HIV-1 Transmission by Dendritic Cell-specific ICAM-3-grabbing Nonintegrin (DC-SIGN) Is Regulated by Determinants in the Carbohydrate Recognition Domain That Are Absent in Liver/Lymph Node-SIGN (L-SIGN)*□

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In this study, we identify determinants in dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) necessary for human immunodeficiency virus, type 1 (HIV-1), transmission. Although human B cell lines expressing DC-SIGN efficiently capture and transmit HIV-1 to susceptible target cells, cells expressing the related molecule liver/lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN) do not. To understand the differences between DC-SIGN and L-SIGN that affect HIV-1 interactions, we developed Raji B cell lines expressing different DC-SIGN/L-SIGN chimeras. Testing of the chimeras demonstrated that replacement of the DC-SIGN carbohydrate-recognition domain (CRD) with that of L-SIGN was sufficient to impair virus binding and prevent transmission. Conversely, the ability to bind and transmit HIV-1 was conferred to L-SIGN chimeras containing the DC-SIGN CRD. We identified Trp-258 in the DC-SIGN CRD to be essential for HIV-1 transmission. Although introduction of a K270W mutation at the same position in L-SIGN was insufficient for HIV-1 binding, an L-SIGN mutant molecule with K270W and a C-terminal DC-SIGN CRD subdomain transmitted HIV-1. These data suggest that DC-SIGN structural elements distinct from the oligosaccharide-binding site are required for HIV-1 glycoprotein selectivity.

Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN)2 is a C-type lectin expressed on myeloid-lineage DC (1–4), activated B cells (5, 6), and some subsets of macrophages (7, 8). By contrast, L-SIGN, a related lectin (also known as DC-SIGNR), is expressed on liver sinusoidal endothelial cells, the lung, and in lymph nodes (9–11). L-SIGN has 77% amino acid sequence identity with DC-SIGN (9, 12). Both lectins have similar structures consisting of a short cytoplasmic domain, transmembrane domain, repeat domain (conserved in DC-SIGN but polymorphic in L-SIGN), and carbohydrate-recognition domain (CRD) (9, 13, 14). DC-SIGN/L-SIGN are type II membrane-spanning proteins, with the N-terminal domain embedded in the cytoplasm and the C-terminal portion in the extracellular matrix (15). On the surface of cells, DC-SIGN/L-SIGN assemble into tetramers, and this configuration appears necessary for interaction with glycoproteins (16–18).

DC-SIGN and L-SIGN interact with a wide spectrum of animal pathogens, including HIV-1/HIV-2, simian immunodeficiency virus, cytomegalovirus virus, dengue virus, Ebola virus, hepatitis C virus, and severe acute respiratory syndrome coronavirus (10, 11, 19–25). Although it has been proposed that DC-SIGN/L-SIGN-mediated capture of microbial pathogens may aid in antigen processing (26), many pathogens captured by DC-SIGN/L-SIGN-expressing cells appear to escape processing and degradation, and their spread through cell cultures is enhanced after capture. Genetic and epidemiological studies have also implicated a role for these lectins in vivo in response to pathogenic infection. Recent studies using DC-SIGN transgenic mice showed protection and prolonged survival of mycobacterial infection (27). In addition, genetic analysis revealed that individuals homozygous for specific polymorphisms in the L-SIGN repeat domain had a lower risk of severe acute respiratory syndrome coronavirus infection (28).

HIV-1 utilizes DC-SIGN as an attachment receptor, and this interaction appears to facilitate either infection in cis or transmission to other susceptible target cells (29, 30). Activated primary B cells expressing DC-SIGN efficiently transmit HIV-1 to susceptible CD4+ T cells (6), and up to 90% of HIV-1 binding to human mucosal tissue explants has been attributed to DC-SIGN-expressing cells (31). DC-SIGN expressed by in vitro differentiated immature monocyte-derived DCs confers signifi-
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Antibodies—Four monoclonal antibodies (mAbs) against human DC-SIGN and L-SIGN were used for FACS analysis. mAbs 120507 (DC-SIGN-specific), 120526 (cross-reactive), 120604 (L-SIGN specific), and 120612 (cross-reactive) were provided by R & D Systems (Minneapolis, Minn.). DC-SIGN monoclonal antibody DC28 from Drs. F. Baribaud, S. Pöhlmann, J. A. Hoxie, and R. W. Doms was used for immunoblots and obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health.

Cells—Stable Raji B cell populations that express DC-SIGN, L-SIGN, or DC-SIGN/L-SIGN chimeras were generated by electroporation with the different chimeric constructs, followed by selection with Geneticin® (500 µg/ml) and two rounds of cell sorting by FACS (FACS Vantage, BD Biosciences) for high cell-surface expression of wild-type DC-SIGN, L-SIGN, or DC-SIGN/L-SIGN chimeras using cross-reactive mAb 612.

Stable DC-SIGN- and L-SIGN-expressing cell lines derived from parental NC-37 and Ramos cells were generated by transduction of the parental cells with MX-DC-SIGN and MX-L-SIGN retroviral vectors, respectively, followed by cell sorting for high levels of DC-SIGN and L-SIGN expression.

The human Hut/CCR5, HEK293T, and HIV indicator GHOST/X4/R5 cell lines have been described previously (34). HEK293T and GHOST/X4/R5 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories). Parental Raji B, NC-37, and Ramos cells and their derivatives as well as Hut/CCL5 cell lines were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS.

Flow Cytometry—To assess surface expression of DC-SIGN, L-SIGN, and chimeric molecules, cells were stained with mAbs 507, 604, and 612, respectively, and compared with the isotype-matched IgG controls. Cells (2 × 10^6) were incubated in cold phosphate-buffered saline containing 2% FBS (FACS buffer) and 2 µg/ml phycoerythrin-conjugated mAb in a total volume of 100 µl. Cells were incubated for 30 min at 4 °C, washed with FACS buffer, and analyzed using FACS Calibur apparatus (BD Biosciences).

ICAM-3 Adhesion Assay—Soluble, recombinant human ICAM-3 was purchased from R & D Systems. Carboxylate-modified TransFluoSpheres (1.0 µm, 488 nm excitation/645 nm emission, Molecular Probes) were coated with ICAM-3 as described previously (44). Briefly, cells (1.5 × 10^5) were resuspended in adhesion buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2, 2% FBS (adhesion buffer), and ICAM-3-coated fluorescent beads were added and incubated at 4 °C for 30 min to minimize internalization of beads. For the blocking experiment, cells were pretreated with mAb 526, mAb 612, or mannan (20 µg/ml, Sigma) for 20 min at room temperature before addition of ICAM-3-coated beads. Cells were washed with adhesion buffer three times. Adhesion to DC-SIGN, L-SIGN, and chimeras was determined by the detectable percentage of cells that bound fluorescent beads, using flow cytometry on a FACS Calibur apparatus (BD Biosciences).

EXPERIMENTAL PROCEDURES

Plasmids—Human DC-SIGN and L-SIGN with five, six, or seven complete repeats in the repeat domain were cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen). Overlap extension PCR with inner primers was used to generate DC-SIGN/L-SIGN-7 chimeras. Swaps between DC-SIGN and L-SIGN were made by exchanging the fragments at the domain junctions as follows: cytoplasmic domain (residues 1–41 in DC-SIGN/1–49 in L-SIGN); transmembrane domain (residues 42–60/50–72); neck domain (residues 61–80/73–92); repeat domain (residues 81–252/93–264); and carbohydrate recognition domain (253–404/265–399). All PCR fragments were gel-purified, subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen), and verified by DNA sequencing.

Mutagenesis—Mutations in DC-SIGN, L-SIGN, and chimeras were introduced using the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Mutations were created in DC-SIGN at amino acids His-254, Pro-255, Trp-258, Glu-259, Ile-281, Lys-285, and Gly-288 and at Lys-270 in L-SIGN. All constructs were confirmed by DNA sequencing.

cant HIV-1 binding and transmission by these cells (32–34), which can be recapitulated through ectopic expression of DC-SIGN in Raji B cells (35). Binding of HIV-1 particles to DC-SIGN is dependent on the interaction of the CRD with the N-linked high mannosyl oligosaccharides that decorate the HIV-1 envelope (Env) glycoprotein (17, 36, 37). HIV-1 capture and transmission can be significantly inhibited by soluble mannan, and deglycosylation of Env can also impair binding to DC-SIGN (29, 34, 38, 39). DC-SIGN-mediated HIV-1 transmission is cell type-dependent and requires cell-cell contact (35, 39–41). Analysis by confocal microscopy has shown that DC-SIGN, HIV-1, and HIV-1 receptor molecules concentrate at cell-cell junctions, facilitating the trans infection of target cells (20, 32, 42). Although DC-SIGN is involved in HIV-1 capture and transmission, the underlying mechanisms that result in selective binding and subsequent release are not fully understood. Studies using deletion and point mutants have revealed that the repeat region and the CRD of DC-SIGN are essential for capture and transmission of HIV-1 and simian immunodeficiency virus (23, 43). However, there has been no detailed description of the mechanism of DC-SIGN CRD interactions with the HIV-1 Env.

Here we compared the HIV-1 transmission efficiency of human B cell lines expressing DC-SIGN or L-SIGN. In contrast to efficient HIV-1 capture and transmission by DC-SIGN-expressing cells, we found that L-SIGN-expressing cells lack this capacity. Given their similarity in sequence and overall protein structure, a subset of chimeras between DC-SIGN and L-SIGN was made to identify determinants necessary for DC-SIGN-mediated HIV-1 transmission. Lectin chimeras were functionally tested for ICAM-3 interactions, virus-like particle (VLP) binding, and HIV-1 transmission. These studies revealed discrete regions within the DC-SIGN CRD that aid in HIV-1 binding, and they indicate that a residue distal from the site of sugar interactions is critical for lectin ligand specificity.
A. expression of DC-SIGN and L-SIGN in Raji cells. Parental cells and CLR transfectants were stained with cross-reactive mAb 612. The broken line represents staining with isotype control antibody (mouse IgG2a), and the filled black curve represents staining with mAb 612 against DC-SIGN/L-SIGN.

B. ICAM-3 adhesion to Raji expressing DC-SIGN and L-SIGN is inhibited by mAb 526, mAb 612, or mannan. DC-SIGN and L-SIGN transfectants were preincubated with mAb 526, mAb 612, or mannan (all at 20 μg/ml) at room temperature for 20 min prior to addition of ICAM-3-coated fluorescent beads. Fluorescent bead binding was performed at 4 °C for 30 min. The percentage of cells bound to ICAM-3-coated beads was measured by FACS. Background binding of ICAM-3-coated beads to Raji cells was ~5% of cells in two experiments. One representative experiment of two is shown.

C. HIV-1 transmission is mediated by DC-SIGN but not L-SIGN in Raji cells. DC-SIGN- and L-SIGN-expressing donor cells were incubated for 3 h with HIV-luc vectors produced from 293T cells, washed, and then cocultured with Hut/CCR5 target cells. Similarly, donor cells were incubated for 3 h with infectious HIV-1 produced in Hut/CCR5 cells, washed, and then cocultured with TZM cells for 3 days. HIV-1 transmission was determined by measuring luciferase activity. Each data set represents the means of duplicate wells. One representative experiment of two is shown. cps, counts/s.

D. binding of VLPs displaying HIV-1 Env to Raji/DC-SIGN but not to Raji/L-SIGN. Cells were preincubated with mAb 612 for 20 min at room temperature prior to addition of GFP-tagged VLPs displaying HIV-1 Env or no Env, washed, and analyzed for virus binding by FACS. Mouse IgG2a (20 μg/ml) was used as a control. One representative experiment of three is shown. MFI, mean fluorescence intensity.
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FIGURE 2. Raji cells expressing different L-SIGN alleles do not transmit R5- or X4-tropic HIV-1. A, HIV-1 transmission is mediated by DC-SIGN but not L-SIGN. Raji cells expressing DC-SIGN or L-SIGN with five, six, or seven repeats were incubated with HIV-luc vectors for 3 h, respectively, washed, and then cocultured with Hut/CCR5 target cells. Parental Raji cells were used as a donor cell control. HIV-1 infection was determined by measuring luciferase activity after 3 days. One representative experiment of two is shown. B, virus binding to DC-SIGN- and L-SIGN-expressing cells. Cells were incubated with GFP-tagged VLPs displaying ADA or HXB2 Env, respectively. To make infectious virus (10^4 infectious units) for 3 h, washed, and then cocultured with Hut/CCR5 target cells (1 × 10^5) in the presence of Polybrene. Cell lysates were collected 2 days after infection and analyzed for luciferase activity with commercial reagents (Promega). For the HIV-1 infection assay using infectious virus, donor cells were incubated with Hut/CCR5-derived infectious virus (10^5 infectious units) for 3 h, washed, and then cocultured with TZM-bl cells (1 × 10^5) in the presence of Polybrene. Cell lysates were collected 2 days after infection.

Virus Stocks—Single-round infectious, pseudotyped HIV-1 stocks (HIV-Luc/ADA, HIV-Luc/HxB2, or HIV-Luc/JRFL) were generated by calcium phosphate cotransfection of HEK293T cells (MBS mammalian transfection kit, Stratagene) with the proviral vector NL-Luc-E−R− (HIV-Luc) containing a firefly luciferase reporter gene and an expression plasmid for the CCR5-tropic HIV-1 ADA-Env, CCR5-tropic HIV-1 JRFL-Env, or CXCR4-tropic HIV-1 HxB2-Env, respectively. To make infectious HIV-1 (AD8 and JRCSF) stocks, Hut/CCR5 cells were infected with virus for 7 days. Culture supernatants were collected and passed through 0.45-μm filters. Viral stocks were titrated by limiting dilution on GHOST/X4/R5 indicator cells in the presence of 10 μg/ml Polybrene.

Generation of HIV-1 Gag VLPs—HEK293T cells were cotransfected with expression plasmids for an HIV-1 Gag-GFP fusion protein,^3^ HIV-1 envelope glycoproteins (CCR5-tropic HIV-1 ADA-Env, HIV-1 JRFL-Env, or CXCR4-tropic HIV-1 HxB2-Env), and HIV-1 Rev protein. VLPs generated in the absence of expression plasmid for HIV-1 Env were used as VLP/No Env control. Culture supernatants containing VLPs were harvested 2 days post-transfection, passed through 0.45-μm filters, aliquoted, and stored at −80 °C. VLPs were analyzed on immunoblots for Gag-GFP and Env content (data not shown). Env-negative and -positive VLP preparations with comparable Gag-GFP protein levels were used in experiments.

HIV-1 VLP Binding Assays—Cells (2 × 10^5) stably expressing DC-SIGN, L-SIGN, or chimeras were incubated with 300 μl of VLPs for 1 h at 37 °C and washed extensively with RPMI 1640 medium containing 2% FBS to remove unbound VLPs. For blocking experiments, cells were preincubated with either cross-reactive mAb 612 (10 μg/ml) or mouse IgG2a control antibody (10 μg/ml) for 30 min at 37 °C prior to VLP addition. Virus binding to DC-SIGN, L-SIGN, or chimeras was determined by measuring the mean fluorescence intensity of cells that bound VLPs using the FACSCalibur.

HIV-1 Infection Assays—HIV-1 capture and transmission assays were performed as described previously (34). In brief, donor cells (2 × 10^5) were incubated with 10^6 infectious units of 293T-derived HIV-luc pseudotyped virus in a total volume of 400 μl for 3 h at 37 °C to allow cellular adsorption of virus. After 3 h, cells were washed with 1 ml of phosphate-buffered saline to remove unbound virus and cocultured with Hut/CCR5 target cells (1 × 10^5) in the presence of 10 μg/ml Polybrene in 1 ml of cell culture medium. Cell lysates were obtained 3 days after infection and analyzed for luciferase activity with commercial reagents (Promega). For the HIV-1 infection assay using infectious virus, donor cells were incubated with Hut/CCR5-derived infectious virus (10^5 infectious units) for 3 h, washed, and then cocultured with TZM-bl cells (1 × 10^5) in the presence of Polybrene. Cell lysates were collected 2 days after infection.

Production of Recombinant Lectin Extracellular Domains—Soluble recombinant extracellular domains of DC-SIGN, L-SIGN, and LLLLD^3(367−404)/K270W were produced using the pET expression system (Novagen). Briefly, DNA fragments encoding the entire extracellular domain (neck, repeat, and CRD sequences) were amplified by PCR, gel-purified, and subcloned into Ndel and BamHI sites of the pET15b bacterial expression vector. The constructs were confirmed by nucleotide sequencing and transformed into bacterial expression host BL21(DE3). The recombinant proteins were linked to a N-terminal hexahistidin tag for subsequent purification.

To produce recombinant protein, bacterial cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Invitrogen) for 4 h. The recombinant proteins were expressed and aggregated as inclusion bodies. Bacterial pellets were washed twice...
with 20 mM Tris-HCl, pH 7.4, and sonicated (1-min on/off cycle) for 5 min. The inclusion bodies were washed with 20 mM Tris-HCl, pH 7.4, and then solubilized in 6 M guanidine HCl, pH 8, at 4 °C for 1 h. Solubilized proteins were refolded by dilution in refolding buffer (550 mM guanidine HCl, 550 mM L-arginine, 55 mM Tris, pH 8.2, 10.6 mM NaCl, 2.2 mM MgCl₂, 2.2 mM CaCl₂, 1 mM reduced glutathione, 0.1 mM oxidized glutathione) and subsequent dialysis (20 mM Tris, pH 8, 50 mM NaCl) as described previously (45).

Recombinant proteins were then purified by nickel charged His-Trap HP column (GE Healthcare), and protein fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

**VLP Blocking Experiments with Recombinant Extracellular Domains**—Soluble extracellular domains (DC-SIGN, L-SIGN, or LLLLD₃(367–404)/K270W) were preincubated with VLPs at a final concentration of 40 μg/ml for 30 min at 37 °C prior to incubation with 2 × 10⁶ Raji or Raji/L-SIGN cells for 1 h at 37 °C. Cells were extensively washed with RPMI 1640 medium containing 2% FBS, and virus binding was analyzed by flow cytometry.

**Western Blot Analysis**—Recombinant proteins and cleared cell lysates from Raji lines and 293T cells transfected with chimeric constructs were analyzed by SDS-PAGE and immunoblotting with anti-DC/L-SIGN (DC28) at 0.5 μg/ml.

**RESULTS**

**Functional Characterization of DC-SIGN and L-SIGN in Raji Cells**—Raji cells expressing DC-SIGN or L-SIGN were established via electroporation of pcDNA3-DC-SIGN or pcDNA3-L-SIGN and several rounds of cell sorting. Staining of Raji/DC-SIGN and Raji/L-SIGN with cross-reactive mAb 612 demonstrated that both lectins were highly expressed in Raji cell transfectants (Fig. 1A). Parental Raji cells were uniformly negative for lectin expression. To functionally assay both C-type lectin receptors (CLR) in Raji cells, ICAM-3 binding was tested as described previously (2, 34). Adhesion to DC-SIGN and L-SIGN was 23 and 19%, respectively, in the presence of mouse isotype control (Fig. 1B). Pretreatment of transfectants with cross-reactive mAbs (526 or 612) or mannan, a soluble ligand of CLRs, significantly reduced ICAM-3 adhesion to DC-SIGN (10% for mAbs and 8% for mannan) or L-SIGN (8% for mAbs and 6% for mannan) (Fig. 1B). These data indicated that both DC-SIGN and L-SIGN were functionally expressed in Raji cells.

**DC-SIGN, but Not L-SIGN, Transfectants Support HIV-1 Binding and Viral Transmission**—We next tested HIV-1 interactions with DC-SIGN and L-SIGN by using a virus transmission assay. Raji cells expressing DC-SIGN or L-SIGN were incubated in the absence or presence of single-round infectious HIV-luciferase vectors pseudotyped with R5-tropic HIV-1ADA or HIV-1JRFL Env and then cocultured with Hut/CCR5 target cells. In addition to 293T cell-derived HIV-luciferase pseudotyped virus, Raji transfectants were incubated with infectious HIV-1 (AD8 or JRCSF) produced in Hut/CCR5 cells, washed, and then cocultured with TZM indicator cells. As shown in the transmission assay using 293T cell- and Hut/CCR5 cell-derived viruses, Raji/DC-SIGN cells efficiently transmitted virus to T cells, whereas Raji/L-SIGN cells did not (Fig. 1C).

We further examined virus binding by Raji/DC-SIGN and Raji/L-SIGN cells. Raji transfectants were incubated with GFP-tagged VLPs pseudotyped with R5-tropic Env (ADA) or R4-tropic Env (HXB2), washed, and analyzed by flow cytometry. VLPs without HIV-1 Env (VLP/No Env) were used as a control in this assay. Consistent with HIV-1 transmission data, DC-SIGN interacted with VLPs in an Env-dependent manner, whereas L-SIGN did not (Fig. 1D).

**Different Allelic Forms of L-SIGN Do Not Support HIV-1 Transmission**—Previous studies demonstrated that repeat domains of DC-SIGN and L-SIGN contribute to the overall integrity of the tetramer on the cell surface (16, 17). To investigate the impact of the repeat region of L-SIGN on HIV-1 trans-
mission, we obtained Raji cells expressing L-SIGN with five, six, or seven repeats and tested these cells in HIV-1 infection assays. For all the allelic forms tested, none could support HIV-1 transmission (Fig. 2A) or VLP binding with particles bearing either X4- or R5-tropic Env (Fig. 2B).

Other Human B Cell Lines Expressing L-SIGN Do Not Support HIV-1 Transmission—To examine whether impaired virus capture and transmission by L-SIGN were restricted to Raji cells, we generated NC-37 and Ramos B cell lines expressing comparable levels of DC-SIGN or L-SIGN and tested these cells for HIV-1 interactions. Both Ramos/DC-SIGN and NC-37/DC-SIGN cells efficiently transmitted virus to target cells (Fig. 3A). Similar to Raji/L-SIGN cells, NC-37/L-SIGN and Ramos/L-SIGN cells did not support HIV-1 transmission (Fig. 3A) or VLP binding (Fig. 3B).

A number of other non-B cell lines expressing high levels of L-SIGN were also developed, yet none gained a significant increase in their capacity to bind and transmit HIV-1 (supplemental Fig. S1). However, many of these cells also displayed limited DC-SIGN-mediated HIV-1 transmission capacity.

DC-SIGN CRD Is Necessary for HIV-1 Binding and Transmission—To identify the regions of DC-SIGN that were missing in L-SIGN and enable HIV-1 transmission, we created chimeras between DC-SIGN and L-SIGN with seven repeats (the most common human isoform of L-SIGN) by making reciprocal exchanges at domain junctions. Both lectins consist of five domains, and the chimeras were named according to domain compositions (Fig. 4A). Chimeras were stably expressed in Raji cells and stained with three mAbs as follows: 507 (DC-SIGN specific), 604 (L-SIGN specific), and 612 (cross-reactive). Staining of parental Raji cells was uniformly negative (data not shown). Cross-reactive mAb 612 recognized all the chimeras tested. Chimeras containing the DC-SIGN CRD were stained positive using mAb 507, whereas mAb 604 only recognized chimeras containing the L-SIGN CRD (Table 1). All lectin chimeras expressed in Raji cells appeared to be functional and showed 18–26% of ICAM-3 binding in an adhesion assay (Fig. 4B).

| Antibody reactivity of DC-SIGN/L-SIGN chimeras |
|-----------------------------------------------|
| mAb 507(D) is anti-DC-SIGN; mAb 604(L) is anti-L-SIGN; mAb 612(X) is anti-DC-SIGN/L-SIGN. + represents positive antibody staining by FACS analysis, and − represents negative antibody staining by FACS analysis. |

| Chimeras | 612(X) | 507(D) | 604(L) |
|----------|--------|--------|--------|
| DC-SIGN  | +      | −      | −      |
| DDDDL    | +      | −      | −      |
| DDDL    | +      | −      | −      |
| DDDL    | +      | −      | −      |
| DLLL    | +      | −      | −      |
| L-SIGN  | +      | −      | −      |
| LLLD    | +      | −      | −      |
| LLLL    | +      | −      | −      |
| LDDDD   | +      | −      | −      |

TABLE 1

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Chimeras were tested in virus binding and infection assays. Because the surface expression levels of the chimeras showed subtle variation, luciferase activity from infection assays and mean fluorescence indices from VLP binding were normalized to mean fluorescence indices for CLR surface expression with cross-reactive antibodies. HIV-1 transmission and VLP binding relative to DC-SIGN-expressing cells are shown (Fig. 4, C and D). Examination of all the chimeras revealed that replacement of the DC-SIGN CRD with that of L-SIGN (i.e. chimeras DDDDL, DDDDL, and DLLLL) impaired both virus transmission and binding (Fig. 4, C and D). By contrast, HIV-1 transmission was restored in chimeras containing the DC-SIGN CRD (i.e. chimeras DDDLD, LLLLD, LLDDL, and LDDDD) and further enhanced by inclusion of the DC-SIGN repeat domain (1.3–2.2-fold in LLLDD and 1.2–3.2-fold in LDDDD), as shown in Fig. 4C. Chimera LLLDL containing the L-SIGN CRD did not support virus binding or transmission (Fig. 4, C and D). These data suggested that the DC-SIGN CRD was both necessary and sufficient for virus binding and transmission, and the DC-SIGN repeat region could enhance virus transmission efficiency. The L-SIGN CRD, although capable of binding ICAM-3, was not functional for HIV-1 capture or transmission from Raji B cells.

*N-terminal DC-SIGN CRD Region (Residues 253–288) Is Essential for HIV-1 Binding and Transmission*—We next examined which region(s) of the DC-SIGN CRD enabled HIV-1 interactions by developing a subset of CRD chimeras (Fig. 5A). All CRD chimeras were stably expressed in Raji cells as assessed by staining with mAb 612 (data not shown). In addition, all CRD chimeras were shown to bind ICAM-3 (15.5–39.9% of cells in positive samples), suggesting that CRD chimeras were functionally expressed in Raji cells (Fig. 5B). We then analyzed these CRD chimeras in infection and virus
binding assays. Relative to the DC-SIGN control line, HIV-1 transmission was modestly reduced in cells expressing DC-SIGN-del16 (80%), DDDDL₃ (80%), and DDDDL₃₋₂₋₃ (38–57%) (Fig. 5C). By contrast, the exchange of fragment between residues 253 and 288 within the DC-SIGN CRD with the corresponding L-SIGN CRD portion (chimera DDDDL₁₋₋₂₋₋₋₋) was sufficient to impair both VLP binding and HIV-1 transmission (Fig. 5, C and D), suggesting that residues 253–288 were important for DC-SIGN-mediated HIV-1 transmission. As expected, chimeras DDDDL₁₋₋₋₋₋₋ and DDDDL containing the complete L-SIGN CRD segment were also unable to bind and transmit HIV-1 (Fig. 5, C and D).

By contrast, in an L-SIGN background, replacement of the L-SIGN CRD segment with the DC-SIGN CRD residues 289–404, which includes 16 residues unique to the C terminus of the DC-SIGN CRD (chimera LLLLLL₁₋₋₋₋₋₋), did not have any effect on virus binding or transmission (Fig. 5, C and D). Notably, LLLLLL₁₋₋₋₋₋₋, which contains the essential DC-SIGN CRD determinant for HIV-1 interaction, was insufficient to transfer these properties to the chimera. However, chimera LLLLLL₁₋₋₋₋₋₋ supported virus binding (20%) and transmission (31–34%) (Fig. 5, C and D). Increased virus binding (33–45%) and transmission (75%) were also observed for chimera LLLLLL₁₋₋₋₋₋₋, of which the HIV-1 interaction determinant from DC-SIGN is combined with a distal DC-SIGN CRD segment, including residues 326–404 into an L-SIGN backbone.

**DC-SIGN Tryptophan 258 Is Required for HIV-1 Binding and Transmission.**—Because the N-terminal DC-SIGN CRD region (residues 253–288) proved essential for virus binding and transmission, we examined the role of the seven residues in this region that differed between DC-SIGN and L-SIGN (Fig. 6A). Double mutants (H254R/P255H and W258K/E259D) and single mutants (W258K, W258A, K285V, K285Q, K285A, G288R, and G288A) in DC-SIGN were constructed and stably expressed in Raji cells. After FACS enrichment for lectin expression, DC-SIGN mutants in Raji lines were verified for expression. Soluble extracellular domains were refolded, purified, and analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 7A, lanes i–j). Purified extracellular domains were functionally tested by the ICAM-3 adhesion assay. Pretreatment of ICAM-3-adhering cells with soluble proteins would block virus binding. Preincubation of ICAM-3 beads with soluble DC-SIGN, L-SIGN, or LLLLLL₃₋₋₋₋₋₋/K270W significantly inhibited ICAM-3 adhesion to Raji/DC-SIGN and Raji/L-SIGN cells (data not shown). We next examined if these soluble proteins would block virus binding. Pretreatment of VLPs with sDC-SIGN and sLLLLL₃₋₋₋₋₋₋/K270W at 40 μg/ml significantly impaired virus binding to Raji/DC-SIGN cells whereas L-SIGN did not block virus binding.
Inhibition by sDC-SIGN and sLLLLD3(367–404)/K270W was seen to be dose-dependent (data not shown). These results further strengthen observations of virus binding and transmission data using the Raji cell model and confirm that minimal changes in L-SIGN enable interaction with HIV-1 particles.

**DISCUSSION**

In contrast to DC-SIGN, we show that L-SIGN does not support HIV-1 transmission when expressed on human B cell lines. This functional difference is primarily due to amino acid differences at homologous positions within the DC-SIGN and L-SIGN CRDs. Several DC-SIGN residues in the N-terminal portion of the CRD appear to contribute to HIV-1 binding and transmission, including Trp-258, which is essential for these functions. Notably, L-SIGN chimeras require the K270W mutation and C-terminal DC-SIGN CRD residues to gain HIV-1 transmission function.

Prior studies have revealed that DC-SIGN-mediated HIV-1 transmission is cell type-dependent (35, 39, 40), and human B cell lines expressing DC-SIGN are as efficient as primary DCs in...
HIV-1 transmission (46). Recent studies have also shown that activated human primary B cells express DC-SIGN and can support trans-infection of CD4+ T cells by both X4- and R5-tropic HIV-1 (6). By contrast, the examination of HIV-1 transmission by L-SIGN/DC-SIGNR has been more limited.

Studies using HEK293T cells transiently expressing DC-SIGN and L-SIGN have suggested that both CLR s are able to bind and transmit HIV-1 (9, 47). Although 293T CLR transfectants can capture and transmit virus better than control transfectants, we have observed that HIV-1 transmission by 293T cells expressing high levels of DC-SIGN is significantly less efficient than by Raji/DC-SIGN cells or primary DCs (data not shown). We thus elected to assay L-SIGN function in human B cell lines that are permissive for DC-SIGN transmission of HIV-1. Under these conditions, we observed that L-SIGN was ineffective in both HIV-1 binding and transmission. It is unlikely that L-SIGN expressed in Raji cells was non-functional, as it was recognized by specific antibodies and supported ICAM-3 interactions. In addition to B cell lines, we generated stable L-SIGN transfectants in HeLa, K562, and human umbilical vein endothelial cell lines and tested these lines for virus binding and transmission. None of the L-SIGN-expressing lines could bind or transmit HIV-1. Production of recombinant extracellular L-SIGN also revealed that this protein was ineffective in impeding Raji/DC-SIGN interactions with VLPs, whereas excess soluble DC-SIGN or a minimally modified L-SIGN chimera could block such interactions.

HIV-1 produced from human T cell lines or peripheral blood mononuclear cells is bound and transmitted more efficiently by CLR s than virus produced from human fibroblasts due to differential glycosylation of HIV-1 Env (37). Nonetheless, similar to data obtained with 293T cell-produced HIV-1, virus released from infected human T cell lines could be transmitted by Raji/DC-SIGN but not Raji/L-SIGN cell lines in our experiments. These data suggest that in our system, the identity of the lectin receptor, not the source of virus, is the critical variable determining the efficiency of HIV-1 transmission. Notably, all HIV-1 transmission data were uniformly corroborated by VLP binding results. Fluorescent VLPs were used to assay binding in experiments, as they incorporate Env trimers, and thus are a suitable facsimile of HIV-1 particles transmitted by DC-SIGN.

The DC-SIGN CRD contains two Ca2+ -binding sites (Asp-330/Glu-334/Asn-350 and Asp-320/Glu-324/Asn-349/Glu-354/Asn-365), which are critical for the recognition of specific carbohydrate structures. Mutation of the residues coordinating Ca2+ abolishes interactions with ligands, including the HIV-1 Env (48, 49). Our findings using CRD chimeras indicate that DC-SIGN residues 253–288 were also required for HIV-1 binding and transmission. Further mutagenesis within this region revealed that Trp-258 is essential for HIV-1 transmission.

According to the crystal structure of the DC-SIGN CRD (36), Trp-258 is distal from the oligosaccharide recognition and Ca2+ -binding sites (Fig. 9A), suggesting that any effect(s) of Trp-258 mutation on either of these functions is likely to be indirect. Instead, this critical residue is located precisely at the RD-CRD junction in a bend immediately N-terminal to a β-strand. As a consequence of this positioning, mutating Trp-258 in DC-SIGN may alter the trajectory of the CRDs as they emerge from the CRDs in the tetrameric receptor. This in turn may change the angle at which the oligosaccharide recognition subdomain is presented (relative to the axis of the receptor), as well as the spatial separation between oligosaccharide recognition subdomains within a given receptor. Although speculative, it is conceivable that either or both of these changes might serve to prevent stable association between the W258K DC-SIGN mutant with HIV-1 at the surface of the cell.

Alternatively, or perhaps in addition, mutation of Trp-258 may affect the interface between adjacent subunits in tet...
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FIGURE 9. DC-SIGN CRD structural features that contribute to HIV-1 transmission. A, DC-SIGN and L-SIGN CRDs were subdivided into three segments based on chimera construction: segment 1 (red), segment 2 (green), and segment 3 (yellow). Ribbon diagrams of DC-SIGN CRD (Protein Data Bank code 1K9I) and L-SIGN CRD (Protein Data Bank code 1K9J) were generated using the Accelrys Discovery Studio 2.0 software. The first α-helix (α1) is in segment 1, and the second α-helix (α2) is in segment 2. Oligosaccharide GlcNAc2Man3 is shown in ball-and-stick representation (orange). Cyan spheres represent Ca2+ ions. Ca2+-binding sites 1 and 2 are shown as dark blue and purple spheres, respectively. CT, cytoplasmic domain; TM, transmembrane domain; ND, neck domain; RD, repeat domain. B, crystal structure of the L-SIGN dimer in a tetramer configuration (Protein Data Bank code 1XAR). Both α1 and α2 helices are boxed. L-SIGN residue Lys-270, which corresponds to Trp-258 in DC-SIGN, is shown in cyan. C, ribbon diagram of DC-SIGN CRD monomer showing determinants (in dark blue) that enable HIV-1 transmission when introduced into L-SIGN. DC-SIGN Trp-258 and residues 367–382 are highlighted.

Direct interaction between amino acid 270 and the C-terminal substituted region (segment 3).

DC-SIGN CRD subunit inter- and intramolecular interactions are both likely to contribute to ligand selectivity. L-SIGN chimeras containing DC-SIGN CRD segment 1 did not support virus transmission unless segment 2 or C-terminal segment 3 (DC-SIGN residues 326–404 shown in yellow) was also present. L-SIGN chimeras containing DC-SIGN segment 2 alone, segment 3 alone, or segments 2 and 3 together did not support virus binding and transmission. Mutagenesis of DC-SIGN CRD α-helix 1 revealed that 1281V and K285A reduced HIV-1 transmission. G288R/G288A, which is at the end of α-helix 1, also showed a significant reduction in virus transmission. These data support a model in which the interface between α-helices 1 and 2 contributes to ligand specificity.

The mechanism by which the combination of DC-SIGN CRD segments 1 and 3 (e.g. in the LLLLD1-L-D3 chimera) facilitates virus binding is less clear. Mutational analysis revealed that Trp-258 in segment 1 was sufficient to function with DC-SIGN segment 3 or even a smaller C-terminal subregion to enable virus transmission by L-SIGN chimeras. By contrast, Trp-258 was not sufficient in combination with segment 2 to enable HIV-1 transmission by L-SIGN chimeras, indicating that other residues with DC-SIGN segment 1 are more important for interaction with segment 2 in conferring the virus transmission phenotype. A direct interaction of Trp-258 with the DC-SIGN N-terminal subportion required for HIV-1 binding is not apparent from the current structures (Fig. 9C). Trp-258 might conformationally influence the C-terminal CRD segment 3, or it might facilitate direct interactions with HIV-1 Env when combined with segment 3, which contains the Ca2+-binding sites. Structural analysis of minimally modified L-SIGN molecules that have acquired the ability to interact with HIV-1 Env might reveal the key characteristics required for the selective interaction.

Biochemical and structural analyses have demonstrated that DC-SIGN and L-SIGN have distinct oligosaccharide binding properties (36, 48). Although both bind N-linked high mannose oligosaccharides, DC-SIGN can also recognize an expanded range of glycans, including fucose containing Lewis* and Lewisx trisaccharides (48). Despite the apparently broader glycan binding range of DC-SIGN, ligands that interact with L-SIGN do not necessarily bind DC-SIGN. For example, West Nile virus (WNV) preferentially interacts with L-SIGN but not with DC-
SIGN (50). WNV infection is mediated by binding of the WNV envelope protein to the L-SIGN CRD. Although our findings demonstrate that HIV-1 selectively interacts with the DC-SIGN CRD but not with the L-SIGN CRD, Raji/L-SIGN cells support WNV infection, further supporting the functional nature of L-SIGN on these cells. It will be interesting to examine the interaction of viruses such as WNV using DC-SIGN/L-SIGN CRD chimeras to understand what structural features they require and how these interactions compare with those of HIV-1.

In summary, we have identified determinants within the DC-SIGN CRD that are necessary for HIV-1 binding and transmission. Further characterization of specific residues within these determinants will be useful in understanding DC-SIGN/L-SIGN ligand binding selectivity, especially for viral pathogens. Our studies also indicate that L-SIGN does not serve as an efficient trans receptor for HIV-1 and suggest that the L-SIGN contribution to HIV-1 infection in vivo may need to be reconsidered.

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REFERENCES

1. Curtis, B. M., Scharnowske, S., and Watson, A. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8356–8360
2. Geijtenbeek, T. B., Toressma, R., van Vliet, S. J., van Duijnhoven, G. C., Adema, G. J., van Kooyk, Y., and Figdor, C. G. (2000) Cell 100, 575–585
3. Turville, S. G., Cameron, P. U., Handley, A., Lin, G., Pöhlmann, S., Doms, R. W., and Cunningham, A. L. (2002) Nat. Immunol. 3, 975–983
4. Poßmann, S., Baribaud, F., Lee, B., Leslie, G., Sanchez, M. D., Hiebenthal, K., Mühl, J., Kirchhoff, F., and Doms, R. W. (2001) J. Virol. 75, 4664–4672
5. Schafer, M., Reiling, N., Fessler, C., Stephehni, J., Taniuchi, I., Hatam, F., Fix, M., Fiebien, O., Fehrenbacher, W., Walter, K., Ruland, J., Wagner, H., Ehlers, S., and Sparwasser, T. (2008) J. Immunol. 180, 6838–6845
6. He, B., Qiao, X., Klasse, P. J., Chiu, A., Chadburn, A., Knowles, D. M., Hart, D., and Cunningham, A. L. (2001) Blood 98, 2482–2488
7. He, B., Qiao, X., Klasse, P. J., Chiu, A., Chadburn, A., Knowles, D. M., Moore, J. P., and Cerrutti, A. (2001) J. Immunol. 167, 3931–3941
8. Rappocciolo, G., Piazza, P., Fuller, C. L., Reinhardt, T. A., Watkins, S. C., Rowe, D. T., Jais, M., Gupta, P., and Rinaldo, C. R. (2006) PLoS Pathog. 2, e70
9. Geijtenbeek, T. B., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I., Hollen, S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000) Cell 100, 587–597
10. Lee, B., Leslie, G., Soilleux, E., E.O’dough, P., Bisk, L., Levonne, E., Flummerfelt, K., Swiggard, W., Coleman, N., Malim, M., and Doms, R. W. (2001) J. Virol. 75, 1208–12038
11. Gurney, K. B., Elliott, J., Nassian, H., Song, C., Soilleux, E., McGowan, I., Anton, P. A., and Lee, B. (2005) J. Virol. 79, 5762–5773
12. McDonald, D., Wu, L., Bohl, S. M., KewalRamani, V. N., Unutmaz, D., and Hope, T. J. (2003) Science 300, 1295–1297
13. Sanders, R. W., de Jong, E. C., Baldwin, C. E., Schuitemaker, J. H., Kapsenberg, M. L., and Berkhout, B. (2002) J. Virol. 76, 7812–7821
14. Wu, L., Martin, T. D., Vaze, L., Unutmaz, D., and KewalRamani, V. N. (2002) J. Virol. 76, 5905–5914
15. Wu, L., Martin, T. D., Carkington, M., and KewalRamani, V. N. (2004) Virology 318, 17–23
16. Feinberg, H., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2001) Science 294, 2163–2166
17. Lin, G., Simmons, G., Pöhlmann, S., Baribaud, F., Ni, H., Leslie, G. J., Haggarty, B. S., Bates, P., Weissman, D., Hoxie, J. A., and Doms, R. W. (2003) J. Virol. 77, 1337–1346
18. Hon, P. W., Flummerfelt, K. B., de Parseval, A., Gurney, K., Elder, J. H., and Lee, B. (2002) J. Virol. 76, 12855–12865
19. Wu, L., Martin, T. D., Han, Y. C., Breun, S. K., and KewalRamani, V. N. (2004) Retrovirology 1, 14
20. Pöhlmann, S., Baribaud, F., Figdor, C. G., and Granelli-Piperno, A. (2003) Int. Immunol. 15, 289–298
Functional Analysis of the DC-SIGN CRD in HIV-1 Transmission

41. Wu, L., and KewalRamani, V. N. (2006) *Nat. Rev. Immunol.* **6**, 859–868
42. Arrighi, J. F., Pion, M., Wiznerowicz, M., Geijtenbeek, T. B., Garcia, E., Abraham, S., Leuba, F., Dutot, V., Ducray-Rundquist, O., van Kooyk, Y., Trono, D., and Pighet, V. (2004) *J. Virol.* **78**, 10848–10855
43. Biggins, J. E., Yu Kimata, M. T., and Kimata, J. T. (2004) *Virology* **324**, 194–203
44. Geijtenbeek, T. B., van Kooyk, Y., van Vliet, S. J., Renes, M. H., Raymakers, R. A., and Figdor, C. G. (1999) *Blood* **94**, 754–764
45. Pokidyheva, E., Zhang, Y., Battisti, A. J., Bator-Kelly, C. M., Chipman, P. R., Xiao, C., Gregorio, G. G., Hendrickson, W. A., Kuhn, R. J., and Rossman, M. G. (2006) *Cell* **124**, 485–493
46. Wang, J. H., Janas, A. M., Olson, W. J., KewalRamani, V. N., and Wu, L. (2007) *J. Virol.* **81**, 2497–2507
47. Pöhlmann, S., Leslie, G. J., Edwards, T. G., Macfarlan, T., Reeves, J. D., Hiebenthal-Millow, K., Kirchhoff, F., Baribaud, F., and Doms, R. W. (2001) *J. Virol.* **75**, 10523–10526
48. Guo, Y., Feinberg, H., Conroy, E., Mitchell, D. A., Alvarez, R., Blixt, O., Taylor, M. E., Weis, W. I., and Drickamer, K. (2004) *Nat. Struct. Mol. Biol.* **11**, 591–598
49. Geijtenbeek, T. B., van Duijnhoven, G. C., van Vliet, S. J., Krieger, E., Vriend, G., Figdor, C. G., and van Kooyk, Y. (2002) *J. Biol. Chem.* **277**, 11314–11320
50. Davis, C. W., Nguyen, H. Y., Hanna, S. L., Sánchez, M. D., Doms, R. W., and Pierson, T. C. (2006) *J. Virol.* **80**, 1290–1301