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## **Trans-dominant cellular inhibition of DC-SIGN-mediated HIV-1 transmission**

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### **Abstract**

**Background:** Dendritic cell (DC) transmission of human immunodeficiency virus (HIV) to CD4+ T cells occurs across a point of cell-cell contact referred to as the infectious synapse. The relationship between the infectious synapse and the classically defined immunological synapse is not currently understood. We have recently demonstrated that human B cells expressing exogenous DC-SIGN, DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin, efficiently transmit captured HIV type I (HIV-1) to CD4+ T cells. K562, another human cell line of hematopoietic origin that has been extensively used in functional analyses of DC-SIGN and related molecules, lacks the principal molecules involved in the formation of immunological synaptic junctions, namely major histocompatibility complex (MHC) class II molecules and leukocyte function-associated antigen-1 (LFA-1). We thus examined whether K562 erythroleukemic cells could recapitulate efficient DC-SIGN-mediated HIV-1 transmission (DMHT).

**Results:** Here we demonstrate that DMHT requires cell-cell contact. Despite similar expression of functional DC-SIGN, K562/DC-SIGN cells were inefficient in the transmission of HIV-1 to CD4+ T cells when compared with Raji/DC-SIGN cells. Expression of MHC class II molecules or LFA-1 on K562/DC-SIGN cells was insufficient to rescue HIV-1 transmission efficiency. Strikingly, we observed that co-culture of K562 cells with Raji/DC-SIGN cells impaired DMHT to CD4+ T cells. The K562 cell inhibition of transmission was not directly exerted on the CD4+ T cell targets and required contact between K562 and Raji/DC-SIGN cells.

**Conclusions:** DMHT is cell type dependent and requires cell-cell contact. We also find that the cellular milieu can negatively regulate DC-SIGN transmission of HIV-1 *in trans*.

### **Background**

DC-SIGN is a DC-expressed HIV-1 attachment receptor that facilitates the *trans* infection of CD4+ T cell targets [1]. Similar to immature DCs isolated from the blood,

monocyte-differentiated dendritic cells (MDDCs) express high levels of DC-SIGN *in vitro*. DC-SIGN is a member of the C-type lectin receptor superfamily and adsorbs HIV particles via interactions with the HIV envelope

glycoprotein (Env) [1-4]. Preventing the DC-SIGN capture of HIV greatly impairs the ability of MDDCs to promote HIV infection and virus proliferation in co-culture [5-7].

The mechanism through which DC-SIGN capture of HIV facilitates the *trans* infection of CD4+ target cells is unclear. Prior studies with MDDCs indicated that cell-cell contact is necessary for efficient stimulation of CD4+ T cell infection [8]. However, DCs possess both DC-SIGN-dependent and -independent mechanisms to facilitate the *trans* infection of CD4+ target cells [5,6,9-11]. Thus, a requirement of cell contact for DC-SIGN-mediated HIV transmission has not been established.

Studies with MDDCs have also revealed that transmission of HIV occurs across a cell-cell junction referred to as the infectious synapse [12]. The intercellular adhesion molecules that form this junction have not been identified, although it has been reasoned that this structure may have similarities to the immunological synapse formed between antigen presenting cells (APCs) and their T cell conjugates. Two significant components of the immunological synapse expressed on APCs include MHC class II molecules and LFA-1, the ICAM-1 receptor [13]. MHC class II molecules play a central role in immune responses through the presentation of processed antigens derived from endogenous and exogenous proteins that access endocytic pathways [14-17]. Antigens presented on MHC class II molecules are scanned by a T cell receptor complex that includes the CD4 molecule. The integrin LFA-1 mediates cell-cell adhesion principally via interactions with the ICAM-1 ligand, a property important in many cellular processes. Significantly, LFA-1/ICAM-1 interactions help initiate formation of the immune synapse. Antibody-blocking experiments have suggested that LFA-1 interactions with ICAM-1 may be important in DC-mediated transmission of HIV [7,8,18]. In addition, LFA-1 interactions with ICAM-1 can contribute to cell-cell transmission of HIV in other cell types [19,20].

Raji/DC-SIGN cells, which were previously misidentified as monocytic "THP-1/DC-SIGN" cells [21], capture and transmit HIV at efficiencies comparable to those of MDDCs [1,5-7,9-11]. Thus, if the DC-SIGN-mediated pathway of HIV transmission requires the participation of other MDDC-associated functions prior to CD4+ T cell infection, these are likely preserved in Raji cells and other human B cells [21]. Human K562 cells, an erythroleukemic line, have been used extensively in previous functional analyses of DC-SIGN and its homologs [22-32]. A screening of cellular markers revealed that K562 cells lack MHC class II and LFA-1 molecules involved in the formation of immunological synaptic junctions. We therefore first examined whether cell-cell contact is essential for DC-SIGN-mediated HIV-1 transmission (DMHT). Using

single-cycle HIV-1 transmission assays, we also quantitatively tested whether K562 cells could recapitulate the efficient DMHT observed using Raji/DC-SIGN cells. Here we describe a new cellular mechanism regulating HIV-1 transmission by DC-SIGN.

## Results

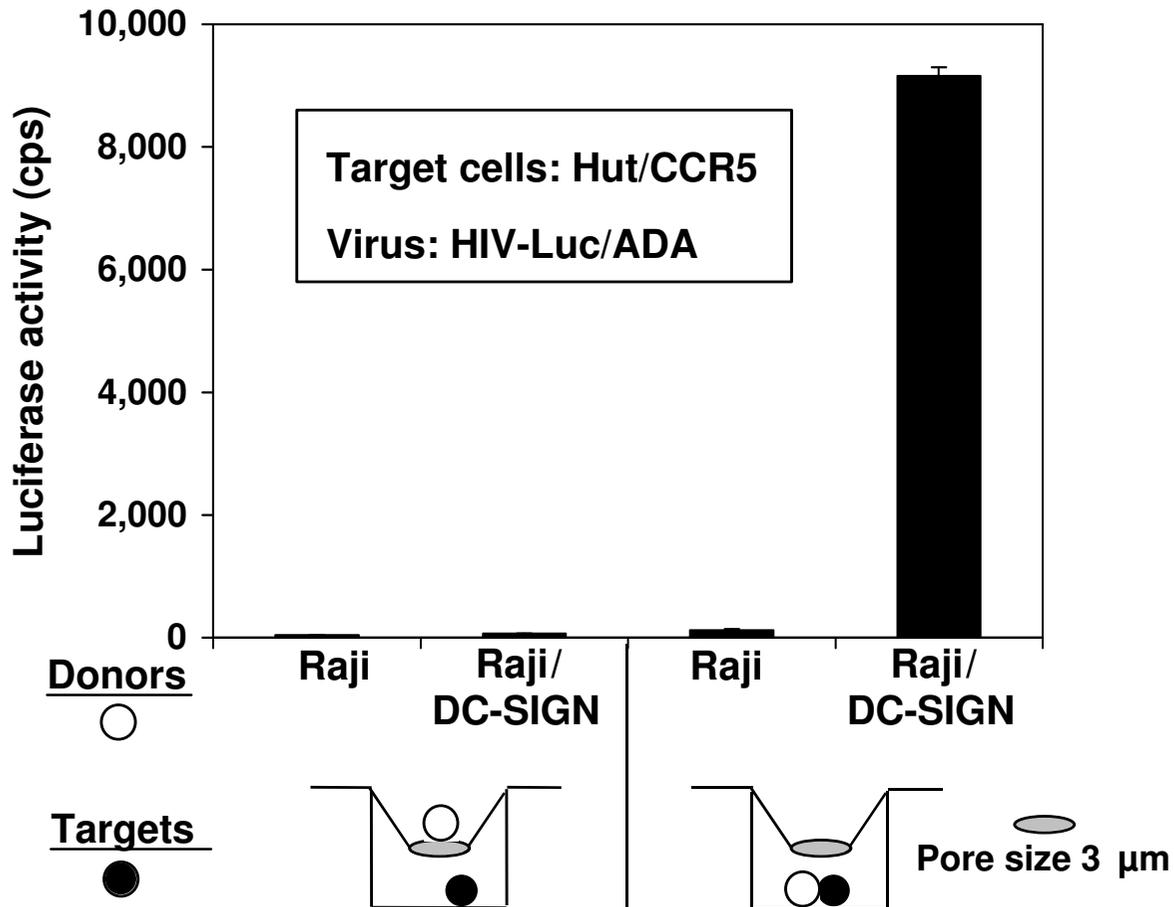
### **DMHT requires donor and target cell contact**

Previous studies indicated that MDDC contact with CD4+ T cells is required for efficient HIV-1 replication [8]. Because MDDCs can transmit HIV-1 independently of DC-SIGN [5,6,9-11], these studies did not establish whether DMHT requires donor and target cell interactions. To evaluate whether cell contact is required for DMHT, Raji or Raji/DC-SIGN donor cells were preincubated with single-round infectious HIV-Luc/ADA, washed, and cocultured with Hut/CCR5 cells, a human T cell line. Alternatively, HIV-Luc/ADA-pulsed donor cells were separated from target cells by using transwell cell culture plates with permeable membranes. Compared with Raji donor cell controls, HIV-1 infection of Hut/CCR5 cells was enhanced significantly when in direct co-culture with Raji/DC-SIGN cells (Figure 1). In contrast, Raji/DC-SIGN cells did not transmit HIV-1 to Hut/CCR5 cells when the donor and target cells were separated in the coculture by a membrane (Figure 1). Placement of HIV-1-pulsed Raji/DC-SIGN donor cells on either the top or bottom of the transwell membrane opposite the target cells had no effect on the infection profiles (data not shown).

### **Functional characterization of DC-SIGN transfectants**

Raji and K562 cells expressing DC-SIGN were established via stable transfection of pcDNA3-DC-SIGN and several rounds of flow cytometry-based fluorescence-activated cell sorting (FACS) for cell populations expressing intermediate levels of DC-SIGN (Table 1 and Figure 2A). Parental Raji and K562 cells were uniformly negative for DC-SIGN expression.

We first examined whether the DC-SIGN transfectants would adsorb greater amounts of HIV-1 relative to the parental cell lines. Although HIV-1 can nonspecifically bind to a number of transformed cell types, high-level DC-SIGN expression increases HIV-1 adsorption by different transformed lines [1,23,33,34]. HIV-1 binding to cell lines was detected by measuring the capture of pseudotyped HIV-Luc/ADA using an enzyme-linked immunosorbent assay (ELISA) for HIV-1 capsid (CA)-p24. Compared with the Raji and K562 parental cell controls, DC-SIGN transfectants bound an increased amount of HIV-1 (Figure 2B). Relative to the corresponding parental cells, results from six independent experiments indicate that HIV-1 binding was enhanced in DC-SIGN-expressing Raji or K562 cells by  $1.5 \pm 0.2$  and  $1.8 \pm 0.2$ , respectively (mean  $\pm$  SD,  $P > 0.05$ ).



**Figure 1**

**DC-SIGN transmission of HIV-1 requires donor and target cell contact** Pseudotyped HIV-Luc/ADA ( $1 \times 10^5$  IU) was preincubated with Raji or Raji/DC-SIGN donor cells ( $2.5 \times 10^5$ ) for 2 h at 37°C; the cells were washed with 1 ml of PBS and then cocultured with Hut/CCR5 target cells ( $1 \times 10^5$ ) in the presence of 10 µg of polybrene in 1 ml of culture medium. Transwell cell culture plates with polycarbonate membrane inserts (3 µm pore size) were used in the capture and transmission assays to separate HIV-1-pulsed donor cells from target cells as illustrated. Cell lysates were obtained 2 days after infection and analyzed for luciferase activity. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown. cps, counts per second.

We next functionally assayed the DC-SIGN expressed in these cell lines. Binding to ICAM-3, a physiological ligand of DC-SIGN, was tested using a previously described flow cytometric assay [2,5]. Low nonspecific binding to cells was observed in this assay. Less than 2% of ICAM-3-coated fluorescent beads bound to parental Raji and K562 cells (Figure 2C). In contrast, the adhesion to Raji/DC-SIGN and K562/DC-SIGN cells was 27% and 25%, respectively (Figure 2C). These data indicate that Raji and K562 transfectants express functionally authentic forms of DC-SIGN.

#### **Variable efficiency of HIV-1 transmission by DC-SIGN-expressing cell lines**

We next tested the efficiency of HIV-1 transmission by the two DC-SIGN-expressing cell lines. Virus donor cells were preincubated with HIV-Luc/ADA and washed to remove unbound virus, after which CD4<sup>+</sup> T cells were added in coculture as infection targets. Raji/DC-SIGN cells stimulated HIV-1 transmission more than 100-fold relative to the Raji parental cells (Figure 2D). K562/DC-SIGN cells captured and transmitted HIV-1 less efficiently. These cells transmitted HIV-1 37-fold less efficiently than Raji/DC-

**Table 1: Description of stable cell populations.<sup>a</sup>**

Cell lines	Expression vectors <sup>b</sup>	Antibiotic selection (concentration, µg/ml)
Raji	NA <sup>c</sup>	NA
Raji/DC-SIGN	pcDNA3-DC-SIGN	Neomycin (500)
Raji/DN-CIITA	pDN-CIITA (300–1130)	Neomycin (500)
Raji/DC-SIGN/DN-CIITA	pMX-DC-SIGN, pDN-CIITA (300–1130)	Neomycin (500)
K562	NA	NA
K562/LFA-1	pCDB1, pCDL1, pCMV-hph	Hygromycin (200)
K562/DC-SIGN	pcDNA3-DC-SIGN	Neomycin (500)
K562/DC-SIGN/LFA-1	pcDNA3-DC-SIGN, pCDB1, pCDL1, pCMV-hph	Neomycin (500) Hygromycin (200)
K562/WT-CIITA	pWT-CIITA	Neomycin (500)
K562/WT-CIITA/LFA-1	pWT-CIITA pCDB1, pCDL1, pCMV-hph	Neomycin (500) Hygromycin (200)
K562/DC-SIGN/WT-CIITA	pBABE-DC-SIGN pWT-CIITA	Puromycin (1) Neomycin (500)
K562/DC-SIGN/WT-CIITA /LFA-1	pMX-DC-SIGN, pWT-CIITA, pCDB1, pCDL1, pCMV-hph	Neomycin (500) Hygromycin (200)

<sup>a</sup> Stable cell lines were transduced with murine leukemia virus (MLV) vectors [44] or stably transfected with other constructs. After vector transduction or selection for antibiotic resistance, cells were subjected to FACS to enrich desired populations. <sup>b</sup> Construct information is provided in the Methods section. <sup>c</sup> NA, not applicable.

SIGN cells and only 10-fold better than the K562 parental line. Using a smaller virus inoculum, we next tested whether the K562/DC-SIGN cells would enhance transmission of HIV-Luc/ADA that remained in co-culture with the CD4+ T cells. Compared with DC-SIGN-negative parental cells, Raji cells expressing moderate levels of DC-SIGN enhanced HIV-1 infection of T cells 10-fold (Figure 2E). In contrast, K562 cells expressing similar levels of DC-SIGN enhanced HIV-1 infection 3-fold under the same conditions. Similar results were observed when HIV-1 pseudotyped with simian immunodeficiency virus (SIV), X4-tropic HIV-1, or different R5-tropic HIV-1 Env proteins were used in transmission assays (data not shown). DMHT could be blocked by pre-incubation of donor cells either with monoclonal antibody against DC-SIGN or with mannan, a soluble ligand of C-type lectins (Figure 2D,2E).

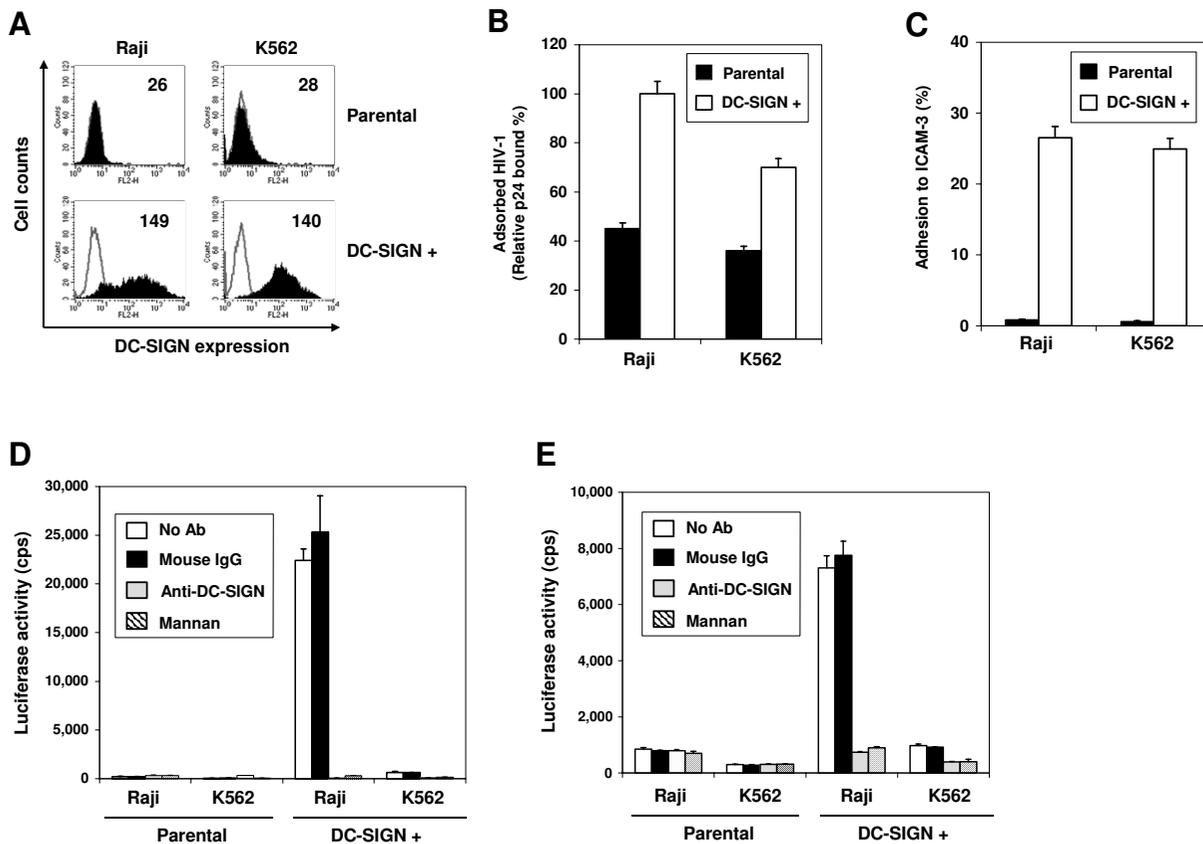
#### **Examination of immune synapse molecules in DMHT**

Our findings reinforced the notion that DMHT is cell type dependent, implying that cell-specific factors account for differences in HIV-1 transmission by different donor cells. We thus screened for differential K562 surface expression of immune synapse and cell adhesion molecules common to immature MDDCs and Raji cells (Table 2). Because cell contact is required for DMHT, we reasoned that the lack of such factors on K562/DC-SIGN cells could impair the efficient transmission of HIV-1. Surveyed ligands included the MHC class I and II complexes involved in antigen presentation; intercellular adhesion molecules and their cognate ligands, such as ICAM-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50), LFA-1 (CD11a/CD18), LFA-3; and molecules involved in T cell activation, such as B7-1 (CD80) and B7-2 (CD86). We also assayed for the presence of HIV-1 receptors on the different donor cell types.

Staining and flow cytometric analysis of surface expression of these molecules is summarized in Table 2. Notably, immature MDDCs and Raji/DC-SIGN cells, which transmit HIV-1 efficiently, expressed similar levels of MHC class II (HLA-DR, -DP and -DQ), and LFA-1. In contrast, neither LFA-1 nor MHC class II molecules were expressed by K562 cells. As expected, Raji and K562 cells did not express CD4 or CCR5, ligands that could compete for interaction with the HIV-1<sub>ADA</sub> Env.

The MHC class II transactivator (CIITA) is a master regulator of the class II locus as well as related proteins that influence MHC class II sorting [35]. Expression of CIITA is sufficient to reconstitute the MHC class II presentation pathway in different cell types. In addition, dominant-negative (DN) versions of CIITA have been developed that are capable of suppressing expression of CIITA-regulated genes in APCs [35]. We took advantage of these tools to manipulate expression of MHC class II molecules in Raji/DC-SIGN and K562/DC-SIGN cell populations.

After stable transfection of K562 and K562/DC-SIGN cells with wild-type (WT) CIITA, MHC class II-positive cells were detected and enriched via FACS (Figure 3A). In contrast, stable transfection of DN-CIITA in Raji and Raji/DC-SIGN cells yielded cells with significantly impaired expression of MHC class II molecules that were further enriched by FACS (Figure 3B). Antibody staining for DC-SIGN and HLA-DR expression confirmed that greater than 95% of K562/DC-SIGN/WT-CIITA cells were double-positive for both molecules, and 93% of the DC-SIGN-positive Raji/DC-SIGN/DN-CIITA cells were largely negative for expression of MHC class II.



**Figure 2**

**Variable efficiency of HIV-1 transmission mediated by different DC-SIGN-expressing cell lines** (A) DC-SIGN expression in Raji and K562 cell lines. Parental cells and DC-SIGN transfectants were stained with MAb against DC-SIGN as described [5]. On all histograms, the gray curve represents staining with an isotype control antibody, whereas the filled black curve represents DC-SIGN MAb staining. The mean fluorescence intensity of DC-SIGN staining is shown in the inset of the histograms. One representative experiment out of three is shown. Cells maintained consistent DC-SIGN expression levels throughout the analyses. (B) Adhesion of HIV-1 to DC-SIGN-expressing cells. Cells were incubated with pseudotyped HIV-Luc/ADA containing 20 ng of CA-p24 for 2 h at 37°C, washed extensively, lysed with 0.5% Triton X-100, and quantified with p24 ELISA kits. HIV-1 adsorbed by Raji/DC-SIGN cells was normalized as 100% (170 pg of recovered CA-p24 in this experiment). The relative percentage of adsorbed p24 was the average of three separate samples. One representative experiment of six is shown. (C) Adhesion of ICAM-3 to DC-SIGN-expressing cells. The percentage of the cells bound to ICAM-3 was measured by flow cytometry using a fluorescent bead adhesion assay as described [5]. Adhesion of ICAM-3 to DC-SIGN-negative parental cells was less than 2%. Mouse IgG represents an isotype control antibody. One representative experiment of three is shown. (D) Capture and transmission of HIV-Luc/ADA by different donor cells. Donor cells pulsed with HIV-1 ( $1 \times 10^5$  IU) were washed before coculturing with Hut/CCR5 target cells as described for Figure 1. DC-SIGN-negative parental cells were used as controls. Donor cells were preincubated with either mannan (20  $\mu$ g/ml) or MAb against DC-SIGN (10  $\mu$ g/ml), respectively, before virus addition. Mouse IgG (10  $\mu$ g/ml) was used as a control antibody. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown. cps, counts per second. (E) DC-SIGN enhancement of *trans*-infection by HIV-Luc/ADA. Donor cells pulsed with HIV-1 ( $1 \times 10^4$  IU) were cocultured with Hut/CCR5 target cells without removing unbound virus present in the culture medium. DC-SIGN-negative parental cells were used as controls. Donor cells were preincubated with either mannan (20  $\mu$ g/ml) or MAb against DC-SIGN (10  $\mu$ g/ml), respectively, before virus addition. Mouse IgG (10  $\mu$ g/ml) was used as a control antibody. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown. cps, counts per second.

**Table 2: Expression of HIV-1 receptors and adhesion molecules on donor cells.<sup>a</sup>**

Surface ligands	Dendritic cells <sup>b</sup>	Raji/ DC-SIGN	K562/ DC-SIGN
CD4	+	-	-
CCR5	+	-	-
CXCR4	+	+	+
Duffy antigen/receptor for chemokines (DARC)	ND	-	+/-
HLA-I (MHC-I)	++	++	+
HLA-DR (MHC-II) <sup>c</sup>	++	++	-
ICAM-1 (CD54)	+	+	+
ICAM-2 (CD102)	+	+	+
ICAM-3 (CD50)	+	-	-
LFA-1 (CD11a/CD18)	+	+	-
LFA-3 (CD58)	+	+	+
B7-1 (CD80)	+	+	+
B7-2 (CD86)	+	+	+

<sup>a</sup> Proteins expressed on the surface of the donor cells were stained and analyzed by FACS. Isotype-matched mouse IgG controls were also examined. Cell population expression levels indicated as ++, +, and +/- demonstrated a mean fluorescence of antibody staining greater than 50, 10, and 7.5, respectively. No antibody and isotype control stainings typically displayed a mean fluorescence intensity of 5 in the FL-2 channel, using a Becton-Dickinson FACSCalibur instrument. ND, not done. Staining of parental Raji and K562 cells was uniformly negative for DC-SIGN and showed a similar expression pattern of these ligands (data not shown). <sup>b</sup> Immature monocyte-derived dendritic cells were stained at day 7 of culture in the presence of cytokines [5]. <sup>c</sup> HLA-DP and -DQ expression was also examined and results were similar to what is shown for HLA-DR.

To assess the role of LFA-1 in DMHT, we generated K562/LFA-1, K562/DC-SIGN/LFA-1, and K562/DC-SIGN/WT-CIITA/LFA-1 cell lines. Comparison of the surface expression of DC-SIGN, HLA-DR, and LFA-1 on these cells is shown in Figure 3C. DC-SIGN expression in the K562-derived cells was similar or even higher than that in Raji/DC-SIGN cells. In addition, three-color staining and FACS analysis of sorted K562/DC-SIGN/WT-CIITA/LFA-1 cells also confirmed that more than 96% of cells were triple-positive for DC-SIGN, HLA-DR, and LFA-1 (data not shown).

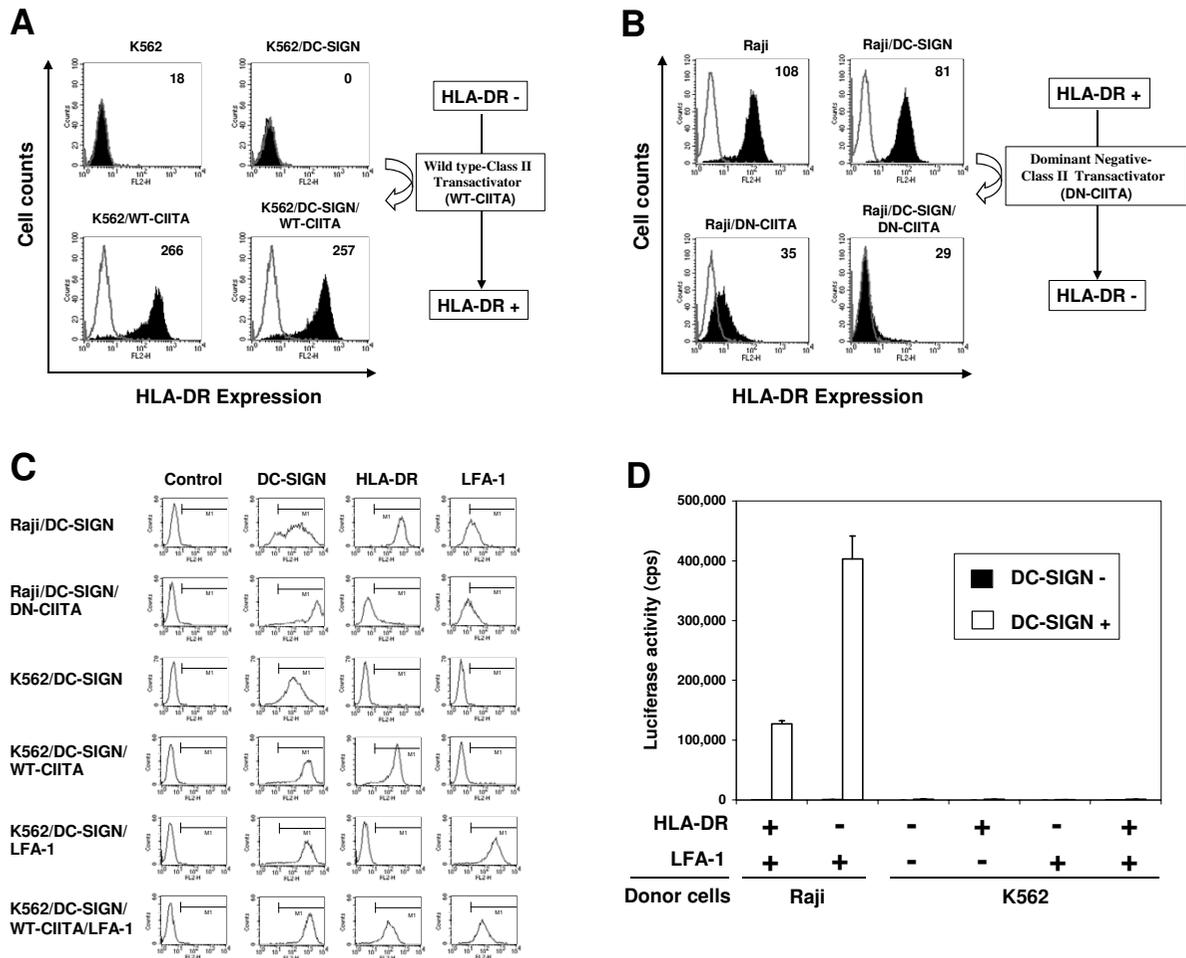
The MHC class II- and LFA-1-manipulated donor cells were next tested for their ability to transmit DC-SIGN-captured HIV-1. Neither induction nor repression of MHC class II expression in K562/DC-SIGN/WT-CIITA cells or Raji/DC-SIGN/DN-CIITA cells, respectively, increased or reduced the efficiency of HIV-1 transmission significantly (Figure 3D). In fact, HIV-1 transmission mediated by Raji/DC-SIGN/DN-CIITA cells was somewhat higher than that mediated by Raji/DC-SIGN cells, potentially due to increased DC-SIGN expression levels (Figure 3C). In addition, expression of LFA-1 in the different DC-SIGN-expressing K562 transfectants did not enhance DMHT (Figure 3D). In contrast, Raji/DC-SIGN cells with equal or lower levels of DC-SIGN relative to K562/DC-SIGN lines transmitted HIV-1 efficiently (Figure 3D).

#### **Trans inhibition of DMHT by K562 cells**

To determine whether K562 cells express negative factors that impair DMHT or lack positive factors necessary for DMHT, we sought to make fusions with K562/DC-SIGN

and Raji cells to test in HIV-1 transmission assays. We first attempted to make cell-cell fusions through a hybridoma protocol that used polyethylene glycol (PEG)-3000. However, this method resulted in a low proportion of cell fusions that had a transient cell culture life. Because DMHT can occur in less than a few hours, we were curious what fraction of DMHT-permissive cells could be detected under conditions simulating a cell fusion experiment. We observed that under conditions where Raji/DC-SIGN cells were 10% or lower in a mixed population with K562 cells, DMHT was inefficient and comparable to the level of DMHT by a uniform K562/DC-SIGN population (data not shown). We next assayed whether HIV-1 transmission could be detected when Raji/DC-SIGN or Raji cells were mixed in equal proportion with K562/DC-SIGN or K562 lines. Strikingly, we found that HIV-1 transmission by Raji/DC-SIGN cells was strongly inhibited in the presence of K562 cells (Figure 4A). This effect was observed irrespective of whether the K562 cells were added before or after HIV-Luc/ADA adsorption to Raji/DC-SIGN cells (data not shown). In contrast, cocultured Raji cells did not significantly affect HIV-1 transmission by Raji/DC-SIGN or K562/DC-SIGN cells (Figure 4A). Compared with the Raji and Raji/DC-SIGN mixture, HIV-1 transmission was 14-fold reduced by the K562 and Raji/DC-SIGN mixture.

To examine whether the K562 cells were exerting a negative effect on the HIV-1 susceptibility of Hut/CCR5 target cells, Hut/CCR5 cells alone or mixed with an equal amount of Raji or K562 cells were infected with HIV-Luc/ADA. Co-culture with K562 cells did not result in any



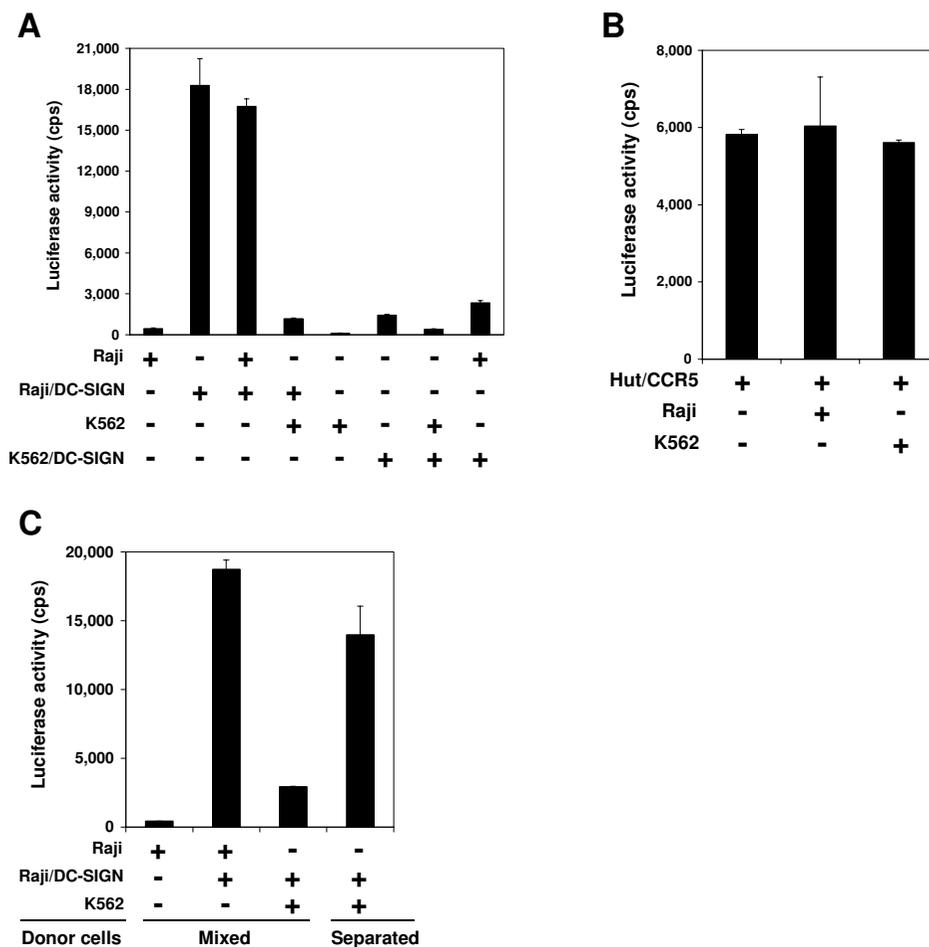
**Figure 3**

**Manipulation of MHC class II and LFA-1 expression does not affect DMHT HLA-DR surface expression of (A) K562 and K562/DC-SIGN cells that were transfected with the construct pWT-CIITA compared with (B) Raji and Raji/DC-SIGN cells that were transfected with the construct pDN-CIITA (300-1130). On all histograms, the gray curve represents staining with an isotype control antibody, whereas the filled black curve represents HLA-DR MAb staining. The mean fluorescence intensity is shown in the inset of the histograms. (C) Expression of DC-SIGN, HLA-DR, and LFA-1 in donor cells. Raji/DC-SIGN and K562/DC-SIGN cells that co-express either HLA-DR, LFA-1, or both were singly stained with PE-conjugated isotype control Ab or MAb against DC-SIGN, HLA-DR, or LFA-1. Antibody staining (FL2) is depicted by the histogram plots along the x axis. (D) Transmission of HIV-Luc/ADA by DC-SIGN-expressing donor cells that were manipulated for HLA-DR and LFA-1 expression. The HIV-1 capture and transmission assay was performed as described for Figure 1. Hut/CCR5 cells were used as targets and DC-SIGN-negative parental cells were used as controls. +, positive expression; -, negative. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown. cps, counts per second.**

detectable inhibition of HIV-1 infection of Hut/CCR5 cells (Figure 4B).

To determine whether the *trans* inhibition of HIV-1 transmission was dependent on direct interactions between K562 and Raji/DC-SIGN cells, we assayed HIV-1 transmission efficiency when the K562 cells were separated from Raji/DC-SIGN cells by a transwell membrane. As expected, K562 cells were able to significantly diminish

Raji/DC-SIGN-mediated HIV-1 transmission when these cells were cultured in the same compartment with Hut/CCR5 target cells (Figure 4C, "mixed" donor cells). However, when K562 cells were placed on the top of the permeable membrane to separate them from cocultured Raji/DC-SIGN and Hut/CCR5 cells on the bottom, no significant inhibition of HIV-1 transmission was observed (Figure 4C, "separated" donor cells).



**Figure 4**

**Trans-dominant cellular inhibition of DC-SIGN transmission of HIV-1** (A) Transmission of HIV-1 by Raji/DC-SIGN cells was inhibited in the presence of K562 cells. Donor cells alone ( $1.25 \times 10^5$ ) or two mixed types of donor cells ( $1.25 \times 10^5$  for each, 1:1 ratio) as indicated were incubated with HIV-Luc/ADA. The HIV-1 capture and transmission assay using transwell plates was performed as described for Figure 1. Hut/CCR5 cells were used as targets and DC-SIGN-negative parental cells were used as controls. +, K562 cells present; -, absent. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown. cps, counts per second. (B) Direct infection of Hut/CCR5 targets cells with HIV-Luc/ADA in the presence of the Raji or K562 cells. Hut/CCR5 cells alone ( $1 \times 10^5$ ) or mixed with Raji or K562 cells ( $1 \times 10^5$  for each, 1:1 ratio) were incubated with HIV-Luc/ADA ( $2.5 \times 10^4$  IU) for 2 h at 37°C, washed with PBS, then cultured 2 days before lysis. One representative experiment out of two is shown. (C) *Trans* inhibition of HIV-1 transmission mediated by Raji/DC-SIGN cells requires contact with K562 cells. The HIV-1 capture and transmission assay using the transwell plates was performed as described for Figure 1. The different types of donor cells were either mixed or separated by the permeable membrane as illustrated. DC-SIGN-negative parental cells were used as controls. Hut/CCR5 cells were used as target cells, which were co-cultured with donor cells below the membrane inserts. K562 cells were placed on top of the membrane inserts in the separated donor cell group. +, K562 cells present; -, absent. Each data set represents the mean of three separate wells of infected cells. One representative experiment out of three is shown.

## Discussion

Here we show that DMHT requires cell-cell contact and is supported by restricted cell types. These findings reinforce the idea that the cellular environment is an important factor when examining transmission of HIV-1 captured by DC-SIGN [21]. In addition, we observe that MHC class II molecules are not required for efficient DMHT, suggesting that virus transmission can occur in the absence of the classically defined immune synapse. Despite similar levels of DC-SIGN expression, K562 transfectants were markedly less efficient in the transmission of HIV-1 when compared with Raji cell transfectants. A previous study has implicated cell type differences in the protection and transmission of HIV-1 by THP-1, 293T, and HOS cells transfectants expressing DC-SIGN [11]. It is likely that the cells identified as "THP-1" in this prior study were actually Raji-derived cells, as THP-1 cells generally do not support DMHT [21]. In contrast to our study, these authors did not observe cell type differences in DMHT of newly captured HIV-1. Instead, cell type differences in HIV-1 transmission were only manifested when target cells were added 2 days after virus inoculation of the DC-SIGN-expressing cells. Although it is possible that K562 cells are especially restrictive in DMHT, it is more likely that differences in assay systems precluded a quantitative comparison of DMHT of newly captured HIV-1 by the 293T and HOS cells in the prior study. We have observed that 293T cells expressing DC-SIGN are significantly less efficient than Raji/DC-SIGN cells in the transmission of newly acquired HIV-1 (data not shown). Notably, K562 cell lines have been used to examine DC-SIGN function in a number of studies [22-32]. However, our study represents the first quantitative comparison of DMHT in a single-replication viral cycle between K562 transfectants and other cells. We have observed that K562 cells are not only impaired in DMHT but also that K562 cells can inhibit virus transmission by other cells in a contact-dependent manner. These data provide the first evidence that DC-SIGN transmission of HIV-1 can be regulated in *trans*.

One model to reconcile K562 cell restriction of cell-cell HIV-1 transmission when DC-SIGN is expressed either in *cis* or in *trans* is that K562 cells express a cell surface molecule that hinders DMHT. It is unlikely that such a molecule competes for occupancy in the DC-SIGN carbohydrate recognition domain, as HIV-1 particles and ICAM-3 bound K562/DC-SIGN and Raji/DC-SIGN cells at comparable efficiencies. In addition, K562 cells did not have a direct detrimental effect on the infectivity of cell-free HIV-1 or on the susceptibility of Hut/CCR5 target cells. Thus, it is more likely that a K562 cell-expressed surface factor influences the fate of DC-SIGN-bound HIV-1 particles or interferes with the formation of an infectious synapse with virus target cells. K562 cells might compete with Hut/CCR5 cells for interaction with the Raji/DC-

SIGN donor lines, preventing synaptic transmission of HIV-1 to the Hut/CCR5 cells. Alternatively, the K562 cells might induce the Raji/DC-SIGN cells to traffic HIV-1 to a degradative compartment within the cells, preventing virus transmission. It is unclear whether negative regulation of DMHT by cells in *trans* is unique to K562 cells or extends to other cell types, including primary cells. Given the presumed erythrocytic origin of K562 cells, it will be interesting to examine the effect of blood erythrocytes on MDDC-mediated HIV-1 transmission. Notably, we observed that K562 cells express a low level of Duffy antigen/receptor for chemokines (DARC), a promiscuous chemokine receptor that may interact with HIV-1 Env [36].

The requirements for DMHT subsequent to HIV-1 binding have not been fully delineated. Others have reported that differentiation of human DCs toward different effector subsets creates cells with different abilities to stimulate HIV-1 replication despite equal levels of DC-SIGN expression [18]. This study had indicated that ICAM-1 expression on DCs might predict the efficiency of HIV-1 transmission. Prior studies have also indicated that the combined interactions of LFA-1/ICAM-1 and LFA-3/CD2 aid in the efficient HIV-1 replication in cocultured DCs and CD4+ T cells [7,8,18]. Consistent with these studies performed with DCs [8,18], we found that HIV-1 transmission mediated by Raji/DC-SIGN cells was impaired when the direct contacts between donor cells and target cells were obstructed by a permeable membrane. Collectively, these data suggest that cell surface ligands could act as cofactors in DMHT and the formation of an infectious synapse.

A preliminary survey of molecules that are expressed on DCs and Raji/DC-SIGN cells and that are important in establishing contact and communication between APCs and CD4+ T cells revealed two potentially significant candidates that are not expressed in K562/DC-SIGN cells, LFA-1 and MHC class II antigens. To investigate the role of MHC class II antigens in the DC-SIGN transmission of infectious HIV-1, we genetically manipulated donor cell lines to alter their MHC class II expression using WT- and DN-CIITA and functionally tested their efficiency of HIV-1 transmission. We observed that HIV-1 transmission mediated by these cell lines was not significantly affected, and coexpression of LFA-1 and MHC class II molecules in K562/DC-SIGN cells was not sufficient to enable efficient HIV-1 transmission by the modified cells. Because of the dominant-negative effect that K562 cells appear to exert on DMHT, it is difficult to conclude the roles of possible positive factors by using these cells. Reduced MHC class II expression in Raji/DC-SIGN/DN-CIITA cells did not have a negative effect on DMHT. However, because MHC class II expression was not fully silenced in Raji/DC-SIGN/DN-

CIITA cells, these data do not *a priori* exclude a contribution of the MHC class II pathway on DMHT.

Despite the presence of a negative factor on K562 cells, K562 cells that express DC-SIGN did modestly stimulate HIV-1 transmission irrespective of MHC class II or LFA-1 expression levels. This stimulation was significantly less than that observed with Raji/DC-SIGN cells, which transmit HIV-1 or other primate lentiviruses at efficiencies comparable to DCs [1,9]. Similar transmission results comparing K562/DC-SIGN and Raji/DC-SIGN cells were obtained with virus particles bearing different HIV-1 or SIV Env proteins (data not shown). Because K562/DC-SIGN adsorption of HIV-1 particles was comparable to that of Raji/DC-SIGN cells, this increased virus binding was not predictive of HIV-1 transmission efficiency. These results suggest that the DC-SIGN effect on HIV-1 transmission is more complex than simple virus binding and provide support for a model put forward by Pöhlmann and colleagues that DC-SIGN binding and transmission functions are dissociable [37].

The cell type specificity of DC-SIGN function in HIV-1 transmission provides a means to explore this mechanism. There are likely positive as well as negative cellular factors involved in DMHT. The examination of additional cell types, particularly those derived from APCs, and their mechanisms of cell-cell communication and of HIV-1 intracellular trafficking may be useful in identifying features that are required for efficient DC-SIGN-mediated transmission. In addition, understanding the negative regulatory mechanism that cells can exert on DC-SIGN transmission of HIV-1 may facilitate the development of immune-modulating therapies to help prevent the dissemination of HIV-1 by DCs *in vivo*.

## Conclusions

DMHT is cell type dependent and requires contact between virus donor and target cells. K562/DC-SIGN donor cells are inefficient in the transmission of captured HIV-1, and this transmission defect cannot be rescued by enforced expression of immune synapse components. This donor cell defect in supporting DC-SIGN transmission may be regulated in part by negative factors. Strikingly, we find that K562 cells in co-culture can impair Raji/DC-SIGN cell transmission of HIV-1 to CD4+ T cells.

## Methods

### Plasmids

PMX-DC-SIGN and pBABE-DC-SIGN expression constructs containing human DC-SIGN cDNA have been previously described [5]. Human DC-SIGN cDNA obtained from pBABE-DC-SIGN was subcloned into the polylinker of the pcDNA3 expression construct (Invitrogen) between the *Bam*HI and *Eco*RV sites to derive pcDNA3-DC-SIGN.

Constructs encoding the WT- and DN-CIITA truncation mutant (300–1130) [35,38] were gifts from Jenny Ting (University of North Carolina, Chapel Hill). Constructs pCDL1 [39] and pCDB1 [40] encoding the  $\alpha$ L and  $\beta$ 2 subunits of LFA-1, respectively, were kindly provided by Timothy Springer (Harvard Medical School, Boston). The pCMV-hph construct encoding hygromycin selection resistance was a gift from Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle).

### Cells

Hut/CCR5 and GHOST/X4/R5 cell lines have previously been described [5]. Raji B cells used in this study have been previously described as B-THP-1 cells [21]. The human erythroleukemic K562 cell line was purchased from the American Type Culture Collection (ATCC number CCL-243). A panel of stable cell lines generated and used in this study is summarized in Table 1. Stable cell lines derived from parental Raji and K562 cells were subjected to FACS to obtain cells with the desired enhanced or reduced expression levels of specific target molecules. Constructs used for expression of target genes and conditions for selective drugs are also listed (Table 1).

Immature DCs were generated from CD14+ monocyte precursors treated with a cocktail of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) as described [5,41]. One week after differentiation, these cells uniformly expressed high levels of HLA-DR, HLA-I, CD11b, CD11c, DC-SIGN, and ICAM-1; moderate levels of LFA-1 and CD86; and low levels of CD14.

Raji, K562-derived cell lines, and Hut/CCR5 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories) and selective drugs as indicated in Table 1. HEK293T and GHOST/X4/R5 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals). DCs were maintained in OptiMEM medium (Invitrogen) supplemented with 20% FBS (HyClone Laboratories) and specific cytokines as previously described [5].

### Antibodies

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), and tri-color or Cy-chrome-conjugated mouse anti-human MAbs against the following molecules were used: DC-SIGN (clone 120526) and CXCR4 (clone 44717) (R&D Systems); LFA-1 (clone MEM-25), HLA-I (clone TÛ 149), HLA-DR (clone TÛ 36), ICAM-1 (clone MEM-111), ICAM-3 (clone TP1/25.1), CD4 (clone S3.5), and goat anti-mouse immunoglobulin (IgG, Caltag Laboratories); CD80 (clone L307.4), CD86 (clone 2331), HLA-DQ (clone TÛ 169), HLA-DR (clone TÛ 36), LFA-3 (clone

1C3), CCR5 (2D7/CCR5), and Blood Group Fy6 (Duffy, clone NaM185-2C3) (PharMingen); ICAM-2 (clone CBR-IC2/2, Biosource) and HLA-DP (clone B7/21, Leinco Technologies). MAb to HLA-1 identify MHC class I expression, and MAbs to HLA-DP, -DQ, and -DR identify MHC class II expression.

#### **Virus stocks**

Single-round infectious, pseudotyped HIV-1 stocks (HIV-Luc/ADA) were generated by calcium phosphate cotransfections of HEK293T cells with the proviral vector plasmid NL-Luc-E-R<sup>-</sup> (HIV-Luc) containing a firefly luciferase reporter gene [42] and an expression plasmid for the R5-tropic HIV-1<sub>ADA</sub> envelope glycoprotein. Viral stocks were evaluated by limiting dilution on GHOST/X4/R5 cells.

#### **Flow cytometry**

To assess the expression of DC-SIGN or other surface molecules, cells ( $2 \times 10^5$ ) were stained with an MAb directed against the specific antigen and compared with isotype-matched IgG controls. For direct staining, cells were incubated at 4°C in phosphate-buffered saline (PBS) containing 2% FBS (FACS buffer) and 2 µg of FITC- or PE-conjugated MAbs per milliliter in a total volume of 100 µl. For indirect staining, after a 30-min incubation with the first specific MAb at 4°C, the cells were washed with FACS buffer and resuspended in 100 µl of FACS buffer containing 2 µg of FITC- or PE-conjugated antibody against mouse IgG per milliliter. DC-SIGN specific MAbs [5] were obtained from R&D Systems. Cells were incubated for 30 min at 4°C, washed with FACS buffer, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

#### **HIV-1 adhesion assay**

DC-SIGN-expressing Raji and K562 transfectants as well as DC-SIGN-negative parental cells ( $2 \times 10^5$ ) were incubated with pseudotyped HIV-Luc/ADA containing 20 ng of CA-p24 for 2 h at 37°C, and washed extensively with PBS to remove unbound virus. Cells were subsequently lysed with 0.5% Triton X-100 to release adsorbed virus, which was quantified using HIV-1 p24 ELISA kits (Coulter Beckman).

#### **ICAM-3 adhesion assay**

Soluble, recombinant ICAM-3 was obtained from R&D Systems. Carboxylate-modified TransFluorSpheres (1.0 µm, 488 nm excitation/645 nm emission, Molecular Probes) were coated with ICAM-3 as described previously [43]. Adhesion of ICAM-3 to DC-SIGN was determined by measuring the detectable percentage of cells that bound fluorescent beads, using flow cytometry on a FACSCalibur instrument. The fluorescent bead adhesion assay has been described in detail previously [5,9].

#### **HIV-1 infection assays**

HIV-1 capture and transmission assays were performed as described previously [5]. In brief, donor cells ( $2.5 \times 10^5$ ) were incubated with  $1 \times 10^5$  infectious units (IU) of pseudotyped HIV-Luc/ADA in a total volume of 400 µl for 2 h at 37°C to allow adsorption of the virus. After 2 h, cells were washed with 1 ml of PBS and cocultured with Hut/CCR5 target cells ( $1 \times 10^5$ ) in the presence of 10 µg of polybrene in 1 ml of culture medium. Cell lysates were obtained 2 days after infection and analyzed for luciferase activity with a commercially available kit (Promega). HIV-1 enhancement assays were performed using limiting amounts of HIV-1 ( $1 \times 10^4$  IU) during incubation with donor cells, and target cells were added directly to the coculture without removing the virus present in the culture medium. To assay DC-SIGN-blocking agents, cells were preincubated with either mannan (20 µg/ml; Sigma) or MAb against DC-SIGN (10 µg/ml) or mouse IgG control (10 µg/ml) for 30 min at 37°C before virus addition.

To investigate whether cell-cell contact between donor cells and target cells was critical for HIV-1 transmission, transwell cell culture plates with inserts of polycarbonate membranes of 3-µm pore size (Costar) were used in HIV-1 capture and transmission assays to separate donor cells from target cells.

To test mixed donor cells for effects on DMHT, equal amounts ( $1.25 \times 10^5$ , 1:1 ratio) of two donor cell types with or without DC-SIGN were mixed prior to the 2-h incubation of HIV-Luc/ADA ( $1 \times 10^5$  IU) at 37°C. After virus incubation, these cells were washed and cocultured with Hut/CCR5 target cells in the presence of polybrene as described above for the HIV-1 capture and transmission assays. As a control for the susceptibility of Hut/CCR5 cells to direct infection by HIV-1, parental Raji or K562 cells were mixed with Hut/CCR5 cells ( $1 \times 10^5$ , 1:1 ratio), then incubated with HIV-Luc/ADA ( $2.5 \times 10^4$  IU) for 2 h at 37°C, washed with PBS, and cultured in the presence of polybrene (10 µg/ml). Cell lysates were obtained 2 days after infection and analyzed for luciferase activity. Hut/CCR5 cells alone ( $1 \times 10^5$ ) were used as a control.

#### **Authors' contributions**

LW developed most of the cell lines described in this study; performed the HIV-1 adhesion assay, HIV-1 infection assays, and most of the flow cytometric analyses; participated in the study design; and drafted the manuscript. TDM carried out cell sorting, the ICAM-3 adhesion assay, and some of the flow cytometric analyses. YCH assisted in generating some of the cell lines and participated in some of the HIV-1 adhesion and infection assays. SKJB generated the pcDNA3-DC-SIGN construct, generated K562 cells transduced with pBABE-DC-SIGN and pcDNA3-DC-SIGN, and participated in the optimization of the ICAM-

3 adhesion assay. VNK conceived the study and formulated the experimental design; coordinated the study; and revised the manuscript. All authors read and approved the final manuscript.

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