

The C Type Lectins DC-SIGN and L-SIGN

Receptors for Viral Glycoproteins

Pierre-Yves Lozach, Laura Burleigh, Isabelle Staropoli, and Ali Amara

Summary

DC-SIGN and L-SIGN are C-type lectins that recognize carbohydrate structures present on viral glycoproteins and function as attachment factors for several enveloped viruses. DC-SIGN and L-SIGN enhance viral entry and facilitate infection of cells that express the cognate entry receptor (*cis*-infection). They are also able to capture viruses and transfer viral infections to other target cells (*trans*-infection). In this chapter, we will give an overview of protocols used to produce soluble viral glycoproteins at high levels and to study the molecular basis of viruses/DC-SIGN and L-SIGN binding and internalization. We will also describe techniques to investigate the molecular mechanisms by which DC-SIGN or L-SIGN spread viral infections.

Key Words: DC-SIGN; L-SIGN; dendritic cells; endothelial cells; viruses; envelope glycoproteins; endocytosis; viral entry; infection; viral transmission.

1. Introduction

DC-SIGN (CD209) and its homolog L-SIGN (also called DC-SIGN-R, CD209L) belong to the C-type (calcium-dependent) lectin family. This large group of proteins which includes the mannose receptor, DEC-205 or langerin, is specialized in the recognition of carbohydrate structures present on cellular and viral proteins and is implicated in several processes such as cell adhesion and antigen presentation (1,2). DC-SIGN and L-SIGN are constitutively expressed by specific cell populations that play a key role in the activation of the innate and adaptive immune responses. DC-SIGN is highly expressed at the surface of dendritic cells (DCs) localized in the lymphoid tissues (thymus, tonsils, or lymph nodes), mucosal surface, and in the dermis (3–5). DC-SIGN is not expressed by Langerhans cells, which are a unique DC subset

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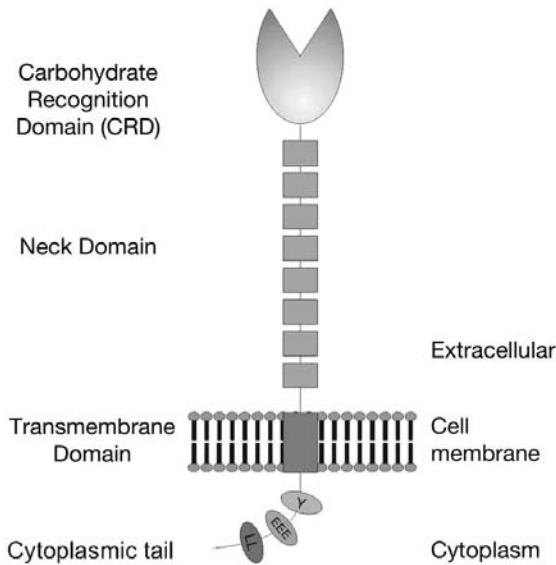


Fig. 1. Structure of DC-SIGN and L-SIGN proteins. The C-type lectins DC-SIGN and L-SIGN are type II transmembrane proteins. Their cytoplasmic tails contain internalization signals (di-leucine, tyrosine, and tri-acidic) which are involved in internalization of the lectin. The extracellular domain is composed of a carbohydrate recognition domain (CRD) and a neck domain (conserved in the case of DC-SIGN, variable for L-SIGN) implicated in the oligomerization of these lectins. The oligomerization is probably important for the orientation and subsequently for the function of the CRDs.

residing in epidermis. Certain macrophages such as Hofbauer cells in the placenta, Kupffer cells in the liver sinusoids, and alveolar macrophages have been shown to express DC-SIGN (3,4). L-SIGN expression is restricted to endothelial cells such as those in lymph nodes, placenta, and, particularly, liver sinusoidal endothelial cells (LSECs) (6,7).

DC-SIGN and L-SIGN share nearly 77% amino acid identity and are closely related in global architecture. Both lectins are type II transmembrane proteins composed of a short cytoplasmic tail responsible for signalling and internalization, a transmembrane region, a neck domain consisting of eight repeat regions of 23 amino acids and a carbohydrate recognition domain (CRD) which binds carbohydrate ligands in a calcium-dependent manner (1,3,4) (Fig. 1). The DC-SIGN CRD recognizes different mannose or fucose-based carbohydrates whereas the L-SIGN CRD appears to interact only with mannose resi-

Table 1
DC-SIGN and L-SIGN Are Receptors for a Broad Range of Viruses

	Virus family	Virus	Mechanisms	Viral envelope protein(s)*
DNA	<i>Herpesviridae</i>	CMV	<i>cis, trans, trans-enh.</i>	gB
RNA	<i>Coronaviridae</i>	SARS	<i>cis ?, trans</i>	Spike
	<i>Filoviridae</i>	Ebola	<i>cis ?, trans</i>	GP
		Marburg	<i>cis ?</i>	GP
	<i>Flaviviridae</i>	Dengue	<i>cis</i>	E
		HCV	<i>trans</i>	E1/E2
	<i>Retroviridae</i>	HIV-1/-2	<i>cis, trans, trans-enh.</i>	gp120
		SIV	<i>cis, trans, trans-enh.</i>	gp120
<i>Togaviridae</i>	Sindbis	<i>cis ?</i>	E1 or E2 (?)	

* Viral envelope protein(s) bound by DC-SIGN or L-SIGN. *cis, cis*-infection; CMV, cytomegalovirus. HCV, hepatitis C virus; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome virus; SIV, simian immunodeficiency virus; *trans, trans*-infection; *trans-enh, trans*-enhancement.

dues on *N*-glycans (Man5GlucNac2 to Man9GlucNac2) (8,9). The repeat regions within the neck domain permit the oligomerization of the lectins which is critical for their biological activities, because only tetramers efficiently capture glycosylated ligands (10–12).

DC-SIGN was originally cloned for its ability to bind and internalize the heavily glycosylated human immunodeficiency virus (HIV) gp120 protein (13). DC-SIGN strongly binds all HIV and simian immunodeficiency virus (SIV) strains examined to date and plays an important role in virus adhesion to DC (14). These studies have paved the way for further investigations into interactions between DC-SIGN and pathogens and it has become clear that many viruses target DC-SIGN and L-SIGN to bind DCs and endothelial cells, respectively. Both lectins recognize high mannose oligosaccharides present on viral glycoproteins and thus function as attachment factors for several viruses including cytomegalovirus (CMV), dengue (DV), Ebola, severe acute respiratory syndrome (SARS), hepatitis C (HCV), Marburg and Sindbis viruses (Table 1) (10, 15–27). Differential glycosylation of viral envelope glycoproteins strongly influences the efficiency of viral capture by DC-SIGN and L-SIGN (10, 19, 22). For example, the DV envelope glycoprotein E has two conserved *N*-linked glycosylation sites at Asn-67 and Asn-153 that mediate DV binding to DCs (22).

Only mannosylated E glycoproteins (which are exposed at the surface of DV virions transmitted to humans by infected mosquitoes), and not E proteins with complex glycosylation (produced in mammalian cells), have been shown to interact with DC-SIGN-expressing cells (22).

DC-SIGN and L-SIGN are endocytic receptors and their cytoplasmic tails carry putative internalisation signals such as a dileucine (LL) motif (which is present in both DC-SIGN and L-SIGN) and a tri-acidic cluster that is believed to be involved in intracellular trafficking (**Fig. 1**) (3). Despite findings showing that a large fraction of viral particles captured by DC-SIGN are rapidly internalized and degraded (28,29), viruses are nevertheless able to hijack DC-SIGN and L-SIGN functions to spread infection. For the viruses examined to date, DC-SIGN and L-SIGN have been shown to act as attachment factors rather than authentic entry receptors involved in membrane fusion. This does not exclude the possibility that some viruses, and particularly those that require targeting to acidified endosomes for membrane fusion, use these lectins as primary entry receptors. DC-SIGN and L-SIGN have also been shown to function as “*cis*-receptors” that enhance infection of target cells. This mechanism, known as *cis*-infection, has been described for DV, CMV, and HIV, and probably relies on the capacity of these lectins to concentrate viral particles at the cell surface, allowing optimal interaction with their cognate receptors and enhanced viral entry (**Table 1**) (18,22,30). Viruses captured by DC-SIGN or L-SIGN can also be transmitted *in trans* to target cells expressing the entry receptors (“*trans*-infection”), as has been proposed for HIV, HCV, and SARS virus (14,16,26,31). For HIV and CMV, DC-SIGN enhances infection of target cells at a low multiplicity of infection (MOI). DC-SIGN-bound viruses infect target cells more efficiently than free viruses and remain infectious for several days (14,18). The molecular mechanisms underlying these processes remain poorly understood.

The contribution of DC-SIGN and L-SIGN to viral transmission and dissemination *in vivo* is currently unknown. Their role as principal attachment factors for a broad range of enveloped viruses and their restricted expression in anatomical site of virus exposure suggest that these two lectins dictate viral tropism for DCs and endothelial cells and consequently may influence viral pathogenesis. DCs are sentinel cells that capture pathogens entering skin or mucosal tissues and then migrate to the lymph nodes where they present processed antigens to T-cells, initiating adaptive immune responses. By interacting with DC-SIGN, viruses that are transmitted sexually (such as HIV) or through introduction into human skin by an insect vector (such as dengue virus or Sindbis virus) may hijack DC function either to modulate the immune response or to assure their dissemination from peripheral tissues to lymphoid organs (32). LSECs also participate in the capture and processing of foreign antigens, in

addition to the elimination of undesirable macromolecules from the blood by transporting them to hepatocytes (6,7). LSECs represent a barrier separating the liver and the blood and could be exploited by viruses such as HCV in order to gain access to hepatocytes (16,33,34). We recommend the two following reviews for an overview of the physiological importance of LSECs in viral infections of the liver (6,7).

In this chapter, we will provide general protocols to study the molecular interactions between viruses and DC-SIGN or L-SIGN and to investigate the functions of these two lectins in viral infection and transmission. We will first describe the methods used to obtain human dermal-like DCs and cells expressing DC-SIGN and L-SIGN. We next present protocols to produce soluble viral envelope proteins using a Semliki forest virus (SFV) vector (35–37) and to study the molecular basis of viral capture and internalization by DC-SIGN or L-SIGN. Finally, we will describe methods required to assess the contribution of DC-SIGN and L-SIGN to *cis*- and *trans*-infection.

2. Materials

2.1. Cell Culture and Antibodies

1. All of the products used for cell culture are purchased from Invitrogen (RPMI 1640, Glasgow's modified Eagle's medium [GMEM], fetal calf serum [FCS], penicillin/streptomycin, HEPES, and tryptose phosphate broth) except cysteine/methionine-free Dulbecco's modified Eagle's medium (DMEM) (ICN Bio-medicals).
2. Phycoerythrin (PE)-conjugated mouse monoclonal antibodies (mAbs) directed against DC-SIGN (FAB161P), L-SIGN (FAB162P), or both lectins (FAB1621P) were purchased from R&D Systems. The anti-DC-SIGN mAb clone 1B10 (IgG2a, κ) has been developed in our laboratory and previously described (18). The anti-DC-SIGN mAb1B10 is directed against the CRD and inhibits DC-SIGN activity (18). The mAb1621 directed against both lectins is purchased from R&D Systems. The mAb1621 blocks the activity of both lectins. Differentiation of human DCs is assessed by fluorescence-activated cell sorting (FACS) analysis using fluorescein isothiocyanate (FITC)-conjugated mouse mAb anti-CD14 (M ϕ P9) and anti-CD1a (HI149) purchased from BD Biosciences.
3. HeLa and 293T cells are maintained in DMEM supplemented with 10% FCS and antibiotics (100 μ g/mL⁻¹ streptomycin and 100 U/mL⁻¹ penicillin). Raji cells are grown in RPMI containing 10% FCS and antibiotics. BHK is cultured in GMEM with 5% FCS, 1% penicillin/streptomycin, 20 mM Hepes, and 10% tryptose phosphate broth.
4. Cell lines expressing DC-SIGN or L-SIGN are generated by transduction with the retroviral TRIP Δ U3 vector (a gift from Pierre Charneau, Pasteur Institute, France) encoding either DC-SIGN or L-SIGN (16). Transduced cells are stained with PE-conjugated anti-DC-SIGN mAb and sorted for high expression level of DC-SIGN or L-SIGN.

2.2. Generation of Monocyte-Derived Dendritic Cells (see Note 1)

1. Human peripheral blood mononuclear cells (PBMC) are isolated from healthy donors by density gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences).
2. Lysis buffer: dissolve 8.3 g of NH_4Cl and 1 g of NaHCO_3 in 1 L of water complemented with 1 mL of EDTA (100 mM, pH ~8.0). Lysis buffer must be autoclaved and can be stored at 4°C for several months.
3. MACS buffer: phosphate-buffered saline (PBS) containing 2 mM EDTA (pH ~8.0) and 0.5% bovine serum albumin (BSA; Sigma Aldrich). This buffer can be kept at 4°C for several months.
4. Filters (cell strainer, 40 μm) used to eliminate cell aggregates are purchased from Falcon.
5. Monocytes are negatively selected using FCR blocking and biotin antibodies (Ab) cocktail and anti-biotin magnetic beads (Miltenyi Biotec).
6. Recombinant human interleukin (IL)-4 and recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) are purchased from PeproTech and Gentaur respectively.

2.3. Recombinant Protein Expression

1. Soluble viral glycoproteins are produced using the SFV vector in BHK mammalian cells. SFV shuts off the cellular translation machinery and so transduced cells produce only the viral protein of interest, which is secreted and accumulates in the supernatant of infected cells. This allows the production of large amounts of soluble proteins with a high degree of purity. The SFV expression vector was originally described in reviews (35–37). Briefly, the SFV genome is a single-stranded, positive RNA which encodes both structural and nonstructural viral proteins. A signal sequence in the 5' RNA domain permits specific viral genome packaging. The SFV vector is made of two cDNAs (pSFV- Δenv and pSFV-helper2) (Fig. 2). The first codes for the SFV RNA genome in which the sequence corresponding to structural proteins is replaced by the soluble glycoprotein of interest (pSFV- Δenv). The structural proteins are encoded by a second cDNA (pSFV-helper2) which lacks the packaging signal. cDNAs are transcribed in vitro and transfected into cells by electroporation. In this way, only RNAs encoding the protein of interest are packaged into new defective viral particles. The SFV envelope proteins are activated by the furin cleavage in the Golgi apparatus. Replacement of the furin site by a chymotrypsin site allows control of SFV particle activation.
2. pSFV-helper2 and pSFV- Δenv have been previously described (35–37). Polymerase chain reaction (PCR) fragments coding for soluble glycoprotein of interest are usually digested either by BssH II (5') and Nsi I (3'), by BssH II (5') and Apa I (3') or by BamH I (5' and 3') and then introduced into pSFV- Δenv (10,22,38).
3. Restriction enzymes Spe I and Sph I are purchased from New England Biolabs. The DNA purification kit (QIAquick PCR purification kit) is purchased from QIAGEN.

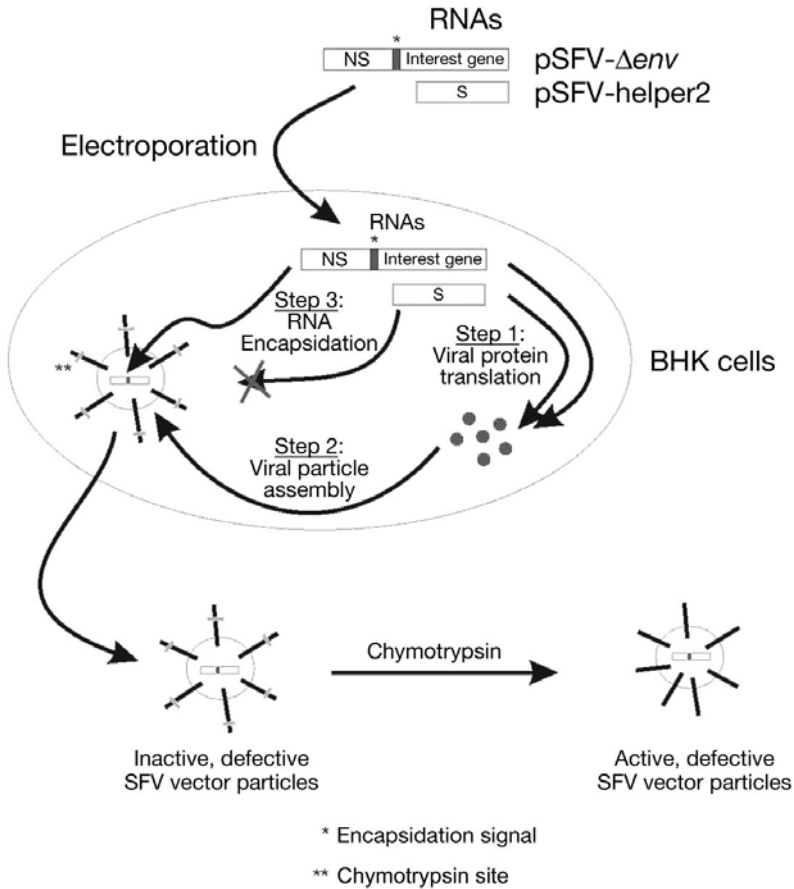


Fig. 2. Semliki forest virus (SFV) expression vector. The SFV vector is composed of two RNAs which are electroporated into BHK cells. New synthesized particles incorporate only the RNA coding for nonstructural proteins and the protein of interest (NS, nonstructural and S, structural) because it is the only one with an encapsidation signal. The furin site of the SFV envelope protein is replaced by a chymotrypsin site so the particles can be activated by chymotrypsin digestion.

4. SP6 RNA polymerase and RNasin are purchased from Roche Applied Science and Promega, respectively.
5. 0.4-cm electroporation cuvettes are purchased from Eurogentec.
6. TNE buffer: 12 mM TrisHCl, 2 M NaCl, and 0.2 M EDTA (pH ~8.0) are prepared in distilled water and can be stored at 4°C for several months.
7. Chymotrypsin and aprotinin are purchased from Sigma Aldrich.

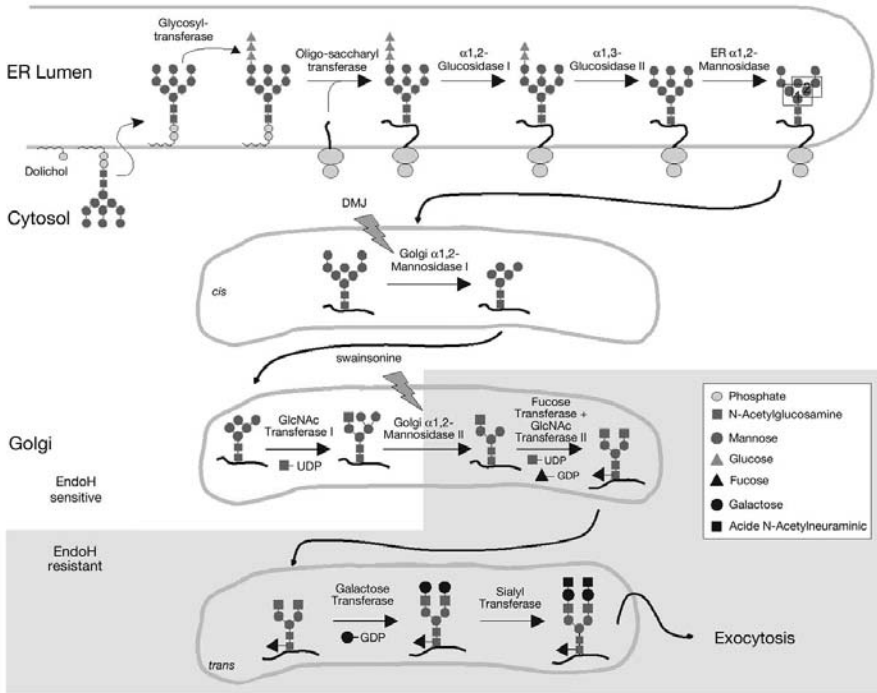


Fig. 3. Carbohydrate maturation in mammalian cells. Proteins with NXS or NXT sites that pass through the endoplasmic reticulum can be potentially glycosylated. Glycoproteins are sensitive to EndoH until they are modified by α 1,2-mannosidase II. Swainsonine and 1-deoxymannojirimycin hydrochloride (DMJ) block maturation steps of glycoprotein carbohydrates. These drugs permit to produce mannosylated glycoproteins in mammalian cells. 1, internal tri-mannose branch recognized by DC-SIGN; 2, external tri-mannose branch; ER, endoplasmic reticulum; GDP, guanosine biphosphate; UDP, uridine biphosphate.

8. Peroxidase-conjugated mAb and diaminobenzidine (DAB) solution kit are obtained from Vector Laboratories (ABCYS Biologie). DAB solution is prepared by adding four drops of DAB solution, two drops of H_2O_2 solution, two drops of nickel solution, and two drops of buffer solution to 5 mL of water.
9. [^{35}S] cysteine and methionine (Pro-Mix ^{35}S) are obtained from Amersham Biosciences.
10. 1-deoxymannojirimycin hydrochloride (DMJ) and swainsonine are purchased from Calbiochem and Sigma respectively. These molecules specifically inhibit α -mannosidase I and II and permit the production of proteins with mannose carbohydrate residues in mammalian cells (**Fig. 3**).

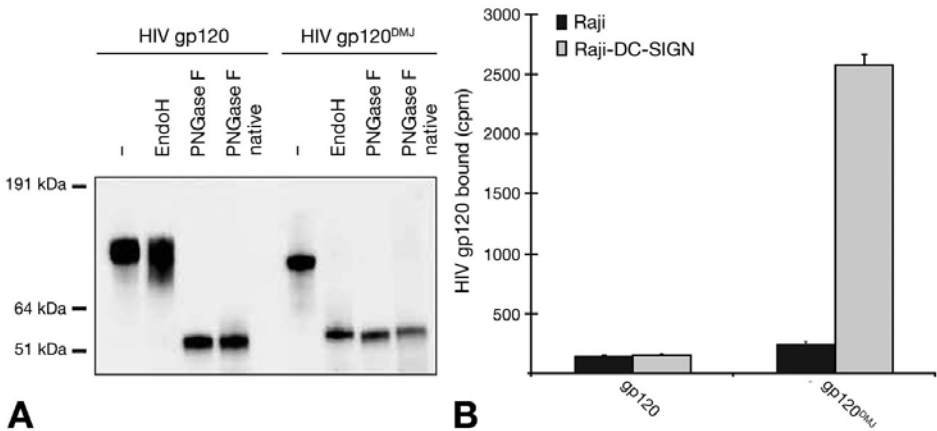


Fig. 4. Production of HIV gp120^{DMJ} and binding to DC-SIGN. **(A)** Soluble HIV gp120 is produced in BHK cells in the presence or absence of mannosidase inhibitors (1 mM DMJ and 5 μ M swainsonine) (HIV gp120^{DMJ} or HIV gp120, respectively) as described under **Subheading 3.2.3**. Secreted proteins were subjected to digestion with EndoH or PNGase F and analyzed by Western blot. Only HIV gp120^{DMJ} is sensitive to EndoH confirming its high mannosylated glycosylation. **(B)** ³⁵S-labeled HIV gp120^{DMJ} and HIV gp120 (20 nM) are bound to Raji and Raji-DC-SIGN cells for 2 h at 4°C. Cells are washed three times before measuring cell-associated radioactivity. Note that only HIV gp120^{DMJ}, which carries only mannosylated *N*-glycans, binds to DC-SIGN.

- Glycoproteins are concentrated through columns (Biomax, Millipore) with an appropriate molecular weight cut-off for the particular protein being produced.
- Endoglycosidase H (EndoH) and peptide: *N*-glycosidase F (PNGase F) are purchased from Roche Applied Science and New England Biolabs respectively. The buffer provided with the PNGase F is used to treat the glycoproteins either with EndoH or with PNGase F. PNGase F is able to digest all *N*-glycans whereas EndoH digests only *N*-glycans unmodified by α -mannosidase I and II (**Figs. 3 and 4A**).

2.4. Binding and Internalization Assays

- Buffer A: PBS containing 1% BSA, 0.2% γ -globulin, 0.1% sodium azide, 1 mM CaCl₂, and 2 mM MgCl₂. Buffer A can be stored at 4°C for several months.
- Buffer B: serum-free RPMI containing 1 mM CaCl₂ and 2 mM MgCl₂. Buffer B can be stored at 4°C for few months.
- Mannan, EDTA (pH ~8.0) and EGTA (pH ~8.0) are all purchased from Sigma Aldrich. Mannan stock solution is dissolved in water (5 mg/mL⁻¹) and can be stored at 4°C for several months.
- Scintillation solution is obtained from Wallac (optiphase supermix solution).

2.5. Viruses

Wild-type viruses or viral particles carrying the reporter genes firefly luciferase (Luc) or green fluorescent protein (GFP) can both be used to study DC-SIGN and L-SIGN-mediated *cis*-infection and *trans*-enhancement of target cell infection. These viruses can be prepared using standard methods, and an example is described in **Note 7**.

3. Methods

3.1. Generation of Dermal-Like DCs Expressing DC-SIGN

3.1.1. Isolation of PBMCs From Blood

1. Fresh blood (450 mL) is completed to 600 mL with PBS.
2. Prepare 20 50-mL tubes containing 15 mL of Ficoll. Slowly add 30 mL of blood into each tube, taking care to avoid mixing, then centrifuge for 20 min at 850g without brake at room temperature.
3. Aspirate the plasma and transfer the PBMCs (white ring) into new tubes containing 20 mL of PBS (two rings per tube). Complete each tube to 50 mL with PBS, and then centrifuge for 10 min at 300g at room temperature. Repeat this washing step twice, each time pooling two tubes.
4. Remove the supernatant and resuspend the pellet in 5 mL of lysis buffer for 4 min. Add 20 mL of PBS 2% FCS and centrifuge at 300g for 5 min at 4°C. Resuspend the pellet in 5 mL of cold MACS buffer.

3.1.2. Monocyte Isolation and Differentiation

1. Isolated PBMCs are passed through a filter placed inside a 50-mL tube (to eliminate cell aggregates) and the filter is rinsed twice with 5 mL of MACS buffer. Centrifuge at 300g for 5 min at 4°C.
2. Resuspend the cells at up to 10^7 cells per 30 μ L of MACS buffer and add 10 μ L each of FCR blocking reagent and biotin antibody cocktail per 10^7 cells. Incubate for 10 min at 4°C.
3. Add 30 μ L of MACS buffer and 20 μ L of anti-biotin microbeads per 10^7 cells and incubate for 15 min at 4°C.
4. Complete to 50 mL with MACS buffer and centrifuge 10 min at 300g, 4°C.
5. Resuspend the cells at 10^8 cells per 500 μ L of MACS buffer. Wash the magnetized column with 3 mL of MACS buffer, then pass cells through the column.
6. Rinse the column three times with 3 mL of MACS buffer and collect the eluate containing monocytes. Cells are cultured at 10^6 cells/mL⁻¹ in RPMI with 10% FCS, 1% penicillin/streptomycin, 50 ng/mL⁻¹ IL-4, and 100 ng/mL⁻¹ GM-CSF for 6 d. GM-CSF and IL-4 are added every 2 d. Differentiation of monocyte-derived DCs is assessed by FACS analysis (CD14 negative and CD1a- and DC-SIGN-positive).

3.2. Expression of Soluble Viral Glycoproteins

3.2.1. Production of Recombinant

Semliki Forest Virus Particles (see **Note 2**)

1. Digest 2 μg of pSFV-helper2 and pSFV- Δenv with Spe I or Sph I for 2 h at 37°C. Linearized DNAs are purified with a QIAquick PCR purification kit and are then transcribed in vitro for 1 h at 37°C using SP6 RNA polymerase (20 UI) in buffer containing RNasin and cap analog. After 1 h of incubation, 1 μL of SP6 RNA polymerase is added to the mixture and incubated for 30 min at 37°C (see **Note 3**).
2. pSFV-helper2 RNA is mixed with equal quantities of pSFV- Δenv RNA (usually 20 μL of each), added to 8×10^6 BHK cells in 800 μL of PBS (without calcium and magnesium) and immediately transferred to a 0.4-cm electroporation cuvet.
3. The RNA-cell mixture is subjected to two 0.4-ms pulses at 830 V and 25 μfarads in a Bio-Rad gene pulser and plated in 75 cm^2 flasks in 15 mL of GMEM medium containing 5% FCS.
4. Supernatants containing recombinant defective SFV particles are harvested 24 h later and cleared by centrifugation (850g, 10 min, room temperature). The supernatant is then concentrated by ultracentrifugation (100,000g, 4 °C, 1 h 45 min).
5. The pellet is covered with 200 μL of TNE buffer and incubated at 4°C for 1 h in a sealed tube to resuspend. The virus is stored in 20- μL aliquots at -80°C.

3.2.2. Titration of Recombinant Defective SFV Particles

1. Before infection, virus aliquots are activated by a chymotrypsin treatment (0.5 mg/mL^{-1} chymotrypsin, 1 mM CaCl_2) for 30 min at room temperature. To inhibit chymotrypsin activity, aprotinin (0.5 mg/mL^{-1}) is added to activated aliquots and incubated at 4°C for 10 min.
2. For infection, BHK cells (5×10^5 cells seeded in six-well plates 24 h before infection) are washed with serum-free GMEM and incubated with dilutions of viral particles in GMEM complemented with 2% FCS (500 μL of dilutions 10^{-2} to 10^{-7}) at 37°C. One hour later, 2 mL of GMEM complemented with 5% FCS are added.
3. At 7 h postinfection, cells are washed with serum-free GMEM medium and fixed with cold methanol for 5 min at -20°C follow by three washes with PBS.
4. Cells are incubated with 500 μL of primary antibody (directed against the soluble glycoprotein of interest) diluted in PBS for 1 h at room temperature or overnight at 4°C. Cells are washed twice with PBS prior to incubation with the secondary peroxidase-conjugated antibody (1/200 in 700 μL PBS per well) for 30 min at room temperature. Cells are then washed three times in PBS and incubated in DAB solution for 2 to 10 min and then rinsed three times in PBS. Stained cells are counted with a micrometric objective to determine the virus titer.

3.2.3. Production of Soluble Glycoproteins

1. Defective SFV particles are activated and cells are infected as described under **Subheading 3.2.2.** (see **Note 4**).

2. At 6 h postinfection, cells are washed five times in serum-free GMEM to eliminate BSA and then maintained in serum-free GMEM for protein production. For the production of radiolabeled protein, cells are starved for 1 h in serum- and methionine/cysteine-free DMEM prior to addition of $100 \mu\text{Ci/mL}^{-1}$ [^{35}S] cysteine and methionine.
3. Synthesis of proteins is continued up to 24 h postinfection in the presence or absence of α -mannosidase I and II inhibitors DMJ (1 mM) and swainsonine (5 μM). This results in the production of soluble glycoproteins carrying only mannose *N*-glycans (Figs. 3 and 4). Supernatants are clarified by centrifugation (10 min, 850 g at room temperature) and concentrated (see Subheading 2.3., item 11).
4. The glycosylation pattern of the proteins produced can be verified by treatment with endoglycosidase H (endoH; 2 mU, Roche) or Peptide: *N*-Glycosidase F (PNGase F; 1000 U, Biolabs). An example is given in Fig. 4A. Only HIV gp120 bearing mannose *N*-glycans (HIV gp120^{DMJ} produced in presence of DMJ and swainsonine) is sensitive to EndoH. In contrast, PNGaseF treatment allows complete de-glycosylation of HIV gp120 produced either in the presence or absence of inhibitors. The molecular weight of the exclusively mannosylated HIV gp120 is inferior to that of gp120 produced in the absence of mannosidase inhibitors. This is a result of the lower molecular weight of mannose residues compared to complex glycosylation.

3.3. Soluble Glycoprotein Binding to DC-SIGN or L-SIGN and Internalization Assays

1. Binding assays are performed in 96-well plates using 5×10^5 DC-SIGN- or L-SIGN-expressing cells in 100 μL of buffer A.
2. Cells are pelleted by centrifugation (300g, 4°C, 5 min) and buffer A is replaced by ^{35}S -labeled viral glycoprotein at desired concentrations in 100 μL of buffer A.
3. Binding was carried out for 2 h at 4°C with gentle agitation. Unbound radioactivity is removed by three washes with 200 μL of buffer A and cell pellets are resuspended in 25 μL of buffer A prior to addition of 175 μL of scintillate solution. Cell-associated radioactivity is counted in a 1450 Microbeta Trilux β counter (Wallac).
4. To assess the specificity of interactions between viral glycoproteins and DC-SIGN or L-SIGN, cells are preincubated for 30 min at 4°C with mannan (a CRD competitor), the neutralizing anti-DC-SIGN mAb (1B10) or anti L-SIGN mAb (mAb1621) (each at 20 $\mu\text{g/mL}^{-1}$), or the calcium chelator EDTA (5 mM) diluted in buffer A for before addition of labelled envelope proteins.
5. For internalization assays, ^{35}S -labeled proteins are bound to parental or DC-SIGN- or L-SIGN-expressing cells as described above except that the buffer A is replaced by the buffer B. Cells are washed three times and incubated for 30 min either at 4°C in 100 μL of cold buffer B or at 37°C in 100 μL of preheated buffer B to initiate endocytosis. To quantify glycoprotein internalization, cells maintained either at 4°C or 37 °C are treated with 200 μL RMPI containing 20 mM EDTA or mock treated in order to remove viral proteins bound to the lectin at the

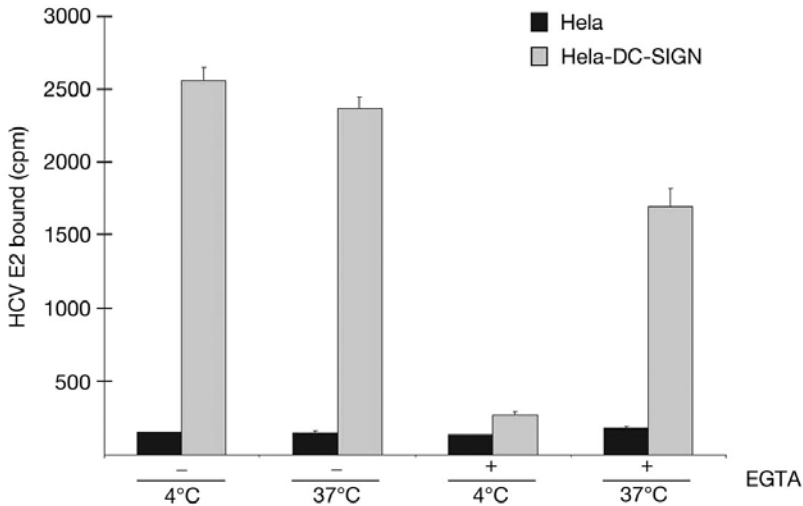


Fig. 5. DC-SIGN induces internalization of viral glycoproteins. HeLa and HeLa-DC-SIGN cells are incubated for 2 h at 4°C with ³⁵S-labeled hepatitis C virus (HCV) E2 glycoprotein. Cells are extensively washed to eliminate unbound material and incubated for 30 min either at 4°C or 37°C. Cells are treated with EDTA or mock treated to distinguish internalized (EDTA-resistant) from cell surface bound HCV-E2 glycoprotein (EDTA-sensitive).

cell surface (*see Fig. 5*). Cells are washed and resuspended in binding buffer prior to addition of optiphase supermix solution. ³⁵S activity is counted as described. For an alternative protocol, *see Note 5*.

3.4. DC-SIGN and L-SIGN-Mediated Infection and Viral Transmission to Target Cells

3.4.1. cis-Infection

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are exposed to viral particles for 2 h at 37°C at varying MOI in FSC-free medium supplemented with 1% penicillin/streptomycin, pH approx 7.5. Cells are washed three times with complete medium to remove excess virus and incubated at 37°C. Viral replication is evaluated 2 to 3 d later, depending on the read-out used (*see Note 7*).

3.4.2. trans-Infection

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are incubated with viral particles at a high MOI for 2 h at 37°C in FCS-

free medium supplemented with 1% penicillin/streptomycin, pH approx 7.5 (see **Note 8**). Cells exposed to virus are washed extensively with cold PBS to remove unbound viral particles, resuspended in 100 μ L of complete medium and co-cultured with an equal number of target cells in 96-well plates. Viral transmission is quantified two or 3 d later.

3.4.3. *trans-Enhancement of Target Cell Infection* (see **Note 8**)

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are incubated with viral particles at a low MOI (insufficient to directly infect target cells) for 2 h at 37°C and immediately co-cultured with target cells without washing. As an important control, viral particles are incubated with medium alone and 2 h later, transferred to target cells.

3.4.4. *Retention of Viral Infectivity by DC-SIGN or L-SIGN*

This assay permits to determine if viral particles bound to DC-SIGN or L-SIGN remain infectious for several days. The protocol is similar to that described under **Subheading 3.4.2.** or **3.4.3.** except that co-culture with target cells is started several days after exposure of cells expressing DC-SIGN or L-SIGN to virus. Multiple points can be tested to establish the kinetic of the virus protection by cells expressing these lectins.

4. Notes

1. Human primary LSECs expressing L-SIGN are difficult to obtain. For this reason, we use cell lines expressing L-SIGN and we describe only the generation of monocyte-derived DCs that constitutively express DC-SIGN.
2. Alternatively, the SFV particle production can be bypassed. The electroporation of BHK cells with pSFV- Δ *env* permits to directly produce the recombinant protein coded by the modified SFV plasmid. This alternative protocol is similar to **Subheading 3.2.1., steps 1 and 2**, except that the amount of pSFV- Δ *env* RNA transfected is doubled. The quantity of RNA electroporated may require adjustment depending on the gene of interest. The maximum volume of RNA we have electroporated is 150 μ L. The next step of the recombinant protein production with this alternative protocol continues at **Subheading 3.2.3., step 2**. The advantage of production of viral particles is that cells can be infected at equal MOI and hence the quantity of protein produced is more reproducible than direct electroporation with pSFV- Δ *env* RNA.
3. The quality of transcribed RNA is verified by agarose gel (1%) electrophoresis. Transcribed RNAs can be stored at -20°C before electroporation.
4. Defective SFV particles are used at a MOI varying from 25 to 100. Some adjustments may be required depending on the gene of interest. The number of cells infected correlates with the quantity of protein produced and the efficiency of protein secreted. For information, the best production we observed is for HCV

envelope protein E2 (**10**). We produce approx 20 μg of purified protein per 10^7 cells infected at a MOI of 50.

5. DC-SIGN- or L-SIGN-mediated viral internalization can also be investigated by confocal microscopy using purified viral glycoproteins (*see Note 6*) or whole virions. HeLa cells expressing DC-SIGN or L-SIGN (5×10^4 cells) are seeded on coverslips. The following day, cells are incubated with viral glycoproteins ($5 \mu\text{g}/\text{mL}^{-1}$) or wild-type viruses diluted buffer B for 1 h at 4°C . Cells are washed three times with ice cold PBS to remove unbound material and shifted to 37°C for different times to allow endocytosis. Cells are fixed with 3.2% paraformaldehyde for 15 min, washed twice in PBS and treated with PBS 0.2 M glycine for 10 min. Cells are then incubated with 500 μL of PBS containing 0.05% saponin and 0.2% BSA for 30 min. Both DC-SIGN and viral protein trafficking can be followed by using specific mAb. Cells are mounted in Vectashield containing DAPI (Vector Laboratories) and imaged on a Zeiss microscope using a Plan Aplanachromat $\times 63/1.4$ oil immersion objective.
6. Optionally, the soluble glycoprotein can be purified by immuno-affinity when a tag peptide is introduced into its sequence. Several commercial tag peptides are available such as the Flag tag peptide which we used (**10**).
7. Read-out is specific for the virus studied. We generally use wild-type viruses or viral particles carrying a reporter gene (GFP or Luc). For example, for HIV or HCV, we use single-cycle pseudotyped viral particles that are generated by co-transfecting 293T cells with an HIV-1 NL ΔEnv Luc or GFP (a HIV provirus lacking the Env gene and carrying the Luc or GFP gene in the place of Nef) and a cDNA plasmid encoding either HIV or HCV envelope proteins. Viral replication is evaluated by measuring luciferase activity in cell lysates (3 d postinfection using a luciferase reporter assay kit [Promega] and a Victor luminometer [Perkin Elmer]) or by counting the GFP-positive cells by FACS. For dengue virus, cell are infected with viral particles produced in insect cells. Viral replication is quantified by flow cytometry 2 d after using Ab specific of dengue virus antigens. For more examples, *see refs. 10,14,16,18,20–26,39*.
8. DC-SIGN- or L-SIGN-expressing cells used for infection *in trans* should not be infectable by the virus studied, to allow evaluation of virus replication that occurs uniquely in the target cell.

Note Added in Proof

While this chapter was in press, four new studies were published concerning the interactions between DC-SIGN and viruses, and the role of DC-SIGN in HIV dissemination (**40–43**).

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