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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 (latrobeads 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·mol^{-1}·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 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.

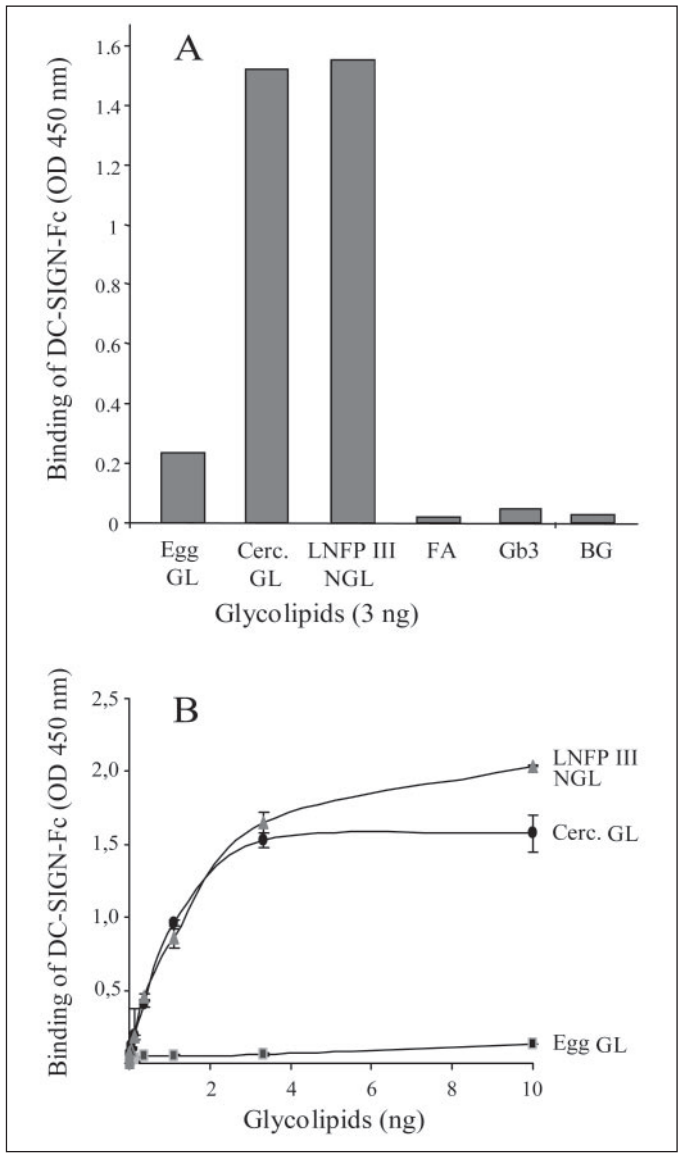

FIGURE 1. **Binding of DC-SIGN to *S. mansoni* cercarial glycolipids and LNFPIII-neoglycolipid.** *A*, ELISA was performed to determine the binding reactivity and specificity of DC-SIGN to egg glycolipids (*Egg GL*), total cercarial glycolipids (*Cerc. GL*), Forssman antigen (*FA*), globotriaosylceramide (*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.

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.M5.5985200

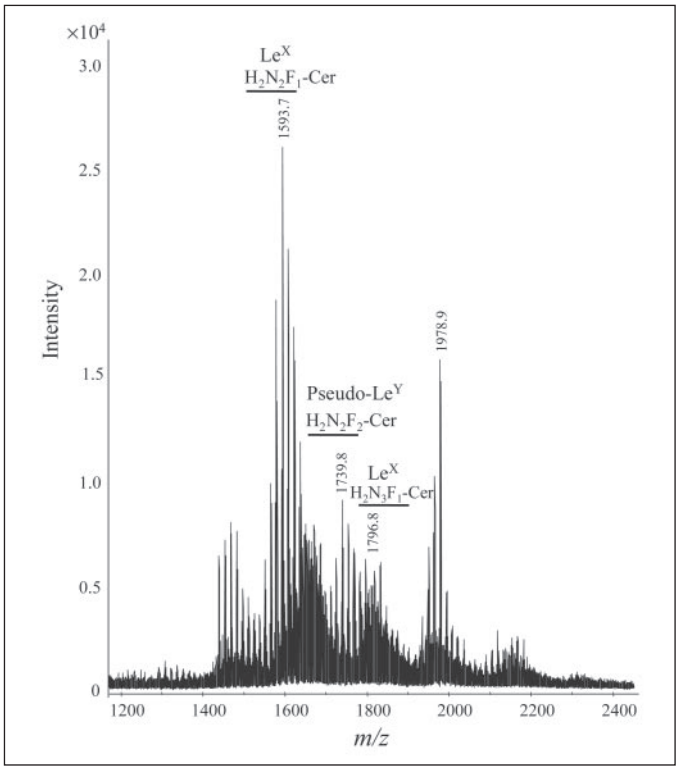

FIGURE 2. **MALDI-TOF-MS analysis of isolated glycolipids from *S. mansoni* cercariae.** Deduced monosaccharide compositions are assigned to major pseudomolecular ions ($[M + Na]^+$) comprising Le^X ($H_2N_2F_1$ at *m/z* 1593.7 or $H_2N_3F_1$ at *m/z* 1796.8) and pseudo-Le^Y epitopes ($H_2N_2F_2$ at *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 $[M\text{-}H + 2Na]^+$ (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 $[M\text{-}H + 2Na]^+$ (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	**$H_2N_2F_1$**	+++	+++
933.32	933.2	H_3N_2	++	—
958.35	958.2	$H_1N_3F_1$	+	+
1063.38	1063.6	**$H_2N_2F_2$**	++	+
1079.2	1079.2	$H_3N_2F_1$	+	—
1104.41	1104.2	$H_1N_3F_2$	+	+
1120.41	1120.3	**$H_2N_3F_1$**	+++	++
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.

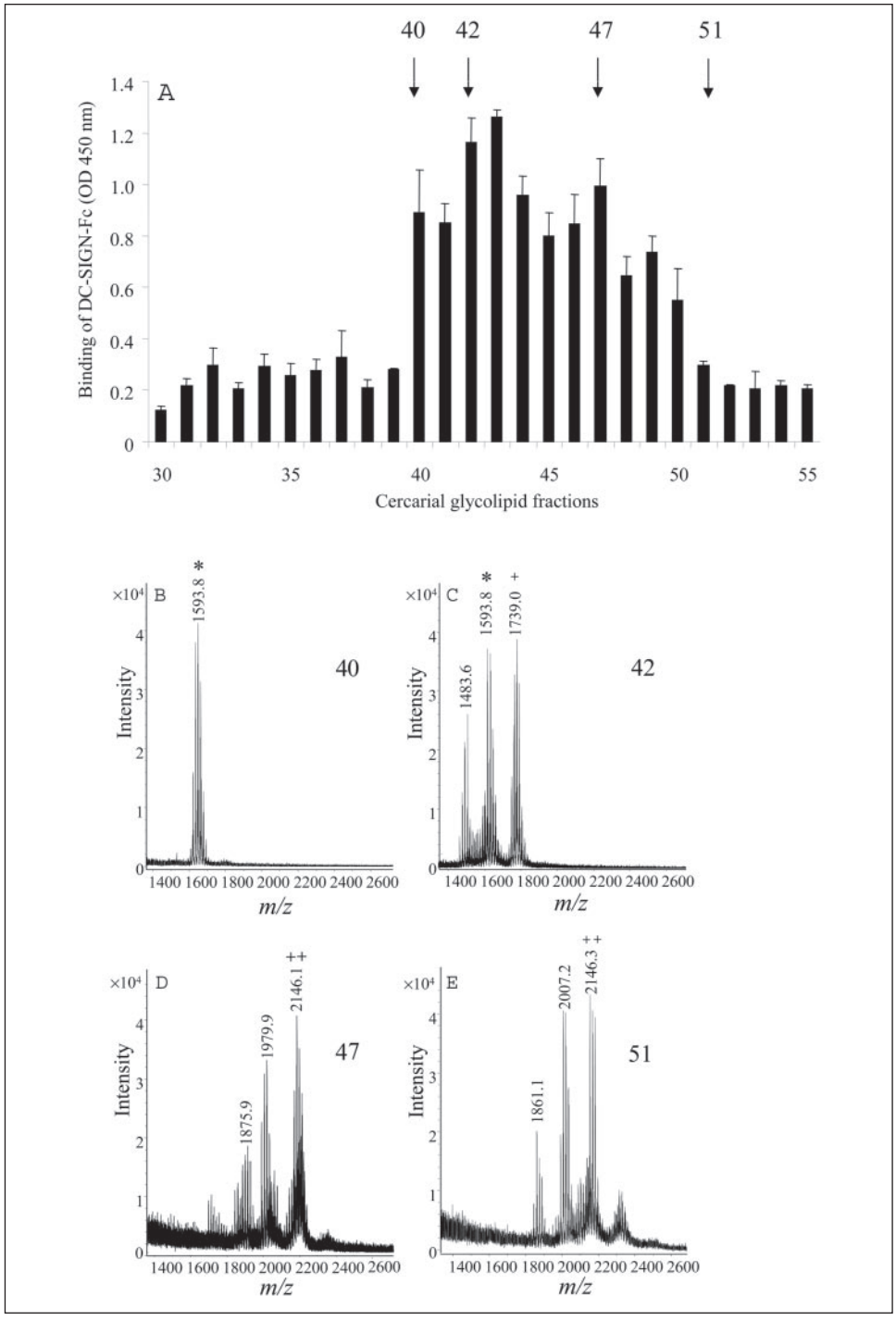

FIGURE 3. **Binding of DC-SIGN to fractionated glycolipids of *S. mansoni* cercariae.** Binding of DC-SIGN to *S. mansoni* cercarial glycolipids was determined by ELISA using soluble DC-SIGN-Fc (*A*). Recognized species occurring in fractions 40–50 are dominated by glycolipids carrying Le^X and pseudo-Le^Y 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-

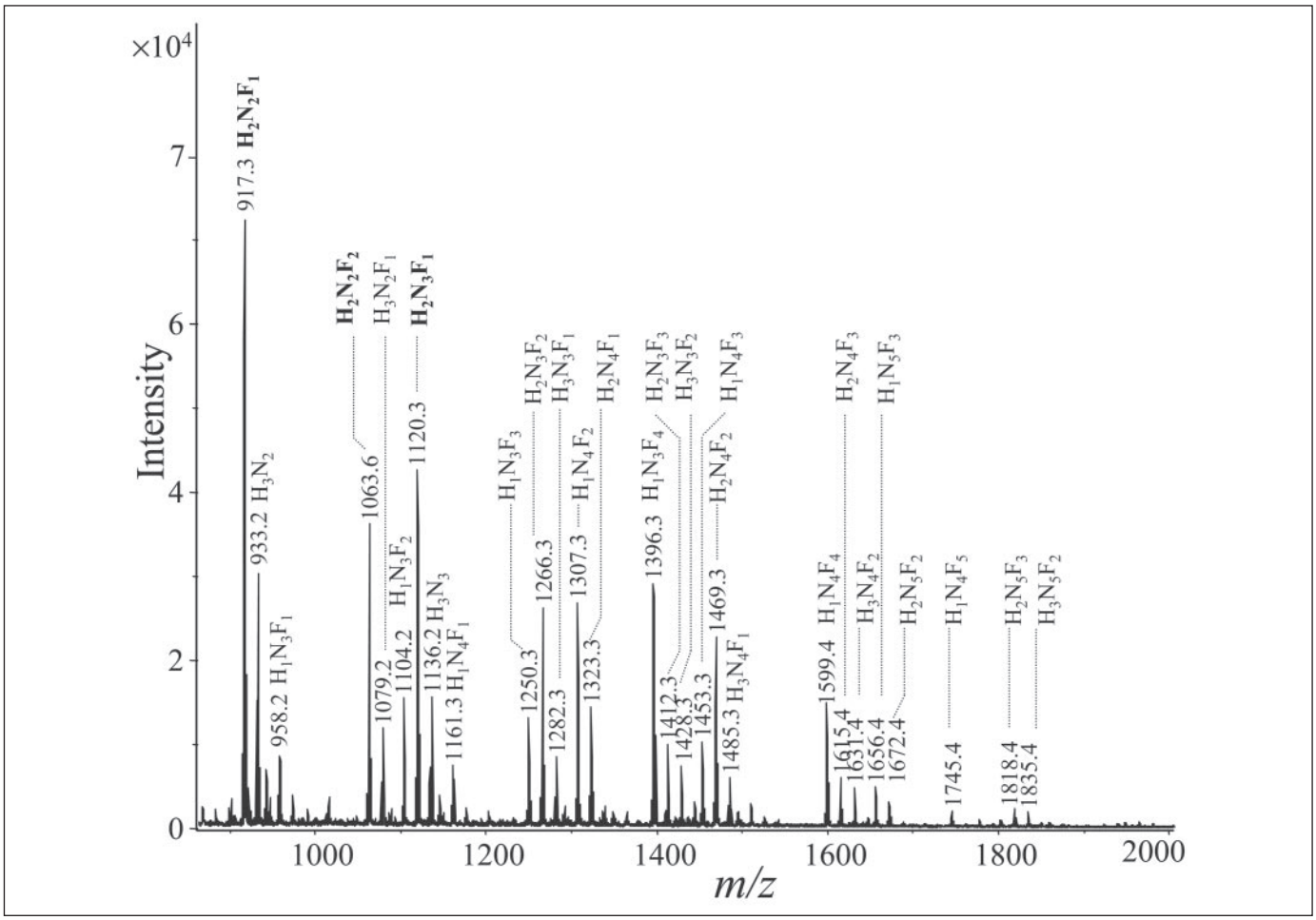

FIGURE 4. **MALDI-TOF-MS analysis of released oligosaccharides.** Oligosaccharides were released from cercarial glycolipids by treatment with endoglycoceramidase and analyzed by MALDI-TOF-MS. Monoisotopic masses of pseudomolecular ions ($[M + Na]^+$) and deduced monosaccharide compositions are assigned. 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).

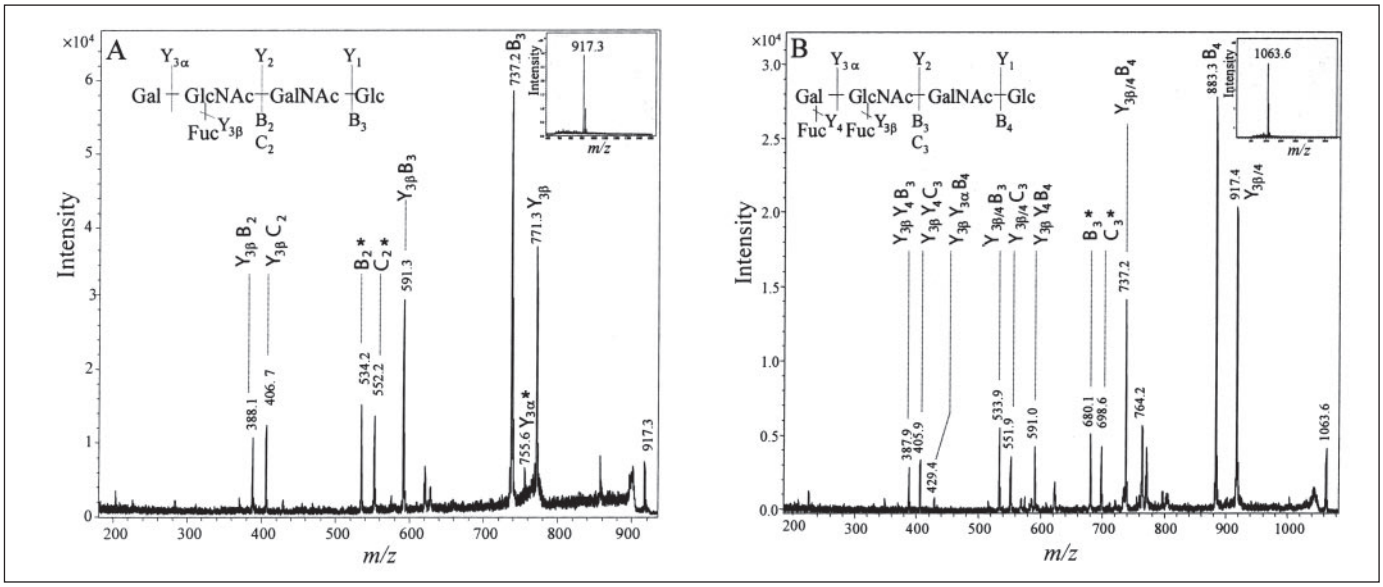

FIGURE 5. **MALDI-TOF-MS and MS/MS analysis of purified glycans with Le^X or pseudo-Le^Y units.** *A*, MALDI-TOF-MS/MS spectrum of the Le^X pentasaccharide (*m/z* 917.3 $[M + Na]^+$). Characteristic Le^X trisaccharide fragment ions (B_2 and C_2) and the diagnostically relevant $Y_{3\alpha}$ fragment 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).

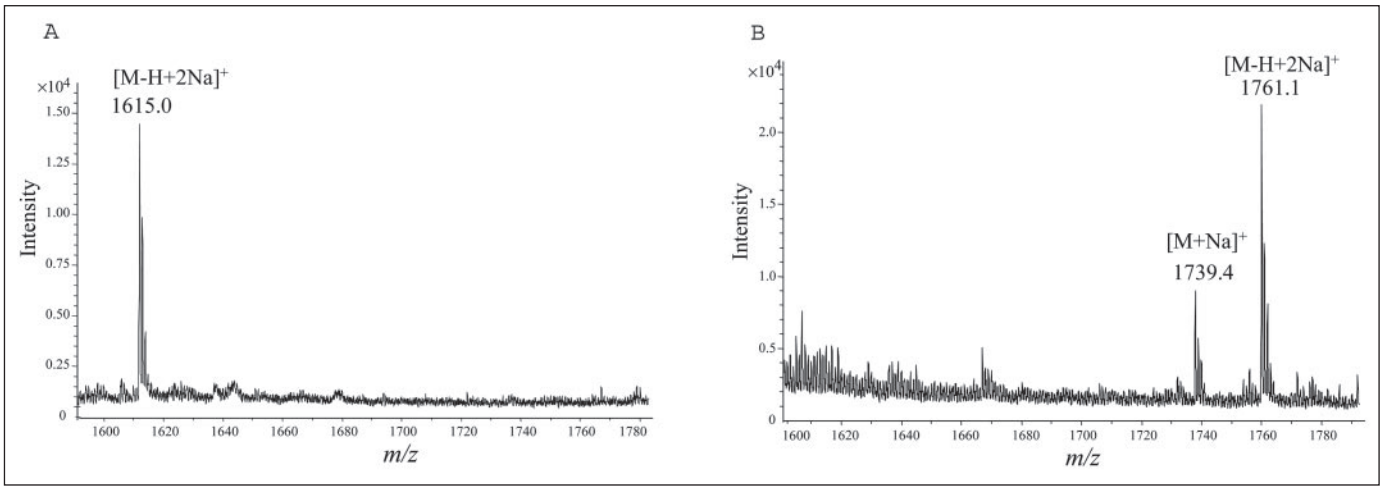

FIGURE 6. **MALDI-TOF-MS analysis of neoglycolipids containing Le^X or pseudo-Le^Y epitopes.** *A* and *B*, MALDI-TOF-MS spectra of Le^X (*m/z* 1615.0 [M-H + 2Na]$^+$) and pseudo-Le^Y (*m/z* 1739.4 [M + Na]$^+$ and 1761.1 [M-H + 2Na]$^+$) neoglycolipids, respectively.

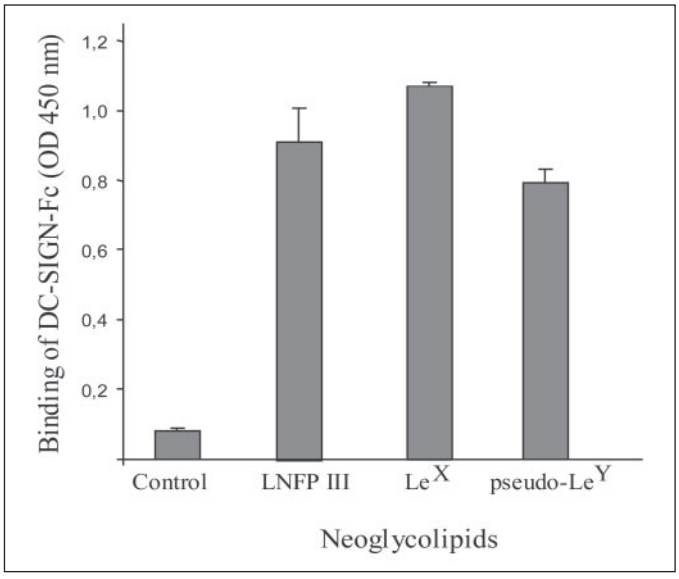

FIGURE 7. **DC-SIGN-Fc binds to Le^X and pseudo-Le^Y neoglycolipids.** Binding of DC-SIGN-Fc to schistosomal neoglycolipids was tested in ELISA. 10 ng of lacto-*N*-fucopentaose III neoglycolipid (*LNFP III*) was coated as positive control. In parallel, 8 ng of Le^X neoglycolipid (*Le^X*) and 8 ng of 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

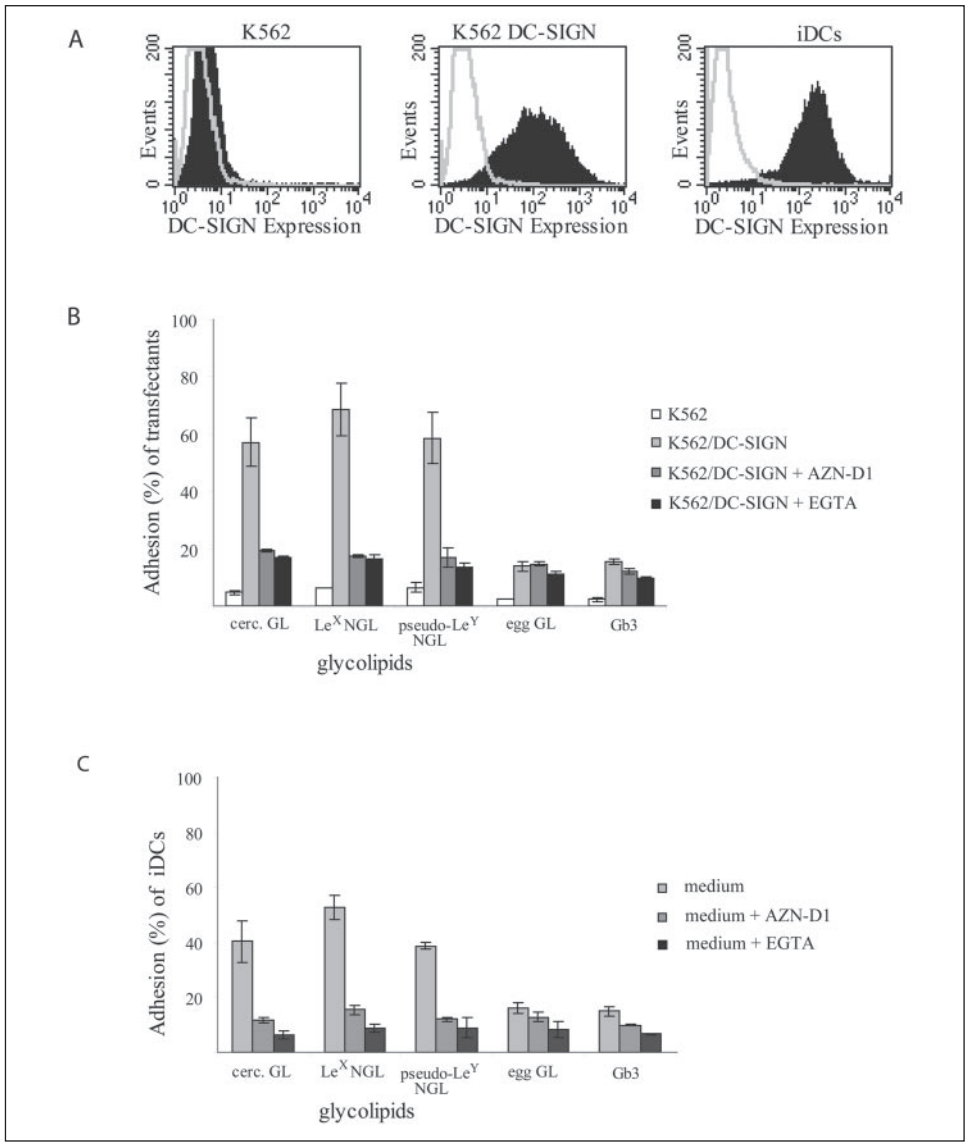

FIGURE 8. **DC-SIGN on human dendritic cells interacts with authentic *S. mansoni* cercarial glycolipids and Le^X and pseudo-Le^Y neoglycolipids.** The expression of DC-SIGN on transfectants and iDCs was determined by flow cytometry (*A*). Binding of DC-SIGN expressed on K562 transfectants (*B*) or iDCs (*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

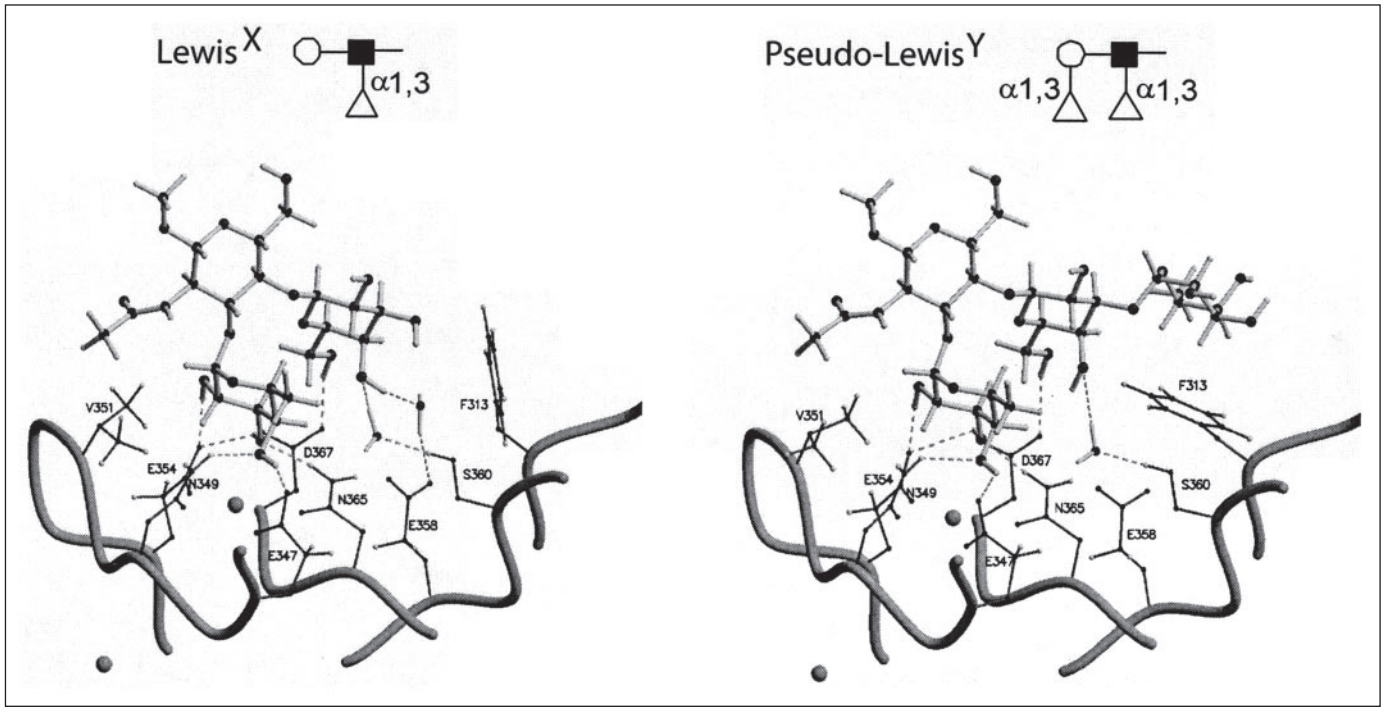

FIGURE 9. **Interaction of DC-SIGN with Le^X and pseudo-Le^Y.** Models of the interaction of DC-SIGN with Le^X trisaccharide (*A*) and pseudo-Le^Y tetrasaccharide (*B*). Calcium ions are represented by *gray spheres*. Only the amino acids interacting directly with the sugars have been displayed.

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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