Biology of monocyte interactions with the endothelium: the platelet factor

da Costa Martins, P.A.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 6

“DC-SIGN mediates adhesion and rolling of dendritic cells on primary human umbilical vein endothelial cells through Lewis\(^\text{Y}\) antigen expressed on ICAM-2”

Juan J. García-Vallejo\(^1\), Ellis van Liempt\(^1\), Paula da Costa Martins\(^2\), Cora Beckers\(^3\), Bert van het Hof\(^4\), Sonja I. Gringhuis\(^1\), Jaap-Jan Zwaginga\(^2,4\), Willem van Dijk\(^1\), Teunis B. H. Geijtenbeek\(^1\), Yvette van Kooyk\(^1\), and Irma van Die\(^1\)

\(^1\)Dept. of Molecular Cell Biology & Immunology, VU University Medical Centre;  
\(^2\)Dept. of Experimental Immunohematology, Sanquin Research, Location CLB;  
\(^3\)Laboratory for Physiology, VU University Medical Centre;  
\(^4\)Dept. of Hematology, Academic Medical Center; Amsterdam, The Netherlands  
Manuscript in preparation
Abstract

Immature dendritic cells (DCs) are recruited from blood into tissues to patrol for foreign antigens. After antigen uptake and processing, DCs mature and migrate to the secondary lymphoid organs where they initiate immune responses. DC-SIGN is a DC-specific C-type lectin that acts both as a pattern recognition receptor and as an adhesion molecule. As an adhesion molecule, DC-SIGN is able to mediate rolling and adhesion over endothelial cells under shear flow. The binding partner of DC-SIGN in endothelial cells is the carbohydrate epitope Lewis\(^\gamma\) (Le\(^\gamma\)), expressed on ICAM-2. ICAM-2 expressed on CHO cells only served as a ligand for DC-SIGN when properly glycosylated, underscoring its function as a scaffolding protein. The expression of Le\(^\gamma\) in endothelial cells is directed by the enzyme FUT1. Silencing of FUT1 results in an inhibition of the rolling and adhesion of immature DCs over endothelial cells. The identification of Le\(^\gamma\) as the carbohydrate ligand of DC-SIGN in endothelial cells opens new possibilities for the manipulation of DC migration.
Introduction

Dendritic cells (DC) have a key role in the control of immunity by surveying peripheral tissues in the search for self or non-self antigens. In order to create a network of tissue-resident DCs, precursor DCs continuously migrate from the blood into peripheral tissues, where they are highly efficient in capturing and processing antigens as immature DCs. Once activated, immature DCs mature and migrate from the peripheral tissues to secondary lymphoid organs in order to interact with specific T-cells and initiate an immune response. The molecular basis for the migratory capacity of DCs is starting to be unraveled, and several molecules have been described to be involved, such as DC-SIGN, MR, and selectins. DC-SIGN (CD209) is a C-type lectin expressed by precursor and immature DCs that was primarily identified through its high affinity interaction with ICAM-3. In addition, DC-SIGN also functions as an HIV-1 trans-receptor important in the dissemination of HIV-1. Importantly, DC-SIGN mediates rolling and adhesion of precursor DC over the endothelium, which is suggested to be mediated through interactions with ICAM-2.

Thus DC-SIGN appears as a molecule with a dual role, acting as a pattern recognition receptor and as an adhesion molecule. As a pattern recognition receptor, the carbohydrate specificity of DC-SIGN has been carefully evaluated. It is now clear that DC-SIGN is able to recognize high-mannose type N-glycans, as well as glycoconjugates carrying non-sialylated, non-sulfated Lewis antigens. This relatively large recognition profile converts DC-SIGN into a sort of broad-spectrum pattern recognition receptor. Many pathogens have been found to be recognized and internalized by DC-SIGN and, although this mechanism is meant to allow the development of an immune response, often is used by the pathogen to escape immune surveillance. As an adhesion molecule, however, the identity of the endogenous carbohydrate ligand(s) of DC-SIGN, especially in endothelial cells, still remains inconclusive.

The present study was undertaken to identify the DC-SIGN ligands that are crucial for the adhesion and rolling of dendritic cells on endothelial cells. We show here that ICAM-2 expressed on endothelial cells constitutes the major scaffold protein ligand for DC-SIGN. Importantly, the interaction of DC-SIGN with ICAM-2 is carbohydrate-dependent, and we provide evidence that LeY antigens within ICAM-2 are of crucial importance for the binding of DC-SIGN to endothelial cells, as well as for the rolling and adhesion of dendritic cells over endothelial cells.
Materials and Methods

**Cell lines and primary cells.** Human umbilical vein endothelial cells (HUVEC) were isolated from 5 healthy donors by a modification of the method of Jaffé et al.\(^{18}\), as previously described\(^{19}\). The cells were resuspended in M199 (Biowhittaker, USA) supplemented with 100 U/mL Penicillin-Streptomycin (Biowhittaker, USA), 10% human serum (Biowhittaker, USA), 10% new born calf serum (Biowhittaker, USA), 5 U/ml heparine (Leo Pharmaceutical Products, The Netherlands), and 150 μg/mL bFGF (Sigma, The Netherlands) and plated in gelatin-coated plates. The cells were cultured to confluency in the mentioned media in a 5% CO\(_2\) atmosphere at 37 °C. When confluency was reached, cells were trypsinized (0.18% trypsin, 10 mM EDTA) and plated again to 1/3 of their density. All endothelial cells used displayed the presence of Von Willebrand factor, platelet endothelial cell adhesion molecule-1 (CD31), and VE-Cadherin\(^ {20}\). No immunoreactivity to the anti-cytokeratin 20 antibody or the anti-α-smooth muscle actin antibody was observed.

Chinese Hamster Ovary (CHO) cells were cultured in RPMI-1640 (Gibco BRL, USA) supplemented with 10% FCS and 100 U/ml Penicillin-Streptomycin. Where indicated, cells were cultured during 5 days in the presence of kifunensine (Kitasatospora kifunense, 2 μg/ml; Calbiochem, USA). Efficiency of treatment was assessed by flow cytometry analysis using Con A as described under Flow Cytometry.

Immature DCs were obtained from a buffycoat as previously described\(^ {21}\). In short, human peripheral blood mononuclear cells (PBMCs) were isolated from a buffycoat by a Ficoll gradient and followed by a CD14 magnetic microbeads isolation (MACS; Miltenyibiotec, USA). The obtained CD14+ monocytes were differentiated into immature DCs in the presence of interleukine-4 and granulocyte-macrophage colony stimulating factor (500 and 800 U/ml, respectively; Schering-Plough, Belgium). At day 6, the phenotype of the cultured DCs was confirmed by flow cytometric analysis. The immature DCs expressed high levels of major histocompatibility complex class I and II, CD11b, CD11c, and ICAM-1; and low levels of CD80 and CD86.

**Western blotting.** 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-plgG1-Fc vector\(^ {22}\). DC-SIGN-Fc was produced in Chinese hamster ovary K1 cells by cotransfection of DC-SIGN-Sig-plgG1 Fc (20 μg) and pEE14 (5 μg) vector. DC-SIGN-Fc concentrations in the supernatant were determined by an anti-IgG1 Fc ELISA. Cells were grown to confluency in a T175 flask (Corning, USA), washed and resuspended in TSM, and lysed in 0.1 M Tris-HCl (pH 7.4), 0.05 M CHAPS. Lysate was centrifuged and the supernatant incubated with DC-SIGN/Fc (0.5 mg/ml) at 4 °C in a rotating device (18 h). Subsequently, 20 μl protA/G-agarose beads (Santa Cruz, USA) were added and incubated at 4 °C in a
rotating device (4 h). The beads were washed twice in TSM, resuspended in Laemmli sample buffer and incubated at 95°C for 5 minutes prior to resolving in 10 % SDS-PAGE, according to Laemmli.23

After electrophoresis, the gel was blotted on to a PVDF (Millipore, The Netherlands) membrane and stained with DC-SIGN/Fc or mouse anti-human-ICAM-2 (12A2) using peroxidase-labeled goat anti-human (Jackson, USA) and rabbit anti-mouse immunoglobulins (DakoCytomation, Denmark). The membrane was developed using SuperSignal WestPico Chemiluminescence substrate (Pierce, USA) and the chemiluminescence detected in an Epi Chemi II Darkroom (UVP, USA) using the Labworks (UVP, USA) software.

**Transient transfection of CHO cells.** Cells were incubated until 50-80 % confluent. The transfection was performed according to the manufacturer's protocol. In short, both DNA (5 μg pcDM8-ICAM2 or pcDNA1-FUT4) and LipofectAMINE (Gibco BRL, USA) were diluted in serum free medium and combined. After 30 min the cells were washed with serum free medium and the complex solution was added to the cells. After 5 h serum-enriched medium (20 %) was added. The medium was replaced with fresh, complete medium 24 h after transfection. 24 h later the cells (5·10⁴) were resuspended, washed with TSM and analysed by flow cytometry as indicated under Flow cytometry. Alternatively, cells (10⁶) were lysed in 0.1 M Tris-HCl (pH 7.4), 0.05 M CHAPS and analyzed by ELISA as indicated under ELISA.

**Capture ELISA.** Goat anti-mouse-Fc was coated on ELISA plates (Nunc, USA; 2 μg/ml, 100 μl/well), followed by mouse anti-human ICAM2 (12A2) antibodies (1 μg/ml, 50 μl/well). Plates were blocked with 1 % ELISA grade BSA (Fraction V, Fatty acid free; Calbiochem, USA), cell lysates were added, and incubated overnight at 4 °C. After washing, the wells were incubated with DC-SIGN-Fc (5 μg/ml), anti-Lewis^Y (5 μg/ml, clone F3, Calbiochem, USA) and digoxin-labeled Con A (5 μg/ml, Roche, Switzerland), in the presence or absence of 50 mM α-D-CH₃-Mannose/α-D-CH₃-Glucose (both Sigma, The Netherlands). Binding was detected using a peroxidase labelled anti-human IgG-Fc, goat anti-mouse IgM (both Jackson, West Grove, PA) or sheep anti-digoxin (Roche, Switzerland), respectively. Color development after adding POD substrate (Roche, Switzerland) was measured in a spectrophotometer (BioRad, USA) at 410 nm.

**Flow Cytometry.** Cells were washed twice in cold TSM (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl₂ and 2 mM MgCl₂), resuspended in 1 % BSA/TSM and incubated 30 minutes at room temperature with 25 μL of 1 % BSA-TSM diluted
primary antibody/lectin (10 μg/ml), washed twice with TSM and incubated 30 minutes at room temperature with secondary antibody according to manufacturers instructions. After the second incubation, cells were washed twice with PBS and resuspended in a final volume of 100 μL 1 % BSA/TSM for analysis in the FACS-Calibur (Becton-Dickinson, USA). The primary monoclonal antibodies (mouse IgM) used were specific for Lewis\(^x\) (DakoCytomation, Denmark), Lewis\(^y\) (clone F3, Calbiochem, USA), Lewis\(^x\) (clone T174, Calbiochem, USA), Lewis\(^b\) (clone T128, Calbiochem, USA). The lectins used were Con A (concanavalin A, digoxin-labeled, Roche, Switzerland), UEA-I (Ulex europeaus agglutinin, biotin-labeled, EY Labs, USA), and AAL (Aleuria aurantia, digoxin-labeled, Roche, Switzerland). The anti-carbohydrate antibodies, were counter-stained with Alexa 488-labeled goat-anti-mouse secondary antibody (Molecular Probes, The Netherlands). For the secondary staining of the biotin-labeled lectins Alexa 488-streptavidin (Vector Laboratories, USA) and FITC-labeled anti-digoxin (Sigma, USA) were used.

Cells were analyzed for immunofluorescence on a FACS-Calibur flow cytometer by collecting data for \(10^4\) cells per histogram. Corresponding negative controls were performed by omitting the antibody or lectin of interest.

**mRNA isolation and cDNA synthesis.** mRNA was isolated by capturing of poly(A+) RNA in streptavidin-coated tubes with an mRNA Capture kit (Roche, Switzerland) and cDNA was synthesized with the Reverse Transcription System kit (Promega, USA) following manufacturer’s guidelines. Cells (2×10\(^5\)/well) were washed twice with ice-cold PBS and harvested with 200 μL of lysis buffer. Lysates were incubated with biotin-labeled oligo(dT)\(_{20}\) for 5 min at 37 °C and then 50 μL of the mix were transferred to streptavidin-coated tubes and incubated for 5 min at 37 °C. After washing 3 times with 250 μL of washing buffer, 30 μL of the reverse transcription mix (5 mM MgCl\(_2\), 1x reverse transcription buffer, 1 mM dNTP, 0.4 U recombinant RNasin ribonuclease inhibitor, 0.4 U AMV reverse transcriptase, 0.5 μg random hexamers in nuclease-free water) were added to the tubes and incubated for 10 min at room temperature followed by 45 min at 42 °C. To inactivate AMV reverse transcriptase and separate mRNA from the streptavidin-biotin complex, samples were heated at 99 °C for 5 min, transferred to microcentrifuge tubes and incubated in ice for 5 min, diluted 1:2 in nuclease-free water and stored at -20 °C until analysis.

**Quantitative real-time PCR.** Oligonucleotides (Table I) have been designed by using the computer software Primer Express 2.0 (Applied Biosystems, USA). All oligonucleotides were synthesized by Invitrogen (Invitrogen, Belgium). Oligonucleotide specificity was computer tested (BLAST, NCBI) by homology search with the human genome and specifically, with all the known galactosyltransferases
(CLUSTALW, EMBL), and later confirmed by dissociation curve analysis and resolving the PCR products in agarose electrophoresis. In the case of FUT3, FUT5 and FUT6, genes with a high homology, the specificity of the primers was tested using plasmids (kindly provided by Dr. JB Lowe) encoding for each of the fucosyltransferases. The efficiency of the oligonucleotides was determined using the computer program LinReg and resulted in an average of 90%. PCR reactions were performed with the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems, USA). The reactions were set on a 96 well-plate by mixing 4 µL of the 2 times concentrated SYBR Green Master Mix (Applied Biosystems, USA) with 2 µL of a oligonucleotide solution containing 5 nmol/µL of both oligonucleotides and 2µL of a cDNA solution corresponding to 1/60 of the cDNA synthesis product. The thermal profile for all the reactions was 2 min at 50 °C, followed by 10 min at 95 °C and then 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. The fluorescence monitoring occurred at the end of each cycle.

Table I. Oligonucleotides used in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>2597</td>
<td>Fwd: aggtcatccctgagctgaacg g  Rev: cgctgttcaccaccttcttg</td>
</tr>
<tr>
<td>FX</td>
<td>7264</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT1</td>
<td>2523</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT2</td>
<td>2524</td>
<td>Fwd: caaatcccatgcagttctgatc  Rev: gtggcctagcttgctgaggt</td>
</tr>
<tr>
<td>FUT3</td>
<td>2525</td>
<td>Fwd: gcctgttcaccaccttcttg  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT4</td>
<td>2526</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT5</td>
<td>2527</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT6</td>
<td>2528</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT7</td>
<td>2529</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT8</td>
<td>2530</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
<tr>
<td>FUT9</td>
<td>10690</td>
<td>Fwd: gcaggccatggactgttggtagc  Rev: gacgtgtggtgggtgacc</td>
</tr>
</tbody>
</table>

Additionally, dissociation curve analysis was performed at the end of every run by increasing the temperature of the block from 60 to 95 °C at a rate of 1.75 °C/min while continuously monitoring fluorescence. The plot of the first derivative of the decrease in fluorescence with respect to temperature showed in all cases one single peak.
peak at the Tm predicted by the Primer Express 2.0 software. The Ct value is defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value, which is fixed above 10 times the standard deviation of the fluorescence during the first 15 cycles and typically corresponds to 0.2 relative fluorescence units. This threshold is set constant throughout the study and corresponds to the log linear range of the amplification curve. The normalized amount of target, or relative abundance, reflects the relative amount of target transcripts with respect to the expression of the endogenous reference gene. Due to the low expression of glycosyltransferases, the results are shown as 100-times the relative abundance. In this study, the endogenous reference gene chosen was GAPDH, based on previous results.

**RNA interference.** The mammalian expression vector, pSUPER.retro.puro (a kind gift of Dr. R. Agami, Netherlands Cancer Institute, Amsterdam, The Netherlands) was used for expression of siRNA in HUVEC. The gene-specific insert identifies a 19-nucleotide sequence corresponding to nucleotides 272-291 (tcagatggccgatgtgacc) of FUT-1 (NM_000148), nucleotides 362-381 (gacgacctaccgatag) of FX (NM_003313), or the sequence 5'-ctgaatgaatcgtgacacg with no significant similarity to any human gene sequence, therefore used as a non-silencing control. The gene-specific insert was separated by a 9-nucleotide non-complementary spacer (ttcaagaga) from the reverse complement of the same 19-nucleotide sequence, and flanked by restriction sites for the enzymes Bgl II and Hind III, producing a final insert of 60 nucleotides. These sequences were inserted into the pSUPER.retro.puro backbone. The different vectors were referred to as pSUPER/FUT-1, pSUPER/FX, and pSUPER/Scrambled, respectively. Plasmids were transfected into HUVEC using the Basic Nucleofector Kit for Primary Mammalian Endothelial Cells (Amaxa, Germany) in an Amaxa Nucleofector (Amaxa, Germany), according to manufacturer’s instructions. Immediately after transfection, cells were seeded in glass coverslips coated with crosslinked gelatin (1 %) and fibronectin (5 mg/ml). Transfection efficiency was higher than 90 % as evaluated by flowcytometry analysis of HUVEC co-transfected pmax/GFP (Amaxa, Germany) and the different pSUPER constructs (data not shown). To test the efficiency of RNA interference, cells were lysed after 48 h, mRNA isolated (mRNA Capture Kit, Roche, Switzerland) and retrotranscribed into cDNA (Reverse Transcription System, Promega, USA), according to manufacturer’s instructions. Gene expression of FUT-1, FX, and ICAM-2 was assessed by means of quantitative real-time PCR in an ABI 7900HT platform (Applied Biosystems, USA) using the SYBR Green I chemistry (Applied Biosystems, USA), as previously described, using the primers described in Table I. The silencing resulted in a decrease in gene expression higher than 80 % (data not shown).
**Immature DC perfusion and evaluation of adhesion and rolling velocity.** Immature DCs in suspension \((2 \times 10^6 \text{ cells/ml in incubation buffer})\) were aspirated from a reservoir through plastic tubing and perfused through a chamber with a Harvard syringe pump (Harvard Apparatus, South Natic, MA). The flow rate through the chamber was precisely controlled and the immature DCs were perfused over endothelial cells at 0.8 dyn/cm\(^2\). During perfusions the flow chamber was mounted on a microscope stage (Axiovert 25, Zeiss, Germany), equipped with a B/W CCD video camera (Sanyo, Osaka, Japan), and coupled to a VHS video recorder. Video images were evaluated for the number of adherent monocytes and the rolling velocity per cell, with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, Silverspring, MD, USA). The immature dendritic cells that were in contact with the surface appeared as bright white-centered cells after proper adjustment of the microscope during recording. The number of surface-adherent immature dendritic cells was measured after 5 min of perfusion at a minimum of 25 randomized high-power fields. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval \((\delta t, \text{with a minimal interval of 80 milliseconds})\) was digitally captured. The position of every cell was detected in each frame, and for all subsequent frames the distance traveled by each cell and the number of images in which a cell appears in focus was measured. The cut-off value to distinguish between rolling and static adherent cells was set at 1 \(\mu\)m/s. With this method, static adherent, rolling and free flowing cells (which were not in focus) could be clearly distinguished.

**Results**

**ICAM-2 is the major DC-SIGN ligand on endothelial cells.** Precursor DCs continuously traffic to peripheral tissues. The migration process is highly dependent on the interaction of DC-SIGN with its ligand \(^1\). In earlier studies, ICAM-2 was identified as a ligand for DC-SIGN that supports binding under shear stress conditions using a chimeric construct produced in CHO cells (ICAM-2/Fc) \(^6\). To identify counter receptors for DC-SIGN on primary endothelial cells, chimeric DC-SIGN/Fc protein was used to immunoprecipitate ligands from a HUVEC cell lysate of which the surface proteins had been labeled by biotin. Subsequently, the immunoprecipitate and the supernatant were subjected to SDS-PAGE under reducing conditions, and the isolated proteins analyzed by western blot. As shown in Figures 1A and 1C, the major surface-labeled protein that was selectively precipitated by DC-SIGN-Fc has an apparent molecular weight of 55-60 KDa, which coincides with the molecular weight of ICAM-2 \(^31,32\). The selective precipitation of ICAM-2 could be confirmed after western blotting analysis using an anti-ICAM-2 antibody for immunodetection (Figure 1B).
**Figure 1. DC-SIGN binds to ICAM-2 in endothelial cells.** HUVECs were labeled with biotin prior to lysis. DC-SIGN ligands were immunoprecipitated in a HUVEC lysate by incubation with DC-SIGN/Fc and Prot-A/G agarose beads as described in Materials and Methods. A. 10 % SDS-PAGE. One major band of approximately 55-60 kDa was detected in the immunoprecipitated fraction. B. Western Blot, immunodetection with anti-ICAM-2 antibody (12A2). The main immunoprecipitated band corresponds to ICAM-2. C. Western Blot, immunodetection with Streptavidin. The immunoprecipitated band identified as ICAM-2, is present in the extracellular membrane of HUVECs. Results are representative of 3 experiments.

**DC-SIGN–ICAM-2 interaction is carbohydrate-dependent.** ICAM-2 is also a ligand for LFA-1 and this interaction is carbohydrate-independent. To investigate whether the DC-SIGN–ICAM-2 interaction is carbohydrate-dependent, CHO cells were transfected with a cDNA coding for ICAM-2, and either grown in the presence of kifunensin or co-transfected with a cDNA coding for FUT4. Kifunensin is an α-mannosidase I inhibitor that stops the processing of N-glycans at the Man$_9$GlcNAc$_2$-Asn stage. Cells grown in the presence of kifunensine produce mainly high-mannose type N-glycans. FUT4 is an α1,3-fucosyltransferase implicated in the synthesis of Le$^x$. Both Le$^x$ and high-mannose N-glycans are recognized by DC-SIGN.

As shown in Figure 2A, the DC-SIGN/Fc chimera does not bind to ICAM-2 expressed in untreated cells, whereas it binds with high affinity to ICAM-2 expressing CHO cells that were grown in the presence of kifunensine. Remarkably, DC-SIGN/Fc also recognized glycoproteins other than ICAM-2 on the CHO cells that were grown in the presence of kifunensine, as can be observed in the mock transfected CHO cells. In an ICAM-2–immobilizing ELISA (Figure 2B), we could show that the kifunensine treatment resulted in an ICAM-2 population that showed increased binding of the lectin Con A, as well as an increased DC-SIGN/Fc binding (Figure 2B).
The co-transfection with FUT4 also resulted in an increase in the binding of DC-SIGN/Fc, which correlated with an increase in the expression of Le^X-containing ICAM-2 (Figure 2A and B). Together, these data demonstrate that binding of DC-SIGN/Fc to ICAM-2 is strictly carbohydrate dependent, and that the glycosylation potential of the cells expressing ICAM-2 determines whether or not ICAM-2 can function as a counter receptor for DC-SIGN.

Figure 2. The DC-SIGN–ICAM-2 interaction is carbohydrate dependent. CHO cells were transfected with the plasmid pcDM8-ICAM-2 and either left untreated, treated with kifunensine or co-transfected with the plasmid pcDNA1-FUT4. Untreated or kifunensine-treated mock transfectant were used as controls. A. Flow cytometry analysis using an anti-ICAM-2 antibody (12A2) or the DC-SIGN/Fc chimeric protein (+ EDTA as a negative control). Gray lines denote the isotype control, while solid areas represent the staining with the above mentioned antibody or chimeric molecule. B. The binding of DC-SIGN/Fc, Con A and anti-Le^X to ICAM-2 captured from the CHO cells transfected with the plasmid pcDM8-ICAM-2 was analyzed by ELISA in plates coated with the antibody 12A2. Results are representative of 3 experiments.

ICAM-2 carries Le^Y in endothelial cells. In order to explore the glycosylation of ICAM-2 in endothelial cells, HUVECs were analyzed by flow cytometry, ELISA, and western blotting. Using antibodies specific for the Lewis antigens Le^X, Le^Y, Le^a,
and Le\(^b\), it was demonstrated that HUVEC expressed significant amounts of Le\(^Y\) (Figure 3A). Presence of the other Lewis antigens could not be detected on HUVECs, whereas all antibodies showed specific binding to control neoglycoconjugates expressing the respective Lewis antigens (data not shown). Con A was also able to bind to endothelial cells (Figure 3A). Con A recognizes unsubstituted hydroxyl groups in mannose, as those present in high-mannose structures, hybrid-type N-glycans and diantennary N-glycans (with this order of affinity)\(^ {36,37}\). Using increasing concentrations of methyl-mannoside it is possible to discriminate whether Con A binds to the high affinity ligand (high-mannose glycans) or to the low affinity ligands (diantennary N-glycans). A priori, as indicated by previous in vitro studies\(^ {11,13}\), both Le\(^Y\) and mannose-rich structures are potential ligands for DC-SIGN.

![Figure 3](image)

**Figure 3. Endothelial cells express Le\(^Y\) and Con A-reactive epitopes.** A. HUVEC were grown to confluency, mechanically detached, incubated with the corresponding lectin or antibody, and analyzed by flow cytometry. Gray lines denote the isotype control, while solid areas represent the staining with the above mentioned lectin or antibody. B. Alternatively, HUVEC were lysed and the binding of DC-SIGN/Fc (+ EDTA as a negative control), anti-Le\(^Y\), Con A, and Con A + 50 mM methylmannoside to captured ICAM-2 was analyzed by ELISA in plates coated with anti-ICAM-2 (12A2). Results are representative of 3 experiments.
To identify the carbohydrates present in ICAM-2, immobilized ICAM-2 from an endothelial cell extract was tested for binding with anti-Le^Y antibodies and Con A in ELISA. The data in Fig. 3B show that both anti-Le^Y antibodies and Con A bound to the captured ICAM-2. Binding of Con A, however, could be completely abolished by addition of low concentrations of methyl-mannoside (Figure 3B), indicating that the low affinity Con A-reactive glycans on ICAM-2 most likely correspond to diantennary N-glycans, rather than high-mannose type N-glycans. This indicates that Le^Y most likely represents the ligand for DC-SIGN on ICAM-2.

**HUVEC express FUT1 and FUT4 as the main α2- and α3-fucosyltransferases, respectively.** The expression of fucosylated carbohydrates depends upon the expression of fucosyltransferases. To identify the fucosyltransferases that are involved in the synthesis of Le^Y structures in HUVEC, a highly sensitive and specific real-time PCR assay was designed. Special care was taken to design oligonucleotides able to discriminate FUT3, FUT5 and FUT6, which have a large sequence identity. Plasmids encoding for FUT3, FUT5 and FUT6 were used as positive control (data not shown). The results showed significant mRNA levels for only three fucosyltransferase genes in HUVEC. Amongst them, FUT4, which encodes an α3-fucosyltransferase involved in the synthesis of Lewis-type structures, as well as the VIM-2 antigen, and FUT1 that encodes an α2-fucosyltransferase, can contribute to the biosynthesis of Le^Y. The third fucosyltransferase, FUT8, encodes for an α6-fucosyltransferase that catalyzes the transfer of fucose to the first N-acetylglucosamine of the chitobiose core of an N-glycan.

**DC-SIGN interacts with the Le^Y structure on ICAM-2 expressed by endothelial cells.** Based on the fucosyltransferase gene expression profile, FUT1 was the a priori candidate to direct the synthesis of the DC-SIGN ligand Le^Y in HUVEC. To further investigate this point, a silencing approach was followed to knock down the expression of the enzymes that are expected to be crucial for the synthesis of Le^Y. HUVECs were transfected with either pSUPER/Scrambled (non-silencing control), pSUPER/FX or pSUPER/FUT-1. The plasmid pSUPER/FX targets the expression of the gene FX, which encodes GDP-4-keto-6-deoxymannose 3,5-epimerase-4-reductase, one of the enzymes necessary for the synthesis GDP-fucose from GDP-mannose. This pathway accounts for the vast majority of cellular GDP-fucose production. GDP-fucose is the sugar donor used in the reactions catalyzed by fucosyltransferases. In the absence of this enzyme, the pool of GDP-Fucose can be rescued by adding fucose to the culture media, which is then metabolized to GDP-Fucose via the salvage pathway. The efficiency of the silencing was evaluated by flow cytometric analysis of the transfected HUVEC using...
an anti-Le\(^\text{Y}\) antibody (data not shown). As shown in Figure 5, the rolling velocity of immature DCs on a cell-layer of primary HUVEC is increased when either FX or FUT1 were silenced. Simultaneously, the tethering and adhesion (B) of the immature DCs to HUVEC is decreased. The degree of increase in rolling velocity and decrease in tethering and adhesion was to the same extent as was achieved using the monoclonal antibody AZN-D1, which is a DC-SIGN blocking antibody. Furthermore, the effects obtained with pSUPER/FX could be rescued by adding fucose to the HUVECs.

![Figure 4. Fucosyltransferase gene expression profile.](image)

HUVEC were grown to confluency and assayed for the expression of a panel of fucosyltransferases (FUT1-9) using real-time PCR. GAPDH was used as an endogenous reference. Results are shown as the average ± SE of 5 experiments.

**Discussion**

Immature DCs use DC-SIGN as an adhesion molecule to recognize a counter-receptor in endothelial cells. As a result of this interaction, immature DCs tether and adhere to endothelial cells, starting the migration to the underlying tissue. In this study we have shown that ICAM-2 expressed in endothelial cells constitutes the major scaffold protein ligand for DC-SIGN. Furthermore, we have demonstrated that Le\(^\text{Y}\) present on ICAM-2 acts as the major carbohydrate ligand that mediates DC-SIGN adhesion and tethering to HUVEC.

ICAM-2 was identified as the major endothelial ligand for DC-SIGN employing in a DC-SIGN/Fc immune precipitation from HUVEC lysates (Figure 1). Although the main visible band is situated around 55-60 KDa (Figure 1A), coinciding with ICAM-2 as
detected by Western-Blot (Figure 1B), two other minor bands (molecular weight > 100 KDa) are visible that are surface located (Figure 1C), indicating that other proteins expressed by HUVEC may contribute to the binding. It was also demonstrated that the ICAM-2–DC-SIGN interaction is carbohydrate-dependent, as ICAM-2 expressed in CHO cells is only able to be recognized by DC-SIGN when properly glycosylated, excluding an integrin-like interaction (Figure 2). This system illustrates a complex model in which both interaction partners perform dual functions, DC-SIGN as a pattern-recognition receptor and an adhesion molecule, and ICAM-2 as an integrin ligand and a scaffold molecule for a lectin-ligand.

In this study, we have identified Le\(^{Y}\) as the major carbohydrate ligand for DC-SIGN on endothelial cell-expressed ICAM-2, as is suggested by the flow cytometry analysis of endothelial cells, and further demonstrated by an ICAM-2 specific capture ELISA (Figure 3), and the FUT-1 silencing experiments (Figure 5). Recently, it was published that ICAM-2 presents high-mannose N-glycans, which serve as carbohydrate ligands for DC-SIGN \(^{45}\).

![Figure 5](image)

**Figure 5.** The binding of DC-SIGN to ICAM-2 in endothelial cells is blocked by silencing the expression of Le\(^{Y}\) using RNA interference. The expression of FX and FUT-1 was silenced in HUVEC. The rolling velocity (A) and adhesion (B) of monocyte-derived DCs over the transfected HUVEC was measured as indicated in Materials and Methods. Results are shown as the average ± SE of 3 experiments.

However, the work of Jiménez et al. has a serious technical disadvantage, the conclusions are based on ICAM-2 produced in large amounts in COS cells. It is very well known that glycosylation is a species and cell-type specific property \(^{46-48}\). This
has also been evidenced in this study (Figure 2). Additionally, our rolling/adhesion assays demonstrate that fucosylation is essential for the rolling and adhesion of monocyte-derived DCs (Figure 5). Additionally, the silencing of FUT-1 results in a reduction in rolling and adhesion analogue to the inhibition obtained with the anti-DC-SIGN antibody AZN-D1 (Figure 5). In our opinion, this unequivocally proves the identity of Le\(^\gamma\) as the carbohydrate ligand of DC-SIGN in HUVEC.

Interestingly, Le\(^\gamma\) is expressed by many endothelial cell glycoproteins (data not shown), however only ICAM-2, and perhaps other high-molecular weight minor ligands, can support the binding of DC-SIGN. This may be explained by spatial considerations, since there is no evidence so far in other post-translational modifications being necessary for the DC-SIGN-carbohydrate ligand interaction \(^{11,12,15}\), as is the case for P-Selectin \(^{49}\). The discovery of FUT-1 as a key enzyme in the synthesis of the endothelial DC-SIGN-ligand opens new possibilities in the manipulation of DC migration.

**Acknowledgements**

We thank Dr J. B. Lowe for the kind gift of the plasmids pCDM7-FUT3, pcDNA1-FUT5, and pcDNA1-FUT6, and Dr. R. Agami for the plasmid pSUPER.retro.puro. We also thank K. van Gisbergen for developing the ELISA assay, Dr. R. Beelen for his help in providing umbilical cords, and Dr. G. Kraal for helpful discussions and critical reading of the manuscript.
Chapter 6. DC-SIGN binds to Lewis\textsuperscript{y} present on ICAM-2

References

7. Leteux C, Chai W, Loveless RW, Yuen CT, Uhlin-Hansen L, Combaroune Y et al. The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and sulfated oligosaccharides of blood group Lewis(a) and Lewis(x) types in addition to the sulfated N-glycans of lutropin. *J.Exp.Med.* 2000; 191 (7): 1117-26.


