-coated beads showed strong binding activity to DCs, whereas binding of Man5-coated beads was not detected. DC–SIGN-mediated

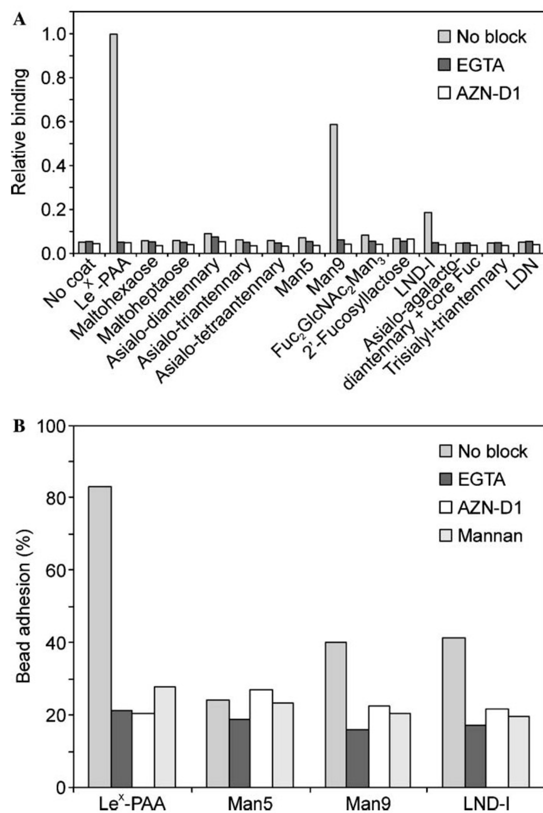

Fig. 4. Binding specificity of the C-type lectin DC–SIGN. (A) Glycan array showing binding specificity of recombinant DC–SIGN-Fc for Man9 and Lewis^b-containing LND-I. Biotinylated carbohydrates were coated in streptavidin-coupled microtiter plates and probed with DC–SIGN-Fc. Peroxidase-labeled anti-human IgG-Fc was used to detect DC–SIGN binding. Binding of DC–SIGN could be blocked by EGTA (10 mM) and the anti-DC–SIGN antibody AZN-D1 (20 µg/ml). Binding levels for each glycan were normalized to the value for the positive control, that is, Lewis^x multivalently coupled to polyacrylamide. (B) Bead adhesion assay demonstrating the binding of DCs to Man9 and LND-I. Streptavidin-coated fluorescent beads were incubated with biotinylated carbohydrates, and binding to DCs was analyzed by flow cytometry and presented as the percentage of cells that had bound the fluorescent beads. Binding could be blocked by EGTA (10 mM), AZN-D1 (20 µg/ml), and yeast mannan (100 µg/ml).

adhesion is completely inhibited by EGTA, AZN-D1, or DC–SIGN-binding yeast mannan (Fig. 4B).

Together, these results demonstrate that the biotinylated carbohydrates can be used effectively in determining the binding specificities of lectins that are expressed on cells.

In situ identification of DC–SIGN on lymph node tissue by carbohydrate binding

In lymph node, DC–SIGN-positive immature DCs are found in the T-cell areas and along the outer zones of the paracortex [36]. In an approach to functionally characterize lectins in tissue, we employed biotinylated Man9 for lectin detection and double stained the tissue with specific anti-DC–SIGN antibodies. Immature DCs could be detected readily in the lymph node tissue, showing scattered DC–SIGN^{pos} cells in the T-cell areas and a rim of DC–SIGN^{pos} DCs at the outer zones of the paracortex (Fig. 5). Strikingly, only the DC–SIGN^{pos} cells in the T-cell areas bound the Man9 carbohydrate structure.

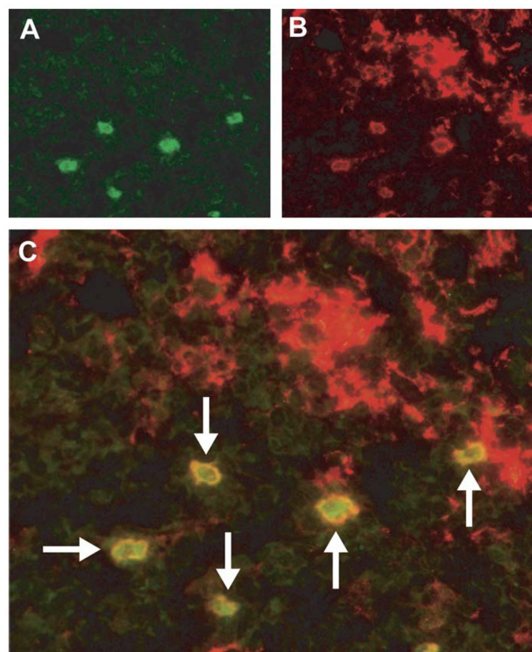

Fig. 5. In situ identification of DC–SIGN on DCs in human lymph node tissue using biotinylated Man9. Tissue sections were stained with biotinylated Man9 (green) (A) and polyclonal anti-DC–SIGN antibodies (red) (B). (C) Colocalization of Man9 with DC–SIGN (yellow, see arrows).

These data indicate that the biotinylated carbohydrates can be used to identify carbohydrate-binding proteins in tissue.

Discussion

We have developed a simple, fast, and efficient method for the incorporation of a biotin group into carbohydrates by coupling biotin-LC-hydrazide to the reducing end of carbohydrates via reductive amination using readily available chemicals and reactants. The method includes a rapid sample cleanup procedure that effectively removes excess labeling reagent without the requirement of sophisticated purification methods such as HPLC. Using this procedure, preparation and purification of biotinylated carbohydrates can be achieved within 3 h.

The reductive amination reaction is principally nonselective for carbohydrates of different structures because the

reaction involves an aldehyde that is present as a semiacetal in all reducing carbohydrates. Indeed, it was demonstrated that the labeling of *N*-glycans with 2-aminobenzamide or 2-anthranilic acid via reductive amination is not influenced by the glycan structure [37]. However, it has been reported that labeling oligogalacturonides with biotin-hydrazide resulted in a decreased labeling efficiency with increasing degrees of polymerization [38]. This may be due to the low solubility of the carbohydrate in the solvent system used for the amination procedure, namely methanol. In the current study, we found DMSO to be a suitable solvent in which the reagents and carbohydrates are readily solubilized, thereby strongly increasing the overall labeling efficiency.

All biotinylated glycans were recognized by lectins with the appropriate specificity, indicating that the biological activity of the glycans was preserved. It should be noted, however, that as a consequence of the reductive amination reaction, the integrity of the reducing monosaccharide is lost [9,11–13], and this may affect binding by those lectins that recognize monosaccharide residues in the vicinity of the reducing monosaccharide. With core α1-6-fucosylated *N*-glycans, for instance, we found that binding of AAL was considerably stronger when omitting the reduction during the biotinylation reaction, indicating that an intact reducing *N*-acetylglucosamine enhanced recognition by the lectin. In contrast, biotinylated 2′-fucosyllactose under nonreducing conditions did not bind to AAL, whereas reduction of the compound strongly increased binding. The latter observations are consistent with those published previously [30]. Omitting the reduction step after biotinylation is not attractive because it results in a rather unstable product that can hydrolyze in solution [38], although we did not encounter large-scale hydrolysis during storage at neutral pH at 4 °C for several days (results not shown).

With the commercial availability of a large number of synthesized or purified naturally occurring carbohydrates, the biotinylation procedure described in the current article enables the detailed characterization of lectins by designing customized glycan arrays. The use of glycan arrays for defining binding specificities of lectins has been growing over the past few years, and the technique has been proved to be exceptionally useful for characterizing (unknown) lectins [35,39–43] (see also www.functionalglycomics.org/static/consortium). We also showed that DC–SIGN expressed on cells or in tissue interacted with appropriate biotinylated carbohydrate ligands, and we propose that the biotinylated carbohydrates can be used for functional analysis of lectins expressed on different cell subsets and during disease development in situ.

Acknowledgment

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References

[1] G.L. Bratthauer, The avidin–biotin complex (ABC) method and other avidin–biotin binding methods, Methods Mol. Biol. 115 (1999) 203–214.
[2] M. Wilchek, E.A. Bayer, The avidin–biotin complex in bioanalytical applications, Anal. Biochem. 171 (1988) 1–32.
[3] H. Heitzmann, F.M. Richards, Use of the avidin–biotin complex for specific staining of biological membranes in electron microscopy, Proc. Natl. Acad. Sci. USA 71 (1974) 3537–3541.
[4] N.M. Green, Avidin: I. The use of [^{14}C]biotin for kinetic studies and for assay, Biochem. J. 89 (1963) 585–591.
[5] N.M. Green, Avidin, Adv. Protein Chem. 29 (1975) 85–133.
[6] D.J. O'Shannessy, M.J. Dobersen, R.H. Quarles, A novel procedure for labeling immunoglobulins by conjugation to oligosaccharide moieties, Immunol. Lett. 8 (1984) 273–277.
[7] E.A. Bayer, H. Ben-Hur, M. Wilchek, Biocytin hydrazide: a selective label for sialic acids, galactose, and other sugars in glycoconjugates using avidin–biotin technology, Anal. Biochem. 170 (1988) 271–281.
[8] B.E. Rothenberg, B.K. Hayes, D. Toomre, A.E. Manzi, A. Varki, Biotinylated diaminopyridine: an approach to tagging oligosaccharides and exploring their biology, Proc. Natl. Acad. Sci. USA 90 (1993) 11939–11943.
[9] D.K. Toomre, A. Varki, Advances in the use of biotinylated diaminopyridine (BAP) as a versatile fluorescent tag for oligosaccharides, Glycobiology 4 (1994) 653–663.
[10] T. Imai, T. Watanabe, T. Yui, J. Sugiyama, Directional degradation of β-chitin by chitinase A1 revealed by a novel reducing end labelling technique, FEBS Lett. 510 (2002) 201–205.
[11] Y. Shinohara, H. Sota, F. Kim, M. Shimizu, M. Gotoh, M. Tosu, Y. Hasegawa, Use of a biosensor based on surface plasmon resonance and biotinyl glycans for analysis of sugar binding specificities of lectins, J. Biochem. (Tokyo) 117 (1995) 1076–1082.
[12] Y. Shinohara, H. Sota, M. Gotoh, M. Hasebe, M. Tosu, J. Nakao, Y. Hasegawa, M. Shiga, Bifunctional labeling reagent for oligosaccharides to incorporate both chromophore and biotin groups, Anal. Chem. 68 (1996) 2573–2579.
[13] B. Xia, Z.S. Kawar, T. Ju, R.A. Alvarez, G.P. Sachdev, R.D. Cummings, Versatile fluorescent derivatization of glycans for glycomic analysis, Nat. Methods 2 (2005) 845–850.
[14] C. Leteux, R.A. Childs, W. Chai, M.S. Stoll, H. Kogelberg, T. Feizi, Biotinyl-L-3-(2-naphthyl)-alanine hydrazide derivatives of *N*-glycans: versatile solid-phase probes for carbohydrate-recognition studies, Glycobiology 8 (1998) 227–236.
[15] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for the determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[16] H. Bakker, P.S. Schoenmakers, C.A.M. Koeleman, D.H. Joziasse, I. van Die, D.H. van den Eijnden, The substrate specificity of the snail *Lymnaea stagnalis* UDP-GlcNAc:GlcNAcβ-R β4-*N*-acetylglucosaminyltransferase reveals a novel variant pathway of complex-type oligosaccharide synthesis, Glycobiology 7 (1997) 539–548.
[17] A.P. Neeleman, W.P.W. van der Knaap, D.H. van den Eijnden, Identification and characterization of a UDP-GalNAc:GlcNAcβ-R β1→4-*N*-acetylgalactosaminyltransferase from cercariae of the schistosome *Trichobilharzia ocellata*: catalysis of a key step in the synthesis of *N,N′*-diacetyllactosediamino (lacdiNAc)-type glycans, Glycobiology 4 (1994) 641–651.
[18] S. Narasimhan, J.R. Wilson, E. Martin, H. Schachter, A structural basis for four distinct elution profiles on concanavalin A–Sepharose affinity chromatography of glycopeptides, Can. J. Biochem. 57 (1979) 83–96.
[19] S. Narasimhan, J.C. Freed, H. Schachter, The effect of a "bisecting" *N*-acetylglucosaminyl group on the binding of biantennary, complex oligosaccharides to concanavalin A, *Phaseolus vulgaris* erythroagglutinin (E-PHA), and *Ricinus communis* agglutinin (RCA-120) immobilized on agarose, Carbohydr. Res. 149 (1986) 65–83.

[20] D.H. Joziasse, W.E.C.M. Schiphorst, D.H. van den Eijnden, J.A. van Kuik, H. van Halbeek, J.F.G. Vliegenthart, Branch specificity of bovine colostrum CMP-sialic acid:Galβ1 → 4GlcNAc-R α2 → 6-sialyltransferase: sialylation of bi-, tri-, and tetraantennary oligosaccharides and glycopeptides of the *N*-acetyllactosamine type, J. Biol. Chem. 262 (1987) 2025–2033.
[21] A. Remoortere, C.H. Hokke, G.J. van Dam, I. van Die, A.M. Deelder, D.H. van den Eijnden, Various stages of *Schistosoma* express Lewis^x, LacdiNAc, GalNAcβ1-4(Fucα1-3)GlcNAc, and GalNAcβ1-4(Fuc1-2Fucα1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins, Glycobiology 10 (2000) 601–609.
[22] H. Geyer, M. Wuhrer, A. Resemann, R. Geyer, Identification and characterization of keyhole limpet hemocyanin (KLH) *N*-glycans mediating cross-reactivity with *Schistosoma mansoni*, J. Biol. Chem. 280 (2005) 40731–40748.
[23] T.B.H. Geijtenbeek, Y. van Kooyk, S.J. van Vliet, M.H. Renes, R.A.P. Raymakers, C.G. Figdor, High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia, Blood 94 (1999) 754–764.
[24] B. Domon, C.E. Costello, A systematic nomenclature for carbohydrate fragmentations in FAB–MS/MS spectra of glycoconjugates, Glycoconj. J. 5 (1988) 397–409.
[25] I.J. Goldstein, C.E. Hollerman, E.E. Smith, Protein–carbohydrate interaction: II. Inhibition studies on the interaction of concanavalin A with polysaccharides, Biochemistry 4 (1965) 876–883.
[26] A. Kobata, T. Endo, Immobilized lectin columns: useful tools for the fractionation and structural analysis of oligosaccharides, J. Chromatogr. 597 (1992) 111–122.
[27] S. Ogata, T. Muramatsu, A. Kobata, Fractionation of glycopeptides by affinity column chromatography on concanavalin A–Sepharose, J. Biochem. (Tokyo) 78 (1975) 687–696.
[28] T. Krusius, J. Finne, H. Rauvala, The structural basis of the different affinities of two types of acidic *N*-glycosidic glycopeptides for concanavalin A–Sepharose, FEBS Lett. 72 (1976) 117–120.
[29] N. Kochibe, K. Furukawa, Purification and properties of a novel fucose-specific hemagglutinin of *Aleuria aurantia*, Biochemistry 19 (1980) 2841–2846.
[30] K. Yamashita, N. Kochibe, T. Ohkura, I. Ueda, A. Kobata, Fractionation of L-fucose-containing oligosaccharides on immobilized *Aleuria aurantia* lectin, J. Biol. Chem. 260 (1985) 4688–4693.
[31] N. Shibuya, I.J. Goldstein, W.F. Broekaert, M. Nsimba-Lubaki, B. Peeters, W.J. Peumans, The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2-6)Gal/GalNAc sequence, J. Biol. Chem. 262 (1987) 1596–1601.
[32] K. Yamashita, K. Totani, T. Ohkura, S. Takasaki, I.J. Goldstein, A. Kobata, Carbohydrate binding properties of complex-type oligosaccharides on immobilized *Datura stramonium* lectin, J. Biol. Chem. 262 (1987) 1602–1607.
[33] R.D. Cummings, S.J. Kornfeld, The distribution of repeating [Galβ 1,4GlcNAcβ1,3] sequences in asparagine-linked oligosaccharides of the mouse lymphoma cell lines BW5147 and PHA^R 2.1, J. Biol. Chem. 259 (1984) 6253–6260.
[34] J.F. Crowley, I.J. Goldstein, J. Amarp, J. Lönngren, Carbohydrate binding studies on the lectin from *Datura stramonium* seeds, Arch. Biochem. Biophys. 231 (1984) 524–533.
[35] O. Blixt, S. Head, T. Mondala, C. Scanlan, M.E. Huflejt, R. Alvarez, M.C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D.J. Stevens, J.J. Skehel, I. van Die, D.R. Burton, I.A. Wilson, R. Cummings, N. Bovin, C.H. Wong, J.C. Paulson, Printed covalent glycan array for ligand profiling of diverse glycan binding proteins, Proc. Natl. Acad. Sci. USA 101 (2004) 17033–17038.
[36] A. Engering, S.J. van Vliet, K. Hebeda, D.G. Jackson, R. Prevo, S.K. Singh, T.B.H. Geijtenbeek, H. van Krieken, Y. van Kooyk, Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes, Am. J. Pathol. 164 (2004) 1587–1595.
[37] J.C. Bigge, T.P. Patel, J.A. Bruce, P.N. Goulding, S.M. Charles, R.B. Parekh, Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid, Anal. Biochem. 230 (1995) 229–238.
[38] B.L. Ridley, M.D. Spiro, J. Glushka, P. Albersheim, A. Darvill, D. Mohnen, A method for biotin labeling of biologically active oligogalacturonides using a chemically stable hydrazide linkage, Anal. Biochem. 249 (1997) 10–19.
[39] Y. Guo, H. Feinberg, E. Conroy, D.A. Mitchell, R. Alvarez, O. Blixt, M.E. Taylor, W.I. Weis, K. Drickamer, Structural basis for distinct ligand-binding and targeting properties of the receptors DC–SIGN and DC–SIGNR, Nat. Struct. Mol. Biol. 11 (2004) 591–598.
[40] S.J. van Vliet, E. van Liempt, E. Saeland, C.A. Aarnoudse, B. Appelmelk, T. Irimura, T.B.H. Geijtenbeek, O. Blixt, R. Alvarez, I. van Die, Y. van Kooyk, Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells, Int. Immunol. 17 (2005) 661–669.
[41] B.S. Bochner, R.A. Alvarez, P. Mehta, N.V. Bovin, O. Blixt, J.R. White, R.L. Schnaar, Glycan array screening reveals a candidate ligand for Siglec-8, J. Biol. Chem. 280 (2005) 4307–4312.
[42] T. Singh, J.H. Wu, W.J. Peumans, P. Rougè, E.J.M. Van Damme, R.A. Alvarez, O. Blixt, A.M. Wu, Carbohydrate specificity of an insecticidal lectin isolated from the leaves of *Glechoma hederacea* towards mammalian glycoconjugates, Biochem. J. 393 (2006) 331–341.
[43] A. Leppänen, S. Stowell, O. Blixt, R.D. Cummings, Dimeric galectin-1 binds with high affinity to α2,3-sialylated and non-sialylated terminal *N*-acetyllactosamine units on surface-bound extended glycans, J. Biol. Chem. 280 (2005) 5549–5562.

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The C-type lectin L-SIGN differentially recognizes glycan antigens on egg glycosphingolipids and soluble egg glycoproteins from *Schistosoma mansoni*

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Recognition of pathogen-derived carbohydrate constituents by antigen presenting cells is an important step in the induction of protective immunity. Here we investigated the interaction of L-SIGN (liver/lymph node specific ICAM-3-grabbing nonintegrin), a C-type lectin that functions as antigen receptor on human liver sinusoidal endothelial cells, with egg-derived glycan antigens of the parasitic trematode *Schistosoma mansoni*. Our data demonstrate that L-SIGN binds both schistosomal soluble egg antigens (SEA) and egg glycosphingolipids, and can mediate internalization of SEA by L-SIGN expressing cells. Binding and internalization of SEA was strongly reduced after treatment of SEA with endoglycosidase H, whereas defucosylation affected neither binding nor internalization. These data indicate that L-SIGN predominantly interacts with oligomannosidic *N*-glycans of SEA. In contrast, binding to egg glycosphingolipids was completely abolished after defucosylation. Our data show that L-SIGN binds to a glycosphingolipid fraction containing fucosylated species with compositions of $Hex_1HexNAc_{5-7}dHex_{3-6}Cer$, as evidenced by mass spectrometry. The L-SIGN "gain of function" mutant Ser363Val, which binds fucosylated Lewis antigens, did not bind to this fucosylated egg glycosphingolipid fraction, suggesting that L-SIGN displays different modes in binding fucoses of egg glycosphingolipids and Lewis antigens, respectively. Molecular modeling studies indicate that the preferred binding mode of L-SIGN to the respective fucosylated egg glycosphingolipid oligosaccharides involves a Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc tetrasaccharide at the nonreducing end. In conclusion, our data indicate that L-SIGN recognizes both oligomannosidic *N*-glycans and multiply fucosylated carbohydrate motifs within *Schistosoma* egg antigens, which demonstrates that L-SIGN has a broad but specific glycan recognition profile.

Keywords: C-type lectin/glycosphingolipids/L-SIGN/parasitic helminth glycans/*Schistosoma mansoni*

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Introduction

Schistosomiasis is a human parasitic disease caused by helminths of the genus *Schistosoma* that affects more than 200 million people worldwide (Pearce and MacDonald 2002). Infection starts when cercariae released by the intermediate host, a snail of the genus *Biomphalaria*, penetrates the skin of its vertebrate host and transforms into schistosomula. They migrate to the portal system and mature to adult worms that produce large amounts of eggs. Eggs that become lodged within host tissues, mainly liver and intestine, are primarily responsible for the development of a strong anti-inflammatory Th2 response that enables parasite survival and induces granuloma formation around the eggs, which is a major cause of pathology (Pearce and MacDonald 2002).

Schistosoma mansoni synthesizes a multitude of complex carbohydrates, which include both parasite-specific glycan antigens, as well as glycan antigens that are shared with the host. One example for a host-like glycan is the Lewis X (Le^X) epitope Galβ1-4(Fucα1-3)GlcNAc, which is expressed in all schistosomal life stages (Cummings and Nyame 1996; Robijn et al. 2005), but also on human leukocytes as CD15 (Fukuda et al. 1986). Glycan antigens expressed by schistosomes induce strong humoral and cellular immune responses in their host (Cummings and Nyame 1999). Especially soluble egg antigens (SEA) have been shown to be highly immunogenic in mice and humans due to the presence of carbohydrate epitopes such as GalNAcβ1-4GlcNAc- (LDN), GalNAcβ1-4(Fucα1-3)GlcNAc- (LDN-F), and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc- (LDN-DF) (see Table I) (van Die and Cummings 2006).

While much remains to be understood about the immunological events triggered by schistosomal glycoconjugates, it is increasingly acknowledged that these biological effects depend on the recognition of these glycans by specific receptors on immune cells. The recognition of carbohydrates is mediated by a family of receptors called lectins, which bind glycan antigens via one or more carbohydrate recognition domains (CRD). We have recently described that both the C-type lectin DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin; CD209) as well as its homologue L-SIGN (liver/lymph node-specific ICAM-3-grabbing nonintegrin; CD209L; DC-SIGN-R) bind to glycans of *S. mansoni* soluble egg antigens (SEA) (van Die et al. 2003; van Liempt et al. 2004; Meyer et al. 2005). The role of DC-SIGN as a broad pathogen receptor has been well established (Geijtenbeek et al. 2000; Alvarez et al. 2002; Colmenares et al. 2002; Cambi et al. 2003; Geijtenbeek et al. 2003; Lozach et al. 2003). In addition, DC-SIGN functions as a cell adhesion receptor mediating the interaction between dendritic cells (DCs) and resting T cells by binding to ICAM-3, and the transendothelial migration of DCs by binding to ICAM-2 (Geijtenbeek et al. 2000).

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Table I. Carbohydrate epitopes mentioned in this study

Carbohydrate epitope	Structure	Structure plot
LacdiNAc (LDN)	GalNAcβ1-4GlcNAc	
Terminally fucosylated LDN (F-LDN)	Fucα1-3GalNAcβ1-4GlcNAc	
Fucosylated LDN (LDN-F)	GalNAcβ1-4(Fucα1-3)GlcNAc	
Fucosylated LDN-F (F-LDN-F)	Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc	
Difucosylated LDN (LDN-DF)	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc	
Fucosylated LDN-DF (F-LDN-DF)	Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc	
Lewis X (Le^{X})	Galβ1-4(Fucα1-3)GlcNAc	
Lewis Y (Le^{Y})	Fucα1-2Galβ1-4(Fucα1-3)GlcNAc	
Lewis A (Le^{A})	Galβ1-3(Fucα1-4)GlcNAc	
Lewis B (Le^{B})	Fucα1-2Galβ1-3(Fucα1-4)GlcNAc	

Respective abbreviations are given in parentheses. Structure plots were generated in the notation of the Consortium for Functional Glycomics (http://www.functionalglycomics.org) using the visual editor of "GlycoWorkbench". This software application is developed and available as part of the EUROCarbDB project (http://www.eurocarbdb.org/applications/ms-tools). Light grey square, *N*-acetylgalactosamine; dark grey square, *N*-acetylglucosamine; triangle, fucose; circle, galactose.

L-SIGN displays 77% amino acid identity with DC-SIGN, and is expressed on endothelial cells in lymph node sinuses, capillary endothelial cells in the placenta and on liver sinusoidal cells (LSECs) (Soilleux et al. 2000; Bashirova et al. 2001; Pohlmann et al. 2001; Engering et al. 2004). In the liver LSECs function as liver-resident antigen presenting cells (Knolle and Gerken 2000) and are important in tolerance induction (Limmer et al. 2000; Knolle and Limmer 2001). LSECs may mediate the clearance of antigens from the circulation in the same manner as DCs do (Bashirova et al. 2001; Karrar et al. 2007). In addition to L-SIGN, LSECs express lectins like the mannose receptor, high levels of adhesion molecules and costimulatory molecules such as MHC class II, CD40, CD80, and CD86 (Adams et al. 1989; McNab et al. 1996). It has been suggested that LSECs can potentially function as cells that are capable of trapping CD4+ and CD8+ T cells (Karrar et al. 2007). DC-SIGN and L-SIGN share a di-leucine motif and a cluster of three acidic amino acids in their cytoplasmic tails, which are known to be essential for antigen uptake (Soilleux et al. 2000; Bashirova et al. 2001; Engering et al. 2002). Recent studies with Ebola virus, Severe Acute Respiratory Syndrome (SARS) virus or antibodies against L-SIGN, clearly demonstrated that L-SIGN indeed is able to internalize antigens (Alvarez et al. 2002; Jeffers et al. 2004; Ludwig et al. 2004; Dakappagari et al. 2006). Likewise, Ludwig et al. monitored the internalization of Hepatitis C virus (HCV) envelope glycoproteins E1 and E2 by L-SIGN and the intracellular localization of these glycoproteins in LSECs by confocal microscopy (Ludwig et al. 2004). Similar to DC-SIGN, L-SIGN can recognize high-mannose type *N*-glycans and the fucosylated glycan epitopes Lewis A (Le^A, Galβ1-3(Fucα1-4)GlcNAc-), Lewis B (Le^B, Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-) and Lewis Y (Le^Y, Fucα1-2Galβ1-4(Fucα1-3)GlcNAc-) (Geijtenbeek et al. 2003; Guo et al. 2004; van Liempt et al. 2004). L-SIGN, however, does not bind to the Le^X epitope, which is one of the major schistosome ligands of DC-SIGN, although the formation of crystals between L-SIGN and Le^X indicates that a weak interaction is possible (Guo et al. 2004). The inability of L-SIGN to bind to Le^X epitopes is mainly due to the presence of a single amino acid in the CRD of L-SIGN, Ser^{363} that prevents interaction with the Fuc(α1-3)GlcNAc unit in Le^X, but supports binding of the Fucα1-4GlcNAc moiety present in Le^A and Le^B antigens. The equivalent amino acid residue Val^{351} in DC-SIGN creates a hydrophobic pocket that strongly interacts with the Fuc(α1-3/4)GlcNAc moiety of Le^X, other Lewis antigens, and probably LDN-F (Guo et al. 2004; van Liempt et al. 2004; van Liempt et al. 2006).

The interaction of L-SIGN with *S. mansoni* egg glycoproteins and its location on liver endothelial cells suggest that L-SIGN may function in the recognition of glycan antigens of eggs that are trapped in the liver, thus contributing to glycan-specific immune responses and/or the immunopathology of schistosomiasis. To increase our understanding of the role of L-SIGN we investigated the binding properties of L-SIGN to both schistosomal egg glycoproteins and glycosphingolipids. Our data revealed that L-SIGN interacts predominantly with oligomannosidic *N*-glycans of SEA. Remarkably, recognition of schistosomal egg glycosphingolipids by L-SIGN was mediated via fucosylated carbohydrate entities, utilizing a binding mode that may be different from the way it binds to Lewis antigens.

Results

Recognition of S. mansoni SEA by L-SIGN

Previous studies have shown that L-SIGN can recognize both high-mannose type *N*-glycans as well as particular fucosylated structures within Lewis antigens (Guo et al. 2004; van Liempt et al. 2004) and may also interact with SEA (van Liempt et al. 2004). Furthermore, we have demonstrated that L-SIGN does not bind to Le^X, a major glycan antigen of SEA (van Liempt et al. 2004). To identify the carbohydrate ligands of SEA that are recognized by L-SIGN, SEA was treated with endo H to remove oligomannosidic *N*-glycans. In parallel, SEA was subjected to HF-treatment to remove fucose residues. Our data show that HF-treated SEA has lost its ability to react with antibodies directed against the fucose-containing epitopes Le^X and LDN-DF (Figure 1A), whereas binding of these antibodies to endo H-treated SEA was hardly affected (results not shown). Hence, it can be concluded that the HF-treatment resulted in a nearly complete removal of fucose residues from SEA. In addition, the reactivity of HF-treated SEA with antibodies recognizing LDN glycan antigens was increased after defucosylation, which shows that the remaining glycan is intact and may expose an increased amount of terminal LDN units.

Subsequent cellular adhesion assays using K562 cells stably transfected with L-SIGN (K562/L-SIGN), revealed that binding of L-SIGN to SEA was apparently not affected by defucosylation, whereas treatment with endo H almost completely abolished SEA recognition by L-SIGN (Figure 1B and C). These data indicate that the binding of L-SIGN to *S. mansoni* SEA is predominantly mediated by oligomannosidic *N*-glycans, whereas the fucose-containing glycan epitopes present on SEA are obviously not or much less involved in this type of interaction. In agreement with this assumption, direct binding assays showed that K562 cells stably transfected with L-SIGN did not bind to neoglycoproteins carrying LDN-F or LDN-DF (Figure 1D), indicating that these glycan antigens, which in addition to Le^X are major fucosylated glycan antigens on SEA, are not ligands of L-SIGN.

Internalization of SEA by L-SIGN expressing cells

To further characterize the interaction between SEA and L-SIGN, we investigated whether SEA is internalized from the cell surface of L-SIGN transfected K562 cells. We found that 64% of the biotinylated SEA disappeared from the cell surface within an incubation time of 15 min at 37°C (Figure 2A), whereas no detectable loss of SEA was observed in the case of paraformaldehyde fixed cells, in which membrane transport had been blocked. These data suggest that SEA is rapidly internalized from the cell surface. The internalization could be significantly inhibited by preincubation of the cells with the L-SIGN specific mAb AZN-D2, thus demonstrating that this process is L-SIGN dependent (Figure 2A). Defucosylation of biotinylated SEA by HF-treatment led only to a minimal reduction of binding and a similar rate of internalization as compared to untreated SEA (Figure 2B). In contrast, binding and internalization of endo H-treated SEA to L-SIGN transfected cells was hardly detectable by flow cytometry analysis (data not shown), in agreement with the previously observed binding properties of L-SIGN to SEA (see Figure 1B).

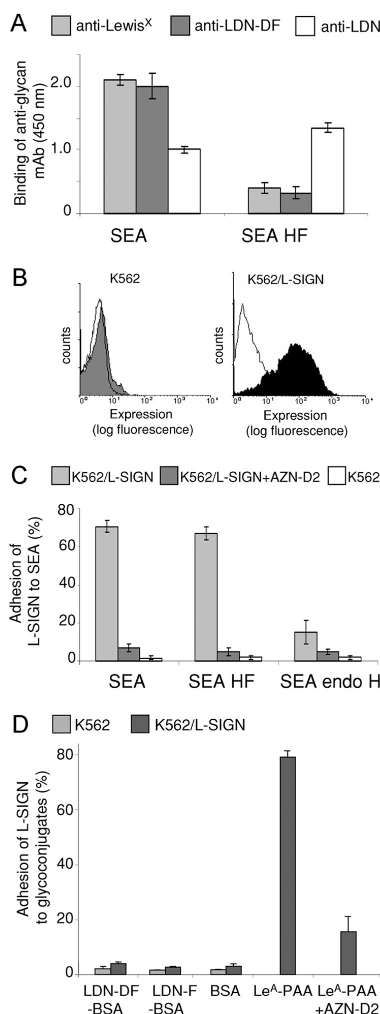

Fig. 1. Binding of *S. mansoni* SEA by L-SIGN transfected cells. (**A**) ELISA was performed to characterize the glycan epitopes of SEA and defucosylated SEA (SEA HF). Similar amounts of SEA (5 μg/mL) were applied in each case. Using antibodies recognizing the fucose-containing glycan epitopes Le^X and LDN-DF the efficacy of HF-treatment was determined. In parallel, an anti-LDN mAb was employed to evaluate the integrity of the remaining glycans. Data represent a typical result out of three experiments performed in duplicate, with error bars indicating standard deviation. (**B**) The expression of L-SIGN on transfected (K562/L-SIGN) and nontransfected K562 cells was determined by flow cytometry using the mAb AZN-D2 that recognizes L-SIGN. The isotype control is shown as lines. (**C**) Binding of L-SIGN to soluble egg antigen (SEA) of *S. mansoni*, defucosylated SEA (SEA HF) and endo H-treated SEA (SEA endo H) was determined by cell adhesion assays using K562/L-SIGN transfected cells in the absence (*light grey bars*) or presence (*dark grey bars*) of a blocking mAb to L-SIGN (AZN-D2). All results are representative of three independent experiments, performed in triplicate, with error bars indicating standard deviation. (**D**) Adhesion of K562/L-SIGN transfected cells to neoglycoconjugates carrying LDN-F (LDN-F-BSA) and LDN-DF (LDN-DF-BSA). The neoglycoconjugate Le^A-PAA was used as a positive control. One representative experiment out of three is shown.

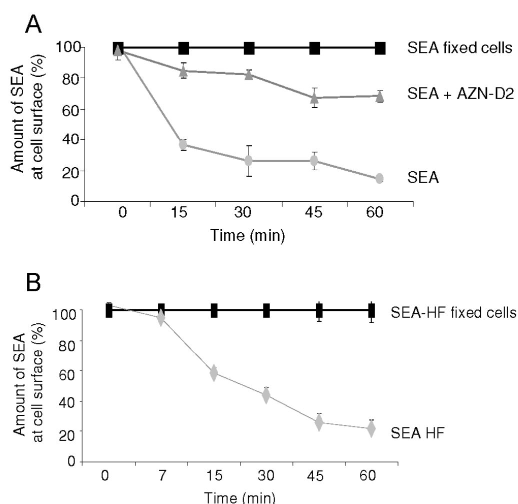

Fig. 2. Internalization of SEA in L-SIGN-transfected cells. (**A**) Internalization of biotinylated SEA (*grey circles*) from the cell surface of K562 cells stably transfected with L-SIGN was analyzed after different time intervals of incubation at 37°C using flow cytometry. Fixed cells (*black squares*) were used to correct for the off-rate of SEA at 37°C. Internalization of biotinylated SEA in the presence of mAb AZN-D2 (*grey triangles*) is clearly reduced. Data represent mean values of duplicate determinations. The representative result of one out of three independent experiments is shown. (**B**) Internalization of defucosylated, biotinylated SEA (SEA-HF, *grey diamonds*) bound to the cell surface of L-SIGN transfected cells was analyzed at different time points of incubation at 37°C using flow cytometry. Fixed cells (black squares) were used to correct for the off-rate of biotinylated SEA-HF at 37°C. Values represent means of duplicates. A representative result out of two independent experiments is shown.

Recognition of S. mansoni egg glycosphingolipids by L-SIGN

To establish whether L-SIGN binds authentic schistosomal glycosphingolipids, we performed cellular adhesion assays using K562 cells stably transfected with L-SIGN. Glycosphingolipids from *S. mansoni* cercariae, adults and eggs were isolated by organic solvent extraction and quantified by compositional analysis with regard to their carbohydrate content to ensure the application of similar, defined amounts of glycosphingolipids in all experiments. The results (Figure 3A) revealed that L-SIGN predominantly recognized egg glycosphingolipids, and to a significantly weaker extent glycosphingolipids from adult worms or cercariae. The blocking antibody AZN-D2 completely inhibited L-SIGN binding of egg glycosphingolipids, indicating that recognition is mediated through the CRD of L-SIGN (Figure 3A). The interaction of L-SIGN transfected cells with egg glycosphingolipids was abolished by adding EDTA (data not shown), thus demonstrating that the binding of egg glycosphingolipids to L-SIGN is calcium-dependent. To investigate whether the recognition of egg glycosphingolipids by L-SIGN is fucose-dependent, egg glycosphingolipids were treated with HF, which resulted in a removal of fucose residues from the glycan moieties, as demonstrated by ELISA using antibodies against the fucose-containing epitopes F-LDN and LDN-DF (Figure 3C). Integrity of the remaining

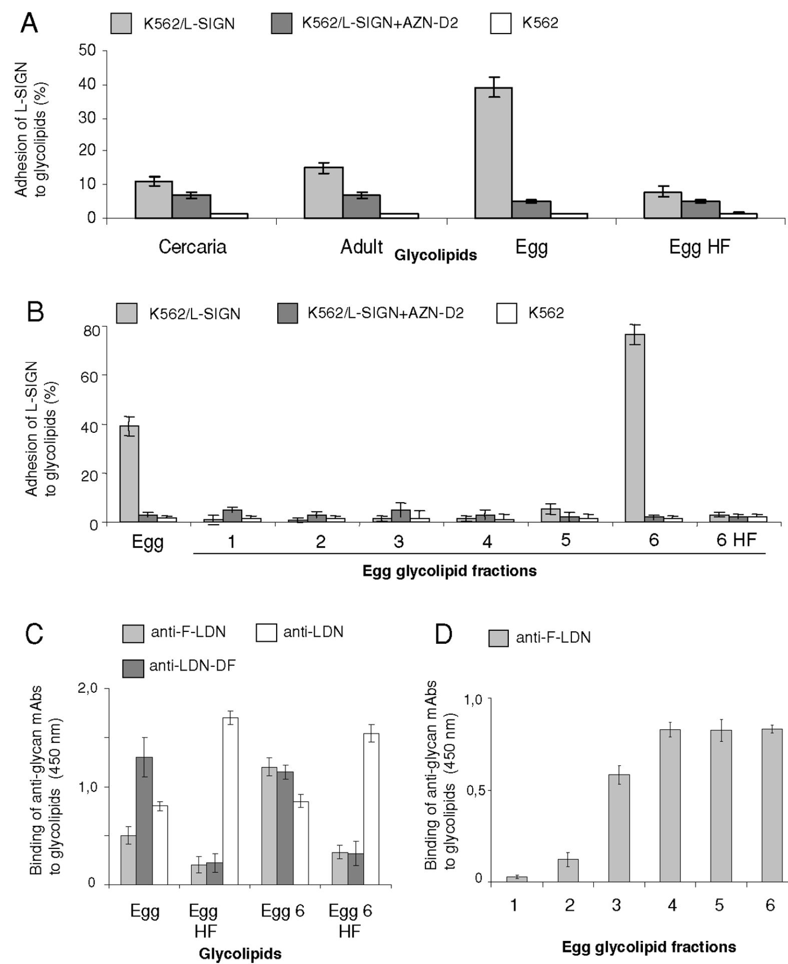

Fig. 3. Binding of L-SIGN-transfected cells to glycosphingolipids of *S. mansoni*. Similar amounts of glycolipids (9 ng/well) were applied in each case. All results are representative of three independent experiments, performed in triplicate (**A**, **B**) or duplicate (**C**, **D**), with error bars indicating standard deviation. (**A**) Binding of L-SIGN transfected cells (K562/L-SIGN) to glycosphingolipids obtained from cercariae (Cercaria), adult worms (Adult), eggs (Egg) and defucosylated egg glycosphingolipids (Egg HF) was determined by cell adhesion assay in the absence (*light grey bars*) or presence (*dark grey bars*) of a blocking mAb to L-SIGN (AZN-D2). (**B**) The binding of L-SIGN transfected cells (K562/L-SIGN) to fractionated egg glycosphingolipids was investigated by cell adhesion assay. Total egg glycosphingolipids (Egg), glycosphingolipid fractions 1 to 6 obtained by silica cartridge fractionation (1–6) and defucosylated fraction 6 egg glycosphingolipids (6 HF) were assayed for their binding to L-SIGN-transfected cells in the absence (*light grey bars*) or presence of AZN-D2 (*dark grey bars*) as blocking antibody. (**C**) Characterization of the glycan epitopes of schistosomal total egg glycosphingolipids (Egg), defucosylated total egg glycosphingolipids (Egg HF) and egg glycosphingolipid fraction 6 (Egg 6) and defucosylated egg glycosphingolipid fraction 6 (Egg 6 HF) by ELISA. Antibodies recognizing the fucose-containing glycan epitopes F-LDN and LDN-DF were tested to determine the efficacy of HF-treatment. In parallel, an anti-LDN mAb was employed to evaluate the integrity of the remaining glycans. (**D**) Reactivity of the monoclonal antibody M2D3H, recognizing F-LDN glycan epitopes, with the schistosomal egg glycosphingolipid fractions 1 to 6, monitored by ELISA.

glycan moieties of these glycosphingolipids was demonstrated by ELISA using an anti-LDN monoclonal antibody (Figure 3C), and by MALDI-TOF-MS (Figure 4). Intriguingly, defucosylation of the egg glycosphingolipids resulted in an almost complete loss of L-SIGN binding (Figure 3A). Hence, these studies demonstrate for the first time that L-SIGN binds to authentic carbohydrate structures expressed by *S. mansoni* egg glycosphingolipids and that this binding is fucose-dependent.

Binding of L-SIGN to individual S. mansoni egg glycosphingolipid fractions

To further characterize the glycans that mediate binding of L-SIGN to egg glycosphingolipids, we have size-fractionated these glycosphingolipids using silica cartridges. The monoclonal antibody M2DH3 reacted with most of these fractions, indicating the presence of terminal F-LDN epitopes on the glycosphingolipids (Figure 3D). L-SIGN, however, showed a very

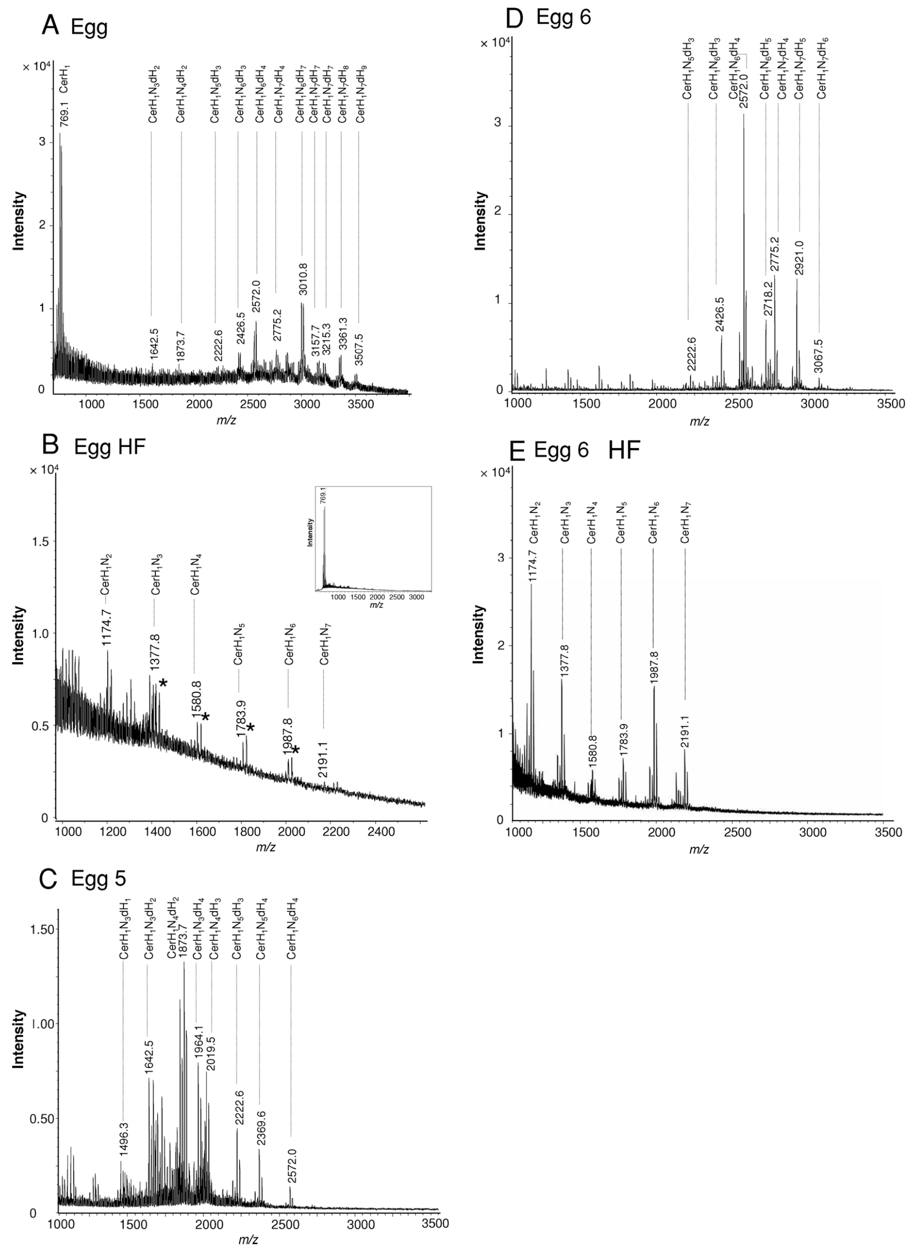

Fig. 4. MALDI-TOF-MS analysis of isolated glycosphingolipids from *S. mansoni* eggs. Total egg glycosphingolipids (**A**), defucosylated egg glycosphingolipids obtained after HF-treatment (**B**) and egg glycosphingolipid fractions 5 (**C**) and 6 (**D**), as well as defucosylated fraction 6 glycosphingolipids (**E**) from *S. mansoni* were analyzed by MALDI-TOF-MS. Relevant mass ranges of the recorded spectra are displayed. Inset in (**B**): Entire mass spectrum of total egg glycosphingolipids after HF-treatment. Deduced compositions are assigned to major pseudomolecular ions ($[M+Na]^+$). Corresponding potassium ions ($[M+K]^+$) are marked by asterisks. The complex pattern of registered signals is due to carbohydrate and ceramide heterogeneity. H: hexose; N: *N*-acetylhexosamine; dH: deoxyhexose (fucose); Cer: ceramide.

specific binding pattern in binding fraction 6 only (Figure 3B). Glycosphingolipid recognition could be completely inhibited using AZN-D2 as a blocking antibody, thus indicating L-SIGN specificity. A weak binding was observed in the case of the preceding fraction 5, which may be due to an overlap of related components. Fractions containing larger glycolipid species (Figure 4A) have not been recovered by this fractionation procedure and have, therefore, not been tested. Treatment of the fraction 6 glycosphingolipids with HF resulted in the removal of the fucose residues, as evidenced by ELISA (Figure 3D) and MALDI-TOF-MS (Figure 4). Defucosylation clearly abolished the binding of L-SIGN to these glycosphingolipids (Figure 3B), as already observed for total egg glycosphingolipids. These data indicated that L-SIGN specifically interacts with fraction 6 egg glycosphingolipid species in a fucose-dependent manner.

Characterization of total and fractionated S. mansoni egg glycosphingolipids

To allow a structural characterization of the glycosphingolipid species that are bound by L-SIGN, isolated egg glycosphingolipids were analyzed by MALDI-TOF-MS (Figure 4A). In agreement with previous studies (Kantelhardt et al. 2002; Wuhrer et al. 2002), the major signal observed for total egg glycosphingolipids corresponded to ceramide monohexoside (Hex_1Cer), with a mass of m/z 769.1 $[M+Na]^+$. In addition, a complex pattern of glycosphingolipids was registered, mainly due to the high heterogeneity of the respective carbohydrate and ceramide moieties. Prevailing species with masses of m/z 2572.0 $[M+Na]^+$ and m/z 3010.8 $[M+Na]^+$ represented monosaccharide compositions of $Hex_1HexNAc_6dHex_4$ and $Hex_1HexNAc_6dHex_7$. As a striking feature, a high number of multiply fucosylated species was observed comprising up to nine dHex units and a mass of m/z 3507.5 $[M+Na]^+$. To remove all fucose residues, egg glycosphingolipids were treated with HF and the resulting products were similarly analyzed by mass spectrometry (Figure 4B). Again, Hex_1Cer (m/z 769.1 $[M+Na]^+$) could be observed as the major species (Figure 4B; inset). A more detailed view of the spectrum, however, displayed the additional presence of complex glycosphingolipids with compositions of $Hex_1HexNAc_{2-7}Cer$ (Figure 4B). Remaining fucosylated glycosphingolipid species were not observed.

Glycosphingolipid fractions that showed binding to L-SIGN were similarly analyzed by MALDI-TOF-MS. The results obtained for fraction 5 and fraction 6 are shown in Figures 4C and D, respectively. The major compound of fraction 5 comprised a mass of m/z 1873.7 $[M+Na]^+$ representing a $Hex_1HexNAc_4dHex_2Cer$ glycosphingolipid. The highest mass registered at m/z 2572.0 $[M+Na]^+$ corresponded to a glycosphingolipid with a carbohydrate composition of $Hex_1HexNAc_6dHex_4$, thus reflecting an overall composition of $Hex_1HexNAc_{3-6}dHex_{1-4}Cer$ of the glycosphingolipids in this fraction. In contrast, fraction 6 (Figure 4D) comprised more complex glycosphingolipids with higher masses. The main compound was registered at a mass of m/z 2572.0 $[M+Na]^+$ in agreement with a composition of $Hex_1HexNAc_6dHex_4Cer$, whereas the overall composition of the major signals observed in this fraction was $Hex_1HexNAc_{5-7}dHex_{3-6}Cer$. MALDI-TOF-MS analysis of fraction 6 glycosphingolipids treated with HF revealed a complete lack of fucose and the presence of glycosphingolipids with a composition of $Hex_1HexNAc_{2-7}Cer$ (Figure 4E). The higher abundance of compounds with two or three HexNAc residues as compared to the starting material indicated that some degradation of the carbohydrate chains has occurred during chemical defucosylation. But as already evidenced by ELISA (Figure 3D) HF-treated fraction 6 glycosphingolipids still carried intact LDN epitopes, which is in agreement with MALDI-TOF-MS data.

MALDI-TOF-MS (/MS) of S. mansoni egg glycans

To simplify compositional and linkage analyses, egg glycosphingolipid fraction 6 was treated with endoglycoceramidase and the resulting oligosaccharides were analyzed by MALDI-TOF-MS (Figure 5A). As already shown for intact egg glycosphingolipids (Figure 3D) the glycan moieties in fraction 6 are multiply fucosylated ($dHex_{2-5}$). The major oligosaccharide consisted of $Hex_1HexNAc_6dHex_4$. Treatment of these glycans with HF yielded defucosylated glycans with the overall compositions of $Hex_1HexNAc_{5-7}$ (see inset in Figure 5A). The major glycan in this fraction 6 with the mass of m/z 2005.6 was further analyzed by MALDI-TOF-MS/MS (LID) (Figure 5B). Obtained data underline the composition of $Hex_1HexNAc_6dHex_4$. Moreover, the characteristic fragments registered in the MS/MS spectrum clearly demonstrated the presence of $HexNAc_1dHex_2$ (m/z 350.0; fragment B2α) and $HexNAc_2dHex_2$ unit (m/z 699.0; fragment B3α) as terminal epitopes and simultaneously excluded the occurrence of $HexNAc_1dHex_2$ and $HexNAc_2dHex_3$ structural elements, thus ruling out the presence of F-LDN-DF moieties in this compound. Hence, these data identify the F-LDN-F unit as major terminal epitope in fraction 6 egg glycosphingolipids.

Linkage analyses of S. mansoni egg glycans

To further corroborate the presence of the F-LDN-F epitope, linkage analysis of the glycan moieties, obtained from the total egg glycosphingolipids and glycosphingolipid fraction 6 was performed (Figure 6 A and 6B). This analysis revealed the presence of two different fucose derivatives (terminal fucose and 2-substituted fucose). The identity of these residues was confirmed by the electron impact mass spectrometry (see insets in Figure 6A and 6B). The linkage data strongly support our hypothesis that the glycosphingolipids in fraction 6 are multiply fucosylated, as already demonstrated by MALDI-TOF-MS. The amount of disubstituted fucose, however, has clearly decreased in fraction 6, compared to the total egg glycosphingolipids. Whereas the ratio of terminal fucose: 2-substituted fucose amounted to 0.43:0.57, in the case of total glycosphingolipid-derived glycans, it was found to be 0.87:0.13 in fraction 6. These data suggest that the terminal α1-3-fucose moieties in fraction 6 are of major importance for recognition by L-SIGN.

Linkage analyses of fraction 6 glycans (Figure 6C) further revealed five differently substituted HexNAc residues (terminal GalNAc; 4-substituted GlcNAc; 3-substituted GlcNAc; 3-substituted GalNAc and 3,4-disubstituted GlcNAc). The ratio of mono-substituted GlcNAc residues to 3,4-disubstituted GlcNAc clearly differed before and after HF-treatment. HF-treated fraction 6 glycan moieties which are not recognized by L-SIGN comprised only trace amounts of 3,4-disubstituted GlcNAc, whereas this type of branched monosaccharide unit represented a major constituent of untreated fraction 6 carbohydrate chains. Hence, linkage data clearly revealed a high branching of

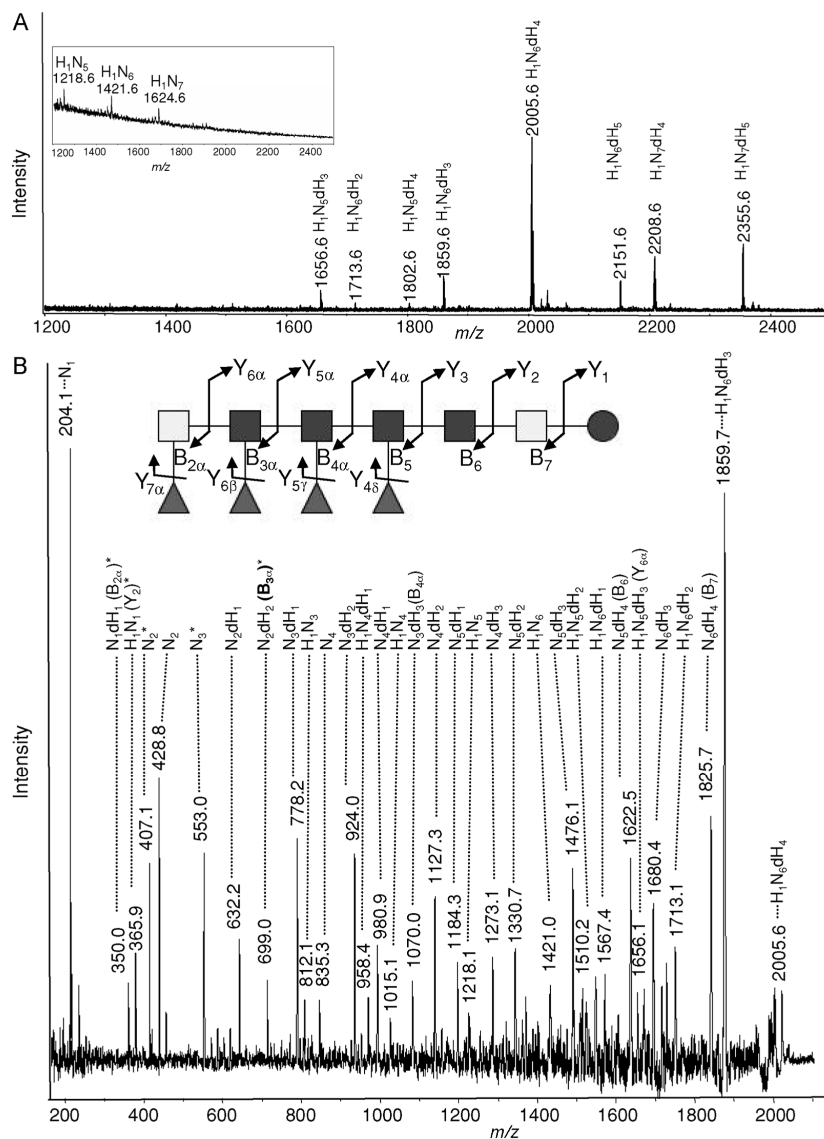

Fig. 5. MALDI-TOF-MS and MS/MS of glycans released by endoglycoceramidase from egg glycosphingolipid fraction 6. Oligosaccharides were released from fraction 6 egg glycosphingolipids by treatment with endoglycoceramidase and analyzed by MALDI-TOF-MS (**A**) and MS/MS (**B**). (**A**) MALDI-MS spectrum of glycans liberated from fraction 6 glycosphingolipids. Monoisotopic masses of pseudomolecular ions ($[M+Na]^+$) and deduced monosaccharide compositions are assigned. H: hexose; N: *N*-acetylhexosamine; dH: deoxyhexose (Fucose). Inset in (**A**): MALDI-TOF-MS of oligosaccharides released from fraction 6 egg glycosphingolipids after subsequent incubation with HF to release fucose residues. (**B**) Sodiated pseudomolecular ions of the glycan species *m/z* 2005.6 $[M+Na]^+$ with a composition of $Hex_1HexNAc_6dHex_4$, obtained by endoglycoceramidase treatment of fraction 6 egg glycosphingolipids, were analyzed by MALDI-TOF-MS/MS in the LID mode. Composition is given in symbols according to the Consortium for Functional Glycomics (see Table I for details); light grey square: *N*-acetylgalactosamine; dark square: *N*-acetylglucosamine; circle: glucose; triangle: deoxyhexose (fucose). Fragments in the structure are assigned using the nomenclature of Domon and Costello (Domon and Costello 1988). For the sake of clarity only the composition and not the putative origin of each fragment is assigned in the spectrum. H: hexose; N: *N*-acetylhexose; dH: deoxyhexose. Protonated ions are marked with an asterisk. Signals resulting from single cleavages are specified. Fragments verifying the presence of a terminal $HexNAc_2dHex_2$ unit are presented in bold-type.

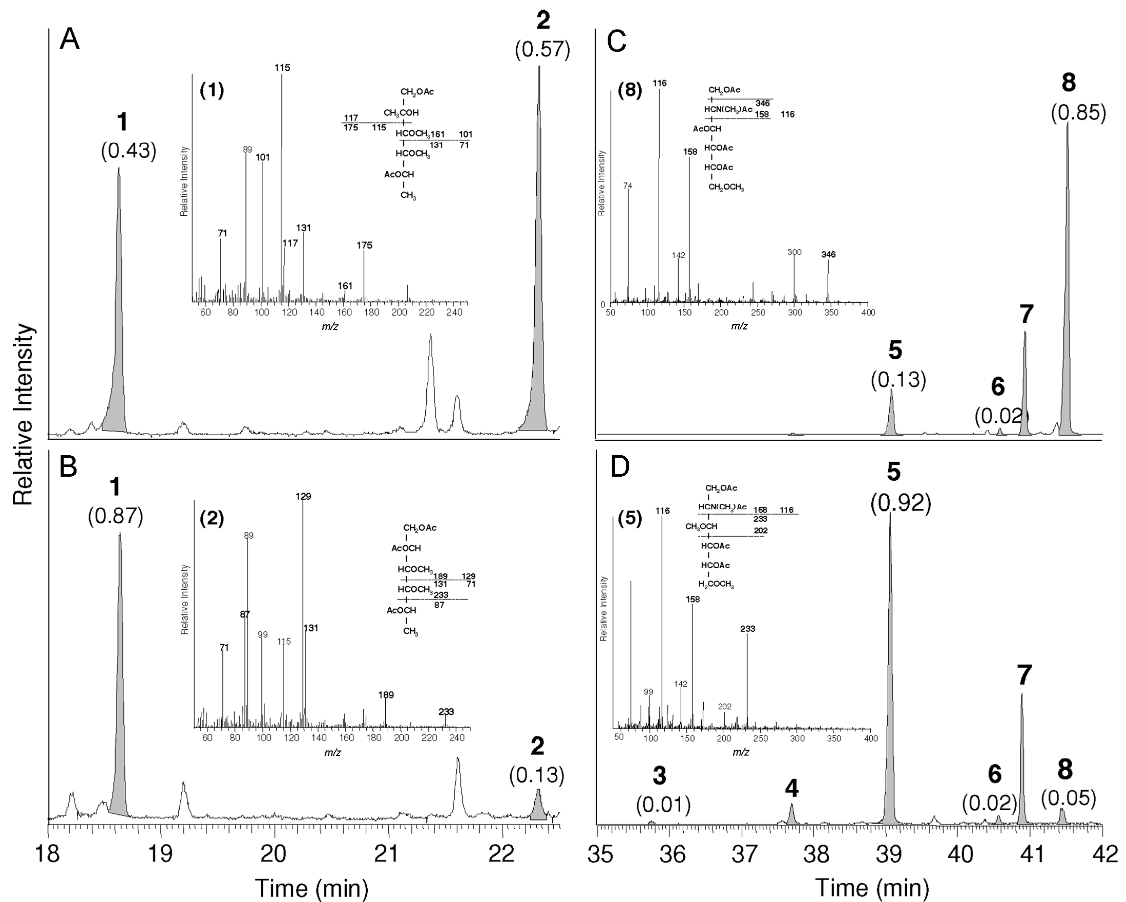

Fig. 6. Linkage analyses of glycans released by endoglycoceramidase from total egg glycosphingolipids and egg glycosphingolipid fraction 6. (**A–D**), linkage analyses of oligosaccharides released from both total and fraction 6 glycosphingolipids before and after HF-treatment. Partially methylated alditol acetates obtained were separated by gas chromatography and registered in the positive ion mode after electron impact ionization. To facilitate understanding, only parts of the entire chromatograms are shown. (**A** and **B**), detection of fucose derivatives obtained from total (**A**) and fraction 6 (**B**) glycans. Peak areas normalized to the sum of terminal fucose (1) and 2-substituted fucose (2) set to 1.0 are shown in parentheses. Inset in (**A**): Electron impact mass spectrum of 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-fucitol (terminal fucose); Inset in (**B**): 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-fucitol (2-substituted fucose). Characteristic primary and some secondary fragment ions are assigned. (**C** and **D**), detection of partially methylated HexNAc-derivatives obtained from fraction 6 glycans before (**C**) and after HF-treatment (**D**). The ratios of terminal GlcNAc (3), 4-substituted GlcNAc (5), 3-substituted GlcNAc (6) and 3,4-disubstituted GlcNAc (8) set to 1.0 are shown in parentheses to underline the clear loss of 3,4-disubstituted GlcNAc after incubation of fraction 6 glycans with HF. Inset in (**C**): Mass spectrum of 2-deoxy-2-(*N*-methyl) acetamido-1,3,4,5,-tetra-*O*-acetyl-6-*O*-methylglucitol. Inset in (**D**): 2-deoxy-2-(*N*-methyl)acetamido-1,4,5,-tri-*O*-acetyl-3,6-di-*O*-methylglucitol. (1) terminal fucose; (2) 2-substituted fucose; (3) terminal GlcNAc; (4) terminal GalNAc; (5) 4-substituted GlcNAc; (6) 3-substituted GlcNAc; (7) 3-substituted GalNAc; (8) 3,4-disubstituted GlcNAc.

fraction 6 glycan species due to multiple fucosylation in agreement with MALDI-TOF-MS. On the basis of MALDI-TOF-MS and linkage data we propose a summarized structure of fraction 6 egg glycosphingolipids, which is shown in Figure 8A.

Binding of the L-SIGN mutant Ser363Val to egg glycosphingolipids and SEA

To characterize in more detail the binding properties of the carbohydrate recognition domain from L-SIGN involved in the interaction with *S. mansoni* SEA and egg glycosphingolipids, we tested the binding capacity of two L-SIGN mutants (Figure 7), in which Ser^{363} has been replaced by Gly (S363G), or Val (S363V), the latter of which is present in DC-SIGN at the equivalent position. The S363G mutant recognized neither SEA nor egg glycosphingolipids, indicating the importance of Ser at this position for both types of recognition. Remarkably, L-SIGN S363V did not interact with the multiply fucosylated egg glycosphingolipid fraction 6 species. As described previously, the single amino acid replacement of Ser by Val (S363V) allows L-SIGN to recognize Le^X (van Liempt et al. 2004). In agreement

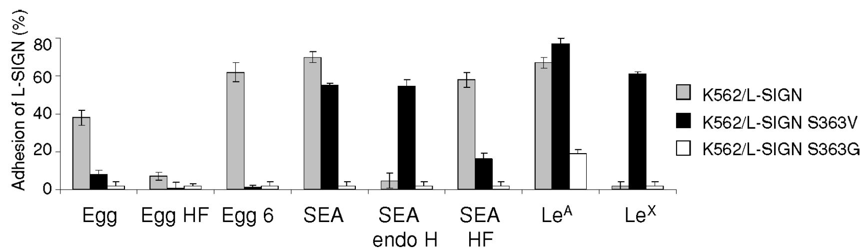

Fig. 7. Binding characteristics of wild-type L-SIGN and L-SIGN mutants. The binding of K562 cells stably expressing wild-type L-SIGN, as well as K562 cells stably expressing the L-SIGN mutants S363V and S363G, to *S. mansoni* egg glycosphingolipids and SEA is shown using cell adhesion assays. Total egg glycosphingolipids (Egg), defucosylated egg glycosphingolipids (Egg HF) and fraction 6 egg glycosphingolipids (Egg 6) as well as SEA, endo H-treated SEA (SEA endo H) and defucosylated SEA (SEA HF) were coated. Le^A-PAA (Le^A) and Le^X-PAA (Le^X) were used as controls. The experiment shown is a representative of three independent experiments, all performed in triplicate, with error bars indicating standard deviation.

with these previous findings, we have demonstrated in this study that the L-SIGN mutant S363V recognizes SEA (Figure 7). In contrast to wild-type L-SIGN, L-SIGN S363V also showed a clear binding to endo H-treated SEA and a strongly reduced recognition of the corresponding defucosylated antigens. These data most likely reflect the gained capacity of L-SIGN S363V to bind to Le^X -epitope of SEA.

Conformational analyses of the major fucosylated oligosaccharide from egg glycosphingolipid fraction 6

The initial conformation of the decasaccharide bearing F-LDN-F at its nonreducing end, i.e., Fucα1-3GalNAcβ1-4[(Fucα1-3)GlcNAcβ1-4]$_4$ (Figure 8A), was built using its structural similarity with Le^X. For this trisaccharide, NMR and conformational studies (Lemieux et al. 1980; Imberty et al. 1999) demonstrated that due to the presence of adjacent branching, the fucose and galactose rings stack one on each other, resulting in a rigid conformation for both the Galβ1-4GlcNAc and Fucα1-3GlcNAc glycosidic linkages, and indeed this corresponds to the conformation observed in the crystal structure of the L-SIGN/Le^X complex (Guo et al. 2004). The situation is similar in the decasaccharide and all Fucα1-3GlcNAc glycosidic linkages can only adopt the conformation that brings the branched fucose above the next GlcNAc (or GalNAc) of the main chain (Figure 8B). Only the fucose located at the nonreducing end, i.e., the one at position 3 of GalNAc appears to be more flexible and conformational analysis indicates that the Ψ torsion angle can vary up to 60°. The conformation displayed in Figure 8B is the lowest energy one.

Docking of glycosphingolipid oligosaccharides in the binding site of L-SIGN

A first docking approach was undertaken by fitting fucose residues of fucosylated oligosaccharides in the binding site of L-SIGN with the orientation observed in the crystal structure of the lectin complexed with Le^X (Guo et al. 2004), which validated the binding mode that we proposed previously by modeling (van Liempt et al. 2004). Three different orientations of the decasaccharide were tested, with either fucose A (α1-3 linked to GalNAc), B or C (α1-3 linked to external or internal GlcNAc), respectively. In all cases, the binding appears possible, with no major steric conflict, but not very favorable, due to the close position of Ser363 to the methyl-group of the *N*-acetyl residue of the adjacent GalNAc or GlcNAc. Only the fucose in the primary binding site establishes hydrogen bonds with the protein. It is therefore proposed that when possible, this binding mode will not result in sufficient affinity to be detected experimentally (by analogy to the nondetection of Le^X binding by L-SIGN).

Another orientation of fucose can be proposed, since it has been observed that the fucoses in several sialyl- and sulfo-Le^X derivatives are bound to mannose-binding protein (MBP) with O-2 and O-3 of fucose involved in calcium coordination (Ng et al. 1996; Ng and Weis 1997; Ng et al. 2002), and not O-3 and O-4 as in the first binding mode described above. Again three different fucose residues of the oligosaccharides were docked in this orientation. Docking of fucose A resulted in a stable binding mode. Geometric optimization of amino acid side chains and ligand yielded to the complex displayed in Figure 8C. In addition to the contact between the fucose, the calcium ion and the protein hydroxyl group in the main binding site, the GalNAc residue directly hydrogen bonds to Ser363 by its O-4 hydroxyl group and to Asn361 and Glu359 by the *N*-acetyl carbonyl. The adjacent GlcNAc does not interact directly with the protein but the fucose residue that it carries (Fucose B) establishes hydrophobic contact with the same region (CH_2 of carbon Cβ of Asn361 and Ser363). The remaining part of the decasaccharide (reducing end) does not interact with the protein and, therefore, has not been displayed in Figure 8C. Docking of the other fucose residues (i.e., the ones linked to GlcNAc) is also possible but, in the absence of an axial group at O-4 in the adjacent residue, the strong stabilisation that involves Ser363 does not occur.

Discussion

The human C-type lectin L-SIGN (liver/lymph node specific ICAM-3-grabbing nonintegrin) is expressed on liver sinusoidal endothelial cells (LSECs), which have a function as antigen-presenting cells in the liver (Bashirova et al. 2001). Since the liver is one of the main organs that is heavily affected during schistosomiasis as a result of the granuloma formation around trapped parasitic eggs (Bashirova et al. 2001; Wynn et al. 2004), L-SIGN is in the perfect position to function as an adhesion and internalization receptor for schistosome egg antigens. We therefore hypothesized that L-SIGN might be involved in the immunobiology and/or liver pathology of schistosomiasis, which implicates that L-SIGN should be able to recognize schistosomal antigens. Here we show that L-SIGN indeed recognizes

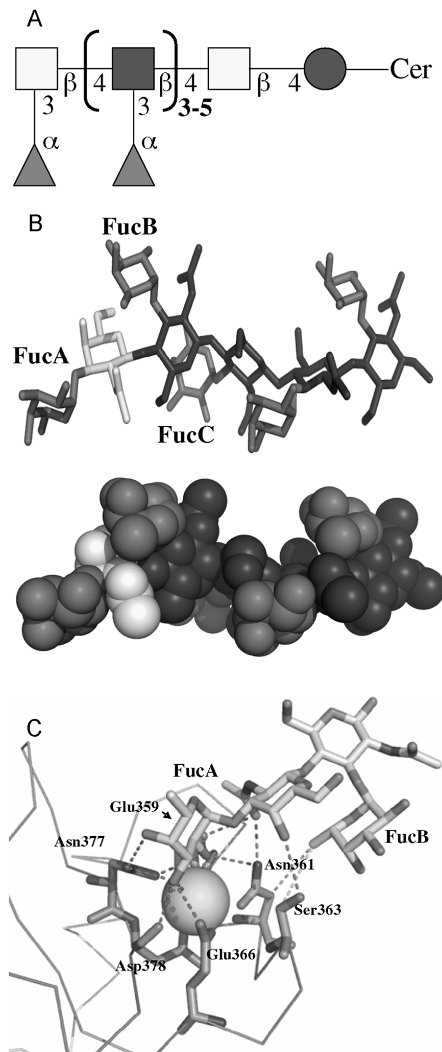

Fig. 8. Modeling of FDLNF and L-SIGN. (**A**) The carbohydrate epitope F-LDN-F, recognized by L-SIGN and present in schistosomal egg glycosphingolipids, is given as a schematic structure, to summarize the data obtained by ELISA, MALDI-TOF-MS and linkage analyses. The nomenclature as used by the EuroCarbDB is utilized (see Table I for details). blue circle: Glucose; yellow square: *N*-acetylgalactosamine; blue square: *N*-acetylglucosamine; red triangle: fucose. (**B**) Stick and space-fill representation of the lowest energy conformation of the terminal decasaccharide of the highly fucosylated glycosphingolipid. Fucose residues are colored in red, GalNAc in yellow and GlcNAc in blue. (**C**) Docking mode of the terminal tetrasaccharide of the highly fucosylated glycosphingolipid in the binding site of L-SIGN. Protein is represented by a line, oligosaccharide and amino acids of interest by stick and calcium ion by green sphere. Carbohydrate atoms are marked in green [C], red [O] and blue [N]. Hydrogen bonds are represented by magenta dot lines, and hydrophobic contacts by blue ones. Drawing has been performed with Pymol software (DeLano Scientific LCC, South San Francisco, CA) and hydrogen atoms have been omitted for clarity.

egg antigens of the human helminth parasite *S. mansoni*, on glycoproteins such as soluble egg antigens (SEA), and egg-derived glycosphingolipids. Remarkably, L-SIGN recognizes completely different glycan entities on egg glycoproteins and glycosphingolipids.

Our data demonstrate that within egg glycoproteins, L-SIGN recognizes primarily oligomannosidic *N*-glycans and shows little interaction with fucose residues. In addition to complex-type *N*-glycans, SEA contains hybride-type *N*-glycans and high-mannose type glycans, but data regarding the precise structures of the glycoprotein-glycans expressed in the egg stage or on SEA are still incomplete (Wuhrer and Geyer 2006).

Here we demonstrate for the first time that L-SIGN is also able to interact with pathogens via recognition of fucosylated glycans. Whereas L-SIGN did not bind to glycosphingolipids from cercarial or adult schistosomal stages, it specifically recognized a multiply fucosylated fraction within egg glycosphingolipids in a fucose dependent manner. This leads to the surprising conclusion that L-SIGN displays a completely different binding pattern to schistosome glycan antigens than DC-SIGN, both in its binding to SEA, as well as in its binding profile to schistosomal glycosphingolipids. It would be of interest to further characterize the relative binding affinities of L-SIGN to high-mannose type glycans and glycan epitopes on egg glycosphingolipids, but such experiments could not be performed so far due to limited amounts of parasite material. DC-SIGN, which contains a CRD domain that is highly similar to the one of L-SIGN, hardly interacts with the total egg glycosphingolipids (Meyer et al. 2005), or with the specific egg glycosphingolipid fraction that is bound by L-SIGN (data not shown). By contrast, DC-SIGN strongly binds to fucosylated cercarial and adult glycosphingolipids (Meyer et al. 2005), which are hardly recognized by L-SIGN. This differential binding mode of L-SIGN and DC-SIGN to fucosylated glycans is also reflected by the results of cell adhesion assays using a L-SIGN S363V mutant, which contains a Val present at a similar position as in DC-SIGN (van Liempt et al. 2004). In contrast to the wild-type L-SIGN, this mutant is able to recognize the fucosylated Le^X carbohydrate epitope in analogy to DC-SIGN. Intriguingly, this mutant is not able to recognize the multiply fucosylated glycan moieties of fraction 6 egg glycosphingolipids. Hence it may be suggested that L-SIGN can recognize fucose-containing Lewis antigens, such as Le^A and Le^B, in a binding mode essentially similar to DC-SIGN, but recognizes multiply-fucosylated glycosphingolipid glycans via an alternative binding-mode. It is remarkable that L-SIGN is able to interact with both oligomannosidic glycans, and differently fucosylated oligosaccharide ligands although comprising only one CRD. It has been reported, however, that the binding pocket of a lectin may change depending on the structural features of the glycan bound (Mitchell et al. 2001; Meyer et al. 2005; Karrar et al. 2007).

Structural data obtained by MALDI-TOF-MS and linkage analysis revealed that *S. mansoni* egg glycosphingolipids consist of a backbone of *N*-acetylhexosamine residues which may be heavily decorated with fucosyl- and oligofucosyl side chains (Khoo et al. 1997; Wuhrer et al. 2002). Our present analysis provided evidence that the minimum requirement for recognition of egg glycosphingolipids by L-SIGN is the presence of terminal Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ (F-LDN-F) tetrasaccharide. To increase our insight in the structural parameters that determine the recognition of multiply fucosylated

egg glycans by L-SIGN, we performed molecular modeling studies in which the docking of different fucosylated entities of the characterized egg glycosphingolipid glycan into the CRD of L-SIGN was investigated. From the present modeling study, it is not possible to state that L-SIGN binds to F-LDN-F or other fucosylated oligosaccharides in only one single way. Different binding modes are possible, involving either fucose on the chito-oligosacccharide backbone, or the one present at the nonreducing end. Nevertheless, based on the number of hydrogen bonds and hydrophobic contacts, one binding mode appears to be strongly preferred. This binding mode involves the terminal Fucα1-3GalNAc unit, and has the particularity that O-2 and O-3 hydroxyl groups of the fucose are coordinated by the calcium ion present in the binding site, instead of O-3 and O-4 as observed in L-SIGN/Le^X crystal structure (Guo et al. 2004).

The binding of the fucose residue linked to position 3 of GalNAc is not only favored in terms of energy, but is also validated by the experimental data since it allows for rationalizing some observations. The mutant Ser363Val, which strongly binds Lewis X, does not bind to the multiply fucosylated glycosphingolipid, which is in agreement with Ser363 being involved in a crucial hydrogen bond with O-4 of GalNAc. In the same manner, this O-4 hydroxyl group has to be in an axial configuration for optimal binding (i.e., Gal configuration over Glc), which would explain the strong observed preference for Fucα1-3GalNAc over Fucα1-3GlcNAc. The *N*-acetyl group of this GalNAc also plays a crucial role since its carbonyl atom is involved in two hydrogen bonds with protein side chains (Glu359 and Asn361). This prediction is in agreement with the absence of binding of pseudo-Le^Y (Fucα1-3Galβ1-4(Fucα1-3)GlcNAc-) that lacks this specific *N*-acetyl group (Wuhrer et al. 2000).

The finding that L-SIGN hardly interacts with multiply fucosylated glycoproteins within SEA is surprising. Many of the fucosylated epitopes in egg glycolipids are also found in SEA glycoproteins (Robijn et al. 2005). We have shown that the SEA glycoprotein preparation that we used in this study reacts with monoclonal antibodies that recognize Lewis X, LDN-F, LDN-DF, and F-LDN epitopes (Figure 1A, and results not shown). We could show that neoglycocojugates carrying Lewis X, LDN-F, and LDN-DF glycan antigens are not recognized by L-SIGN (van Liempt et al. 2004) and this study). Since the anti-F-LDN monoclonal antibody M2DH3 strongly recognizes SEA, cercarial glycolipids, and fraction 3 to fraction 6 egg glycolipid (Figure 3D; results not shown; (Kantelhardt et al. 2002)), it is unlikely that L-SIGN displays more than a weak interaction with F-LDN. In addition, our modeling data show that the GlcNAc of the F-LDN-F moiety does not interact directly with the protein. The α1-3fucose residue that it carries (Fucose B, see Figure 8C), however, establishes hydrophobic contact with the CH_2 of carbon Cβ of Asn361 and Ser363, thus contributing to the binding. Interestingly, from this model it can be deduced that an additional fucose α1-2-linked to fucose B, resulting in the epitope F-LDN-DF, would not sterically interfere with the binding of L-SIGN to the F-LDN-F unit. Thus, although we do not have experimental data to support this type of binding, we cannot exclude the possibility that the glycan F-LDN-DF can be bound by L-SIGN. The minor binding that we observed for L-SIGN to fucosylated species within SEA may be due to the presence of small amounts of F-LDN-(D)F epitopes within SEA, or to the binding to unknown fucosylated species.

Several studies have demonstrated that glycosylation of schistosome antigens plays an important role in immunological processes during schistosome infection (Faveeuw et al. 2002, 2003), such as the induction of hepatic granuloma formation by SEA or schistosome eggs that leads to severe fibrosis, hepatosplenomegaly and portal hypertension usually accompanying schistosomiasis (Okano et al. 1999; Pearce and MacDonald 2002; Sneller 2002). Schistosomal fucosylated glycoproteins and glycosphingolipids can be highly antigenic and act as potent immunomodulators (Velupillai et al. 2000; Okano et al. 2001; Van der Kleij et al. 2002) during parasite infection, indicating a major role of schistosomal egg carbohydrates in the initiation and homeostasis of the inflammatory response (Dyatlovitskaya and Bergelson 1987; Ziegler-Heitbrock et al. 1992; Lochnit et al. 1998; Eberl et al. 2001; Van der Kleij et al. 2002; van Die and Cummings 2006).

Although the involvement of glycans in immunomodulation is clearly established in schistosome infection, not much is known about the receptors involved in these processes. The present study is the first description of a lectin binding the stage-specific multiply fucosylated glycosphingolipids from schistosome eggs. In addition, our data demonstrate that L-SIGN has the potential to rapidly internalize egg glycoproteins. This may indicate that L-SIGN may capture and present antigens that are secreted by the eggs during schistosome infection, which in turn, may lead to the stimulation of T-cells. It is attractive to speculate that the uptake and the presentation of schistosome egg antigens by L-SIGN contributes to the high levels of glycan-specific antibodies found after egg-laying in schistosome infection.

Alternatively, or in addition to a proposed function in the presentation of schistosome egg antigens to T cells, L-SIGN may play a role in the actual trapping of schistosome eggs in the liver. LSECs, on which L-SIGN is constitutively expressed, are in permanent contact with the blood stream in the liver. Eggs, released from schistosome couples located in the liver portal veins, circulate through the bloodstream and L-SIGN is in the ideal position to interact with passing eggs. Whereas such eggs are obviously much too large to be internalized, they could be trapped by L-SIGN.

It will be important to establish whether the differential interaction of L-SIGN with either oligomannose-type *N*-glycans, and/or multiply fucosylated egg glycosphingolipids, controls the functional activity of L-SIGN in schistosome infections. Understanding of the molecular mechanisms by which the interaction between human lectins and parasitic glycans modulate the host immune response, and contribute to the severe pathology that is observed in schistosomiasis, may open novel ways to develop improved treatment of this infection.

Material and methods

Cell lines, antibodies and neoglycoconjugates

Human K562 cells stably expressing L-SIGN (K562/L-SIGN) have been described by Bashirova et al. (Bashirova et al. 2001) and K562 cells stably expressing the L-SIGN mutant S363V, in which Ser^{363} has been replaced by Val, by van Liempt et al. (van Liempt et al. 2004). The following antibodies were used: AZN-D2 (anti-DC-SIGN/anti-L-SIGN) (Bashirova et al. 2001), anti-Lewis X (G8G12) (Bickle and Andrews 1988), anti-LDN-DF (mAb 114-5B1-A) (van Remoortere et al. 2000), anti FLDN (mAb M2D3H) (Bickle and Andrews 1988),

anti-LDN-F (SmLDNF1) (Nyame et al. 2000) and anti-LDN (mAb SMLDN1.1) (Nyame et al. 1999). Neoglycoconjugates containing Le^X or Le^A multivalently coupled to biotinylated polyacrylamide (PAA) were from Lectinity (Lappeenranta, Finland). Neoglycolipids containing the LNFP III-epitope were synthesized as described before (Meyer et al. 2005). Neoglycoproteins consisting of BSA carrying LDN-DF and LDN-F antigens, respectively, were synthesized as described previously (van Remoortere et al. 2000).

Preparation of schistosome SEA and glycosphingolipid fractions

Crude *S. mansoni* SEA extract was centrifuged at 100,000 × *g* for 90 min at 4°C and sterilized by passing through a 0.2 μm filter (Nyame et al. 2003). Purified glycosphingolipids were isolated from lyophilized *S. mansoni* adult worms, eggs and cercariae by organic solvent extraction, saponification, desalting and anion-exchange chromatography as described previously (Wuhrer et al. 2000).

Neutral glycosphingolipids were fractionated chromatographically. Samples were dissolved in chloroform and applied after sonication on a 1 mL or 5 mL, depending on the amount of sample, silica cartridge (Waters, Eschborn, Germany), equilibrated with chloroform. Subsequent elution was achieved with chloroform:methanol (CM) and chloroform:methanol:water (CMW). Eluents used were CM 90:10 (v/v); CM 60:40 (v/v); CM 50:50 (v/v); CM 40:60 (v/v), CMW 65:25:4 (v/v/v) and CMW 10:70:20 (v/v/v). Resulting fractions (named 1–6) were analyzed by MALDI-TOF-MS and their carbohydrate content was quantified by compositional analyses using derivatization with anthranilic acid (Anumula 1994).

Matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS and MS/MS)

MALDI-TOF-MS and MS/MS analysis was performed on an Ultraflex instrument (Bruker-Daltonik, Bremen, Germany) equipped with a nitrogen laser and a LIFT-MS/MS facility as described previously (Geyer et al. 2005; Lehr et al. 2007). The instrument was operated in the positive-ion reflector mode using 6-aza-2-thiothymine (Sigma-Aldrich, München, Germany) as matrix throughout. About 100 to 500 individual spectra were summarized in each case.

Release and linkage analysis of schistosomal glycans

Carbohydrate moieties were liberated from egg glycosphingolipids by treatment with recombinant endoglycoceramidase II (from *Rhodococcus spp.,* Takara Shuzu Co., Otsu, Shiga, Japan). Released glycans were separated from ceramide residues by reverse-phase (RP-) chromatography as described previously (Wuhrer et al. 2000). For linkage analysis oligosaccharides were permethylated with methyl iodide after deprotonation with lithium methylsulfinyl carbanion and hydrolyzed (4 M aqueous trifluoroacetic acid, 100°C, 4 h). Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by capillary gas–liquid chromatography followed by electron impact ionization mode (single ion monitoring), using a PTV injector, fused-silica bonded-phase capillary columns of different polarity (60 m VF5MS and 30 m VF200MS; Varian Inc., CA) and helium as carrier gas as described elsewhere (Geyer and Geyer 1994).

Deglycosylation of schistosome egg glycoconjugates

To remove fucose residues from the egg glycoconjugates, dried samples of SEA or egg glycosphingolipids were treated with 48% (v/v) fluoric acid (HF) (Merck, Darmstadt, Germany) at 4°C for 48 h. HF was removed by a stream of nitrogen and the resulting pellet washed twice with methanol as described previously (Wuhrer et al. 2002). The degree of defucosylation and the integrity of the remaining glycan antigens, were assessed by MALDI-TOF-MS analysis and ELISA using monoclonal antibodies recognizing specific glycan epitopes.

To remove oligomannosidic *N*-glycans, SEA was treated with endoglycosidase H (endo H from *Streptomyces plicatus*, recombinant, *E. coli*; Calbiochem, Merck Darmstadt, Germany), which cleaves the chitobiose units of asparagine-linked oligomannose and hybrid, but not complex-type oligosaccharides of glycoproteins, as described by the manufacturer. In brief, SEA (100 μg) was denaturated by 5 min heating at 100°C in denaturating solvent (1% SDS, 2M β-mercaptoethanol). After cooling down, the enzyme (10 milliunits) was added and incubated at 37°C for 3 h.

Enzyme-linked immunosorbent assay (ELISA) with antiglycan antibodies

Solutions of total egg or HF-treated glycosphingolipids were diluted with ethanol, applied (6 or 9 ng/well) to NUNC maxisorb plates (Roskilde, Denmark) and incubated for 60 min at 37°C to coat the glycosphingolipids to the plate. SEA was diluted to 5 μg/mL in coating buffer (50 mM $NaHCO_3$) and coated 1 h at 37°C or at 4°C overnight. Plates were blocked with 1% ELISA grade BSA (Fraction V, fatty acid free; Calbiochem, San Diego, CA) in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and incubated with anti-glycan antibodies recognizing Le^X, LDN or LDN-DF (see Table I). Binding was registered using a horseradish peroxidase-labeled goat-anti-mouse antibody (Dako, Glostrup, Denmark). After coloring (4.5 mL 0.2 M Na_2HPO_4, 4.5 mL 0.1 M Citric acid, 1 mL TMB-solution (3,3′,5,5′-tetramethylbenzidine; 1 mg/mL H_2O), 10 μL H_2O_2) the optical density was measured at a wavelength of 450 nm using an ELISA reader (BioRad, Hercules, CA).

Enzyme-linked immunosorbent assay (ELISA) with DC-SIGN-Fc

Total cercarial and egg glycosphingolipids, as well as separated egg glycosphingolipid fractions 5 and 6 (9 ng) were diluted in ethanol on NUNC maxisorb plates (Roskilde, Denmark), and incubated for 60 min at 37°C to coat the glycosphingolipids to the plate. Plates were blocked with 1% ELISA grade BSA (Fraction V, Fatty acid free; Calbiochem, San Diego, CA) in TSM (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) and incubated with DC-SIGN-Fc (3 μg/mL) (Geijtenbeek et al. 2002). Binding was detected using a peroxidase labeled goat-anti-human IgG-Fc (Jackson, West Grove, PA). EDTA (10 mM, Roth, Karlsruhe, Germany) was added when indicated to investigate whether the binding was calcium dependent.

Cell adhesion assays with L-SIGN transfected cells

Ninety-six-well plates (NUNC maxisorb) were coated overnight at 4°C with *S. mansoni* SEA or neoglycoconjugates (5 μg/mL

in 50 mM $NaHCO_3$) or schistosomal glycosphingolipids (6 or 9 ng/well in ethanol, dried at 37°C). Blocking (30 min at 37°C) was performed with 1% BSA in TSM. Cells labeled with Calceine AM (25 μL/7 × 10^6 cells; Molecular Probes, Eugene, OR), were added for 1.5 h at 37 °C in the presence or absence of mAb AZN-D2 (20 μg/mL). Nonadherent cells were removed by gently washing with TSM. Adherent cells were lysed with 50 mM Tris–HCl, pH 7.4, 0.1% SDS and fluorescence was quantified using a Fluostar spectrofluorimeter (BMG Labtech, Offenburg, Germany) at 485/520 nm. Results are expressed as the mean percentage of adhesion of triplicate wells. All experiments are performed at least three times.

Internalization of SEA in L-SIGN transfected cells

Internalization of SEA was assessed as described previously (van Liempt et al. 2007). Shortly, K562/L-SIGN transfected cells (0.5 × 10^6 per sample) were incubated with biotinylated-SEA, or endo H-treated or HF-treated biotinylated-SEA (10 μg/mL) in TSA (TSM with 1% BSA) for 1 h on ice. Unbound ligand was washed off twice with ice-cold TSA. Specificity of the binding was established using nontransfected K562 cells, or by inhibition with the mAb AZN-D2 that recognizes L-SIGN. To this end K562/L-SIGN transfected cells were preincubated with the mAb for 30 min at 37°C. To control the off-rate of SEA at 37°C, cells were fixed with paraformaldehyde (2% in PBS) for 20 min at room temperature, prior to SEA binding. All cells were then incubated at 37°C with slight horizontal shaking (500 rpm) to enable internalization. At different time points (15–60 min) aliquots were taken and stored on ice. After washing with TSA, cells were incubated with Alexa 488-labeled avidin (Molecular Probes, Eugene, OR) for 30 min at 4°C, washed and analyzed by flow cytometry on a BD FACS Calibur or BD FACSScan (Beckton Dickinson, San Jose, CA). The relative differences in mean fluorescence intensity were determined in relation to the fluorescence observed in the case of fixed cells.

Molecular modeling

The coordinates of the crystal structure of human L-SIGN interacting with Le^X containing trisaccharide (Guo et al. 2004) (code 1SL6) were taken from the Protein Data Bank. The structure was edited using the Sybyl software (Tripos Inc., St Louis, MO), in order to contain only one protein monomer together with calcium ions and the Le^X trisaccharide. Protein hydrogen atoms were added, the peptide atoms partial charges were calculated using the Pullman procedure and the calcium ions were given a charge of two.

The tetrasaccharide Fucα1-3GalNAcβ1-4(Fucα1-3) GlcNAcβ was built by graphically editing the galactose residue of Le^X extracted from the above mentioned crystal structure into a GalNAc residue and adding a fucose residue on its 3 position. A decasaccharide with three additional Fucα1-3GlcNAcβ units on the reducing end was also built. Atom types and charges for oligosaccharides were defined using the PIM parameters developed for carbohydrates. A conformational search with the TRIPOS force-field allowed for defining the preferred conformations of the oligosaccharides.

Docking studies were performed by homology with L-SIGN/Le^X complex or with other C-type lectin interacting with fucose (see results). In all cases, one of the fucose residues of the oligosaccharide of interest was superimposed on the fucose in the crystal structure and the structures were merged. When needed, subsequent energy minimizations were performed using the Tripos force-field (Clark et al. 1989) with geometric optimization of the sugar and the side chains of amino acids in the binding sites. A distance-dependent dielectric constant was used in the calculations. Energy minimizations were carried out using the Powell procedure until a gradient deviation of 0.05 kcal/mol/Å was attained.

Conflict of information

None declared.

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Abbreviations

CD, cluster of differentiation; CRD, carbohydrate recognition domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrindHex, deoxyhexose; Egg 6, silica gel fraction 6 of *S. mansoni* egg glycolipids; Egg 6 HF, HF-treated silica gel fraction 6 of *S. mansoni* egg glycolipids; EI, electron impact; FA, Forssman Antigen; FLDN-F (Fucα1-3), GalNAcβ1-4(Fucα1-3)GlcNAc; Gb3, globotriaosylceramide; HCV, Hepatitis C Virus; Hex, hexose; HexNAc, *N*-acetylhexosamine; ICAM, intercellular adhesion molecule; LDN, GalNAcβ1-4GlcNAc; LDN-DF, GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAc; LID, laser-induced dissociation; LSECs, liver sinusoidal cells; L-SIGN, liver/lymph node-specific ICAM-3-grabbing nonintegrin; mAb, monoclonal antibody; MALDI-TOF-MS, matrix-assisted laser-desorption/ionization-time-of-flight mass spectrometry; PAA, polyacrylamide; SARS, severe acute respiratory syndrome; SEA, soluble egg antigens.

References

Adams DH, Hubscher SG, Shaw J, Rothlein R, Neuberger JM. 1989. Intercellular adhesion molecule 1 on liver allografts during rejection. Lancet. 2:1122–1125.

Alvarez CP, Lasala F, Carrillo J, Muniz O, Corbi AL, Delgado R. 2002. C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in *cis* and in *trans*. J Virol. 76:6841–6844.

Anumula KR. 1994. Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection. Anal Biochem. 220:275–283.

Bashirova AA, Geijtenbeek TB, van Duijnhoven GC, van Vliet SJ, Eilering JB, Martin MP, Wu L, Martin TD, Viebig N, Knolle PA, KewalRamani VN, van Kooyk Y, Carrington M. 2001. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. J Exp Med. 193:671–678.

Bickle QD, Andrews BJ. 1988. Characterisation of *Schistosoma mansoni* monoclonal antibodies which block in-vitro killing: Failure to demonstrate blockage of immunity in vivo. Parasite Immunol. 10:151–168.

Cambi A, Gijzen K, de Vries JM, Torensma R, Joosten B, Adema GJ, Netea MG, Kullberg BJ, Romani L, Figdor CG. 2003. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. Eur J Immunol. 33:532–538.

Clark M, Cramer RDI, Van Den Opdenbosch N. 1989. Validation of the general purpose Tripos 5.2 force field. J Comput Chem. 10:982–1012.

Colmenares M, Puig-Kroger A, Pello OM, Corbi AL, Rivas L. 2002. Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for Leishmania amastigotes. J Biol Chem 277:36766–36769.

Cummings RD, Nyame AK. 1996. Glycobiology of schistosomiasis. FASEB J. 10:838–848.

Cummings RD, Nyame AK. 1999. Schistosome glycoconjugates. Biochim Biophys Acta. 1455:363–374.

Dakappagari N, Maruyama T, Renshaw M, Tacken P, Figdor C, Torensma R, Wild MA, Wu D, Bowdish K, Kretz-Rommel A. 2006. Internalizing antibodies to the C-type lectins, L-SIGN and DC-SIGN, inhibit viral glycoprotein binding and deliver antigen to human dendritic cells for the induction of T cell responses. J Immunol. 176:426–440.

Domon B, Costello C. 1988. A systematic nomenclature for carbohydrate fragmentation in FAB-MS/MS spectra of glycoconjugates. Glycoconjugate J. 5:253–257.

Dyatlovitskaya EV, Bergelson LD. 1987. Glycosphingolipids and antitumor immunity. Biochim Biophys Acta. 907:125–143.

Eberl M, Langermans JA, Vervenne RA, Nyame AK, Cummings RD, Thomas AW, Coulson PS, Wilson RA. 2001. Antibodies to glycans dominate the host response to schistosome larvae and eggs: Is their role protective or subversive?. J Infect Dis 183:1238–1247.

Engering A, Geijtenbeek TB, van Vliet S J, Wijers M, van Liempt E, Demaurex N, Lanzavecchia A, Fransen J, Figdor CG, Piguet V, van Kooyk Y. 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J Immunol. 168:2118–2126.

Engering A, van Vliet SJ, Hebeda K, Jackson DG, Prevo R, Singh SK, Geijtenbeek TB, van Krieken H, van Kooyk Y. 2004. Dynamic populations of dendritic cell-specific ICAM-3 grabbing nonintegrin-positive immature dendritic cells and liver/lymph node-specific ICAM-3 grabbing nonintegrin-positive endothelial cells in the outer zones of the paracortex of human lymph nodes. Am J Pathol. 164:1587–1595.

Faveeuw C, Angeli V, Fontaine J, Maliszewski C, Capron A, Van Kaer L, Moser M, Capron M, Trottein F. 2002. Antigen presentation by CD1d contributes to the amplification of Th2 responses to Schistosoma mansoni glycoconjugates in mice. J Immunol. 169:906–912.

Faveeuw C, Mallevaey T, Paschinger K, Wilson IB, Fontaine J, Mollicone R, Oriol R, Altmann F, Lerouge P, Capron M, Trottein F. 2003. Schistosome *N*-glycans containing core a3-fucose and core b2-xylose epitopes are strong inducers of Th2 responses in mice. Eur J Immunol. 33:1271–1281.

Fukuda MN, Dell A, Tiller PR, Varki A, Klock JC, Fukuda M. 1986. Structure of a novel sialylated fucosyl lacto-*N*-norhexaosylceramide isolated from chronic myelogenous leukemia cells. J Biol Chem. 261:2376–2383.

Geijtenbeek TB, Krooshoop DJ, Bleijs DA, van Vliet SJ, van Duijnhoven GC, Grabovsky V, Alon R, Figdor CG, van Kooyk Y. 2000. DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol. 1:353–357.

Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR, Figdor CG, van Kooyk Y. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell. 100:587–597.

Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, Figdor CG. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell. 100:575–585.

Geijtenbeek TB, van Duijnhoven GC, van Vliet SJ, Krieger E, Vriend G, Figdor CG, van Kooyk Y. 2002. Identification of different binding sites in the dendritic cell-specific receptor DC-SIGN for intercellular adhesion molecule 3 and HIV-1. J Biol Chem. 277:11314–11320.

Geijtenbeek TB, van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, van Kooyk Y. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med. 197:7–17.

Geyer H, Wuhrer M, Resemann A, Geyer R. 2005. Identification and characterization of keyhole limpet hemocyanin *N*-glycans mediating cross-reactivity with *Schistosoma mansoni*. J Biol Chem. 280:40731–40748.

Geyer R, Geyer H. 1994. Saccharide linkage analysis using methylation and other techniques. Methods Enzymol. 230:86–107.

Guo Y, Feinberg H, Conroy E, Mitchell DA, Alvarez R, Blixt O, Taylor ME, Weis WI, Drickamer K. 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat Struct Mol Biol. 11:591–598.

Imberty A, Bettler E, Karababa M, Mazeau K, Petrova P, Pérez S. 1999. Building sugars: The sweet part of structural biology. Vijayan M., Yathindra N., et al., editors. Perspectives in Structural Biology. Hyderabad: Indian Academy of Sciences and Universities Press,392–409.

Jeffers SA, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, Thomas WD, Jr., Thackray LB, Young MD, Mason RJ, Ambrosino DM, Wentworth DE, Demartini JC, Holmes KV. 2004. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc Natl Acad Sci USA. 101:15748–15753.

Kantelhardt SR, Wuhrer M, Dennis RD, Doenhoff MJ, Bickle Q, Geyer R. 2002. Fucα1-3GalNAc-: The major antigenic motif of *Schistosoma mansoni* glycolipids implicated in infection sera and keyhole-limpet haemocyanin cross-reactivity. Biochem J. 366:217–223.

Karrar A, Broome U, Uzunel M, Qureshi AR, Sumitran-Holgersson S. 2007. Human liver sinusoidal endothelial cells induce apoptosis in activated T cells: A role in tolerance induction. Gut. 56:243–252.

Khoo KH, Chatterjee D, Caulfield JP, Morris HR, Dell A. 1997. Structural characterization of glycosphingolipids from the eggs of *Schistosoma mansoni* and *Schistosoma japonicum*. Glycobiology. 7:653–661.

Knolle PA, Gerken G. 2000. Local control of the immune response in the liver. Immunol Rev. 174:21–34.

Knolle PA, Limmer A. 2001. Neighborhood politics: The immunoregulatory function of organ-resident liver endothelial cells. Trends Immunol. 22:432–437.

Lehr T, Geyer H, Maass K, Doenhoff MJ, Geyer R. 2007. Structural characterization of *N*-glycans from the freshwater snail *Biomphalaria glabrata* cross-reacting with *Schistosoma mansoni* glycoconjugates. Glycobiology. 17:82–103.

Lemieux RU, Bock K, Delbaere LTJ, Koto S, Rao VSR. 1980. The conformations of oligosaccharides related to the ABH and Lewis human blood group determinants. Can J Chem. 58:631–653.

Limmer A, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, Momburg F, Arnold B, Knolle PA. 2000. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. Nature Medicine. 6:1348–1354.

Lochnit G, Nispel S, Dennis RD, Geyer R. 1998. Structural analysis and immunohistochemical localization of two acidic glycosphingolipids from the porcine, parasitic nematode, *Ascaris suum*. Glycobiology. 8:891–899.

Lozach PY, Lortat-Jacob H, de Lacroix de Lavalette A, Staropoli I, Foung S, Amara A, Houles C, Fieschi F, Schwartz O, Virelizier JL, Arenzana-Seisdedos F, Altmeyer R. 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J Biol Chem. 278:20358–20366.

Ludwig IS, Lekkerkerker AN, Depla E, Bosman F, Musters RJ, Depraetere S, van Kooyk Y, Geijtenbeek TB. 2004. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J Virol. 78:8322–8332.

McNab G, Reeves JL, Salmi M, Hubscher S, Jalkanen S, Adams DH. 1996. Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. Gastroenterology. 110:522–528.

Meyer S, van Liempt E, Imberty A, van Kooyk Y, Geyer H, Geyer R, van Die I. 2005. DC-SIGN mediates binding of dendritic cells to authentic

3 Discussion

In this chapter a synopsis of the obtained results will be presented together with an outlook on ongoing and future work.

In this thesis we have been interested in the question as to how human cells recognize an invading parasite, a process that involves both signature molecules of the pathogen and specific receptors on the host cell. Therefore, we investigated how specific glycoconjugates of the parasite *S. mansoni* are recognized by the two C-type lectins DC-SIGN and L-SIGN. The first publication resulting from this thesis describes the binding of authentic cercarial glycolipids from *S. mansoni* to the C-type lectin DC-SIGN. It could be further demonstrated that this interaction is mediated by Lewis X and pseudo-Lewis Y carbohydrate motifs. These epitopes are also present in the glycolipids of excretory/secretory (ES) products released from cercariae (104). In a second publication, the binding specificity of L-SIGN was investigated, demonstrating that this C-type lectin recognizes high-mannose type N-glycans of SEA as well as fucosylated glycolipids of schistosome eggs (103). The third publication reports on a new method allowing a rapid biotinylation of carbohydrates to simplify their usage for ligand/lectin studies which is applicable not only for parasitic ligands.

The early phase of schistosome infection and the concomitant role of carbohydrates

The parasite *S. mansoni* has evolved different strategies to survive in its hosts by down-regulating the immune response to benefit its own survival (117). In this context, the eggs are the main inducers of the shift from an early Th1 to a strong Th2 response (57). Notwithstanding the egg-induced immune modulation, there is an increasing number of reports claiming that immune regulation begins before egg deposition already in the cercarial stage (70). The role of carbohydrates in this process has been further established in the last years (65; 147). Likewise, studies on the function of lectins recognizing host-like or parasite-specific glycans in the immune processes of schistosome infection have gained increasing importance. However, before analyzing the different signaling pathways involved, the precise structures of the glycan-epitopes bound by the C-type lectins need to be elucidated. We could demonstrate that DC-SIGN recognizes authentic cercarial glycolipids carrying the Lewis X or the pseudo-Lewis Y epitope. Our observation, that in

contrast to cercarial glycolipids, egg glycolipids interacted only poorly with DC-SIGN is in agreement with previous studies, showing that the pseudo-Lewis Y epitope is solely found in cercarial glycosphingolipids, and Lewis X containing glycosphingolipids represent only a very small fraction of total egg stage glycosphingolipids (104; 165). In addition to Lewis X and pseudo-Lewis Y epitopes expressed on cercarial glycolipids (104), DC-SIGN also recognizes Lewis X and LDN-F motifs as well as high-mannose type glycans of schistosomal egg glycoproteins (148; 151). To understand how different glycan antigens can interact with DC-SIGN, the binding mode was determined by molecular modeling based on the crystal structure of DC-SIGN complexed with a Lewis X oligosaccharide. Interestingly the binding domain of DC-SIGN seems to be flexible due to the ability of the Phe313 side-chain to change its orientation, thus allowing an interaction with both Lewis X trisaccharide and pseudo-Lewis Y tetrasaccharide. It has been reported earlier that the binding pocket of a lectin may change depending on the structural features of the glycan bound (75; 105). Possibly pathogens such as *S. mansoni* may use this flexibility to target DC-SIGN.

Excretory/secretory products from cercariae

Schistosomes invade humans in the cercarial stage, and these cercariae transform into schistosomula directly after penetration of the skin by shedding their glycocalyx and releasing ES products. The latter compounds are rich in components of the highly glycosylated glycocalyx and products from the acetabular gland, containing serine proteases (70). Said ES products enter the surrounding human tissue and may be encountered by surveying DCs, such as the DC-SIGN positive CD1a negative dermal DCs, which are found mostly in the upper dermis (39; 53; 124). The immunomodulatory effect of these ES products has long been realized, but still little is known about the specific glycans leading to an immune response to ES products (91). Analyses performed in this thesis revealed that glycolipids present in these ES products contain species with Lewis X and pseudo- Le Y determinants (104). In contrast to Lewis X, which is found in both mammals and several pathogens, pseudo-Lewis Y is so far only described for schistosomes. Hence, is this pseudo-Lewis Y motif is the first parasite-specific ligand described for DC-SIGN. These results may enable follow-up studies to further investigate the role of the interaction between DC-SIGN and schistosomal glycolipids in host immunity. The schistosomula-released antigens stimulate not only innate immune cells, but are also

effective in limiting strong host immune responses. It is proposed that the active shedding of surface antigens may serve as an important source of parasite antigens available to the immune system in a form in which they can be taken up and processed by antigen-presenting dendritic cells, macrophages and certain B cells for presentation to T helper cells (91). Vieira *et al.* reported, that material released from cercariae could inhibit the *in vitro* proliferation of human PBMCs from schistosome infected patients in response to adult worm antigens (157). Moreover, several studies have shown that cercarial ES products, lead to the formation of edema and influx of neutrophiles when they are used to stimulate naïve skin (42; 137). Taken together these results led to the suggestion that products released by cercariae have the ability to modulate the host's immune response and promote the survival of the parasite. DC-SIGN recognizes cercarial glycolipids, but other molecules may be additionally involved.

Glycolipid signaling

Studies on the presentation of glycolipids to DCs have not been performed yet. Since it is unlikely that such a complex mixture of secreted glycoconjugates is recognized only by one lectin, a complex pattern of receptors on dendritic cells may be involved in antigen presentation. The role of glycolipids in signaling is still poorly understood. Faveeuw *et al.* have shown, however, that compounds of SEA are presented on CD1d which is also involved in glycolipid presentation for other pathogens (43). The antigen presentation of parasitic glycoconjugates to Cd1d-expressing T cells may be important for the induction of a Th2 response and the egg-induced pathology of schistosome infection. As DC-SIGN recognizes cercarial glycolipids carrying Lewis X or pseudo-Lewis Y motifs, it would be of high interest to investigate, as to whether these glycolipids are also presented to CD1d molecules. As pseudo-Lewis Y is a parasite-specific epitope, it could play a prominent role in the induction of an immune response to schistosomes.

The Th1 to Th2 switch and the role of DCs

It is known that schistosomes switch the immune response from a primarily Th1 to a strong Th2 response in the moment of egg deposition. The molecular mechanisms by which eggs or their secreted products induce a Th2 answer are not clear. In this context, a key role has been suggested for both human and murine dendritic cells, since DCs pulsed with SEA are able to elicit a Th2 response (96). But the response of

murine DCs to SEA led to surprising results, as they failed to up-regulate MHC II and co-stimulatory molecules. Moreover, murine SEA-pulsed DCs did not produce cytokines, whereas human DCs pulsed with SEA were described to fail in conventional maturation (152), but they are able to induce a Th2 response. These findings may lead to the suggestion that, besides DC activation, other DC-linked mechanisms may play a role in Th2 priming. We are currently testing, in cooperation with the group of Irma van Die and Yvette van Kooyk, whether DCs stimulated with schistosomal glycolipids are able to induce a Th2 response in naïve T cells. Preliminary results have shown that the induction of a Th2 response by schistosome glycolipids seems to be fucose-dependent, as a shift to Th2 was not observed with defucosylated HF-treated glycolipids.

The cytokine profile induced by schistosomes

The tendency of DCs to promote Th2 response, after being stimulated with helminth products, may reflect their inability to produce Th1-promoting cytokines like IL-12; IL-23 and IL-27 (115). In this respect it has been already shown that neither SEA nor products released by larvae 0-3 hours after transformation (0-3 hRP) are able to induce IL-12p70 production (71). Furthermore, 0-3 hRP inhibits the IL12-p40 release by macrophages when co-stimulated with LPS or Zymosan A, normally strong IL-12 and thereby Th1 inducers (70). In the same studies, however, levels of IL-6 and IL-10 were increased. In the case of DCs, the stimulation with 0-3 hRP expanded the amount of IL-12 in response to CD154-transfected fibroblasts (71). This indicates that 0-3 hRP has different effects on DCs and macrophages, maybe due to the expression of different surface receptors. As we are already analyzing the response of DCs to schistosomal glycolipids, it would be of further interest to investigate, how macrophages respond to schistosomal glycolipids.

The role of TLRs in schistosome infection

The role of Toll-like receptors (TLRs) during schistosome infection represents currently a broad field of interest. The group of Francois Trottein analyzed distinct TLR members with regard to their ability to respond to living schistosome eggs, demonstrating for example that double-stranded RNA (dsRNA) from schistosome eggs is able to activate TLR3 resulting in the activation of NF-kappa B. In addition they showed that stimulation of DCs with schistosome eggs results in type I interferon (IFN) and IFN-stimulated gene (ISG) expression. Furthermore, *S. mansoni*

eggs were shown to activate myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways in DCs (2). The Lewis X epitope, present in Lacto-N-fucopentaose III (LNFP III), can directly activate murine bone marrow-derived DCs by a TLR 4-dependent mechanism. Lewis X functions as an innate Th2 promoter via its action on murine dendritic cells, and it has been demonstrated that the α1-3-linked fucose constituent is required for this activity (138). Lyso-phosphatidyl-serine (lyso-PS), isolated from *S. mansoni* eggs, was shown to activate human monocyte-derived DCs through TLR 2, thus leading to the development of IL-10 producing regulatory T cells and the induction of a Th2 response (145). In some publications lyso-PS is called glycolipid (2; 22), which is wrong. Until now no investigation of the interaction on glycolipids from schistosomes and TLRs has been published.

Other CLRs involved during schistosomiasis

Besides TLRs and the CLRs, DC-SIGN and L-SIGN, mentioned above schistosome-derived glycans can be recognized by further CLRs present on DCs. The macrophage galactose-type lectin (MGL), for example, binds to LDN and LDN-F moieties of SEA (154). For the mannose receptor (MR) the specific glycans mediating binding to SEA have not yet been identified, but they may also be assumed mannose-containing epitopes (92).

The signaling of CLRs

CLRs contain signaling motives in their cytoplasmic domains which might be able to activate intracellular signaling cascades after ligand binding (45). This field of research has to be further investigated, but for some signaling motifs their general function has already been elucidated. DC-SIGN contains a tyrosine-based motif which is involved in the rapid internalization of ligands from the cell surface (13). Both DC-SIGN and L-SIGN possess a di-leucine motif in their cytoplasmic tail which is responsible for the targeting to lysosomal/endosomal vesicles and is also involved in receptor-mediated endocytosis (99). In addition DC-SIGN and L-SIGN comprise a tri-acidic cluster which plays a role in the transport to MHC II-positive, late endosomes and lysosomes (14). The occurrence of these signaling motifs explains the targeting of SEA to MHC II-positive, lysosomal compartments (152). Hence SEA captured by C-type lectins might be presented on MHC II, thereby inducing a SEA-specific Th2 response. However, further work is required to

understand the exact mechanism leading to this type of immune. The signaling cascades induced by ligation of CLRs are still poorly investigated. One recent paper describes that the binding of ligands to DC-SIGN induced the phosphorylation of Extracellular-signal regulated kinase (ERK 1/2) and Akt (protein kinase B), without the concurrent activation of p38 MAP Kinase (18). Furthermore, ligation of DC-SIGN has been shown to rigger the phosphorylation of phospholipase Cγ in dendritic cells. Since pathogens stimulate Th2 responses mainly via activation of ERK1/2, these data provide a molecular explanation for the ability of DC-SIGN-interacting pathogens to preferentially evoke Th2-type immune responses (18). Agrawal and co-workers investigated the function of MAP Kinase-mediated signaling in the Th2 response to schistosomal SEA. They demonstrated that SEA is only a weak inducer of p38, but leads to the phosphorylation of ERK1/2 which suppresses the production of IL-12p70. As blocking of ERK1/2 did not result in an increase of IL-12p70, the authors assume that additional mechanisms regulate the suppression of IL-12p70 by SEA. Hence SEA-induced suppression of IL-12p70 might be important in the generation of the Th2 response (18).

The hygiene-hypothesis

It is the long lasting Th2 immune response that is characteristic for helminth infections. Our immune system has been shaped over time by a continuous exposure to infectious agents suggesting a co-evolution of parasites and humans (35). A key feature of parasite infection is that the invaders are able to down-modulate the host's immune response (35). The so-called "hygiene-hypothesis" claims that the lapse of parasite infection in our high-civilized world leads to a strong boost of allergies and autoimmune diseases (170). Knowledge on the molecular mechanisms in which helminths are able to modulate the immune response, as well as on the chemical nature of parasite-derived molecules, known to elicit an anti-inflammatory response, will help to understand the relevant immune processes in this challenging field of autoimmune disease and allergy.

Schistosomiasis and HIV infection

Furthermore, results achieved by studying the function of DC-SIGN in schistosomiasis might help to define the mechanisms involved in HIV infection, as DC-SIGN mediates the HIV infection *in trans* (52). Only a small number of studies is dealing with co-infection of HIV and schistosomes, but it has been already

described ten years ago that the secretion of eggs is clearly reduced in HIV patients (74). It has been further reported that *S. mansoni* co-infection with SHIV (simian-human immunodeficiency virus) led to significant increase of viral replication (7). Intriguingly, *S. mansoni* co-infection enhanced the expression of Th2-associated cytokine responses and SHIV replication during both acute and chronic phases of SHIV infection. These results support the hypothesis that concomitant schistosomiasis up-regulates replication of immunodeficiency viruses in co-infected hosts, raising the possibility that parasite-infected individuals may also be more susceptible to acquisition of HIV-1 infection (20).

The glycan-binding profile of L-SIGN

The pathology of schistosome infection results mainly from granuloma in the liver due to entrapped eggs. Therefore, we have investigated, whether the C-type lectin L-SIGN which is present on liver resident antigen-presenting cells called LSECs (liver sinusoidal endothelial cells), is able to recognize schistosomal glycans. Remarkably, we could demonstrate that L-SIGN recognizes different glycan entities on egg glycoproteins and glycosphingolipids. Within egg glycoproteins of SEA, L-SIGN recognized primarily high-mannose type N-glycans and showed little interaction with fucose residues. It is known that, in addition to complex-type N-glycans, SEA contains hybrid-type N-glycans and high-mannose type N-glycans, but knowledge on the precise structures of the glycoprotein-glycans expressed in the egg stage or on SEA is still incomplete (166). In addition to the insights in the binding of L-SIGN to SEA, we were able to demonstrate for the first time that L-SIGN is able to interact with fucosylated glycans of schistosomal glycolipids. Whereas L-SIGN was found to bind only weakly to glycosphingolipids from cercarial or adult schistosomal stages, it specifically recognized a fucosylated fraction within egg glycosphingolipids in a fucose-dependent manner. Remarkably, L-SIGN exhibited a completely different binding pattern to schistosome glycan antigens than DC-SIGN, both in its interaction with SEA as well as in its binding profile to schistosomal glycosphingolipids. It would be of high interest to characterize the relative binding affinities of L-SIGN to high-mannose type glycans and glycan epitopes on egg glycosphingolipids, but such experiments could not be performed so far due to limited amounts of parasite material. Nevertheless, we could clearly identify the epitope bound by L-SIGN present in the egg glycolipids. Using a combination of several analytical techniques, we could demonstrate that F-LDN-F

represents a carbohydrate epitope mediating binding to L-SIGN. Intriguingly, F-LDN-F is also one epitope causing serological cross-reactivity of SEA with keyhole limpet-hemocyanine (KLH) (56).

The role of glycans in the formation of liver granulomata

In *in vitro* studies it could be demonstrated that beads coated with SEA or KLH are able to induce granuloma formation similar to schistosome eggs. The destruction of the integrity of the glycans by sodium periodate completely abolished the immunological and functional properties of SEA tested in these experiments (143). In a follow-up study from the same group the granulogenic property of SEA was referred to LN or LDN moieties, but not to fucosylated glycans (142). However, previous observations showed that sensitization with Lewis X containing neoglycoconjugates increases the size of the granulomata formed around SEA beads suggesting an opposite effect (68). Further work has to be performed to define precisely the role of the different glycans like LDN, F-LDN-F or Lewis X on SEA in their granulogenic activity.

The Lewis X epitope

The Lewis X epitope is widespread in the pathogen world. Already 4 decades ago the "molecular mimicry" hypothesis was developed, claiming that parasites share antigens with their host (24). The blood group antigen Lewis X occurs not only in schistosomes, but is, for example, also present on the ruminant parasite *Haemonchus contortus* and the human gastric pathogen *Helicobacter pylori* which is able to switch in its LPS the Lewis antigens on and off. Only the Lewis-positive variants are able to bind to DC-SIGN and this interaction is responsible for blocking the Th1 response (11). Hence, this is an additional example to show how the binding to DC-SIGN modulates the balance between Th1 and Th2 response. Another case demonstrating how pathogens are able to shift the Th1/Th2 balance towards Th2 is provided by *Mycobacterium tuberculosis* which binds via its cell wall component ManLAM to DC-SIGN. ManLAM inhibits the TLR-induced maturation and induces the production of IL-10 (54). Altogether these studies provided clear evidence that the interaction of a pathogen with DC-SIGN may influence the fate of naïve T cells which might be a common mechanism for the mode of action of CLRs.

The binding specificities of L-SIGN and DC-SIGN

It is interesting that the two highly related CLRs, such as DC-SIGN and L-SIGN, differ in their ability to recognize Lewis X. DC-SIGN, the CRD of which is strongly similar to the one of L-SIGN, hardly interacts with the total egg glycosphingolipids (104), and does not recognize the F-LDN-F epitope bound by L-SIGN. In contrast, DC-SIGN binds to fucosylated cercarial and adult glycosphingolipids which are hardly recognized by L-SIGN, as could be shown in this thesis. This differential binding mode of L-SIGN and DC-SIGN to fucosylated glycans has been also demonstrated by cell adhesion assays using a L-SIGN S363V mutant, containing a Valine residue present at a similar position as in DC-SIGN (151). In contrast to the wild-type L-SIGN, this mutant is able to recognize the fucosylated Lewis X carbohydrate epitope in analogy to DC-SIGN. Remarkably this mutant is no longer able to bind to F-LDN-F moieties on egg glycosphingolipids. Hence, we concluded that L-SIGN can recognize fucose-containing Lewis antigens, such as the Lewis antigens Lewis A and Lewis B, in a way similar to DC-SIGN, but recognizes multiply-fucosylated glycosphingolipid glycans via an alternative binding mode. It is astonishing that L-SIGN is able to interact with both high-mannose type glycans and differently fucosylated oligosaccharide ligands although comprising only one CRD. It has been reported several times, however that the binding pocket of a lectin may change depending on the structure of the glycan bound (75; 105). Maybe both C-type lectins, L-SIGN as well as DC-SIGN, use this described flexibility in their CRD to recognize different ligands.

Detailed analyses of the interactions of defined glycoconjugates structures and their corresponding lectins are still in the beginning. But the results achieved so far show that parasites have co-evolved with us and are, therefore, successfully able to challenge our highly developed immune system.

4 Summary

In this thesis the interactions of glycoconjugates, mainly glycosphingolipids, of the parasitic helminth *Schistosoma mansoni* with the human C-type lectins DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) and L-SIGN (liver/lymph node specific ICAM-3-grabbing nonintegrin) have been investigated. Since DC-SIGN is known to act as a pathogen recognition receptor at an early stage of infection, glycolipids of cercariae, the human infectious larvae, have been isolated, and the glycan moieties have been released enzymatically. After separation into single glycan species via HPLC and subsequent structural characterization, neoglycolipids, carrying either Lewis X or pseudo-Lewis Y epitopes, were attached to a synthetic lipid anker by reductive amination. Solid phase assays using a recombinant chimeric DC-SIGN-Fc and binding studies with dendritic cells revealed that both carbohydrate species are recognized by DC-SIGN. Hence, pseudo-Lewis Y is the first parasite-specific ligand described for DC-SIGN so far. Molecular modeling further revealed that the observed binding of this schistosome-specific pseudo-Lewis Y motif to DC-SIGN is not directly compatible with the published model described for Lewis X. To fit pseudo-Lewis Y into the model, the orientation of the side chain of Phe313 in the secondary binding site of DC-SIGN was slightly changed, resulting in an energetically perfect stacking of Phe313 with the hydrophobic side of the galactose-linked fucose of pseudo-Lewis Y. We propose that pathogens such as *S. mansoni* may use this observed flexibility in the secondary binding site of DC-SIGN to target DCs, which may contribute to an escape from the host's immune response. Furthermore, we were able to detect the presence of both Lewis X and pseudo-Lewis Y carbohydrate epitopes in glycolipids derived from cercarial *S. mansoni* excretory/secretory products, underlining their role in the immunobiology of schistosome infection.

L-SIGN, i.e., the second human C-type lectin investigated, functions as antigen receptor on human liver sinusoidal endothelial cells. As the eggs of *S. mansoni* are the main inducers of a Th2 response, we were interested, as to whether glycans expressed on egg glycoproteins or egg glycolipids may play a role in this immune response, e.g., via binding to L-SIGN. Our data demonstrate that L-SIGN binds both schistosomal soluble egg antigens (SEA) and egg glycosphingolipids, and can mediate internalization of SEA by L-SIGN expressing cells. After treatment of SEA with endoglycosidase H to remove high-mannose type N-glycans, binding to L-SIGN

and internalization by L-SIGN expressing cells was clearly reduced, whereas defucosylation affected neither binding nor internalization. These data indicate that L-SIGN interacts with high-mannose type N-glycans of SEA. In parallel, L-SIGN was also tested for binding to egg glycosphingolipids. To this end, isolated glycolipids were fractionated and tested in binding studies using L-SIGN transfected cells. The results revealed that L-SIGN binds to a glycosphingolipid fraction containing fucosylated species with compositions of $Hex_1HexNAc_{5-7}dHex_{3-6}Cer$, as confirmed by mass spectrometry. Subsequent linkage analyses, tandem mass spectrometry and molecular modeling studies demonstrated that binding of L-SIGN to the respective fucosylated egg glycosphingolipid species involves a Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc tetrasaccharide (F-LDN-F) at the non-reducing end of the carbohydrate chain. The L-SIGN "gain of function" mutant Ser363Val, which recognizes fucosylated Lewis antigens, did not bind to this fucosylated egg glycosphingolipid fraction, suggesting that L-SIGN exhibits different modes of binding to fucoses of egg glycosphingolipids and Lewis antigens. Taken together, our data indicate that L-SIGN recognizes both high-mannose type N-glycans and fucosylated carbohydrate motifs within schistosomal egg antigens, demonstrating that L-SIGN has a broad but specific glycan recognition profile.

It is astonishing that such highly related C-type lectins differ in their carbohydrate recognition properties. Clearly, further studies are needed to gain deeper insights in the manifold ways parasitic glycans are able to bind to human C-type lectins and thereby influence the host's immune response.

5 Zusammenfassung

In der vorliegenden Arbeit wurden die Interaktionen von Glykokonjugaten, hauptsächlich Glykolipiden, des Humanparasiten *Schistosoma mansoni* mit den humanen C-typ Lektinen DC-SIGN (dendritic cell-specific ICAM-3 grabbing nonintegrin) und L-SIGN (liver/lymph node specific ICAM-3-grabbing nonintegrin) untersucht. Da DC-SIGN bereits im frühen Stadium der Infektion als Pathogenrezeptor fungiert, wurden Glykolipide aus *S. mansoni* Cercarien, den humaninfektiösen Larven, isoliert und deren Kohlenhydratanteile enzymatisch freigesetzt. Nach Auftrennung dieser Glykane durch verschiedene HPLC-Techniken wurden die resultierenden Fraktionen mittels MALDI-TOF-MS charakterisiert. Aus Glykanen, die das Lewis X [Galβ1-4(Fucα1-3)GlcNAc] oder das pseudo-Lewis Y Epitop [Fucα1-3Galβ1-4[Fucα3]GlcNAcβ-] tragen, wurden Neoglykolipide durch reduktive Aminierung an einen Lipidanker synthetisiert. Verschiedene Bindungsstudien mit rekombinantem, chimärem DC-SIGN-Fc und humanen dendritischen Zellen (DCs) zeigten, dass diese beiden Kohlenhydratepitope von DC-SIGN gebunden werden. Somit ist das schistosomenspezifische pseudo-Lewis Y Motiv der erste pathogenspezifische Ligand welcher für DC-SIGN entdeckt wurde. Durch molekulares Modellieren konnte gezeigt werden, dass zur Bindung von pseudo-Lewis Y die Orientierung der Seitenkette der Aminosäure Phe313 in der „secondary binding site“ von DC-SIGN leicht geändert werden musste, was jedoch in einer energetisch optimalen Bindung von Phe313 mit der hydrophobe Seite der an die Galaktose gebundenen Fucose des pseudo-Lewis Y resultierte. Daher wurde postuliert, dass Pathogene wie *S. mansoni* diese, auch von anderen Autoren beobachtete Flexibilität in der „secondary binding site“ von DC-SIGN, nutzen, um mit DCs zu interagieren, was zur Immunmodulation beitragen könnte. Des Weiteren konnte das Vorkommen von Lewis X und pseudo-Lewis Y Epitopen auf Glykolipiden, welche aus den exkretorischen/sekretorischen (E/S)- Produkten von Cercarien gewonnen wurden, aufgezeigt werden, was die Rolle dieser beiden Epitope in der Immunmodulation durch *S. mansoni* unterstreicht.

L-SIGN, das zweite untersuchte humane C--typ Lektin, fungiert als Antigenrezeptor auf humanen sinusoidalen Endothelzellen der Leber (liver sinusoidal endothelial cells; LSECs). Da insbesondere die Eier von *S. mansoni* stark immunogen und ein Hauptauslöser der Th2 Immunantwort sind, interessierte uns, in wiefern deren Kohlenhydrate, vorliegend als Glykoproteine oder Glykolipide, in der

schistosomenspezifischen Immunantwort beteiligt sind, und welche Kohlenhydratepitope von L-SIGN gebunden werden. Die gewonnenen Daten belegen, dass L-SIGN sowohl Glykoproteine aus löslichen Eiantigenen (SEA, soluble egg antigens) als auch Glykolipide aus Eiern bindet. Des Weiteren konnte gezeigt werden, dass von L-SIGN gebundenes SEA auch in die Zelle internalisiert wird. Nach Behandlung von SEA mit Endoglykosidase H, welche oligomannosidische N-Glykane („high-mannose type“) abspaltet, waren jedoch keine Bindung an L-SIGN und folglich keine Internalisierung von SEA in L-SIGN-exprimierenden Zellen mehr beobachtbar. Im Gegensatz dazu wurde durch Flusssäure (HF) defucosyliertes SEA weiterhin von L-SIGN exprimierenden Zellen gebunden und vergleichbar schnell wie unbehandeltes SEA in diese Zellen internalisiert. Demzufolge bindet L-SIGN SEA nicht über ein fucosyliertes Epitop, sondern über oligomannosidische N-Glykane. Parallel zu den Glykoproteinen aus SEA wurden auch Glykolipide aus *S. mansoni* Eiern auf ihre Ligandenspezifität zu L-SIGN untersucht. Dazu wurden Glykolipide aus Eiern isoliert und über Kieselgelsäulen chromatographisch aufgetrennt. Die resultierenden Glykolipidfraktionen wurden in Bindungsstudien mit L-SIGN-exprimierenden Zellen eingesetzt. Diese führten zu dem Ergebnis, dass L-SIGN nur eine Glykolipidfraktion bindet. Diese enthält zahlreiche fucosylierte Spezies mit der massenspektrometrisch bestimmten allgemeinen Zusammensetzung $Hex_1HexNAc_{5-7}dHex_{3-6}Cer$. Mit dieser Glykolipidfraktion durchgeführte Methylierungsanalysen zur Klärung der Kohlenhydratverknüpfungspositionen sowie Tandem Massenspektrometrie und molekulares Modellieren zeigten, dass die Bindung von L-SIGN zu dieser Glykolipidfraktion am nicht-reduzierenden Ende der Kohlenhydratkette das F-LDN-F Tetrasaccharid [Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc] als minimalen Liganden benötigt. Die L-SIGN „gain of function” Mutante Ser363Val, welche auch fucosylierte Lewis Antigen erkennt, bindet jedoch diese F-LDN-F enthaltene Glykolipidfraktion nicht. Dies führte zu der Vermutung, dass L-SIGN über verschiedene Bindungsmodi für Fucosen in Ei Glykolipiden (F-LDN-F) und Fucosen in Lewis Antigenen verfügen muss. Zusammengefasst zeigen diese Daten zu L-SIGN, dass dieses Lektin sowohl oligomannosidische N-Glykane als auch fucosylierte Kohlenhydratepitope innerhalb der Schistosomen Eiantigene erkennt. Dies zeigt, dass L-SIGN zwar ein breites, aber zugleich auch ein kohlenhydratepitopspezifisches Ligandenprofil besitzt.

Es ist bemerkenswert, dass zwei biochemisch so ähnliche und hoch konservierte C-typ Lektine wie DC-SIGN und L-SIGN dennoch in ihrer Ligandenspezifität variieren, welches in dieser Arbeit aufgezeigt werden konnte. Weitere Studien sind nötig, um die Funktionen dieser beiden Lektine innerhalb der vielfältigen Möglichkeiten, wie parasitische Glykane die Immunantwort des Wirtes beeinflussen können, aufzuklären.

6 References

[1] Abdeen, H.H., Attallah, A.-F.M., El-Mohamady, H.I., Harrison, R.A., Mansour, M.M., 1998. *Schistosoma mansoni*: the circulating cathodic antigen forms an abundant product of 41/42 kDa in the urine of infected patients. Experimental Parasitology 90, 286-289.

[2] Aksoy, E., Zouain, C.S., Vanhoutte, F., Fontaine, J., Pavelka, N., Thieblemont, N., Willems, F., Ricciardi-Castagnoli, P., Goldman, M., Capron, M., Ryffel, B., Trottein, F., 2005. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. J Biol Chem 280, 277-283.

[3] Amiri, P., Locksley, R.M., Parslow, T.G., Sadick, M., Rector, E., Ritter, D., McKerrow, J.H., 1992. Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. Nature 356, 604-607.

[4] Angeli, V., Faveeuw, C., Roye, O., Fontaine, J., Teissier, E., Capron, A., Wolowczuk, I., Capron, M., Trottein, F., 2001. Role of the parasite-derived prostaglandin D2 in the inhibition of epidermal Langerhans cell migration during schistosomiasis infection. J Exp Med 193, 1135-1147.

[5] Appelmelk, B.J., Van Die, I., Van Vliet, S.J., Vandenbroucke-Grauls, C.M., Geijtenbeek, T.B., Van Kooyk, Y., 2003. Cutting Edge: Carbohydrate Profiling Identifies New Pathogens That Interact with Dendritic Cell-Specific ICAM-3-Grabbing Nonintegrin on Dendritic Cells. J Immunol 170, 1635-1639.

[6] Attallah, A.M., Ismail, H., El Masry, S.A., Rizk, H., Handousa, A., El Bendary, M., Tabll, A., Ezzat, F., 1999. Rapid detection of a *Schistosoma mansoni* circulating antigen excreted in urine of infected individuals by using a monoclonal antibody. Journal of Clinical Microbiology 37, 354-357.

[7] Ayash-Rashkovsky, M., Chenine, A.L., Steele, L.N., Lee, S.J., Song, R., Ong, H., Rasmussen, R.A., Hofmann-Lehmann, R., Else, J.G., Augostini, P., McClure, H.M., Secor, W.E., Ruprecht, R.M., 2007. Coinfection with Schistosoma mansoni reactivates viremia in rhesus macaques with chronic simian-human immunodeficiency virus clade C infection. Infect Immun 75, 1751-1756.

[8] Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245-252.

[9] Bashirova, A.A., Geijtenbeek, T.B., van Duijnhoven, G.C., van Vliet, S.J., Eilering, J.B., Martin, M.P., Wu, L., Martin, T.D., Viebig, N., Knolle, P.A., KewalRamani, V.N., van Kooyk, Y., Carrington, M., 2001. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. J Exp Med 193, 671-678.

[10] Beall, M.J., Pearce, E.J., 2001. Human transforming growth factor-beta activates a receptor serine/threonine kinase from the intravascular parasite Schistosoma mansoni. J Biol Chem 276, 31613-31619.

[11] Bergman, M.P., Engering, A., Smits, H.H., van Vliet, S.J., van Bodegraven, A.A., Wirth, H.P., Kapsenberg, M.L., Vandenbroucke-Grauls, C.M., van Kooyk, Y., Appelmelk, B.J., 2004. Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. J Exp Med 200, 979-990.

[12] Bergwerff, A.A., van Dam, G.J., Rotmans, J.P., Deelder, A.M., Kamerling, J.P., Vliegenthart, J.F.G., 1994. The immunologically reactive part of immunopurified circulating anodic antigen from *Schistosoma mansoni* is a threonine-linked polysaccharide consisting of -6)-(β-D-Glc*p*A-(1–3))-β-D-Gal*p*NAc-(1- repeating units. Journal of Biological Chemistry 269, 31510-31517.

[13] Bonifacino, J.S., Dell'Angelica, E.C., 1999. Molecular bases for the recognition of tyrosine-based sorting signals. J Cell Biol 145, 923-926.

[14] Bonifacino, J.S., Traub, L.M., 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72, 395-447.

[15] Brown, D.R., Fowell, D.J., Corry, D.B., Wynn, T.A., Moskowitz, N.H., Cheever, A.W., Locksley, R.M., Reiner, S.L., 1996. Beta 2-microglobulin-dependent NK1.1+ T cells are not essential for T helper cell 2 immune responses. J Exp Med 184, 1295-1304.

[16] Cambi, A., de Lange, F., van Maarseveen, N.M., Nijhuis, M., Joosten, B., van Dijk, E.M., de Bakker, B.I., Fransen, J.A., Bovee-Geurts, P.H., van Leeuwen, F.N., Van Hulst, N.F., Figdor, C.G., 2004. Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. J Cell Biol 164, 145-155.

[17] Cambi, A., Gijzen, K., de Vries, J.M., Torensma, R., Joosten, B., Adema, G.J., Netea, M.G., Kullberg, B.J., Romani, L., Figdor, C.G., 2003. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. Eur J Immunol 33, 532-538.

[18] Caparros, E., Munoz, P., Sierra-Filardi, E., Serrano-Gomez, D., Puig-Kroger, A., Rodriguez-Fernandez, J.L., Mellado, M., Sancho, J., Zubiaur, M., Corbi, A.L., 2006. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. Blood 107, 3950-3958.

[19] Cella, M., Engering, A., Pinet, V., Pieters, J., Lanzavecchia, A., 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. Nature 388, 782-787.

[20] Chenine, A.L., Buckley, K.A., Li, P.L., Rasmussen, R.A., Ong, H., Jiang, S., Wang, T., Augostini, P., Secor, W.E., Ruprecht, R.M., 2005. Schistosoma mansoni infection promotes SHIV clade C replication in rhesus macaques. Aids 19, 1793-1797.

[21] Chiaramonte, M.G., Schopf, L.R., Neben, T.Y., Cheever, A.W., Donaldson, D.D., Wynn, T.A., 1999. IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by Schistosoma mansoni eggs. J Immunol 162, 920-930.

[22] Comin, F., Speziali, E., Martins-Filho, O.A., Caldas, I.R., Moura, V., Gazzinelli, A., Correa-Oliveira, R., Faria, A.M., 2007. Ageing and Toll-like receptor expression by innate immune cells in chronic human schistosomiasis. Clin Exp Immunol 149, 274-284.

[23] Cummings, R.D., Nyame, A.K., 1996. Glycobiology of schistosomiasis. FASEB Journal 10, 838-848.

[24] Damian, R.T., 1964. Molecular mimicry; antigen sharing by parasite and host and its consequences. Am Naturalist 98, 129-149.

[25] Damian, R.T., 1989. Molecular mimicry: parasite evasion and host defense. Current Topics in Microbiology and Immunology 145, 101-115.

[26] Davis, A., 1996. Schistosomiasis. In: Manson´s Tropical Diseases, WB Saunders, London, pp. 1413-1456

[27] de Jesus, A.R., Silva, A., Santana, L.B., Magalhaes, A., de Jesus, A.A., de Almeida, R.P., Rego, M.A., Burattini, M.N., Pearce, E.J., Carvalho, E.M., 2002. Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni. J Infect Dis 185, 98-105.

[28] de Jong, E.C., Vieira, P.L., Kalinski, P., Schuitemaker, J.H., Tanaka, Y., Wierenga, E.A., Yazdanbakhsh, M., Kapsenberg, M.L., 2002. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J Immunol 168, 1704-1709.

[29] de Jonge, N., Kremsner, P.G., Krijger, F.W., Schommer, G., Fillié, Y.E., Kornelis, D., van Zeyl, R.J., van Dam, G.J., Feldmeier, H., Deelder, A.M., 1990. Detection of the schistosome circulating cathodic antigen by enzyme immunoassay using biotinylated monoclonal antibodies. Trans R Soc Trop Med Hyg 84, 815-818.

[30] Deelder, A.M., Kornelis, D., 1980. *Schistosoma mansoni*: characterization of two circulating polysaccharide antigens and the immunological response to these antigens in mouse, hamster, and human infections. Experimental Parasitology 50, 16-32.

[31] Deelder, A.M., van Dam, G.J., Kornelis, D., Fillié, Y.E., 1996. *Schistosoma*: analysis of monoclonal antibodies reactive with the circulating antigens CAA and CCA. Parasitology 112, 21-35.

[32] Dennis, R.D., Geyer, H., Geyer, R., 2006. Glycoconjugates of Parasitic Helminth Infections. In: Kamerling, J.P., Boons, G.-J., Lee, Y.C., Suzuki, A., Taniguchi, N., Voragen, A.G.J., Comprehensive Glycoscience: From Chemistry to System Biology, 4. Elsevier, pp. 473-494

[33] Dissous, C., Grzych, J.M., Capron, A., 1986. *Schistosoma mansoni* shares a protective oligosaccharide epitope with freshwater and marine snails. Nature 323, 443-445.

[34] Dunne, D.W., Agnew, A.M., Mohda, J., Doenhoff, M.J., 1986. *Schistosoma mansoni* egg antigens: preparation of rabbit antisera with monospecific immunoprecipitating activity, and their use in antigen characterization. Parasite Immunology 8, 575-586.

[35] Dunne, D.W., Cooke, A., 2005. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. Nat Rev Immunol 5, 420-426.

[36] Dunne, D.W., Jones, F.M., Doenhoff, M.J., 1991. The purification, characterization, serological activity and hepatotoxic properties of two cationic glycoproteins (α_1 and ω_1) from *Schistosoma mansoni* eggs. Parasitology 103, 225-236.

[37] East, L., Isacke, C.M., 2002. The mannose receptor family. Biochim Biophys Acta 1572, 364-386.

[38] Eberl, M., Langermans, J.A., Vervenne, R.A., Nyame, A.K., Cummings, R.D., Thomas, A.W., Coulson, P.S., Wilson, R.A., 2001. Antibodies to glycans dominate the host response to schistosome larvae and eggs: is their role protective or subversive? J Infect Dis 183, 1238-1247.

[39] Ebner, S., Ehammer, Z., Holzmann, S., Schwingshackl, P., Forstner, M., Stoitzner, P., Huemer, G.M., Fritsch, P., Romani, N., 2004. Expression of C-type lectin receptors by subsets of dendritic cells in human skin. Int Immunol 16, 877-887.

[40] Engering, A., Geijtenbeek, T.B., van Vliet, S.J., Wijers, M., van Liempt, E., Demaurex, N., Lanzavecchia, A., Fransen, J., Figdor, C.G., Piguet, V., van Kooyk, Y., 2002. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J Immunol 168, 2118-2126.

[41] Fallon, P.G., Richardson, E.J., McKenzie, G.J., McKenzie, A.N., 2000. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol 164, 2585-2591.

[42] Fallon, P.G., Teixeira, M.M., Neice, C.M., Willams, T.J., Hellewell, P.G., Doenhoff, M.J., 1996. Enhancement of *Schistosoma mansoni* infectivity by intradermal injections of larval extracts: a putative role for larval proteases. Journal of Infectious Diseases 173, 1460-1466.

[43] Faveeuw, C., Angeli, V., Fontaine, J., Maliszewski, C., Capron, A., Van Kaer, L., Moser, M., Capron, M., Trottein, F., 2002. Antigen presentation by CD1d contributes to the amplification of Th2 responses to Schistosoma mansoni glycoconjugates in mice. J Immunol 169, 906-912.

[44] Feinberg, H., Mitchell, D.A., Drickamer, K., Weis, W.I., 2001. Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. Science 294, 2163-2166.

[45] Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat Rev Immunol 2, 77-84.

[46] Fitzsimmons, C.M., Schramm, G., Jones, F.M., Chalmers, I.W., Hoffmann, K.F., Grevelding, C.G., Wuhrer, M., Hokke, C.H., Haas, H., Doenhoff, M.J., Dunne, D.W., 2005. Molecular characterization of omega-1: a hepatotoxic ribonuclease from Schistosoma mansoni eggs. Mol Biochem Parasitol 144, 123-127.

[47] Fox, N., Damjanov, I., Knowles, B.B., Solter, D., 1983. Immunohistochemical localization of the mouse stage-specific embryonic antigen 1 in human tissues and tumors. Cancer Research 43, 669-678.

[48] Fukuda, M., Hiraoka, N., Yeh, J.C., 1999. C-type lectins and sialyl Lewis X oligosaccharides. Versatile roles in cell-cell interaction. J Cell Biol 147, 467-470.

[49] Fukuda, M., Spooncer, E., Oates, J.E., Dell, A., Klock, J.C., 1984. Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. J Biol Chem 259, 10925-10935.

[50] Fulford, A.J., Butterworth, A.E., Ouma, J.H., Sturrock, R.F., 1995. A statistical approach to schistosome population dynamics and estimation of the life-span of *Schistosoma mansoni* in man. Parasitology 110 (Pt 3), 307-316.

[51] Geijtenbeek, T.B., Krooshoop, D.J., Bleijs, D.A., van Vliet, S.J., van Duijnhoven, G.C., Grabovsky, V., Alon, R., Figdor, C.G., van Kooyk, Y., 2000. DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol 1, 353-357.

[52] Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., Figdor, C.G., van Kooyk, Y., 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100, 587-597.

[53] Geijtenbeek, T.B., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Adema, G.J., van Kooyk, Y., Figdor, C.G., 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell 100, 575-585.

[54] Geijtenbeek, T.B., Van Vliet, S.J., Koppel, E.A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C.M., Appelmelk, B., Van Kooyk, Y., 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 197, 7-17.

[55] Geyer, H., Wuhrer, M., Kurokawa, T., Geyer, R., 2004. Characterization of keyhole limpet hemocyanin (KLH) glycans sharing a carbohydrate epitope with *Schistosoma mansoni* glycoconjugates. Micron 35, 105-106.

[56] Geyer, H., Wuhrer, M., Resemann, A., Geyer, R., 2005. Identification and characterization of keyhole limpet hemocyanin N-glycans mediating cross-reactivity with Schistosoma mansoni. J Biol Chem 280, 40731-40748.

[57] Grzych, J.M., Pearce, E., Cheever, A., Caulada, Z.A., Caspar, P., Heiny, S., Lewis, F., Sher, A., 1991. Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. J Immunol 146, 1322-1327.

[58] Guo, Y., Feinberg, H., Conroy, E., Mitchell, D.A., Alvarez, R., Blixt, O., Taylor, M.E., Weis, W.I., Drickamer, K., 2004. Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. Nat Struct Mol Biol 11, 591-598.

[59] Haas, W., 1998. Host finding - a physiological effect. In: Mehlhorn, H., Parasitology in Focus: Facts and Trends, Springer Verlag, Berlin, pp. 454-464

[60] Haas, W., vandeRoemer, A., 1998. Invasion of the vertebrate skin by cercariae of *Trichobilharzia ocellata*: penetration processes and stimulating host signals. Parasitology Research 84, 787-795.

[61] Harris, D.P., Haynes, L., Sayles, P.C., Duso, D.K., Eaton, S.M., Lepak, N.M., Johnson, L.L., Swain, S.L., Lund, F.E., 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol 1, 475-482.

[62] Haslam, S.M., Coless, G.C., Munn, E.A., Smith, T.S., Smith, H.F., Morris, H.R., Dell, A., 1996. *Haemonchus contortus* glycoproteins contain *H*-linked oligosaccharides with novel highly fucosylated core structures. Journal of Biological Chemistry 271, 30561-30570.

[63] Herbert, D.R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., Leeto, M., Kirsch, R., Hall, P., Mossmann, H., Claussen, B., Forster, I., Brombacher, F., 2004. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity 20, 623-635.

[64] Hesse, M., Modolell, M., La Flamme, A.C., Schito, M., Fuentes, J.M., Cheever, A.W., Pearce, E.J., Wynn, T.A., 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol 167, 6533-6544.

[65] Hokke, C.H., Yazdanbakhsh, M., 2005. Schistosome glycans and innate immunity. Parasite Immunol 27, 257-264.

[66] Huang, H.H., Tsai, P.L., Khoo, K.H., 2001. Selective expression of different fucosylated epitopes on two distinct sets of *Schistosoma mansoni* cercarial O-glycans: identification of a novel core type and Lewis X structure. Glycobiology 11, 395-406.

[67] Huang, H.H., Tsai, P.L., Khoo, K.H., 2001. Selective expression of different fucosylated epitopes on two distinct sets of Schistosoma mansoni cercarial O-glycans: identification of a novel core type and Lewis X structure. Glycobiology 11, 395-406.

[68] Jacobs, W., Deelder, A., Van Marck, E., 1999. Schistosomal granuloma modulation. II. Specific immunogenic carbohydrates can modulate schistosome-egg-antigen-induced hepatic granuloma formation. Parasitology Research 85, 14-18.

[69] Jankovic, D., Kullberg, M.C., Caspar, P., Sher, A., 2004. Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. J Immunol 173, 2419-2427.

[70] Jenkins, S.J., Hewitson, J.P., Jenkins, G.R., Mountford, A.P., 2005. Modulation of the host's immune response by schistosome larvae. Parasite Immunol 27, 385-393.

[71] Jenkins, S.J., Mountford, A.P., 2005. Dendritic cells activated with products released by schistosome larvae drive Th2-type immune responses, which can be inhibited by manipulation of CD40 costimulation. Infect Immun 73, 395-402.

[72] Kalinski, P., Hilkens, C.M., Wierenga, E.A., Kapsenberg, M.L., 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 20, 561-567.

[73] Kane, C.M., Cervi, L., Sun, J., McKee, A.S., Masek, K.S., Shapira, S., Hunter, C.A., Pearce, E.J., 2004. Helminth antigens modulate TLR-initiated dendritic cell activation. J Immunol 173, 7454-7461.

[74] Karanja, D.M., Colley, D.G., Nahlen, B.L., Ouma, J.H., Secor, W.E., 1997. Studies on schistosomiasis in western Kenya: I. Evidence for immune-facilitated excretion of schistosome eggs from patients with Schistosoma mansoni and human immunodeficiency virus coinfections. Am J Trop Med Hyg 56, 515-521.

[75] Karrar, A., Broome, U., Uzunel, M., Qureshi, A.R., Sumitran-Holgersson, S., 2007. Human liver sinusoidal endothelial cells induce apoptosis in activated T cells: a role in tolerance induction. Gut 56, 243-252.

[76] Kawasaki, N., Kawasaki, T., Yamashina, I., 1983. Isolation and characterization of a mannan-binding protein from human serum. J Biochem (Tokyo) 94, 937-947.

[77] Khoo, K.H., 2001. Structural variations in schistosomal glycans. Trends in Glycoscience and Glycotechnology 31, 493-506.

[78] Khoo, K.H., Chatterjee, D., Caulfield, J.P., Morris, H.R., Dell, A., 1997. Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences. Glycobiology 7, 663-677.

[79] Khoo, K.H., Huang, H.H., Lee, K.M., 2001. Characteristic structural features of schistosome cercarial N-glycans: expression of Lewis X and core xylosylation. Glycobiology 11, 149-163.

[80] Khoo, K.H., Sarda, S., Xu, X., Caulfield, J.P., McNeil, M.R., Homans, S.W., Morris, H.R., Dell, A., 1995. A unique multifucosylated -3GalNAc β1-4GlcNAc β1-3Gal α1-motif constitutes the repeating unit of the complex *O*-glycans derived from the cercarial glycocalyx of *Schistosoma mansoni*. Journal of Biological Chemistry 270, 17114-17123.

[81] Knolle, P.A., Gerken, G., 2000. Local control of the immune response in the liver. Immunol Rev 174, 21-34.

[82] Knolle, P.A., Limmer, A., 2001. Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells. Trends Immunol 22, 432-437.

[83] Ko, A.I., Drager, U.C., Harn, D.A., 1990. A Schistosoma mansoni epitope recognized by a protective monoclonal antibody is identical to the stage-specific embryonic antigen 1. Proc Natl Acad Sci U S A 87, 4159-4163.

[84] Koppel, E.A., van Gisbergen, K.P., Geijtenbeek, T.B., van Kooyk, Y., 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. Cell Microbiol 7, 157-165.

[85] Koster, B., Strand, M., 1994. Schistosoma mansoni: immunolocalization of two different fucose-containing carbohydrate epitopes. Parasitology 108 (Pt 4), 433-446.

[86] Kusel, J.R., 1970. The penetration of human epidermal sheets by the cercariae of *Schistosoma mansoni* and the collection of schistosomula. Parasitology 60, 89-96.

[87] La Flamme, A.C., MacDonald, A.S., Pearce, E.J., 2000. Role of IL-6 in directing the initial immune response to schistosome eggs. J Immunol 164, 2419-2426.

[88] Langerhans, P., 1868. Ueber die Nerven der menschlichen Haut. Archiv fuer Pathologische Anatomie und Physiologie und fuer Klinische Medizin 44.

[89] Lehr, T., Geyer, H., Maass, K., Doenhoff, M.J., Geyer, R., 2006. Structural characterization of N-glycans from the freshwater snail Biomphalaria glabrata cross-reacting with Schistosoma mansoni glycoconjugates. Glycobiology 17, 82-103.

[90] Ley, K., Kansas, G.S., 2004. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol 4, 325-335.

[91] Lightowlers, M.W., Rickard, M.D., 1988. Excretory-secretory products of helminth parasites: effects on host immune responses. Parasitology 96 Suppl, S123-166.

[92] Linehan, S.A., Coulson, P.S., Wilson, R.A., Mountford, A.P., Brombacher, F., Martinez-Pomares, L., Gordon, S., 2003. IL-4 receptor signaling is required for mannose receptor expression by macrophages recruited to granulomata but not resident cells in mice infected with Schistosoma mansoni. Lab Invest 83, 1223-1231.

[93] Lu, J., Teh, C., Kishore, U., Reid, K.B., 2002. Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. Biochim Biophys Acta 1572, 387-400.

[94] Ludwig, I.S., Lekkerkerker, A.N., Depla, E., Bosman, F., Musters, R.J., Depraetere, S., van Kooyk, Y., Geijtenbeek, T.B., 2004. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J Virol 78, 8322-8332.

[95] MacDonald, A.S., Araujo, M.I., Pearce, E.J., 2002. Immunology of parasitic helminth infections. Infect Immun 70, 427-433.

[96] MacDonald, A.S., Straw, A.D., Bauman, B., Pearce, E.J., 2001. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. J Immunol 167, 1982-1988.

[97] MacDonald, A.S., Straw, A.D., Dalton, N.M., Pearce, E.J., 2002. Cutting edge: Th2 response induction by dendritic cells: a role for CD40. J Immunol 168, 537-540.

[98] Makaaru, C.K., Damian, R.T., Smith, D.F., Cummings, R.D., 1992. The human blood fluke *Schistosoma mansoni* synthesizes a novel type of glycosphingolipid. Journal of Biological Chemistry 267, 2251-2257.

[99] Marks, M.S., Woodruff, L., Ohno, H., Bonifacino, J.S., 1996. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. J Cell Biol 135, 341-354.

[100] Martin, D.L., King, C.L., Pearlman, E., Strine, E., Heinzel, F.P., 2000. IFN-gamma is necessary but not sufficient for anti-CD40 antibody-mediated inhibition of the Th2 response to Schistosoma mansoni eggs. J Immunol 164, 779-785.

[101] Marzi, A., Gramberg, T., Simmons, G., Moller, P., Rennekamp, A.J., Krumbiegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H., Pohlmann, S., 2004. DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. J Virol 78, 12090-12095.

[102] McKee, A.S., Pearce, E.J., 2004. CD25+CD4+ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development. J Immunol 173, 1224-1231.

[103] Meyer, S., Tefsen, B., Imberty, A., Geyer, R., van Die, I., 2007. The C-Type Lectin L-Sign Differentially Recognizes Glycan Antigens on Egg Glycosphingolipids and Soluble Egg Glycoproteins from Schistosoma Mansoni. Glycobiology.

[104] Meyer, S., van Liempt, E., Imberty, A., van Kooyk, Y., Geyer, H., Geyer, R., van Die, I., 2005. DC-SIGN mediates binding of dendritic cells to authentic pseudo-LewisY glycolipids of Schistosoma mansoni cercariae, the first parasite-specific ligand of DC-SIGN. J Biol Chem 280, 37349-37359.

[105] Mitchell, D.A., Fadden, A.J., Drickamer, K., 2001. A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR. Subunit organization and binding to multivalent ligands. J Biol Chem 276, 28939-28945.

[106] Murphy, K.M., Reiner, S.L., 2002. The lineage decisions of helper T cells. Nat Rev Immunol 2, 933-944.

[107] Nyame, A.K., Pilcher, J.B., Tsang, V.C., Cummings, R.D., 1996. Schistosoma mansoni infection in humans and primates induces cytolytic antibodies to surface Le(x) determinants on myeloid cells. Exp Parasitol 82, 191-200.

[108] Nyame, A.K., Pilcher, J.B., Tsang, V.C.W., Cummings, R.D., 1996. *Schistosoma mansoni* infection in humans and primates induces cytolytic antibodies to surface Le^x determinants on myeloid cells. Experimental Parasitology 82, 191-200.

[109] Nyame, K., Cummings, R.D., Damian, R.T., 1987. *Schistosoma mansoni* synthesizes glycoproteins containing terminal *O*-linked *N*-acetylglucosamine residues. Journal of Biological Chemistry 262, 7990-7995.

[110] Nyame, K., Cummings, R.D., Damian, R.T., 1988. Characterization of the high mannose asparagine-linked oligosaccharides synthesized by *Schistosoma mansoni* adult male worms. Molecular and Biochemical Parasitology 28, 265-274.

[111] Nyame, K., Cummings, R.D., Damian, R.T., 1988. Characterization of the *N*- and *O*-linked oligosaccharides in glycoproteins synthesized by *Schistosoma mansoni* schistosomula. Journal of Parasitology 74, 562-572.

[112] Nyame, K., Smith, D.F., Damian, R.T., Cummings, R.D., 1989. Complex-type asparagine-linked oligosaccharides in glycoproteins synthesized by *Schistosoma mansoni* adult males contain terminal β-linked *N*-acetylgalactosamine. Journal of Biological Chemistry 264, 3235-3243.

[113] Okano, M., Satoskar, A.R., Nishizaki, K., Abe, M., Harn, D.A., Jr., 1999. Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on Schistosoma mansoni egg antigens. J Immunol 163, 6712-6717.

[114] Pearce, E.J., 2005. Priming of the immune response by schistosome eggs. Parasite Immunol 27, 265-270.

[115] Pearce, E.J., C, M.K., Sun, J., J, J.T., McKee, A.S., Cervi, L., 2004. Th2 response polarization during infection with the helminth parasite Schistosoma mansoni. Immunol Rev 201, 117-126.

[116] Pearce, E.J., Caspar, P., Grzych, J.M., Lewis, F.A., Sher, A., 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. J Exp Med 173, 159-166.

[117] Pearce, E.J., MacDonald, A.S., 2002. The immunobiology of schistosomiasis. Nat Rev Immunol 2, 499-511.

[118] Pearce, E.J., Sher, A., 1987. Mechanisms of immune evasion in schistosomiasis. Contrib Microbiol Immunol 8, 219-232.

[119] Sabin, E.A., Kopf, M.A., Pearce, E.J., 1996. Schistosoma mansoni egg-induced early IL-4 production is dependent upon IL-5 and eosinophils. J Exp Med 184, 1871-1878.

[120] Sallusto, F., Lanzavecchia, A., 1999. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J Exp Med 189, 611-614.

[121] Schmidt, G.S., Roberts, L.S., 2000. Foundations of Parasitology. McGraw-Hill Education.

[122] Schramm, G., Gronow, A., Knobloch, J., Wippersteg, V., Grevelding, C.G., Galle, J., Fuller, H., Stanley, R.G., Chiodini, P.L., Haas, H., Doenhoff, M.J., 2006. IPSE/alpha-1: a major immunogenic component secreted from *Schistosoma mansoni* eggs. Mol Biochem Parasitol 147, 9-19.

[123] Soilleux, E.J., Barten, R., Trowsdale, J., 2000. DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13. J Immunol 165, 2937-2942.

[124] Soilleux, E.J., Morris, L.S., Leslie, G., Chehimi, J., Luo, Q., Levroney, E., Trowsdale, J., Montaner, L.J., Doms, R.W., Weissman, D., Coleman, N., Lee, B., 2002. Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro. J Leukoc Biol 71, 445-457.

[125] Sol-Foulon, N., Moris, A., Nobile, C., Boccaccio, C., Engering, A., Abastado, J.P., Heard, J.M., van Kooyk, Y., Schwartz, O., 2002. HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. Immunity 16, 145-155.

[126] Soong, G., Reddy, B., Sokol, S., Adamo, R., Prince, A., 2004. TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. J Clin Invest 113, 1482-1489.

[127] Spooncer, E., Fukuda, M., Klock, J.C., Oates, J.E., Dell, A., 1984. Isolation and characterization of polyfucosylated lactosaminoglycan from human granulocytes. J Biol Chem 259, 4792-4801.

[128] Srivatsan, J., Smith, D.F., Cummings, R.D., 1992. The human blood fluke *Schistosoma mansoni* synthesizes glycoproteins containing the Lewis X antigen. Journal of Biological Chemistry 267, 20196-20203.

[129] Srivatsan, J., Smith, D.F., Cummings, R.D., 1992. *Schistosoma mansoni* synthesizes novel biantennary Asn-linked oligosaccharides containing terminal β-linked *N*-acetylgalactosamine. Glycobiology 2, 445-452.

[130] Staudacher, E., Altmann, F., Wilson, I.B., Maerz, L., 1999. Fucose in N-glycans: from plant to man. Biochim Biophys Acta 1473, 216-236.

[131] Steinman, R.M., Cohn, Z.A., 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137, 1142-1162.

[132] Symington, F.W., Hedges, D.L., Hakomori, S., 1985. Glycolipid antigens of human polymorphonuclear neutrophils and the inducible HL-60 myeloid leukemia line. J Immunol 134, 2498-2506.

[133] Tailleux, L., Schwartz, O., Herrmann, J.L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L.P., Gluckman, J.C., Lagrange, P.H., Gicquel, B., Neyrolles, O., 2003. DC-SIGN is the major Mycobacterium tuberculosis receptor on human dendritic cells. J Exp Med 197, 121-127.

[134] Takeda, K., Akira, S., 2003. Toll receptors and pathogen resistance. Cell Microbiol 5, 143-153.

[135] Tchuem Tchuente, L.-A., 1996. Mating behaviour in schistosomes: are paired worms always faithful? Parasitology Today 12, 231-236.

[136] Teixeira, M.M., Almeida, I.C., Gazzinelli, R.T., 2002. Introduction: innate recognition of bacteria and protozoan parasites. Microbes Infect 4, 883-886.

[137] Teixeira, M.M., Doenhoff, M.J., McNeice, C., Williams, T.J., Hellewell, P.G., 1993. Mechanisms of the inflammatory response induced by extracts of *Schistosoma mansoni* larvae in guinea pig skin. J Immunol 151, 5525-5534.

[138] Thomas, P.G., Carter, M.R., Atochina, O., Da'Dara, A.A., Piskorska, D., McGuire, E., Harn, D.A., 2003. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a toll-like receptor 4-dependent mechanism. J Immunol 171, 5837-5841.

[139] Triantafilou, M., Miyake, K., Golenbock, D.T., Triantafilou, K., 2002. Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation. J Cell Sci 115, 2603-2611.

[140] van Dam, G.J., Bergwerff, A.A., Thomas-Oates, J.E., Rotmans, J.P., Kamerling, J.P., Vliegenthart, J.F., Deelder, A.M., 1994. The immunologically reactive *O*-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke *Schistosoma mansoni* have Lewis x as repeating unit. European Journal of Biochemistry 225, 467-482.

[141] van Dam, G.J., Deelder, A.M., 1996. Glycoproteins of parasites - Schistosome glycoconjugates and their role in host-parasite pathological interactions. In: Montreuil, J., Vliegenthart, J.F.G., Schachter, H., Glycoproteins and Disease, 1. Elsevier Science, pp. 123-142

[142] Van de Vijver, K.K., Deelder, A.M., Jacobs, W., Van Marck, E.A., Hokke, C.H., 2006. LacdiNAc- and LacNAc-containing glycans induce granulomas in an in vivo model for schistosome egg-induced hepatic granuloma formation. Glycobiology 16, 237-243.

[143] van de Vijver, K.K., Hokke, C.H., van Remoortere, A., Jacobs, W., Deelder, A.M., Van Marck, E.A., 2004. Glycans of Schistosoma mansoni and keyhole limpet haemocyanin induce hepatic granulomas in vivo. Int J Parasitol 34, 951-961.

[144] van den Berg, T.K., Honing, H., Franke, N., van Remoortere, A., Schiphorst, W.E., Liu, F.T., Deelder, A.M., Cummings, R.D., Hokke, C.H., van Die, I., 2004. LacdiNAc-glycans constitute a parasite pattern for galectin-3-mediated immune recognition. J Immunol 173, 1902-1907.

[145] van der Kleij, D., Latz, E., Brouwers, J.F., Kruize, Y.C., Schmitz, M., Kurt-Jones, E.A., Espevik, T., de Jong, E.C., Kapsenberg, M.L., Golenbock, D.T., Tielens, A.G., Yazdanbakhsh, M., 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. J Biol Chem 277, 48122-48129.

[146] Van der Kleij, D., Van Remoortere, A., Schuitemaker, J.H., Kapsenberg, M.L., Deelder, A.M., Tielens, A.G., Hokke, C.H., Yazdanbakhsh, M., 2002. Triggering of innate immune responses by schistosome egg glycolipids and their carbohydrate epitope GalNAc beta 1-4(Fuc alpha 1-2Fuc alpha 1-3)GlcNAc. J Infect Dis 185, 531-539.

[147] van Die, I., Cummings, R.D., 2006. Glycans modulate immune responses in helminth infections and allergy. Chem Immunol Allergy 90, 91-112.

[148] van Die, I., van Vliet, S.J., Nyame, A.K., Cummings, R.D., Bank, C.M., Appelmelk, B., Geijtenbeek, T.B., van Kooyk, Y., 2003. The dendritic cell-specific C-type lectin DC-SIGN is a receptor for Schistosoma mansoni egg antigens and recognizes the glycan antigen Lewis x. Glycobiology 13, 471-478.

[149] van Kooten, C., Banchereau, J., 1997. Functions of CD40 on B cells, dendritic cells and other cells. Curr Opin Immunol 9, 330-337.

[150] van Kooyk, Y., Geijtenbeek, T.B., 2003. DC-SIGN: escape mechanism for pathogens. Nat Rev Immunol 3, 697-709.

[151] Van Liempt, E., Imberty, A., Bank, C.M., Van Vliet, S.J., Van Kooyk, Y., Geijtenbeek, T.B., Van Die, I., 2004. Molecular basis of the differences in binding properties of the highly related C-type lectins DC-SIGN and L-SIGN to Lewis X trisaccharide and Schistosoma mansoni egg antigens. J Biol Chem 279, 33161-33167.

[152] van Liempt, E., van Vliet, S.J., Engering, A., Garcia Vallejo, J.J., Bank, C.M., Sanchez-Hernandez, M., van Kooyk, Y., van Die, I., 2007. Schistosoma mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. Mol Immunol 44, 2605-2615.

[153] van Remoortere, A., Hokke, C.H., van Dam, G.J., van Die, I., Deelder, A.M., van den Eijnden, D.H., 2000. Various stages of schistosoma express Lewis(x), LacdiNAc, GalNAcbeta1-4 (Fucalpha1-3)GlcNAc and GalNAcbeta1-4(Fucalpha1-2Fucalpha1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. Glycobiology 10, 601-609.

[154] van Vliet, S.J., van Liempt, E., Saeland, E., Aarnoudse, C.A., Appelmelk, B., Irimura, T., Geijtenbeek, T.B., Blixt, O., Alvarez, R., van Die, I., van Kooyk, Y., 2005. Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells. Int Immunol 17, 661-669.

[155] Velupillai, P., dos Reis, E.A., dos Reis, M.G., Harn, D.A., 2000. Lewis(x)-containing oligosaccharide attenuates schistosome egg antigen- induced immune depression in human schistosomiasis. Hum Immunol 61, 225-232.

[156] Velupillai, P., Harn, D.A., 1994. Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosome-infected mice: a mechanism for regulation of CD4+ T-cell subsets. Proc Natl Acad Sci U S A 91, 18-22.

[157] Vieira, L.Q., Gazzinelli, G., Kusel, J.R., De Souza, C.P., Colley, D.G., 1986. Inhibition of human peripheral blood mononuclear cell proliferative responses by released materials from Schistosoma mansoni cercariae. Parasite Immunol 8, 333-343.

[158] Webster, J.P., Woolhouse, M.E.J., 1998. Heritability and strain specifity in compatibility between snail intermediate hosts and their parasitic schistosomes. Evolution 52, 1627-1634.

[159] Weis, W.I., Kahn, R., Fourme, R., Drickamer, K., Hendrickson, W.A., 1991. Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. Science 254, 1608-1615.

[160] Weiss, J.B., Magnani, J.L., Strand, M., 1986. Identification of *Schistosoma mansoni* glycolipids that share immunogenic carbohydrate epitopes with glycoproteins. Journal of Immunology 136, 4275-4282.

[161] Wuhrer, M., Balog, C.I., Catalina, M.I., Jones, F.M., Schramm, G., Haas, H., Doenhoff, M.J., Dunne, D.W., Deelder, A.M., Hokke, C.H., 2006. IPSE/alpha-1, a major secretory glycoprotein antigen from schistosome eggs, expresses the Lewis X motif on core-difucosylated N-glycans. Febs J 273, 2276-2292.

[162] Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Bickle, Q., Lochnit, G., Geyer, R., 1999. Immunochemical characterisation of *Schistosoma mansoni* glycolipid antigens. Mol Biochem Parasitol 103, 155-169.

[163] Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Geyer, R., 2000. A fucose-containing epitope is shared by keyhole limpet haemocyanin and *Schistosoma mansoni* glycosphingolipids. Mol Biochem Parasitol 110, 237-246.

[164] Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Geyer, R., 2000. Stage-associated expression of ceramide structures in glycosphingolipids from the human trematode parasite *Schistosoma mansoni*. Biochim Biophys Acta 1524, 155-161.

[165] Wuhrer, M., Dennis, R.D., Doenhoff, M.J., Lochnit, G., Geyer, R., 2000. *Schistosoma mansoni* cercarial glycolipids are dominated by Lewis X and pseudo-Lewis Y structures. Glycobiology 10, 89-101.

[166] Wuhrer, M., Geyer, R., 2006. Glycoconjugate Structures. In: Maule, A.G., Marks, N.J., Parasitic Flatworms - Molecular Biology, Biochemistry, Immunology and Parasitology, CAB International, Wallingford, pp. in press

[167] Wuhrer, M., Kantelhardt, S.R., Dennis, R.D., Doenhoff, M.J., Lochnit, G., Geyer, R., 2002. Characterization of glycosphingolipids from *Schistosoma mansoni* eggs carrying Fuc(α1-3)GalNAc-, GalNAc(β1-4)[Fuc(α1-3)]GlcNAc- and Gal(β1-4)[Fuc(α1-3)]GlcNAc- (Lewis X) terminal structures. Eur J Biochem 269, 481-493.

[168] Yang, Z.Y., Huang, Y., Ganesh, L., Leung, K., Kong, W.P., Schwartz, O., Subbarao, K., Nabel, G.J., 2004. pH-dependent entry of severe acute respiratory syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN. J Virol 78, 5642-5650.

[169] Yazdanbakhsh, M., Deelder, A.M., 1998. Advances in schistosomiasis research. Curr. Opin. Infect. Dis. 11, 541-546.

[170] Yazdanbakhsh, M., Kremsner, P.G., van Ree, R., 2002. Allergy, parasites, and the hygiene hypothesis. Science 296, 490-494.

[171] Zaccone, P., Fehervari, Z., Jones, F.M., Sidobre, S., Kronenberg, M., Dunne, D.W., Cooke, A., 2003. Schistosoma mansoni antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. Eur J Immunol 33, 1439-1449.

7 Supplement

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7.2 Curriculum vitae

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7.3 Erklärung

Ich erkläre:
Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

Gießen, den 4. Oktober 2007 ______________________________

(Sandra Meyer)