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CUTTING EDGE

Cutting Edge: Carbohydrate Profiling Identifies New Pathogens That Interact with Dendritic Cell-Specific ICAM-3-Grabbing Nonintegrin on Dendritic Cells

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Dendritic cells (DC) are instrumental in handling pathogens for processing and presentation to T cells, thus eliciting an appropriate immune response. C-type lectins expressed by DC function as pathogen-recognition receptors; yet their specificity for carbohydrate structures on pathogens is not fully understood. In this study, we analyzed the carbohydrate specificity of DC-specific ICAM-3-grabbing nonintegrin (SIGN)/CD209, the recently documented HIV-1 receptor on DC. Our studies show that DC-SIGN binds with high affinity to both synthetic mannose- and fucose-containing glycoconjugates. These carbohydrate structures are abundantly expressed by pathogens as demonstrated by the affinity of DC-SIGN for natural surface glycans of the human pathogens Mycobacterium tuberculosis, Helicobacter pylori, Leishmania mexicana, *and* Schistosoma mansoni. *This analysis expands our knowledge on the carbohydrate and pathogen-specificity of DC-SIGN and identifies this lectin to be central in pathogen-DC interactions.* The Journal of Immunology, *2003, 170: 1635–1639.*

Dendritic cells (DC) are professional APCs that induce cellular immunity upon pathogen recognition and are therefore important in the defense against many pathogens (1–3). Immature DC are seeded throughout peripheral tissues to act as sentinels against invading pathogens (4). Upon pathogen capture, DC are activated, process pathogens for Ag presentation on MHC class II molecules, and migrate to the secondary lymphoid organs where they activate naive T cells to initiate adaptive immune responses (1, 3, 4). Depending on the pathogen that is recognized by the DC, differentiation of naive T cells into Th1 cells is triggered by DC in response to intracellular microbes, whereas Th2-mediated responses are generated to eliminate pathogens residing extracellularly (3). Thus, DC play an important role in both innate and cellular

immune responses against viral, bacterial, and parasitic infections (1, 5). Knowledge about cell surface receptors on DC that are involved in recognition of pathogens is only starting to emerge, and include Toll-like receptors (6, 7) and C-type lectins (8). Toll-like receptors recognize specific pathogen-derived components, such as lipoproteins, LPS, and bacterial DNA, and relay this information through intracellular signaling cascades leading to the production of regulatory cytokines and up-regulation of MHC and costimulatory molecules (6). In contrast, C-type lectins recognize pathogen-derived carbohydrate structures and upon binding internalize pathogens for Ag processing and presentation to T cells (8–10). In classical calcium-dependent lectins, conserved amino acid residues in the carbohydrate recognition domain are involved in calcium binding and sugar specificity (11). A growing number of C-type lectins has been described to be specifically expressed by DC, but detailed knowledge about pathogenic targets as well as cellular ligands, including the identity of the carbohydrate structure they recognize, is lacking for most of these receptors (8). The DC-specific C-type lectin DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN, CD209) is involved in binding of the HIV-1 envelope glycoprotein by DC to enhance infection of T cells (12), whereas the mannose receptor is involved in recognition of mycobacteria and fungi/protozoa (13). In addition to their function as pathogen receptors, some C-type lectins like DC-SIGN can also interact with carbohydrate-bearing self-glycoproteins (ICAM-2 and ICAM-3) to mediate cellular adhesion processes (14, 15).

We here set out to further investigate the carbohydrate specificity of the C-type lectin DC-SIGN to predict and subsequently determine whether DC-SIGN might function as a receptor for pathogens other than HIV-1 and Ebola (12, 16, 17). Based on recent reports that demonstrated interactions of DC-SIGN with high mannose-containing glycoconjugates (18, 19) and on known structural similarity between mannose and fucose, we hypothesized that DC-SIGN would bind to both mannosylated and fucosylated surface glycans of human pathogens.

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[2] Abbreviations used in this paper: DC, dendritic cell; PMN, polymorphonuclear leukocyte; SIGN, specific ICAM-3-grabbing nonintegrin.

Materials and Methods

Abs, natural and synthetic glycoconjugates

The following mAbs were used: anti-DC-SIGN (mAbs AZN-D1 and AZN-D2 and anti-CD107a (=Lamp-1; mAbH4A3, BD PharMingen, San Diego, CA). Mannan purified from *Saccharomyces cerevisiae* (50 μg/ml) and recombinant gp120 (0.50 μg/ml) were obtained from Sigma-Aldrich (St. Louis, MO) and the AIDS Resource Foundation (Rockville, MD), respectively. *Schistosoma mansoni* extract was kindly provided by Dr. A. K. Nyame (Oklahoma University Health Science Center, Oklahoma City, OK). Purified lipophosphoglycan from *L. mexicana* was kindly donated by Dr. M. Wiese (Bernard Nocht Institute of Tropical Medicine, Hamburg, Germany). Purified LPS of *H. pylori* was obtained from M. Monteiro (National Research Council, Ottawa, Canada). The glycolipid mannose-capped lipoarabinomannan was obtained from J. Belisle (Colorado State University, Fort Collins, CO). A sonicate of bacterial cells of a clinical isolate of *Mycobacterium tuberculosis* was donated by A. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands). Clinical isolates of *H. pylori*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were obtained from Vrije Universiteit Medical Center Hospital (Amsterdam, The Netherlands). Synthetic glycoconjugates were obtained from Syntesome (Munich, Germany) and comprise mono- and oligosaccharides multivalently linked to a biotinylated polyacrylamide carrier (molecular mass, 40,000 kDa).

Soluble DC-SIGN-Fc adhesion assay

DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (aa residues 64–404) fused at the C terminus to a human IgG1-Fc fragment. DC-SIGN-Fc was produced in Chinese hamster ovary K1 cells by cotransfection of DC-SIGN-Sig-pIgG1-Fc (20 μg) and the pEE14 (5 μg) vector. DC-SIGN-Fc concentrations in the supernatant were determined by an anti-IgG1-Fc ELISA. The DC-SIGN-Fc binding assay was performed as follows. Glycoconjugates and sonicated mycobacteria were coated onto ELISA plates at 5 μg/well; intact bacterial cells were coated at 5×10^7/ml; and coating took place for 18 h at room temperature, followed by blocking with 1% BSA for 30 min at 37°C in TSM (20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM CaCl$_2$, and 2 mM MgCl$_2$). Soluble DC-SIGN-Fc (~2 μg/ml in TSM buffer) was added and the adhesion was performed for 120 min at room temperature. Unbound DC-SIGN-Fc was washed away and binding was determined by an anti-IgG1-Fc ELISA using a peroxidase conjugate of goat anti-human-Fc. Specificity was determined in the presence of either 20 μg/ml blocking Abs, 50 μg/ml mannan, or 5 mM EGTA.

Cells

Immature DC were cultured from monocytes in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium). At day 7, the phenotype of the cultured DC was confirmed by flow cytometric analysis. K562 transfectants expressing wild-type DC-SIGN were generated by transfection of K562 cells with 10 μg pRc/CMV-DC-SIGN plasmid by electroporation as previously described (14).

Fluorescent bead adhesion assay

Streptavidin was covalently coupled to the beads (carboxylate-modified TransFluorSpheres;488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) as previously described (14) and were incubated with biotinylated polyacrylamide-linked glycoconjugates (50 pM; Syntesome). Ligand-coated fluorescent beads (20 beads/cell) were added to the cells for 45 min at 37°C, washed, and adhesion was analyzed by flow cytometry (FACSCalibur; BD Immunocytometry, San Jose, CA), by measuring the percentage of cells that had bound fluorescent beads, as previously described (14).

Binding of mycobacteria and *Helicobacter* by DC-SIGN-expressing cells was evaluated using FITC-conjugated *Mycobacterium bovis* bacillus Calmette-Guérin *H. pylori*. Bacteria (10^9/ml) were labeled by incubation of 0.5 mg FITC/ml in PBS (pH 7.4) at room temperature for 1 h and extensively washed. Binding was determined by measuring the percentage of cells that bound FITC-conjugated bacteria using flow cytometry.

Results and Discussion

For in vitro binding studies, we generated a chimeric protein of DC-SIGN with a human IgG1-Fc tag, which we used to screen in an ELISA format for reactivity with a panel of synthetic glycoconjugates containing mannose or fucose residues and their derivatives in multimeric form (Figs. 1 and 2). As reported earlier, DC-SIGN-Fc binds to purified yeast-derived mannan and the high mannose-containing HIV-1 gp120 (12), but also to
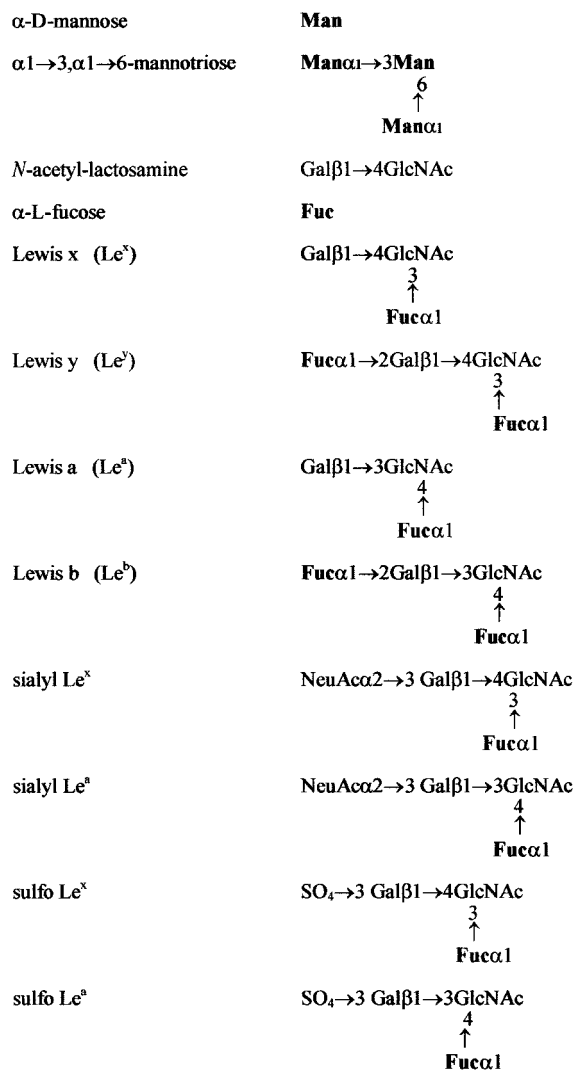
α-D-mannose	**Man**
α1→3,α1→6-mannotriose	**Manα1→3Man** $\uparrow$6 **Manα1**
N-acetyl-lactosamine	Galβ1→4GlcNAc
α-L-fucose	**Fuc**
Lewis x (Le^x)	Galβ1→4GlcNAc 3 $\uparrow$ **Fuc**α1
Lewis y (Le^y)	**Fuc**α1→2Galβ1→4GlcNAc 3 $\uparrow$ **Fuc**α1
Lewis a (Le^a)	Galβ1→3GlcNAc 4 $\uparrow$ **Fuc**α1
Lewis b (Le^b)	**Fuc**α1→2Galβ1→3GlcNAc 4 $\uparrow$ **Fuc**α1
sialyl Le^x	NeuAcα2→3 Galβ1→4GlcNAc 3 $\uparrow$ **Fuc**α1
sialyl Le^a	NeuAcα2→3 Galβ1→3GlcNAc 4 $\uparrow$ **Fuc**α1
sulfo Le^x	SO$_4$→3 Galβ1→4GlcNAc 3 $\uparrow$ **Fuc**α1
sulfo Le^a	SO$_4$→3 Galβ1→3GlcNAc 4 $\uparrow$ **Fuc**α1

FIGURE 1. Schematic diagram of the covalent structures of the glycan chains of synthetic glycoconjugate used in the DC-SIGN binding assay. Gal, D-galactose; Fuc, L-fucose; Man, D-mannose; GlcNAc, N-acetyl-D-glucosamine; NeuAc, N-acetylneuraminic acid. Mannose and fucose are depicted in bold.

less complex mannose-containing glycoconjugates, i.e., mannose and α1→3,α1→6-mannotriose (18, 19). Strikingly, DC-SIGN binds to Lewis blood group Ags (Le^x, Le^y, Le^a, Le^b) that contain fucose residues in different anomeric linkages (Figs. 1 and 2). Sialylation of Le^x (yielding sialyl-Le^x, a L-, E-, and P-selectin ligand) completely abrogates the recognition by DC-SIGN, indicating that DC-SIGN has a carbohydrate specificity that is distinct from that of the selectins that mediate leukocyte rolling (20). Sulfation reduced the binding affinity of DC-SIGN for Le^x strongly and, to a lesser extent, for Le^a (Fig. 3A). To compare in more detail the affinity of DC-SIGN binding to Le^x and α1→3,α1→6-mannotriose, titration studies were performed with the different DC-SIGN binding glycoconjugates (Fig. 3A). Strikingly, DC-SIGN binds with much higher affinity to the fucose-containing carbohydrate Le^x than to mannotriose. The binding activity of DC-SIGN-Fc to these glycan structures was specific, since anti-DC-SIGN Abs blocked the interaction (Fig. 3A).

To determine whether DC-SIGN-Fc exhibits a similar carbohydrate recognition profile as cell surface-expressed DC-SIGN, both DC-SIGN transfectants and monocyte-derived
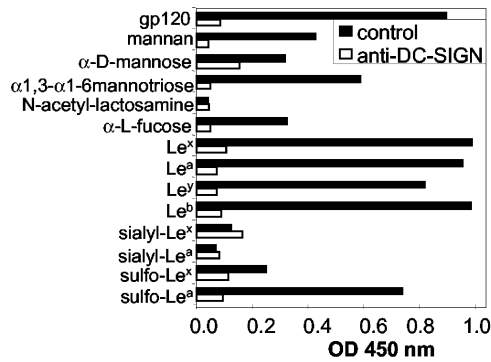
FIGURE 2. Le^x-neoglycoconjugates bind with high affinity to DC-SIGN. Glycoconjugates were coated and binding of recombinant DC-SIGN-Fc was measured after incubation with peroxidase-labeled goat anti-human-Fc. Anti-DC-SIGN mAb AZN-D1 were used to block binding.

DC were studied for carbohydrate binding activity using a fluorescent beads adhesion assay with different glycoconjugates ($\alpha1\rightarrow3,3f\alpha1\rightarrow6$-mannotriose, Le^x and sulfo-Le^a; Fig. 3B). Indeed, DC-SIGN expressed by K562 transfectants bound similarly to the glycoconjugates as DC-SIGN-Fc and the binding was completely inhibited by anti-DC-SIGN Abs (Fig. 3B). Even though DC express many other C-type lectins on their cell surface, our data demonstrate that the glycoconjugates containing Le^x and $\alpha1\rightarrow3,\alpha1\rightarrow6$-mannotriose are preferentially bound by DC-SIGN. The interaction is specific since anti-DC-SIGN Abs almost completely inhibited the binding activity. This illustrates that DC-SIGN is the major receptor on DC for these carbohydrate structures. Binding of sulfo-Le^a to DC could only be partially blocked by anti-DC-SIGN Abs, indicating that other C-type lectins on DC compete with DC-SIGN for binding of sulfo-Le^a. Our data show that DC-SIGN recognizes a wider range of glycan structures, including Lewis blood group Ags, than hitherto realized. Thus, DC-SIGN may be an important receptor for recognition of novel biologically relevant targets expressed by the host or alternatively by human pathogens.

DC-SIGN is known to interact with ICAM-2 and ICAM-3; however, the glycan ligands on these molecules have not yet been identified (14, 15). The blood group Ag Le^x (CD15) is expressed by gastric mucosal epithelial cells and by polymorphonuclear leukocytes (PMNs); indeed DC-SIGN binds
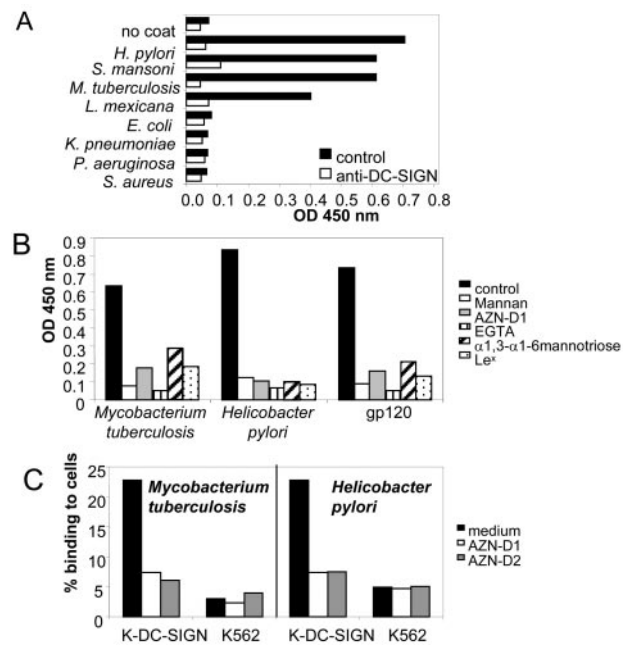

FIGURE 4. DC-SIGN binds four novel pathogens. A, Pathogens that consist out of Le^x-rich *H. pylori* and *S. mansoni* and mannose-capped lipoarabinnanomannan of *M. tuberculosis* and mannose-capped lipophosphoglycan of *L. mexicana*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* were coated and binding of recombinant DC-SIGN-Fc was measured with peroxidase-labeled goat anti-human Fc. B, The Le^x-rich *H. pylori* LPS and mannose-rich cell wall component lipoarabinnan of *M. tuberculosis* and gp120 binding to DC-SIGN-Fc can be inhibited by several glycans, such as polymannose mannan (50 mM), Le^x (20 μg/ml), $\alpha1,3$-$\alpha1,6$-mannotriose (50 μg/ml), that occupy the DC-SIGN lectin domain in a similar manner as anti-DC-SIGN Abs (10 μg/ml) or EGTA (5 mM) that deprives Ca^{2+} from the lectin domain, which is essential in glycan coordination. C, Both FITC-labeled bacteria of *H. pylori* and *M. tuberculosis* strongly bind to DC-SIGN-expressing K562 cells.

PMNs strongly through a novel glycoprotein (data not shown). Le^y expression is increased on many carcinomas including ovary, pancreas, prostate, breast, colon, and non-small cell lung cancers (21), while sulfo-Le^a is present on certain tumors that express mucins. This indicates that recognition of distinct carbohydrate structures by DC-SIGN may allow DC-mediated cell adhesion to T cells, to endothelial cells, as well as to PMNs and tumor cells.
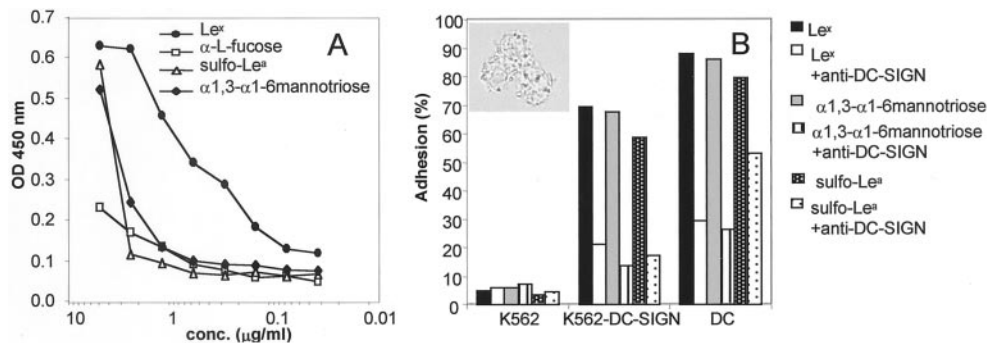

FIGURE 3. Cellular DC-SIGN displays a binding specificity similar to that of soluble DC-SIGN-Fc. A, Titration of the glycoconjugates revealed that soluble DC-SIGN binds with high affinity to Le^x-glycoconjugates, whereas it has a lower binding affinity for α-L-fucose, sulfo-Le^a, and $\alpha1\rightarrow3,\alpha1\rightarrow6$-mannotriose. B, Binding of DC-SIGN-expressing K562 transfectants and immature monocyte-derived DC to glycoconjugate-coated fluorescent beads was measured by FACScan analysis. Binding was inhibited by anti-DC-SIGN mAb AZN-D2. One representative experiment of three is shown. SD is <2%. Inset, Photograph of Le^x-coupled fluorescent beads that bind to DC-SIGN-expressing DC.
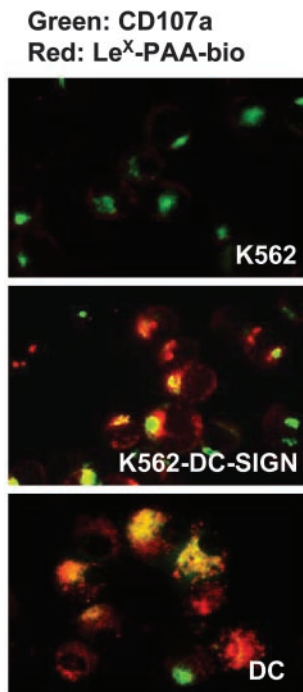
Green: CD107a
Red: Le^x-PAA-bio

FIGURE 5. DC-SIGN rapidly internalizes Le^x glycans to lysosomal compartments. Synthetic biotinylated Le^x-polyacrylamide were added to DC-SIGN-expressing K562 cells or immature monocyte-derived DC for 30 min incubation at 37°C. Cells were stained with anti-CD107a (=LAMP-1, green) as a lysosomal marker and avidin-Alexa 594 for localization of Le^x after permeabilization of the cells. Colocalization of LAMP-1 and Le^x results in a yellow staining.

We subsequently investigated the binding of DC-SIGN to human pathogens that express mannose- or fucose-containing glycans often expressed in a multivalent form. The Gram-negative bacterium *H. pylori*, which induces peptic ulcers and gastric carcinoma (22), and the worm parasite *S. mansoni* (the causal agent of schistosomiasis) both heavily express Le^x (23). In *H. pylori*, Le^x is present on surface-located LPS, whereas in *S. mansoni* Le^x is expressed by all stages of the parasite, including soluble egg Ag (23). Binding of DC-SIGN to Le^x-positive *H. pylori* lysate and to extract of *S. mansoni* was strong and completely inhibited by anti-DC-SIGN Abs (Fig. 4A); DC-SIGN also bound to purified LPS of *H. pylori* and the mannose-capped lipoarabinomannan cell wall component of *M. tuberculosis* (Fig. 4B). Detailed future studies need to identify the exact glycan component present in *S. mansoni* that interact with DC-SIGN.[3] Binding activity of these pathogens could be blocked by any of the glycans that binds to the lectin domain of DC-SIGN, such as Le^x or mannotriose, indicating that they inhibit DC-SIGN-pathogen interaction by occupying the ligand binding site (Fig. 4B). Strong binding activity of *H. pylori* and *M. tuberculosis* to DC-SIGN-expressing cells was observed which could be completely blocked with anti-DC-SIGN mAb, indicating that cell surface-expressed DC-SIGN exhibits a similar pathogen recognition profile as DC-SIGN-Fc (Fig. 4, A and C).

The exact glycan conformation present on the distinct pathogens that interact with DC-SIGN needs to be further identified, but several findings hint to the involvement of the mannose-capped surface glycan, lipoarabinomannan of *M. tuberculosis* (24, 25), and Le^x containing LPS from *H. pylori* (Fig. 4B) to contain the recognition site for DC-SIGN. Binding of DC-SIGN to *Leishmania* was reported very recently (26); however, we identified here that similar to *M. tuberculosis* the mannose-capped surface lipophosphoglycan expressed by *L. mexicana* is the DC-SIGN binding structure of *Leishmania*, an unicellular parasite that causes leishmaniasis (27). No binding of DC-SIGN to three clinically relevant Gram-negative bacterial human pathogens (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) was observed nor to Gram-positive *S. aureus*. The broader carbohydrate specificity of DC-SIGN may also identify more DC-SIGN binding pathogens than presently described, such as *Candida*, *K. pneumoniae* LPS serotype O3 that contains a polymannose, or *Mycobacterium luteus* that expresses a lipomannan. Alternatively, also trypanosomes that express cell surface saccharides such as α-D-mannose may interact with DC-SIGN. The finding that different clinical isolates from *Klebsiella* pneumonia may contain or lack polymannose may have great implications for the efficiency by which these pathogens are recognized by DC-SIGN on DC and thus may have consequences for the clinical manifestations. These findings indicate that binding of DC-SIGN to pathogens is selective and that the carbohydrate specificity of DC-SIGN governs a broader pathogen recognition than only viruses such as HIV-1, Ebola virus, and CMV (12, 16, 28). To determine whether DC-SIGN may capture and internalize the carbohydrate-coated pathogens, we analyzed whether binding of glycoconjugates to DC led to rapid internalization into cells. Time kinetic experiments with Le^x-containing glycoconjugates demonstrated that within 15 min after initial binding Le^x was completely internalized and was targeted into the lysosomes of DC (Fig. 5). Similar internalization profiles were observed upon binding of *M. tuberculosis* to DC (25).

A common feature of the pathogens that interact with DC-SIGN is that they cause chronic infections that may last a lifetime and also that parasite manipulation of the Th1/Th2 balance is central to this persistence. The interaction between these pathogens and DC-SIGN may greatly influence Ag presentation as well as cytokine secretion by DC, and may thereby contribute to the persistence of these pathogens. For infection with *M. tuberculosis*, it has already been demonstrated that interaction of the mannose-capped surface glycan, lipoarabinomannan of *M. tuberculosis* to DC, reduces IL-12 production and enhances IL-10 production and shifts the immune response toward Th2, which results in immune evasion and persistence of infection (29). Likewise, a Th1 to Th2 shift, associated with a decrease in IL-12 concentrations, is crucial to virulence and persistence of *L. mexicana* and its surface LPS has a role in inhibiting DC function by blocking the migration of DC from skin to lymph nodes. Also, for *S. mansoni*, a Th2 immune response is associated with persistence of the pathogen. Soluble egg Ag and its major glycan Ag Le^x are able to cause a switch toward a Th2-mediated immune response (30). Therefore, we consider it likely that the interaction of pathogen glycans with DC-SIGN shifts the Th1/Th2 balance in favor of persistence.

[3] I. van Die, S. J. van Vliet, A. Kwame Nyame, R. D. Cummings, C. M. C. Bank, B. J. Appelmelk, T. B. H. Geijtenbeek, and Y. van Kooyk. The dendritic cell-specific C-type lectin DC-SIGN is a receptor for *Schistosoma mansoni* egg antigens and recognizes the fucose-containing glycan antigens Lewis^x and LDNF. *Submitted for publication.*

The identification of DC-SIGN as a novel pathogen receptor on DC for *M. tuberculosis*, *H. pylori*, *L. mexicana*, and *S. mansoni* opens up new opportunities for the exploitation of carbohydrate-based inhibitors to reduce pathogen-driven inflammatory responses. Future experiments will reveal whether these pathogens that interact with DC-SIGN preferentially modulate the Th1/Th2 balance by affecting cytokine secretion by DC. Therefore, anti-DC-SIGN Abs or antagonists may be used as tool to manipulate Th1/Th2 balance, favoring immune activation, attack, and elimination of the pathogen.

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