

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

Sandra Meyer<sup>2</sup>, Boris Tefsen<sup>3</sup>, Anne Imberty<sup>4</sup>,  
Rudolf Geyer<sup>2</sup>, and Irma van Die<sup>1,3</sup>

<sup>2</sup>Institute of Biochemistry, Medical Faculty, Justus-Liebig-University Giessen, Friedrichstrasse 24, D-35392 Giessen, Germany; <sup>3</sup>Department of Molecular Cell Biology and Immunology, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands; and <sup>4</sup>Centre de Recherches sur les Macromolécules Végétales, CNRS (affiliated with Université Joseph Fourier), 38041 Grenoble, Cedex 09, France

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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<sub>1</sub>HexNAc<sub>5–7</sub>dHex<sub>3–6</sub>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 Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)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*

### 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<sup>X</sup>) epitope Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)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 GalNAc $\beta$ 1-4GlcNAc- (LDN), GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc- (LDN-F), and GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-2Fuc $\alpha$ 1-3)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).

<sup>1</sup>To whom correspondence should be addressed; Tel: +31-2-04-44-81-57; Fax: +31-2-04-44-81-44; e-mail: im.vandie@vumc.nl

**Table I.** Carbohydrate epitopes mentioned in this study

Carbohydrate epitope	Structure	Structure plot
LacdiNAc (LDN)	GalNAc $\beta$ 1-4GlcNAc	
Terminally fucosylated LDN (F-LDN)	Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4GlcNAc	
Fucosylated LDN (LDN-F)	GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc	
Fucosylated LDN-F (F-LDN-F)	Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc	
Difucosylated LDN (LDN-DF)	GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-2Fuc $\alpha$ 1-3)GlcNAc	
Fucosylated LDN-DF (F-LDN-DF)	Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-2Fuc $\alpha$ 1-3)GlcNAc	
Lewis X (Le <sup>X</sup> )	Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc	
Lewis Y (Le <sup>Y</sup> )	Fuc $\alpha$ 1-2Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc	
Lewis A (Le <sup>A</sup> )	Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc	
Lewis B (Le <sup>B</sup> )	Fuc $\alpha$ 1-2Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)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<sup>A</sup>, Galβ1-3(Fucα1-4)GlcNAc-), Lewis B (Le<sup>B</sup>, Fucα1-2Galβ1-3(Fucα1-4)GlcNAc-) and Lewis Y (Le<sup>Y</sup>, 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<sup>X</sup> epitope, which is one of the major schistosome ligands of DC-SIGN, although the formation of crystals between L-SIGN and Le<sup>X</sup> indicates that a weak interaction is possible (Guo et al. 2004). The inability of L-SIGN to bind to Le<sup>X</sup> epitopes is mainly due to the presence of a single amino acid in the CRD of L-SIGN, Ser<sup>363</sup> that prevents interaction with the Fuc(α1-3)GlcNAc unit in Le<sup>X</sup>, but supports binding of the Fucα1-4GlcNAc moiety present in Le<sup>A</sup> and Le<sup>B</sup> antigens. The equivalent amino acid residue Val<sup>351</sup> in DC-SIGN creates a hydrophobic pocket that strongly interacts with the Fuc(α1-3/4)GlcNAc moiety of Le<sup>X</sup>, 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

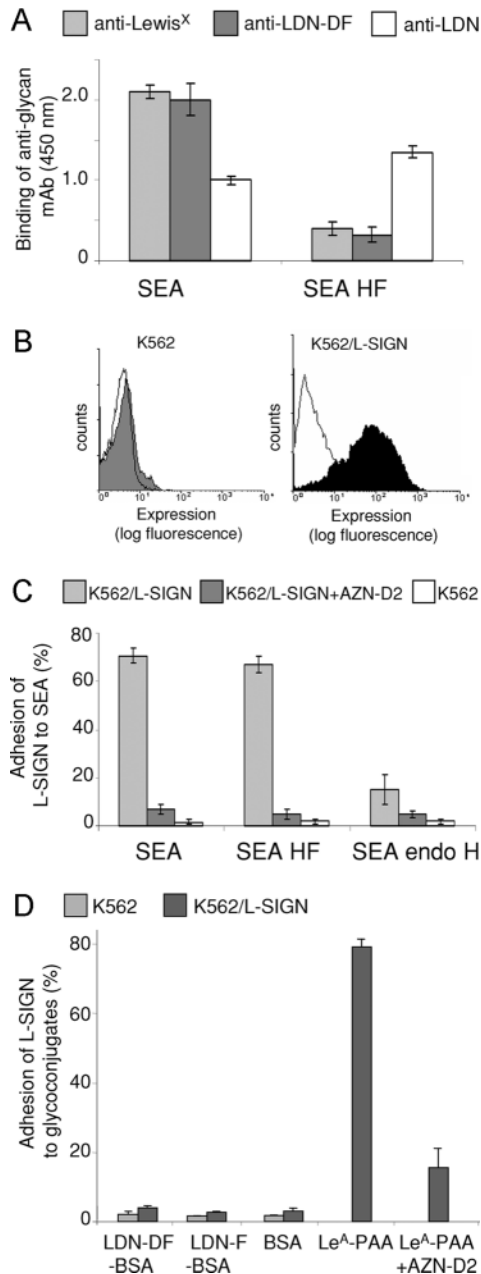
### Recognition of *S. mansoni* SEA by L-SIGN

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<sup>X</sup>, 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<sup>X</sup> 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.

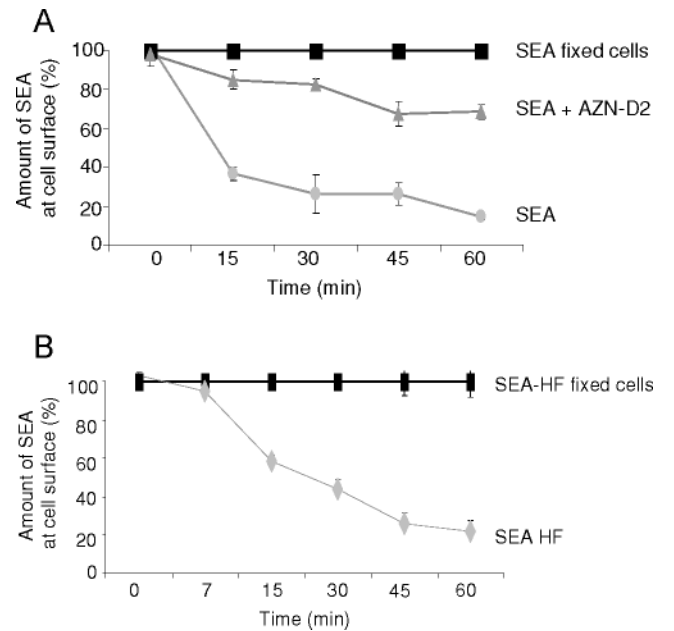
Subsequent cellular adhesion assays using K562 cells stably transfected with L-SIGN (K562/L-SIGN), revealed that binding of L-SIGN to SEA was apparently not affected by defucosylation, whereas treatment with endo H almost completely abolished SEA recognition by L-SIGN (Figure 1B and C). These data indicate 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<sup>X</sup> 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).



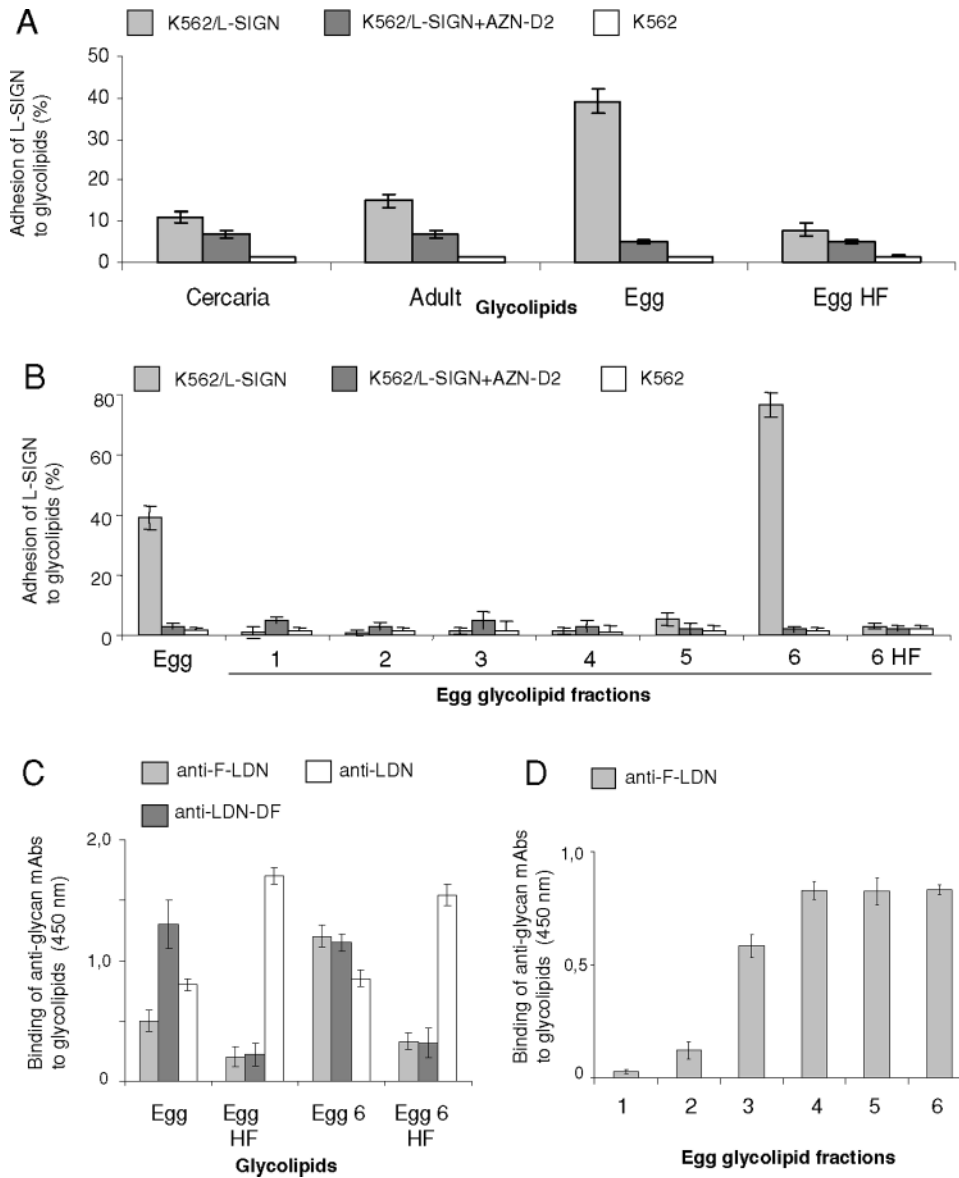
**Fig. 1.** Binding of *S. mansoni* SEA by L-SIGN transfected cells. (A) ELISA was performed to characterize the glycan epitopes of SEA and defucosylated SEA (SEA HF). Similar amounts of SEA (5  $\mu$ g/mL) were applied in each case. Using antibodies recognizing the fucose-containing glycan epitopes Le<sup>x</sup> 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<sup>A</sup>-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

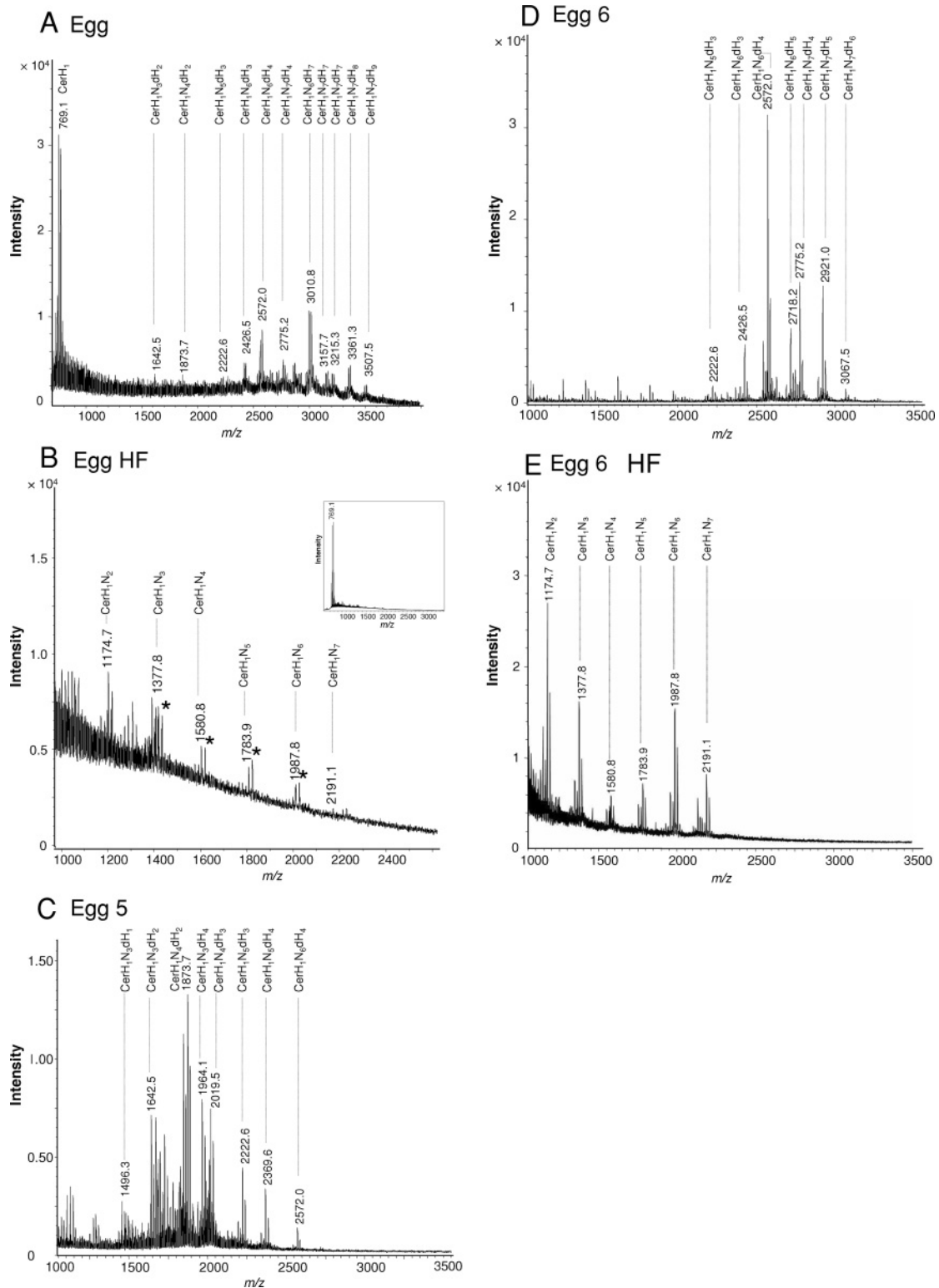


**Fig. 3.** Binding of L-SIGN-transfected cells to glycosphingolipids of *S. mansoni*. Similar amounts of glycolipids (9 ng/well) were applied in each case. All results are representative of three independent experiments, performed in triplicate (**A**, **B**) or duplicate (**C**, **D**), with error 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; Wuhler et al. 2002), the major signal observed for total egg glycosphingolipids corresponded to ceramide monohexoside ( $\text{Hex}_1\text{Cer}$ ), with a mass of  $m/z$  769.1  $[\text{M}+\text{Na}]^+$ . 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  $[\text{M}+\text{Na}]^+$  and  $m/z$  3010.8  $[\text{M}+\text{Na}]^+$  represented monosaccharide compositions of  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_4$  and  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_7$ . 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  $[\text{M}+\text{Na}]^+$ . To remove all fucose residues, egg glycosphingolipids were treated with HF and the resulting products were similarly analyzed by mass spectrometry (Figure 4B). Again,  $\text{Hex}_1\text{Cer}$  ( $m/z$  769.1  $[\text{M}+\text{Na}]^+$ ) 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  $\text{Hex}_1\text{HexNAc}_{2-7}\text{Cer}$  (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  $[\text{M}+\text{Na}]^+$  representing a  $\text{Hex}_1\text{HexNAc}_4\text{dHex}_2\text{Cer}$  glycosphingolipid. The highest mass registered at  $m/z$  2572.0  $[\text{M}+\text{Na}]^+$  corresponded to a glycosphingolipid with a carbohydrate composition of  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_4$ , thus reflecting an overall composition of  $\text{Hex}_1\text{HexNAc}_{3-6}\text{dHex}_{1-4}\text{Cer}$  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  $[\text{M}+\text{Na}]^+$  in agreement with a composition of  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_4\text{Cer}$ , whereas the overall composition of the major signals observed in this fraction was  $\text{Hex}_1\text{HexNAc}_{5-7}\text{dHex}_{3-6}\text{Cer}$ . 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  $\text{Hex}_1\text{HexNAc}_{2-7}\text{Cer}$  (Fig-

ure 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.

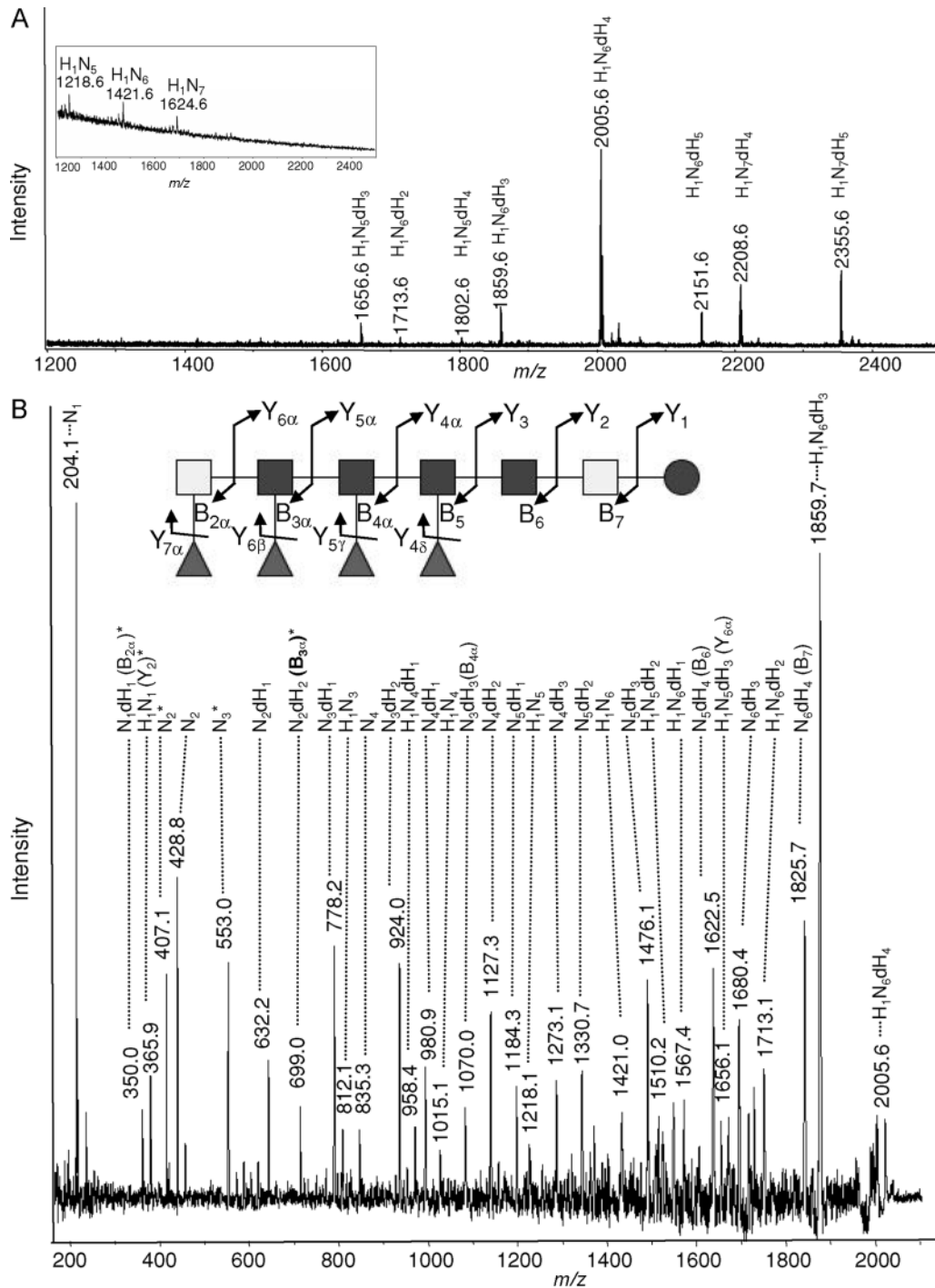
#### *MALDI-TOF-MS (IMS) of S. mansoni egg glycans*

To simplify compositional and linkage analyses, egg glycosphingolipid fraction 6 was treated with endoglycosidase 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 ( $\text{dHex}_{2-5}$ ). The major oligosaccharide consisted of  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_4$ . Treatment of these glycans with HF yielded defucosylated glycans with the overall compositions of  $\text{Hex}_1\text{HexNAc}_{5-7}$  (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  $\text{Hex}_1\text{HexNAc}_6\text{dHex}_4$ . Moreover, the characteristic fragments registered in the MS/MS spectrum clearly demonstrated the presence of  $\text{HexNAc}_1\text{dHex}_2$  ( $m/z$  350.0; fragment B2 $\alpha$ ) and  $\text{HexNAc}_2\text{dHex}_2$  unit ( $m/z$  699.0; fragment B3 $\alpha$ ) as terminal epitopes and simultaneously excluded the occurrence of  $\text{HexNAc}_1\text{dHex}_2$  and  $\text{HexNAc}_2\text{dHex}_3$  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*

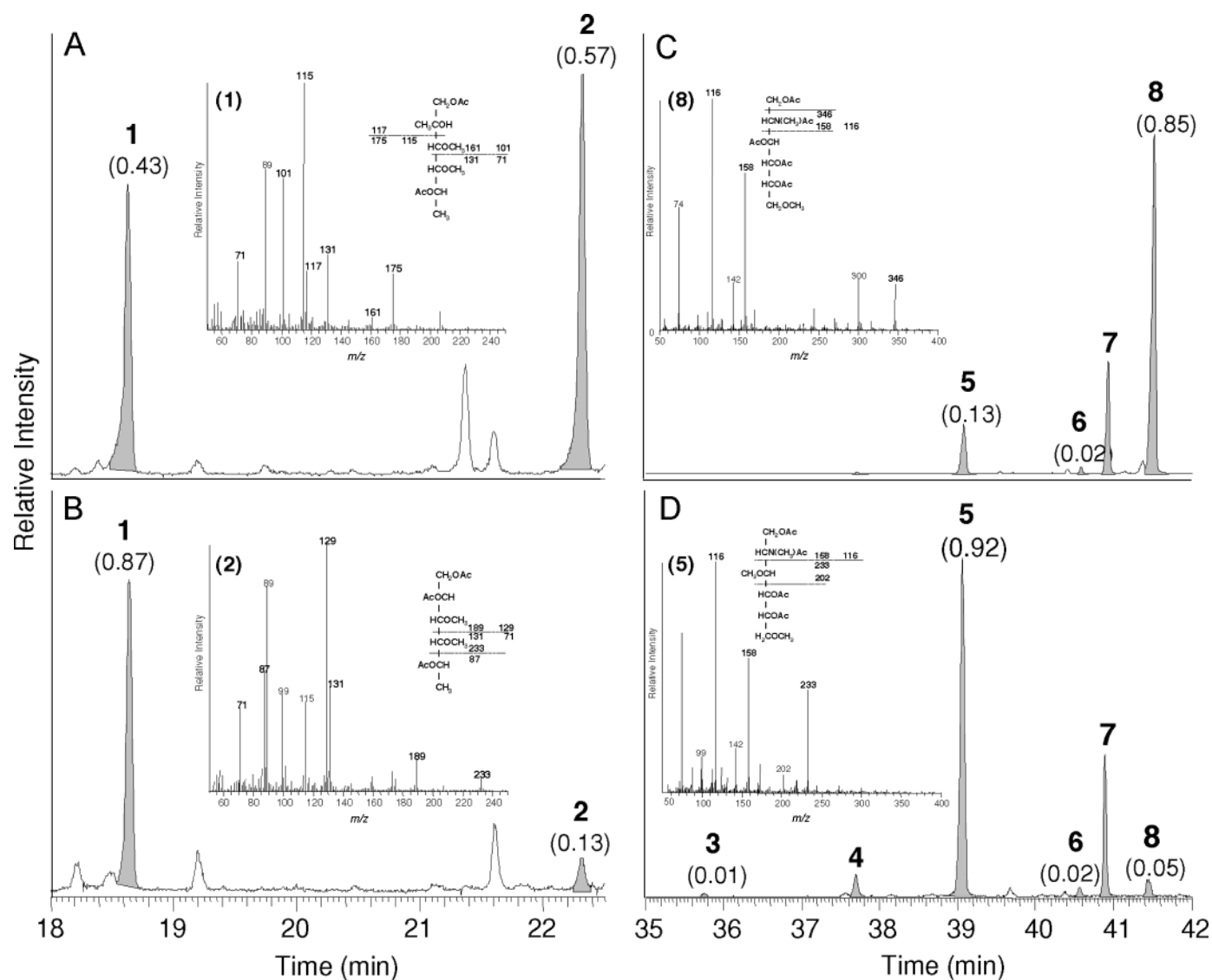
To further corroborate the presence of the F-LDN-F epitope, linkage analysis of the glycan moieties, obtained from the total egg glycosphingolipids and glycosphingolipid fraction 6 was performed (Figure 6 A and 6B). This analysis revealed the presence of two different fucose derivatives (terminal fucose 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  $\alpha$ 1-3-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 endoglycosidase from egg glycosphingolipid fraction 6. Oligosaccharides were released from fraction 6 egg glycosphingolipids by treatment with endoglycosidase 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<sub>1</sub>HexNAc<sub>6</sub>dHex<sub>4</sub>, obtained by endoglycosidase 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<sub>2</sub>dHex<sub>2</sub> unit are presented in bold-type.





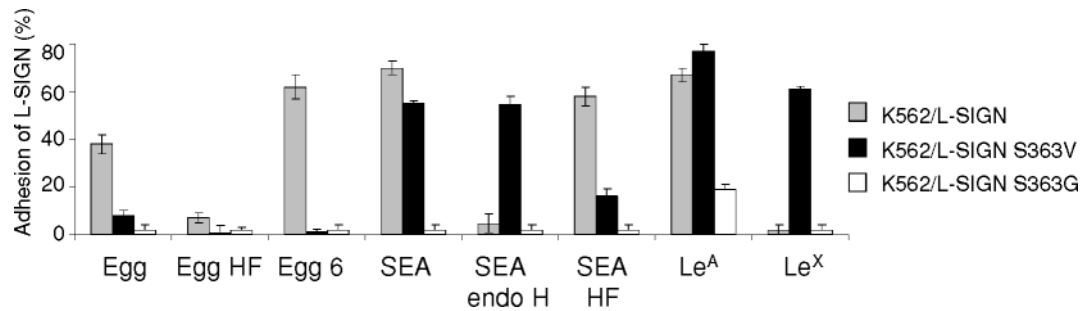
**Fig. 6.** Linkage analyses of glycans released by endoglycosceramidase from total egg glycosphingolipids and egg glycosphingolipid fraction 6. (A–D), linkage analyses of oligosaccharides released from both total and fraction 6 glycosphingolipids before and after HF-treatment. Partially methylated alditol 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<sup>363</sup> 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<sup>x</sup> (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<sup>A</sup>-PAA (Le<sup>A</sup>) and Le<sup>X</sup>-PAA (Le<sup>X</sup>) 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<sup>X</sup>-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., Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4[(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-4]<sub>4</sub> (Figure 8A), was built using its structural similarity with Le<sup>X</sup>. 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 Gal $\beta$ 1-4GlcNAc and Fuc $\alpha$ 1-3GlcNAc glycosidic linkages, and indeed this corresponds to the conformation observed in the crystal structure of the L-SIGN/Le<sup>X</sup> complex (Guo et al. 2004). The situation is similar in the decasaccharide and all Fuc $\alpha$ 1-3GlcNAc 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  $\Psi$  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*

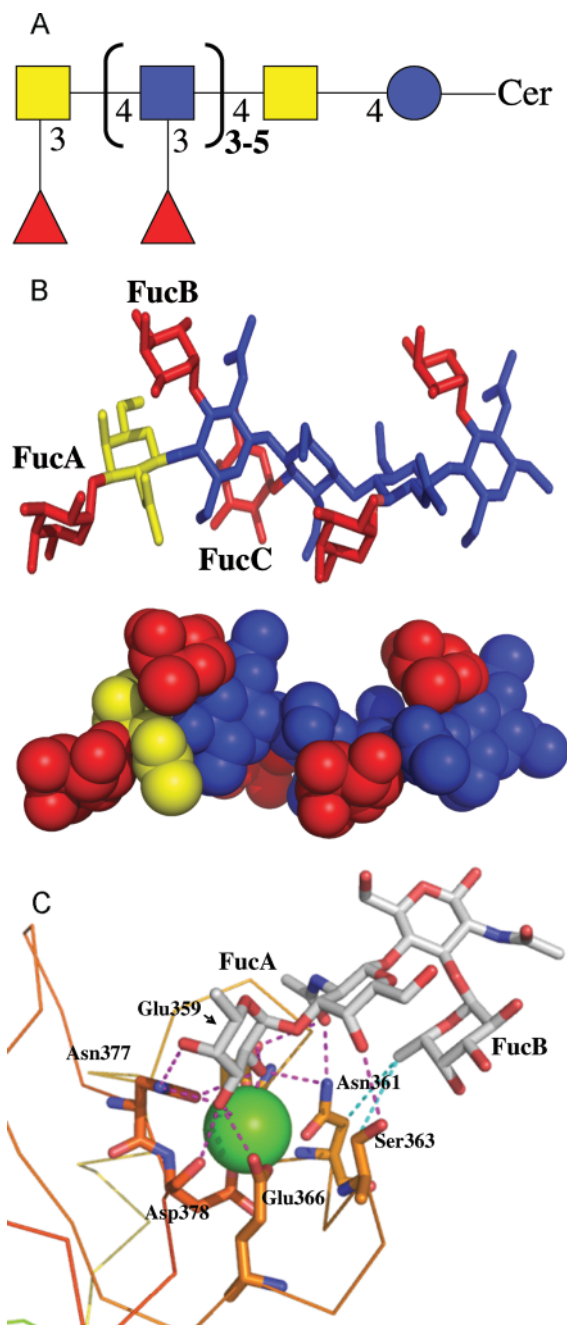
A first docking approach was undertaken by fitting fucose residues of fucosylated oligosaccharides in the binding site of L-SIGN with the orientation observed in the crystal structure of the lectin complexed with Le<sup>X</sup> (Guo et al. 2004), which validated the binding mode that we proposed previously by modeling (van Liempt et al. 2004). Three different orientations of the decasaccharide were tested, with either fucose A ( $\alpha$ 1-3 linked to GalNAc), B or C ( $\alpha$ 1-3 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<sup>X</sup> 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<sup>X</sup> 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<sub>2</sub> of carbon C $\beta$  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 deca-saccharide 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 hybrid-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<sup>x</sup> 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<sup>A</sup> and Le<sup>B</sup>, 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 Fuc $\alpha$ 1-3GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$  (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-oligosaccharide 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 Fuc $\alpha$ 1-3GalNAc 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<sup>X</sup> 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 Fuc $\alpha$ 1-3GalNAc over Fuc $\alpha$ 1-3GlcNAc. 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<sup>Y</sup> (Fuc $\alpha$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)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 neoglycoconjugates 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  $\alpha$ 1-3fucose residue that it carries (Fucose B, see Figure 8C), however, establishes hydrophobic contact with the CH<sub>2</sub> of carbon C $\beta$  of Asn361 and Ser363, thus contributing to the binding. Interestingly, from this model it can be deduced that an additional fucose  $\alpha$ 1-2-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<sup>363</sup> 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<sup>X</sup> or Le<sup>A</sup> 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 denaturing 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<sub>3</sub>) 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<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>) and incubated with anti-glycan antibodies recognizing Le<sup>X</sup>, 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<sub>2</sub>HPO<sub>4</sub>, 4.5 mL 0.1 M Citric acid, 1 mL TMB-solution (3,3',5,5'-tetramethylbenzidine; 1 mg/mL H<sub>2</sub>O), 10 µL H<sub>2</sub>O<sub>2</sub>) 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<sub>2</sub>, 2 mM MgCl<sub>2</sub>) 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<sub>3</sub>) 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<sup>6</sup> 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<sup>6</sup> 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<sup>X</sup> 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<sup>X</sup> 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<sup>X</sup> 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<sup>X</sup> 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.

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#### Conflict of interest statement

None declared.

#### Abbreviations

CD, cluster of differentiation; CRD, carbohydrate recognition domain; DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin; 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.



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