Glycodendrimers prevent HIV transmission via DC-SIGN on dendritic cells

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Abstract

Dendritic cells (DCs) are antigen-presenting cells efficient in capturing pathogens, and processing their antigenic determinants for presentation to antigen-specific T cells to induce robust immune responses. Their location at peripheral tissues and the expression of pattern-recognition receptors, among them DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), facilitates the capture of pathogens before spreading. However, some pathogens have developed strategies to escape the immune system. One of the most successful is HIV-1, which targets DC-SIGN for transport to the lymph node where the virus infects CD4+ T cells. Contact of HIV-1 with DC-SIGN is thus the first event in the pathogenic cascade and, therefore, it is the primary target point for therapies aimed at HIV infection prevention. DC-SIGN recognizes specific glycans on HIV-1 and this interaction can be blocked by competitive inhibition through glycans. Although the affinity of glycans is relatively low, multivalency may increase avidity and the strength to compete with HIV-1 virions. We have designed multivalent dendrimeric compounds based on Lewis-type antigens that bind DC-SIGN with high selectivity and avidity and that effectively block gp120 binding to DC-SIGN and, consequently, HIV transmission to CD4+ T cells. Binding to DC-SIGN and gp120 inhibition was higher on glycodendrimers with larger molecular diameter, indicating that the geometry of the compounds is an important factor determining their functionality. Our compounds elicited DC-SIGN internalization, a property of the receptor upon triggering, but did not affect the maturation status of DCs. Thus, LeX glycodendrimers could be incorporated into topical prophylactic approaches for the prevention of HIV-1 transmission.

Keywords: competitive inhibition, gp120, human, infectious disease, LewisX

Introduction

Dendritic cells (DCs) represent a collection of mononuclear phagocytes located in both lymphoid organs and non-lymphoid peripheral tissues that are crucial in the early recognition of pathogens (1). Thanks to their strategic position at the boundaries of the organism, especially the skin and the respiratory and digestive mucosa, DCs are the first cells of the innate immune system to detect invading pathogens. This step is essential in the development of robust adaptive immune responses, as DCs are the most efficient cells of the immune system in processing and presenting antigens to effector T cells (2). Pathogen recognition is achieved with the help of several families of germline-encoded receptors. Since these receptors often recognize pathogens on the basis of relatively broad molecular patterns, they are called pattern-recognition receptors. Four families of pattern-recognition receptors have been described to date: Toll-like receptors, Nod-like receptors, RIG-I-like receptors and C-type lectin receptors (CLRs). The molecular patterns recognized by these receptors are diverse and range from lipids to proteins, carbohydrates and nucleic acids, but often converge on common intracellular signaling pathways, including those mediated by nuclear factor-κB, mitogen-activated protein kinases and interferon-regulatory factors (3). The CLR family is characterized by the presence in their structure of a C-type lectin-like domain, a conserved structural motif that confers Ca2+-dependent glycan binding to some members of this superfamily of receptors (4). Contrary to other pattern-recognition receptors, CLRs are often able to mediate internalization and induce and modulate signaling (5, 6). Although pathogen internalization is beneficial for the host because it allows the processing of pathogens into their
antigenic determinants for presentation to antigen-specific T cells, some pathogens have developed strategies to escape immune surveillance by targeting CLRs. This is best highlighted by the HIV-1 (7). The HIV-1 envelope glycoprotein gp120 is decorated with multiple N-glycans that mediate binding to both DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (8) and langerin (9). However, interaction with each of these CLRs leads to opposite effects. HIV-1 capture by DC-SIGN results in first-phase transmission by internalization of the virus to endosomes or multivesicular bodies where it remains protected from degradation and is subsequently transferred to T cells in the lymph node (10). On the other hand, langerin captures HIV-1 and mediates its internalization into Birbeck granules, which results in rapid degradation of the virus (11). The exclusive localization of langerin in Langerhans cells ensures the first contact with HIV-1, and prevents interaction of the virus with mucosal DC-SIGN+ DCs. Thus, only when the langerin barrier is surpassed, infection proceeds through DC-SIGN. The affinity of DC-SIGN and langerin for HIV-1 can be explained at the molecular level by the glycan specificity of these lectins. Both CLRs have been shown to recognize high-mannose structures on HIV-1. However, although overlapping in their selectivity for Lewis-type antigens, it appears that langerin prefers Leb and LeY and has lower affinity for the monofucosylated Lewis-type antigens, it appears that langerin prefers Leb and LeY and has lower affinity for the monofucosylated Lewis-type antigens (12). Additionally, langerin binds N-acetyl-glucosamine (GlcNAc)-terminated glycans, irrespective of the glycosidic linkage to the sugar residue preceding in the oligosaccharide chain (12).

Due to the protective function of langerin in HIV-1 infection and the role of DC-SIGN in the trans-infection of this virus, it has been proposed that anti-infective strategies should be aimed at the selective inhibition of HIV-1 capture by DC-SIGN without altering langerin recognition of the virus (13). On the basis of the glycan specificity of these CLRs, Lewis-type antigens are interesting candidates in the design of DC-SIGN-specific antagonists. One important aspect to consider in the design of such compounds is that glycan recognition by CLRs is characterized by poor affinity for the glycans that is often enhanced when glycans are presented in multivalent form (14). This is of special relevance in the case of the interaction of DC-SIGN and gp120, a glycoprotein that is known to present clustered subdomains of high-mannose glycans, which explains its high affinity/avidity for DC-SIGN (15). Thus, competitive inhibition of HIV-1 binding to DC-SIGN can only be achieved by using highly multivalent molecular systems. Several chemical scaffolds exist to prepare such compounds. Among them, polyamido amine (PAMAM) dendrimers (16) could be ideal candidates, as these compounds have a defined globular shape, are available with different levels of multivalency and can be functionalized for direct conjugation to activated glycans through their reducing end.

Here, we conjugated Lewis-type antigens to different generations of PAMAM dendrimers in order to investigate the effect of increasing multivalency in selective binding to DC-SIGN and inhibition of HIV-1 trans-infection. We show here that the spacing of glycans on the surface of the glycodendrimer is an important factor determining high-avidity interaction with DC-SIGN and, therefore, warrants effective and selective inhibition of HIV-1 binding to DC-SIGN but not langerin.

### Methods

**Glycodendrimer synthesis**

Lacto-N-difucoxhexasose I (L602, Lewis\(^\text{b}\)); Le\(^\text{b}\)), lacto-N-fuco-pentaose III (L504, Lewis\(^\text{a}\)); Le\(^\text{a}\)), lacto-N-fucopentaose II (L503, Lewis\(^\text{c}\)); Le\(^\text{c}\)), N,N',N''-tetraacetyl chitotetraose (C8004, Chito) and maltotetraose (G602, MH) were obtained from Dextra Laboratories (UK). Dimethylsulfoxide (DMSO), acetic acid (AcOH), 2-picoline borane, Atto488 NHS-ester, triethylamine (TEA) and PAMAM amino-dendrimers were obtained from Sigma-Aldrich (Germany); disposable PD10 desalting columns were purchased from GE Healthcare (The Netherlands). The PAMAM amino-dendrimers of generations 3, 4 and 5 were conjugated to five different glycans through reductive amination between the free reducing end of the glycans and the amino moieties of the dendrimers. Briefly, the glycans (1.25 equivalents per arm) dissolved in DMSO/AcOH were added to a solution of PAMAM amino-dendrimers at the following concentration: 30 nmoles for generation 3 (32 arms), 25 nmoles for generation 4 (64 arms) and 20 nmoles for generation 5 (128 arms) in DMSO to a final volume of 200 µl. Reaction was allowed to proceed at 65°C with frequent stirring. After 30 min, 2-picoline borane (10 equivalents per arm) was added and the reaction continued with frequent stirring at 65°C for 5 h. The reaction products were purified by disposable PD10 desalting columns over 50 mM NH\(_4\)HCO\(_3\) and then freeze-dried twice. The lyophilized PAMAM glycodendrimers were then dissolved in 150 µl of DMSO and three equivalents of Atto488 NHS-ester were added, followed by 6.4 µl of TEA. Reactions were allowed to proceed overnight at room temperature, with frequent stirring and protected from light. Reaction products were recovered by disposable PD10 desalting columns using 50 mM NH\(_4\)HCO\(_3\) as eluent. The fluorescently labeled glycodendrimers were freeze-dried twice and then dissolved in PBS to reach a stock concentration of 4 mg ml\(^{-1}\). The mass and purity of the conjugates were determined in a Microflex LRF matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Germany). The characteristics of the glycan-conjugated dendrimers are shown in Table 1.

**Cells**

Human monocyte-derived DCs were generated from monocytes as previously described (17). Briefly, monocytes were isolated from the blood of healthy donors (Sanquin, Netherlands). The PAMAM amino-dendrimers of generations 3, 4 and 5 were conjugated to five different glycans through reductive amination between the free reducing end of the glycans and the amino moieties of the dendrimers. Briefly, the glycans (1.25 equivalents per arm) dissolved in DMSO/AcOH were added to a solution of PAMAM amino-dendrimers at the following concentration: 30 nmoles for generation 3 (32 arms), 25 nmoles for generation 4 (64 arms) and 20 nmoles for generation 5 (128 arms) in DMSO to a final volume of 200 µl. Reaction was allowed to proceed at 65°C with frequent stirring. After 30 min, 2-picoline borane (10 equivalents per arm) was added and the reaction continued with frequent stirring at 65°C for 5 h. The reaction products were purified by disposable PD10 desalting columns over 50 mM NH\(_4\)HCO\(_3\) and then freeze-dried twice. The lyophilized PAMAM glycodendrimers were then dissolved in 150 µl of DMSO and three equivalents of Atto488 NHS-ester were added, followed by 6.4 µl of TEA. Reactions were allowed to proceed overnight at room temperature, with frequent stirring and protected from light. Reaction products were recovered by disposable PD10 desalting columns using 50 mM NH\(_4\)HCO\(_3\) as eluent. The fluorescently labeled glycodendrimers were freeze-dried twice and then dissolved in PBS to reach a stock concentration of 4 mg ml\(^{-1}\). The mass and purity of the conjugates were determined in a Microflex LRF matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Germany). The characteristics of the glycan-conjugated dendrimers are shown in Table 1.

**Table 1. Characteristics of the glycodendrimers**

<table>
<thead>
<tr>
<th>Generation</th>
<th>Radius of gyration (nm)</th>
<th>Mean mass (kDa)</th>
<th>Glycans/dendrimer</th>
<th>Average distance between adjacent glycans (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.22–1.57 (58)</td>
<td>23.1 ± 2.1</td>
<td>16 ± 2</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>1.55–2.06 (58)</td>
<td>27.2 ± 1.4</td>
<td>14 ± 1</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>1.90–2.53 (58)</td>
<td>45.5 ± 3.3</td>
<td>16 ± 3</td>
<td>2.7</td>
</tr>
</tbody>
</table>
The Atto488-conjugated glycodendrimers and K562/DC-SIGNLL/Y dendrimer binding to cellular DC-SIGN was evaluated using a Cellular DC-SIGN binding assay. Systems) on a FACScan flow cytometer (BD Biosciences). Langerin was confirmed using PE-labeled anti-langerin (BD Biosciences). and granulocyte macrophage colony-stimulating factor (GM-CSF) (800 IU ml−1; BioSource) for 7 days (18). Monocyte differentiation into DC was confirmed by flow cytometric analysis (FACScan, BD Biosciences, USA) of the expression of DC-SIGN using the mAb AZN-D1 (19) followed by staining with a secondary FITC-labeled anti-mouse antibody (Zymed, San Francisco, CA, USA).

Stable K562/DC-SIGN and K562/DC-SIGN

Cellular DC-SIGN binding assay

Dendrimer binding to cellular DC-SIGN was evaluated using Atto488-conjugated glycodendrimers and K562/DC-SIGN1

The IC50 was calculated with the help of the GraphPAD v4.0 software (GraphPAD Software Inc., USA).

Gp120 binding assay

Fluorescently labeled gp120 microbeads were generated in a similar way as was described for Intercellular adhesion molecule 1 (ICAM-1) beads (23). In brief, carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 μm, Invitrogen) were coupled to streptavidin (Sigma, Germany) followed by incubation with a biotinylated F(ab′)2 fragment of goat-anti-human IgG Fc fragment of (0.5mg ml−1, Jackson ImmunoResearch, USA) for 2h at 37°C. Next, the beads were washed and coupled to purified HIV-1 gp120-Fc protein (1 μg ml−1) by an overnight incubation at 4°C. Cells (5 x 10⁴ per well) were incubated with glycodendrimers or polyacrilamide-coupled (PAA-coupled) LeX or GlcNAc for 45min at 37°C. Next, gp120 fluorescent beads were added to the cells and incubated for another 45min at 4°C. The percentage of gp120 bead-positive cells was analyzed on a FACScan flow cytometer (BD Biosciences). Both PAA-LeX and PAA-GlcNAc were from Lectinity (Russian Federation).

Maturation assay

Immature DCs were incubated with glycodendrimers (5mg ml−1) or with LPS (10ng ml−1) overnight at 37°C. Cells were washed and incubated with PE-labeled anti-CD1c, anti-CD40, anti-HLA-DR, anti-CD80, anti-CD83 and anti-CD86 for 60min at 4°C. All antibodies except anti-CD40 were from BD Biosciences. Anti-CD40 was a kind gift of Dr T. D. de Gruijl (VUmc, Amsterdam, The Netherlands). Fluorescence was measured and analyzed on a FACScan flow cytometer (BD Biosciences).

ELISA

Glycodendrimers or PAA-LeX⁺, PAA-Le⁺ or PAA-Le⁺ (Lectinity) were coated at the indicated concentrations onto Nunc maxisorb 96-well plates (Nunc, Denmark) (100 µl per well) in 0.05M NaHCO₃ buffer overnight at 4°C. Plates were then washed twice in TSM buffer (20mM Tris–HCl pH 8.0, 150mM NaCl, 1mM CaCl₂, 1mM MgCl₂) followed by blocking with 1% BSA in TSM for 30 min at 37°C. Soluble DC-SIGN/Fc or langerin/Fc supernatants were added for 90min and incubated under mild shaking at room temperature. Unbound DC-SIGN/Fc or langerin/Fc were washed away and binding was determined by anti-IgG ELISA (HRP-labeled goat-anti-human IgG, Fcγ fragment specific, from Jackson ImmunoResearch). Detection was performed using a 3,3',5,5'-tetramethylbenzidine (100mg ml⁻¹, Sigma-Aldrich)-containing substrate and 0.2M H₂SO₄ to stop the reaction. Optical density was determined using a microplate absorbance spectrophotometer at 450nm (Bio-Rad, USA). DC-SIGN/Fc consists of the extracellular portion of DC-SIGN (residues 64–404) fused at the C-terminus to a human IgG/Fc fragment into the Sig-pIgG₁-Fc vector (24). The carbohydrate-recognition domain (CRD) region of human langerin (residues 193–328) fused to a human IgG/Fc fragment into the Sig-pIgG₁-Fc vector (24). DC-SIGN/Fc and langerin/Fc were produced in Chinese hamster ovary K1 cells by co-transfection of DC-SIGN-Sig-pIgG₁-Fc (20 μg) or langerin-Sig-pIgG₁-Fc (20 μg) and pEE14 (5 μg) vector.
Internalization assay

K562/DC-SIGN transfectants were incubated in the presence of glycodendrimers at either 4 or 37°C for 60 min. Cells were then washed, fixed and prepared for acquisition on the ImageStreamX (Amnis Corp., Seattle, WA, USA) imaging flow cytometer. The following laser powers were used: 488 nm at 20 mW and 785 nm at 4.5 mW. Brightfield illumination was set at 800 mW before the acquisition of each sample. Brightfield images were collected in channels 1 and 9. Channels 2 (Atto488) and 6 (granularity) were habituated for the internalization assay. Cells were acquired at x40 magnification and on the basis of their area (area = the number of pixels in an image reported in square microns). The minimum area for acquisition was set to 50 pixels. A minimum of 15 x 10^3 cells was acquired per sample at a flow rate ranging between 50 and 100 cells s⁻¹. At least 2 x 10^4 cells were acquired from single-stained samples to allow for compensation. Compensation samples were acquired with all channels habituated and with the brightfield illumination and the 785 nm laser switched off. A minimum of 5 x 10^4 cells from the single-stained samples were acquired with the same settings as experimental samples to control for over/under compensation.

Analysis was performed using the IDEAS v5.0 software (Amnis Corp.). A compensation table was generated using the compensation macro built in the software. Single-stained samples were manually gated for accurate calculation of spectral overlap coefficients (25). Once the compensation table was calculated, it was applied to the single-staining samples that were acquired using the same settings as experimental samples. A template analysis file was generated, then which include an area versus aspect ratio intensity plot and a gradient root mean square (RMS) histogram of one of the brightfield channels (channels 1 and 9). Area is the number of squared microns of the cells, while the aspect ratio intensity index is the result of dividing the minor axis (intensity weighted) by the major axis (intensity weighted) and describes how round or oblong an object is; but also indicates if there are doublets in a population of normally circular cells. The gradient RMS feature measures the sharpness quality of an image by detecting large changes of pixel values in the image and is useful for the selection of focused objects. The gradient RMS feature is computed using the average gradient of a pixel normalized for variations in intensity levels. Using these features a population of focused single cells (SC/F) was gated. This template, together with the corresponding compensation table was applied to all the experimental samples acquired. Each of the data files generated was opened and the SC/F population gated to a new compensated image file. Compensated image files were then merged into the final analysis file. This file allows for the direct comparison of features among the different glycodendrimers.

To calculate the internalization of the glycodendrimers, a mask was designed that characterizes only the intracellular space of the cells. This mask was based on the use of the morphology feature applied to the brightfield image on channel 1, and then eroded until the membrane was left out of the mask. Since cells are gated on a certain level of focusing, it is possible to assume that the image acquired represents, in all cells, a 4 μm cross-section of the major circumference (26). At this location, the thickness of the membrane is similar in all cells and allows us to design a mask based exclusively on brightfield images. This is a major advantage over the use of an extracellular fluorescent marker, which introduces new challenges in the experiment: the use of an additional channel complicates the compensation process and the selection of a marker that is exclusively located in the extracellular membrane during the internalization process of the glycodendrimers is a difficult task. The intracellular mask was then used to calculate the feature internalization applied to channel 2 (Atto488). The internalization score is a log-scaled ratio of the intensity inside the cell (intracellular mask) with respect the intensity of the entire cell. Cells that have internalized antigen typically have positive scores, while cells that show the antigen still on the membrane have negative scores. Cells with scores around zero have similar amounts of antigen on the membrane and in intracellular compartments.

Immunofluorescence microscopy

Immature DCs were incubated with the Atto488-labeled glycodendrimers (5 μg ml⁻¹) for 30 min at 37°C. Cells were then washed twice in ice-cold PBS and fixed in 2% PFA in PBS for 15 min in ice. Cells were then washed twice in PBS and incubated with the biotin-labeled plant lectin Con A (VectorLabs, USA) at 10 μg ml⁻¹ for 60 min at room temperature and the monoclonal anti-DC-SIGN antibody AZN-D1, also at 10 μg ml⁻¹. After two wash steps in PBS, cells were incubated with an AlexaFluor555-labeled goat-anti-mouse IgG Fc antibody and AlexaFluor647-labeled streptavidin for 30 min at room temperature. Cells were washed twice and allowed to adhere onto poly-L-lysine-coated glass slides and mounted with anti-bleach reagent. Samples were analyzed for immunofluorescence using a 63x/1.4 HCX PL APO CS oil objective on a TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems GmbH). Images were acquired using LCS 2.61 (Leica Microsystems GmbH) and processed using Adobe Photoshop CS4.

HIV transmission assay

Raji/SIGN cells (5 x 10⁴ cells per well) were pre-treated with different concentrations of glycodendrimers (ranging from a final concentration of 0.6 up to 10 μg ml⁻¹) for 1 h at 37°C. Mannan (100 μg ml⁻¹) and a monoclonal blocking anti-DC-SIGN antibody (AZN-D1, 20 μg ml⁻¹) were used as blocking controls. Cells were incubated with the R5-tropic HIV-1 NL4-3-Bal virus; (multiplicity of infection, 1 x 10⁻⁴) for 4 h. Cells were washed extensively (four times) with fresh medium. About 50 x 10⁴ Jurkat CCR5 T cells (target cells) were added and co-cultured for 5 days. At day 2 and day 5, post-transmission co-cultured cells were harvested, fixed, permeabilized and stained for the p24 HIV capsid protein. Stained cells were analyzed by flow cytometry and transmission assessed as the percentage of p24+ target cells. Experiments were repeated twice. Data are indicated by the mean and standard deviation of the duplicated measurements. For each sample, two positive controls of transmission (no glycodendrimers) were used to demonstrate the accuracy and robustness of the transmission model.
Results

LeX, Leb and Lea glycodendrimers bind to DC-SIGN

LeX, Leb and Lea glycodendrimers were synthesized by the conjugation of activated glycans to PAMAM dendrimers via the coupling of the reducing end of glycans to the functional groups of the dendrimers (primary amines). In order to achieve increasing levels of multivalency, three PAMAM dendrimers were chosen, generations 3, 4 and 5 containing 32, 64 and 128 functional group available, respectively. Although the reactions were carried out in sufficient excess of glycan moieties per available functional group of the PAMAM dendrimers, we did not achieve a complete substitution of all the reactive groups (Table 1). On the basis of the MALDI-TOF data and the molecular weight of each of the unsubstituted PAMAM dendrimers (Fig. 1), we concluded that the dendrimers had been conjugated with similar amounts of glycans, 16 ± 2, 14 ± 1 and 16 ± 3 for generations 3, 4 and 5, respectively. Unfortunately, the goal of achieving glycodendrimers with increasing levels of glycan valency was not reached, but instead we obtained a set of compounds that differed in the molecular diameter (Table 1), but had a comparable amount of glycans on the surface. Assuming an even distribution of glycans on the surface of the PAMAM dendrimers and a rigid dendrimer structure, the distance between adjacent glycans was calculated to range between 1.8 and 2.7 nm, approximately (Table 1). Because the influence of glycan spacing in the recognition of ligands by DC-SIGN has never been investigated, we decided to proceed with testing our set of glycodendrimers.

Firstly, DC-SIGN binding was tested by ELISA. Glycodendrimers were coated on to ELISA plates and binding of DC-SIGN was tested using a soluble DC-SIGN/Fc chimera. Detection was achieved using a goat-anti-human IgG, Fc ELISA. The results were similar for the three glycodendrimer sets tested, LeX (Fig. 2A), Leb (Fig. 2B) and Lea (Fig. 2C), and no binding could be observed to the negative control MH (data not shown). Surprisingly, DC-SIGN binding increased in parallel with the glycodendrimer generation, even though the amount of glycan per dendrimer was similar, indicating that the glycan spacing on the surface of the glycodendrimers plays a decisive role in determining the binding avidity. Interestingly, binding of glycodendrimers to DC-SIGN was much higher than the binding of the comparable polyacrylamide conjugates (Fig. 2A–C). PAA-glycans carry typically 15–25 glycan units per conjugate (27). However, the polyacrylamide carrier is organized as a random coil in which the distance between two oligosaccharides is not fixed and, therefore, the conjugate can adjust itself to a target molecule or cell. Keeping this in consideration, it appears obvious that DC-SIGN prefers its ligands in a spherical configuration rather than a linear or coiled structure.

Because DC-SIGN/Fc functions as a dimeric carbohydrate-binding domain, but DC-SIGN exists on cells as a tetramer (28), we investigated the binding of the glycodendrimers to DC-SIGN-expressing cells by flow cytometry. Since the fluorescence labeling of the glycodendrimers was performed after glycan conjugation of the PAMAM dendrimers, we could not assume a comparable amount of fluorochrome per molecule. In order to compare the binding properties of the differently Atto488-labeled glycodendrimers, we used a mannose inhibition assay on cells expressing a mutant form of DC-SIGN that is unable to mediate internalization (20). K562/DC-SIGNLL/Y transfectants were incubated with Atto488-labeled glycodendrimers for 1 h at 4°C, washed and the cell-associated fluorescence measured by flow cytometry. As shown in Fig. 2D–F, the IC50 of LeX glycodendrimers to cellular bound DC-SIGN was clearly higher for generation 5 versus generations 4 and 3, and it was the lowest for LeX-PAA. MH glycodendrimers did not show any binding to K562/DC-SIGNLL/Y and, therefore, were not tested for IC50. Thus, despite the amount of glycans among the different glycodendrimer generations was the same, binding appeared to be higher for the larger glycodendrimers (generation 5) as compared with the smaller glycodendrimers (generation 3).

Fig. 1. MALDI-TOF spectrum of generations 3, 4 and 5 LeX glycodendrimers.
Lewis-type glycodendrimers inhibit gp120 interaction with DC-SIGN

Since our glycodendrimers demonstrated a very strong binding to DC-SIGN, we speculated that they could be efficient inhibitors of HIV, a known DC-SIGN-targeting pathogen (8). To test this, we prepared gp120/Fc-coated fluorescent beads, a well-characterized model to investigate the interaction of HIV with DC-SIGN (8). Even at the lowest concentration, all glycodendrimer generations were able to mediate a drastic reduction in the binding of gp120 particles to K562/DC-SIGN-transfectants (Fig. 3B) or DCs (Fig. 3D). The most efficient glycodendrimer generation appeared to be, as expected,
generation 5, which achieved a total blockade of gp120 binding at approximately 0.5 µg ml⁻¹ (Fig. 3B). Also, Lewis-type glycodendrimers were more efficient than Lewis-type PAA conjugates (Fig. 3A and C) in blocking gp120 binding to DC-SIGN. Similar results were obtained for Leα- and Leβ-conjugated glycodendrimers (data not shown). These results indicate that not only the spacing but also the geometric distribution of the DC-SIGN ligand determines the success in achieving an efficient binding inhibition.

**Glycodendrimer uptake/binding does not activate DCs**

DC-SIGN is an efficient internalization receptor (20). Both carbohydrate ligands and antibodies are able to induce the internalization of the receptor in DCs (29, 30). We, therefore, tested whether binding of glycodendrimers to DC-SIGN resulted in the internalization of these compounds. Internalization was quantified by imaging flow cytometry on K562/DC-SIGN cells. Imaging flow cytometry is an attractive new technology that allows the quantitative characterization of different aspects of cellular morphology and fluorescent probe localization in single cells, among them internalization (31). Using this technology, we observed that at 4°C, as expected, localization of the glycodendrimers was exclusively extracellular, while incubation at 37°C resulted in the internalization of the glycodendrimers (Fig. 4A). Internalization was maximal for all glycodendrimer generations, as more than 95% of the cells displayed internalization scores higher than zero. No significant differences were found regardless of the glycan (data not shown) or the generation tested (Fig. 4). Representative images of K562/DC-SIGN cells incubated with generations 3, 4 and 5 Leα glycodendrimers are shown in Fig. 4B. Internalization was further confirmed in DCs by confocal laser scanning microscopy (Fig. 4C). For this experiment, DCs were incubated with generation 5 Leα glycodendrimers for only 30 min. The fluorescence pattern obtained at 30 min corresponds to small vesicles in close proximity with the membrane (Fig. 4C), a pattern that resembles that of early endosomes, whereas in prolonged incubations, the fluorescence pattern shows large compact vesicular aggregates (Fig. 4B), compatible with lysosomes. Since no binding could be observed for MH glycodendrimers, we could not assess internalization.

**Fig. 3. Lewis-type glycodendrimers inhibit gp120 binding to DC-SIGN.** (A) Binding of gp120-coated beads to K562/DC-SIGN cells in the presence of PAA-GlcNAc or PAA-Leα. (B) Binding of gp120-coated beads to K562/DC-SIGN cells in the presence of generations 3, 4 or 5 Leα glycodendrimers. (C) Binding of gp120-coated beads to DCs in the presence of PAA-GlcNAc or PAA-Leα. (D) Binding of gp120-coated beads to DCs in the presence of generations 3, 4 or 5 Leα glycodendrimers. Data represent the mean of triplicate wells, and the coefficient of variation was in all cases below 5%. This experiment is representative of at least three independent experiments.
Fig. 4. Lewis-type glycodendrimers are internalized by DCs. (A) Internalization score of K562/DC-SIGN cells incubated with Atto488-labeled generations 3, 4 or 5 Le\textsuperscript{x} glycodendrimers at either 4 or 37°C for 60 min. An internalization score < 0 reflects that most of the probe is localized to the membrane, whereas an internalization score > 0 indicates that most of the signal has an intracellular localization. Equal distribution of the signal over the membrane and the intracellular space is interpreted as an internalization score of zero. (B) Representative examples of K562/DC-SIGN cells incubated with generations 3, 4 or 5 Le\textsuperscript{x} glycodendrimers at either 4 or 37°C for 60 min. (C) Confocal laser scanning micrographs of DCs incubated with Atto488-labeled generation 5 MH or Le\textsuperscript{x} glycodendrimers (green). DCs were stained for DC-SIGN (red) and the membrane marker Con A (blue).
by imaging flow cytometry. Confocal laser scanning micrographs confirmed the absence of internalization of MH glycodendrimers (Fig. 4C). Similar results were obtained for the remaining Le\(^a\) glycodendrimer generations and the Le\(^b\) and Le\(^a\) glycodendrimers (data not shown).

An important issue when using competitive inhibitors of C-type lectins for the prevention of pathogen entry in DCs is that these molecules should not interfere with the maturation state of DCs. To exclude the possibility that binding of the glycodendrimers to DCs causes cellular activation and maturation, we determined the expression of several maturation markers on DCs exposed to glycodendrimers in an overnight incubation. LPS, a known activator of DCs, induced an up-regulation in the expression of CD1c, CD40, HLA-DR, CD80, CD83 and CD86 (Fig. 5A). Interestingly, none of the glycodendrimers was able to significantly induce the up-regulation of the abovementioned markers or affect the LPS-mediated DC maturation phenotype (Fig. 5B and C and data not shown). Similarly, Le\(^a\) and Le\(^b\) glycodendrimers did not induce DC maturation (data not shown). Thus, although Lewis-type glycodendrimers are efficiently internalized in DCs, they do not affect their activation/maturation status. Furthermore, DC viability was unaffected by exposure to glycodendrimers (Fig. 5D).

Lewis-type glycodendrimers do not inhibit gp120 binding to langerin

Whereas HIV-1 exploits DC-SIGN on DCs for its transmission, the entry of virus through mucosal tissues may also be affected by interaction of langerin on Langerhans cells. Contrary to DC-SIGN, it has been shown that langerin does

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**Fig. 5.** Lewis-type glycodendrimers do not affect DC maturation or viability. (A) Expression of the maturation markers CD1c, CD40, HLA-DR, CD80, CD83 and CD86 on DCs incubated with medium alone or LPS (10 ng ml\(^{-1}\)). (B) Effect of generations 3, 4 or 5 Le\(^a\) or MH glycodendrimers on CD80 expression in immature or mature DCs. (C) Effect of generations 3, 4 or 5 Le\(^a\) or MH glycodendrimers on HLA-DR expression in immature or mature DCs. (D) DC viability after exposure to generations 3, 4 or 5 Le\(^a\) or MH glycodendrimers. Data are shown as mean ± SD of three independent experiments. Flow cytometry histograms (A) are representative of three independent experiments.
not mediate HIV transmission, but rather functions to capture and destroy the virus (11). Therefore, we tested whether our glycodendrimers were also able to bind to langerin. In contrast to DC-SIGN, generation 5 Le\(^{\alpha}\) glycodendrimers did not bind langerin/Fc in ELISA (Fig. 6A). We also investigated whether our glycodendrimers influenced the binding of gp120 beads to primary human Langerhans cells. As shown in Fig. 6B, Le\(^{\alpha}\)-coupled glycodendrimers did not inhibit gp120 binding to Langerhans cells. Binding of gp120 to Langerhans cells was also unaffected by PAA-coupled Le\(^{\alpha}\) (Fig. 6C).

**Le\(^{\alpha}\) glycodendrimers block HIV transmission via DC-SIGN**

Based on the previous experiments, Lewis-type glycodendrimers are able to block binding of gp120 to DCs, without affecting the interaction of gp120 with langerin, or inducing an unwanted activation of the DCs. To determine whether our glycodendrimers are also able to block viral transmission, we exposed DC-SIGN-expressing Raji cells (Raji/DC-SIGN) to HIV in the presence of our glycodendrimers and measured the transmission of HIV to T cells. In this setting, HIV transmission is completely dependent on DC-SIGN, as it can be fully inhibited by a blocking antibody against DC-SIGN (AZN-D1) (8) or the DC-SIGN ligand mannan (Fig. 7A). Because generation 5 Le\(^{\alpha}\) glycodendrimers were the most effective in blocking gp120 binding, we focused only on this set of glycodendrimers. As shown in Fig. 7B, Le\(^{\alpha}\) glycodendrimers completely blocked HIV transmission 2 days after exposure (Fig. 7A), whereas transmission in the presence of MH glycodendrimers did not deviate from the positive control (Fig. 7B). In summary, our generation 5 Le\(^{\alpha}\) glycodendrimers are efficient in blocking the transmission of HIV to T cells.

**Discussion**

In this study, we designed and developed multivalent compounds for the competitive inhibition of HIV-1 binding to DC-SIGN but not langerin. These multivalent compounds were built using PAMAM dendrimers as scaffolds by direct conjugation of the glycans through their reducing end to the dendrimer functional groups, ensuring proper centrifugal orientation of the DC-SIGN-binding motif of the glycans. The glycans chosen for this study, Lewis-type antigens Le\(^{\alpha}\), Le\(^{\alpha}\) and Le\(^{\alpha}\), are well-known DC-SIGN ligands commonly found in both the host and several pathogens. Multivalent presentation of these glycans should enhance avidity for the receptor and facilitate competitive inhibition over host and pathogen DC-SIGN ligands. In order to define the appropriate level of multivalency needed to achieve optimal DC-SIGN binding and gp120 inhibition, we aimed to develop three generations of glycodendrimers. The three PAMAM dendrimer generations chosen differed in the amount of functional groups from 16 (generation 3) to 64 (generation 5) (16). Although the amount of activated glycan added to the dendrimer preparations was calculated based on the glycan equivalents needed to saturate all dendrimer functional groups, we did not achieve the goal of obtaining glycodendrimers that differed in the

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**Fig. 6.** Lewis-type glycodendrimers do not bind langerin and do not block gp120 binding to langerin. (A) langerin/Fc binding to generation 5 Le\(^{\alpha}\) or MH glycodendrimers. (B) Binding of gp120 protein to Langerhans cells in the absence (thick gray line) or presence of generations 3, 4 or 5 Le\(^{\alpha}\) glycodendrimers. (C) Binding of gp120 protein to Langerhans cells in the absence (thick gray line) or presence of PAA-Le\(^{\alpha}\) or PAA-GlcNAc. Data represent the mean of triplicate wells, and the coefficient of variation was in all cases below 5%. This experiment is representative of at least three independent experiments.

**Fig. 7.** Le\(^{\alpha}\) glycodendrimers inhibit DC-SIGN mediated HIV transmission. (A) HIV transmission from infected Raji-SIGN cells to Jurkat CCR5\(^{+}\) T cells in the presence or absence of a blocking antibody against DC-SIGN (AZN-D1) or the competitive inhibitor mannan 2 days after exposure. (B) HIV transmission from infected Raji-SIGN cells to Jurkat CCR5\(^{+}\) T cells in the presence of a titration of generation 5 MH or Le\(^{\alpha}\) glycodendrimers 2 days after exposure. Data represent the mean ± SD of triplicate wells. This experiment is representative of at least three independent experiments.
amount of glycan moieties. Indeed, all glycodendrimers prepared had an average glycan content in the range 14–16 glycan units per glycodendrimer. PAMAM dendrimers were the first complete dendrimer family to be synthesized, characterized and commercialized (32). These compounds are synthesized by a divergent method that involves a two-step iterative reaction sequence that produces concentric shells of dendritic β-alanine units around a central initiator core (32). The PAMAM core-shell architecture grows linearly in diameter as a function of added shells, while the surface groups amplify exponentially at each generation according to dendritic-branching mathematics (33). Since these compounds are synthesized in sequential reactions, a potential pitfall in their synthesis is, therefore, the accumulation of side products in the final preparation, especially in the higher generations. Unfortunately, our PAMAM dendrimers were of a commercial source that provided a poor analytical product description. We, therefore, performed mass spectrometric analysis of the unconjugated compounds and found a high level of polydispersity and contamination with low-molecular-weight compounds that increased with the generation number (data not shown). We interpreted that the high level of polydispersity owed to the presence of reaction side products and incompletely synthesized dendrimers in the dendrimer preparation, which might have acted as scavengers for the activated glycans. After glycan conjugation, glycodendrimers were purified by size-exclusion chromatography and all incompletely synthesized dendrimers and other side products, presumably carrying conjugated glycans were eliminated from the preparation. This would explain why increasing in dendrimer generation did not result in more glycan units per dendrimer. Of note, size-exclusion chromatography ensured that our compounds had an acceptable low level of polydispersity, even though the initial goal of achieving increasing levels of multivalency was not reached. Still, the synthesized compounds allowed us to investigate a different, yet still important, aspect of DC-SIGN biology, i.e. the effect of glycan distance in the avidity effect of multivalent competitive inhibitors.

DC-SIGN exists a tetramer on the surface of DCs (28). DC-SIGN tetramerization is mediated by lateral interactions of α-helical domains in the 23-amino acid repeats present in the neck region (34). This configuration allows the stabilization and projection of the carbohydrate-binding region of DC-SIGN away from the membrane and oriented toward the exterior, in order to facilitate multivalent interaction with its ligands. In this configuration, the distance between binding sites has been calculated to approximately 5nm, although a certain degree of flexibility that allows conformational changes upon ligand binding has been demonstrated (35). Other, alternative measurements have demonstrated the diameter of the tetramer-binding site to be approximately 5–8nm, indicating that the distance between adjacent binding sites might be lower than 5nm (36). Although this phenomenon provides DC-SIGN with an unrivaled capacity to recognize glycans on pathogens displayed in heterogeneous configurations, it also allows us to develop multivalent ligands based on their geometric properties. The glycodendrimers generated in the present study have a calculated adjacent glycan linear distance ranging between 1.8 and 2.7 nm, approximately, based on their molecular diameter (Table 1). Additionally, a certain level of flexibility in the branches is expected. The data presented in this study demonstrate that glycodendrimers with larger molecular diameter performed better in solid-phase-based and cellular-binding assays and were more efficient in inhibiting gp120 binding to DC-SIGN. It remains to be determined if dendrimers with larger molecular diameter have better inhibitory properties. Although glycan spacing seems the most logical explanation for our findings, other alternative plausible explanations include the sheer differences in dendrimer size among generations, as well as differences in charge or in shape. A beneficial aspect of the architecture of glycodendrimers is their globular shape. Compared with other multivalent compounds arranged over a linear scaffold (PAA-glycans), our glycodendrimers showed up to a 100-fold better DC-SIGN binding and gp120 inhibitory activity. This demonstrates that spherical multivalent compounds are superior as competitive inhibitors for DC-SIGN.

Monovalent glycomimetic DC-SIGN antagonists have been developed, which mimic the bioactive function of DC-SIGN ligands. The main advantage of these compounds is that they can be designed and screened to achieve higher affinity constants and improved pharmacokinetic properties (37). Several mannose-based glycomimetics have been reported, which showed inhibitory activity at the micromolar (38–42) and low micromolar range (43). However, these compounds could also bind longer and are, therefore, not ideal for preventing HIV-1 transmission. Fucose-based glycomimetics, on the other hand, are specific for DC-SIGN, but showed a relatively low affinity (44, 45). In order to increase affinity, multimeric glycomimetics have been developed, which enhance affinity to the sub-micromolar range and efficiently inhibit gp120 binding to DC-SIGN (46–53). Unfortunately, there is little information of their toxicity, immunogenicity and their biological effects on DCs. Our compounds are based on glycans that are present on the host and, therefore, toxicity and immunogenicity should be minimal, although effects derived from the PAMAM dendrimer cannot be discarded (54). Importantly, we could not observe any signs of activation or maturation on DCs exposed to our glycodendrimers.

HIV-1 continues to be a global health problem of unprecedented dimensions. Since the discovery of HIV-1 and the recognition of its associated immunodeficiency syndrome in the early 1980s, 60 million people have become infected with HIV, of whom 25 million have died. Although the percentage of people living with HIV globally has stabilized since 2000, the overall number of people infected with HIV has steadily increased to an estimated 33.3 million in 2009 (55). The current standard in HIV infection is anti-retroviral therapy, which requires a daily and expensive medication to prevent disease progression and re-emergence of the virus, but does not cure the disease (56). Significant efforts are therefore aimed at the prevention of infection. Among the proposed pre-exposure prophylactic methods for the sexual transmission of HIV are microbicides. Microbicide development for HIV prophylaxis is an active area of research and focuses in both the destruction of the virus and the blockade of its interaction with DCs. A successful HIV infection inhibitor should respect the first line of defense against the virus,
the Langerhans cells (11), but efficiently block recognition by DC-SIGN on the underlying mucosal DCs (8). Given the partially overlapping glycan specificity of DC-SIGN and langerin, the receptor for HIV on Langerhans cells, we hypothesized that monofucosylated Lewis-type antigens, such as Le\(^a\), would appear to be selective for DC-SIGN (12). Thus, Le\(^a\) glycodendrimers would be ideal candidates to be used as competitive antagonists of DC-SIGN for the prevention of HIV-1 infection. However, since multivalent display enhances avidity for CLR\(_s\) (14) and low-affinity interaction of langerin for Le\(^a\) has been reported (57), we tested whether Le\(^a\) glycodendrimers were also recognized by langerin. We could not observe any significant binding to langerin on solid-phase-dendrimers were also recognized by langerin. We could not observe any significant binding to langerin on solid-phase-dendrimers, suggesting that Le\(^a\) glycodendrimers would be ideal candidates for the competitive inhibition of HIV interaction with DC-SIGN. This was confirmed in an HIV transmission assay, in which generation 5 Le\(^a\) glycodendrimers were able to induce a complete suppression of HIV transmission to CD4\(^+\) T cells after 2 days. It remains to be determined in ex vivo models (11) whether Langerhans cells are capable of clearing the virus efficiently in the context of uptake inhibition by DCs.

In conclusion, we present here a set of compounds that are of natural origin and efficiently and selectively inhibit HIV-1 interaction with DC-SIGN but not with langerin and, therefore, constitute interesting candidates as microbicides for the prevention of sexually transmitted HIV infection.

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