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Efficient Capture of Antibody Neutralized HIV-1 by Cells Expressing DC-SIGN and Transfer to CD4$^+$ T Lymphocytes[1,2]

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Infection of CD4$^+$ T lymphocytes is enhanced by the capture and subsequent transfer of HIV-1 by dendritic cells (DCs) via the interaction with C-type lectins such as the DC-specific ICAM-grabbing nonintegrin (DC-SIGN). Numerous HIV-1 envelope-directed neutralizing Abs have been shown to successfully block the infection of CD4$^+$ T lymphocytes. In this study, we find that HIV-1-neutralized with the mAb 2F5 is more efficiently captured by immature monocyte-derived DCs (iMDDCs) and DC-SIGN-expressing Raji cells (Raji-DC-SIGN). Furthermore, a 2F5-neutralized virus captured by these cells was able to subsequently infect CD4$^+$ T lymphocytes upon the release of HIV-1 from iMDDCs, thereby enhancing infection. We show that upon transfer via DC-SIGN-expressing cells, HIV-1 is released from immune-complexes with the Abs 2F5 and 4E10 (gp41-directed) and 2G12, 4.8D, and 1.7b (gp120-directed). The nonneutralizing V3-21 (V3 region of the gp120-directed) Ab enhanced HIV-1 infection upon capture and transfer via Raji-DC-SIGN cells, whereas no infection was observed with the neutralizing b12 Ab (gp120-directed), indicating that different Abs have variant effects on inhibiting HIV-1 transfer to CD4$^+$ T lymphocytes. The increased capture of the 2F5-neutralized virus by iMDDCs was negated upon blocking the Fc receptors. Blocking DC-SIGN on iMDDCs resulted in a 70–75% inhibition of HIV-1 capture at 37°C, whereas at 4°C a full block was observed, showing that the observed transfer is mediated via DC-SIGN. Taken together, we propose that DC-SIGN-mediated capture of neutralized HIV-1 by iMDDCs has the potential to induce immune evasion from the neutralization effects of HIV-1 Abs, with implications for HIV-1 pathogenesis and vaccine development. ***The Journal of Immunology,*** **2007, 178: 3177–3185.**

Dendritic cells (DCs)[5] have been implicated in the transmission of HIV-1 following sexual exposure (1, 2). Immature DCs (iDCs) are believed to capture the virus that crosses the mucosal surface and transport HIV-1 to replication-competent sites in lymphoid tissues where, upon maturation to a mature DCs, the virus can be transferred to CD4$^+$ T lymphocytes (3, 4). DCs can transmit HIV-1 to CD4$^+$ T lymphocytes in a biphasic mode: 1) capture and release of HIV-1 by DCs, which occurs within a few hours with or without internalization; and 2) infection of the DC resulting in de novo production of the virus, which takes a few days and is zidovudine sensitive (5). Importantly, a small number of HIV-1 particles captured by DCs can survive degradation for several days in vitro and can be released as an infectious virus, whereas a free virus loses infectivity rapidly (6–8). The transfer of HIV-1 from DCs to CD4$^+$ T lymphocytes occurs in the infectious synapse, where HIV-1 is recruited to the contact zone with the T cell. Formation of this synapse results in a high concentration of HIV-1 on the DC side, whereas HIV-1 receptors like CD4 and CCR5 are partially enriched on the T cell side (9, 10). This mode of transmission is believed to be the mediator of enhanced viral replication in DC-T cell clusters.

A number of C-type lectins expressed on DCs have been shown to efficiently capture HIV-1 and enhance the viral infectivity of CD4$^+$ T lymphocytes (6). The most potent receptor to capture HIV-1 by iDCs is the C-type lectin DC-specific ICAM-grabbing nonintegrin (DC-SIGN), although the in vivo implications of such an interaction has remained controversial. Importantly, formation of the infectious synapse is impaired in DCs in which DC-SIGN expression has been knocked down, resulting in the severely impaired transmission of HIV-1 (11). The expression of DC-SIGN on immature monocyte-derived DCs (iMDDCs) is strongly induced upon IL-4 stimulation, whereas other cytokines such as IFN-α, IFN-γ, and TGF-β down-regulate DC-SIGN expression (12). The expression of DC-SIGN, however, is not essential for HIV-1 capture by these cells and other receptors may be involved, because non-IL-4 treated iMDDCs with low DC-SIGN expression are able to establish virus replication on CD4$^+$ T lymphocytes upon transmission (13). DC-SIGN expression down-regulated through small interfering RNA (siRNA) treatment of DCs only moderately inhibited HIV-1 capture (13), again suggesting that DC-SIGN is not required for the capture and transmission of HIV-1 to CD4$^+$ T lymphocytes and that other receptors could mediate these effects.

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[2] T.v.M. designed and performed the experiments, A.A.N. designed experiments and supplied dendritic cells, T.G. and G.P. contributed vital reagents, and W.A.P. supervised the study and participated in writing of the paper.

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[5] Abbreviations used in this paper: DC, dendritic cell; DC-SIGN, DC-specific ICAM-grabbing nonintegrin; iDC, immature DC; iMDDC, immature monocyte-derived DC; IC, immune-complex; siRNA, small interfering RNA; $TCID_{50}$, 50% tissue culture infectious dose.

Neutralizing Abs directed against specific epitopes on the envelope gp120 and gp41 are likely to be the first agents to block HIV-1 infection and are therefore important for vaccine purposes. Abs against HIV-1 block the viral attachment to HIV-1 receptors or inhibit the membrane fusion required for replication in $CD4^+$ T lymphocytes and iDCs (14–16). Nevertheless, the efficacy of Ab responses blocking HIV-1 infection in patients appears to be limited, because most Abs only partially block HIV-1 infection or are otherwise nonneutralizing. Furthermore, HIV-1 has been shown to rapidly escape Ab neutralization (17). Only a few mAbs against gp41 (2F5 and 4E10) (18–22) or gp120 (2G12 and IgG1b12) (23, 24), have been shown to efficiently protect HIV-1 infection in vitro and animal models in vivo (23, 25). A study of six acutely and eight chronically infected patients demonstrated a strong repression of viral load after passive immunization with the 2F5, 4E10, and 2G12 mAbs; however, viral rebound was observed within 12 wk in most patients. No change in viral sensitivity was found for 2F5 and 4E10, whereas insensitive strains were found for 2G12, suggesting that this Ab provided a selection pressure on virus replication (26).

Follicular DCs can transfer Ab-neutralized HIV-1 to $CD4^+$ T lymphocytes and initiate HIV-1 replication by an unknown mechanism (27). In this work we have studied the capture and transfer of neutralized HIV-1 by DCs and a Raji cell line stably transfected with DC-SIGN (Raji-DC-SIGN). We show that specific mAbs in immune complexes (ICs) with HIV-1 can enhance capture by iMDDCs and Raji-DC-SIGN cells. We demonstrate that DC-SIGN is responsible for the efficient capture of HIV-1 within complexes and that FcγRs can increase capture by DC-SIGN. Furthermore, we demonstrate that HIV-1 neutralized by mAbs and captured by DC-SIGN regains its infectivity when transferred to $CD4^+$ T lymphocytes, although HIV-1 can be directly reneutralized by Abs after release from the DC-SIGN-expressing cell. These results indicate that the interaction of HIV-1 through C-type lectins such as DC-SIGN can result in immune evasion from HIV-1-neutralizing Abs.

Materials and Methods

Antibodies and reagents

The mouse DC-SIGN-specific mAb AZN-D1 was purified and used to block HIV-1 from interacting with DC-SIGN. The mouse mAb anti-human FcγR I clone10.1, FcγR II clone 3D3, and FcγR III clone 3G8 (BD Pharmingen) were used to block the FcγRs. To control Ab specificity, the IgG1 isotype mouse mAb (ITK Diagnostics) was used. All mAbs were used at 20 μg/ml. The human mAbs 2F5 and 4E10 directed against the HIV-1 envelope gp41, the mAbs 2G12, 4.8D, 1.7b, and b12 directed against gp120, and the mAb V3-21 directed against the V3 loop of gp120 were obtained from the National Institute for Biological Standards and Control (Potters Bar, U.K.) and used at 20 μg/ml. Human sera obtained from a patient with an IgG1 B cell lymphoma was used as an HIV-1-nonspecific control Ab; the sera contained IgG1-λ M protein at 83 mg/ml and no detectable IgA or IgM (<0.06 mg/ml). Sera were obtained from four HIV-1-negative donors and four HIV-1 subtype B-positive individuals from the Amsterdam cohort studies. Indinavir (obtained from National Institute for Biological Standards and Control) was used at 1 μM to block HIV-1 reverse transcription and replication. Secondary Ab AffiniPure donkey anti-mouse-Cy5 was obtained from Jackson ImmunoResearch Laboratories and used at a 200-fold dilution. PE-labeled DC-SIGN, CD3-labeled allophycocyanin (BD Pharmingen), and CA-p24 FITC were used for FACS analyses at 100-, 50-, and 200-fold dilutions, respectively.

Cells

The Raji cell line and the Raji cell line expressing DC-SIGN (Raji-DC-SIGN) were generated and used as previously described (28). Both cell lines were cultured in RPMI 1640 medium containing 10% FCS and penicillin and streptomycin, both at 100 U/ml. DC-SIGN expression by Raji-DC-SIGN cells was positively selected with neomycin (2 μg/ml) and routinely monitored by FACS analysis using the PE-labeled DC-SIGN Ab. The iMDDCs were prepared as previously described (29). In short, human blood monocytes were isolated from buffy coats by use of a Ficoll gradient and a subsequent CD14 selection step using the MACS system (Miltenyi Biotec). Purified monocytes were differentiated into iMDDCs in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough). On day 6, the phenotype of the cultured iMDDCs was confirmed by flow cytometry. The cells expressed high levels of MHC class I and II molecules, CD11b, CD11c, and ICAM-1 and low levels of CD80 and CD86. PBMCs were isolated from fresh buffy coats by standard Ficoll-Hypaque density centrifugation and checked by PCR screening for the CCR5 Δ32 deletion. PBMCs from three donors were pooled, frozen in multiple vials, and, when required, thawed and activated with PHA at 2 μg/ml and cultured in RPMI 1640 supplemented with rIL-2 at 100 U/ml. On day 3 of culture, $CD4^+$ T lymphocytes were enriched by the depletion of $CD8^+$ T lymphocytes using CD8 immunomagnetic beads (Dynal) and the cells were cultured for 2 days in RPMI 1640 with rIL-2.

Virus

The molecular cloned dual-tropic HIV-1 (5′X.10ΔgV3) consisting of the Lai backbone with the HXB2 envelope was produced on C33A cells (30). Mutations in the HXB2 envelope were made within the V1V2 and V3 regions according to mutations identified in the envelope of a patient from the Amsterdam cohort studies (ACH168) (30). The virus stock was assayed for tissue culture infectious dose ($TCID_{50}$) on enriched $CD4^+$ T lymphocytes. A GFP-fluorescent variant of HIV-1 (HIV-1-GPF) was produced by transfecting C33A cells with a GFP-VPR plasmid and the 5′X.10ΔgV3 backbone in a 1:1 ratio. The virus produced was collected from the supernatant three days after transfection and concentrated with Amicon Ultra filters (100,000 molecular weight cutoff). The concentration of viral capsid (CA-p24) was determined with ELISA and HIV-1-GFP was frozen in aliquots.

Virus neutralization and capture

HIV-1 or HIV-1-GPF was incubated with 20 μg/ml neutralizing mAbs for 1 h. The control virus was incubated with medium or 20 μg/ml non-HIV-1-pecific human IgG1 Ab to control either volume or Ab specificity. Subsequently, iMDDCs, Raji-DC-SIGN, or Raji cells were incubated with the HIV-1 mixture for 2 h and the unbound virus was removed by washing three times with PBS or medium.

HIV-1 capture

Confocal analyses of virus capture at 37°C. Neutralized or control HIV-1-GFP at 100 ng/ml was captured by Raji, Raji-DC-SIGN, or iMDDCs for 2 h at 37°C. Washed cells were seeded on pretreated poly-L-lysine coated coverslips (1 mg/ml). Cells were fixed after 20 min in 3.7% paraformaldehyde, permeabilized with 0.1% saponin, 10 mM NH_4Cl, and 2% BSA in PBS for 30 min, and stained for DC-SIGN with the AZN-D1 mAb and the Cy5-coupled donkey anti-mouse secondary Ab. Nuclear DNA was stained with Hoechst 33258. Excess Ab was removed by washing twice with permeabilization buffer, once with PBS, and twice with H_2O. Cells were embedded in Vectashield and fluorescence was analyzed by confocal microscopy. Virus particles/cell were counted and plotted in column bars or scatters as medians ± SEM.

Confocal analyses of virus capture at 4°C. iMDDCs were pretreated with Abs against FcγRI, FcγRII, FcγRIII, or AZN-D1 (all at 20 μg/ml) for 30 min at 37°C and used to capture 100 ng/ml neutralized or control HIV-1-GFP at 4°C for 1 h. Washed cells were seeded on coverslips and stained for DC-SIGN and nuclei, and fluorescence was analyzed with confocal microscopy.

Intracellular CA-p24 ELISA

Preneutralized or control HIV-1 at 100 ng/ml was captured by 1.0×10^6 Raji, Raji-DC-SIGN, or iMDDCs for 2 h at 37°C. Cells were lysed at 56°C in 1% Empigen for 1 h. The cell debris was pelleted and the supernatant was analyzed for CA-p24 in quadruplicate.

Confocal analyses

Fluorescent images were made with a Leica DM SP2 AOBS confocal microscope with a ×63 HCX PL APO 1.32 oil objective. Images (512 × 512) with a pixel size of 232 nm and a step size of 340 nm were acquired with the Leica confocal imaging processing software with a line average of four scans and were depicted as maximum intensity projections.

$CD4^+$ T lymphocyte infection

PHA-activated $CD4^+$ T lymphocytes (1.5×10^5 per well) were cocultured with Raji, Raji-DC-SIGN, or iMDDCs treated with or without AZN-D1 or

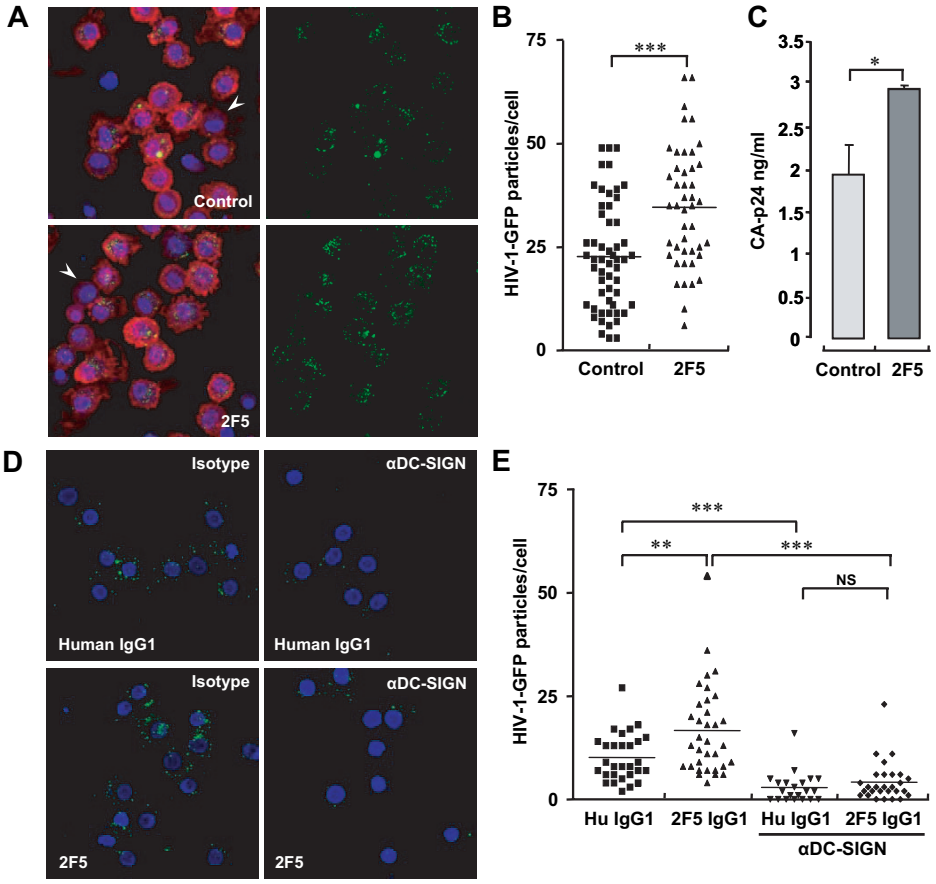

FIGURE 1. Capture of 2F5-neutralized virus by iMDDCs. *A*, iMDDCs were inoculated with 2F5-preneutralized or nonneutralized HIV-1-GFP (green). iMDDCs were stained for DC-SIGN (red) and nuclear DNA (blue). Three-dimensional images were made with confocal microscopy and layers were plotted in one picture. *Right panels* show overlay of virus capture, DC-SIGN, and nuclei staining. *Upper panel* shows the control virus and *lower panel* shows the 2F5-treated virus. White arrows indicate DCs with low DC-SIGN expression and low virus capture. *B*, Analyses of the number of HIV-1-GFP particles per cell ($n = 50$) from *A*. *C*, 2F5-neutralized or nonneutralized HIV-1 captured by iMDDCs was measured by intracellular CA-p24 ELISA. *D*, capture of HIV-1 pretreated with 2F5 or non-HIV-1-specific human IgG1 by iMDDCs. *Right panel*s, iMDDCs treated with AZN-D1 Ab to block DC-SIGN (αDC-SIGN); *left panels*, iMDDCs treated with matched isotype control. *E*, Analyses of the number of HIV-1 particles/cell ($n = 40$) from *D*. Statistical analyses on data were performed with the Student *t* test, and the data represent three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$. Hu, Human.

$CD4^+$ T lymphocytes as control (0.3×10^5/well) and inoculated with a mixture of control or mAb preneutralized HIV-1 (200 $TCID_{50}$). The medium was removed after 48 h and cells were cultured in fresh RPMI 1640 containing rIL-2 (2 μg/ml) and indinavir (1 μM) for 3 days. Cells were washed three times with cold PBS and fixed in 3.7% PFA and 0.1 M PIPES in PBS for 20 min. Fixative was quenched with 20 mM glycine in PBS for 10 min and cells were permeabilized in 0.1% saponin and 2% BSA in PBS for 30 min and subsequently stained with FITC-labeled CA-p24 and CD3-labeled APC for 1 h. Excess Ab was removed by a double wash with permeabilization buffer and once with PBS supplemented with 2% BSA. Subsequently, cells were resuspended and maintained in PBS and analyzed by FACS cytometry. Cells positive for CA-p24 and CD3 were plotted as medians of triplicates ± SD.

Transfer of R5X4 HIV-1 to $CD4^+$ T lymphocytes

Transfer of captured neutralized virus at 37°C. Control or preneutralized HIV-1 (200 $TCID_{50}$) with a 2F5-, 4E10-, 2G12-, 4.8D-, b12-, or V3-21-directed envelope Ab, captured by 1.0×10^5 iMDDCs or Raji-DC-SIGN cells for 2 h at 37°C, were cocultured with 1.5×10^5 $CD4^+$ T lymphocytes in a 96-well plate in triplicate. The medium was removed after 48 h and the cells were cultured in fresh RPMI 1640 containing rIL-2 (2 μg/ml) and indinavir (1 μM) for 3 days. $CD4^+$ T lymphocyte infection was measured by following intracellular CA-p24 expression with FACS flow cytometry.

Transfer of captured neutralized virus at 4°C. iMDDCs treated with buffer or Abs against FcγRI, FcγRII, FcγRIII, and or AZN-D1 for 30 min at 37°C were used to capture 100 ng/ml control or preneutralized HIV-1 at 4°C for 1 h. iMDDCs (1.0×10^5) were cocultured with $CD4^+$ T lymphocytes (1.5×10^5) in a 96-well plate in triplicate. The infection of $CD4^+$ T lymphocytes was measured with FACS flow cytometry.

Statistical analysis

Significance was determined with unpaired *t* test (two tailed) and indicated in figures with stars. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

Results

Enhanced capture of 2F5-neutralized virus by iMDDCs

We aimed to identify whether HIV-1, neutralized with the gp41-directed 2F5 mAb, could be captured by iMDDCs. The virus used in our assays efficiently uses both the CCR5 and CXCR4 coreceptor (30) and is fully neutralized with the 2F5 mAb at 20 μg/ml (31). iMDDCs were inoculated with control or HIV-1-GFP preneutralized with 2F5 mAb. Nonneutralized as well as 2F5-preneutralized HIV-1-GFP could be visualized below the cell membrane stained by DC-SIGN (shown in red), indicating that virus is endocytosed (Fig. 1*A*). Cells with low DC-SIGN expression appeared to capture less HIV-1-GFP than cells with a high DC-SIGN expression (Fig, 1*A*). A high distribution of HIV-1-GFP particles per iMDDC was observed; however, a 2F5-neutralized GFP virus (mean particles/cell, 34.67 ± 2.16) was more efficiently captured than nonneutralized virus (mean particles/cell, 22.69 ± 1.75) ($p < 0.0005$) (Fig. 1*B*). To confirm the capture of 2F5-neutralized HIV-1 vs nonneutralized virus by iMDDCs, a CA-p24 ELISA was performed. In line with the confocal analyses, intracellular CA-p24 capture was significantly higher for the 2F5-neutralized virus (mean CA-p24, 2.93 ng/ml ± 0.03 SD; $p < 0.05$) with a 1.5-fold increase compared with nonneutralized virus (mean CA-p24, 1.92 ng/ml ± 0.36 SD) (Fig. 1*C*). Heightened capture of the virus by iMDDCs was also observed when 2F5-treated HIV-1-GFP (mean particles/cell, 16.68 ± 1.85 SEM) was compared with virus treated with HIV-1-nonspecific human IgG1 Ab (mean particles/cell, 10.10 ± 1.06 SEM; $p < 0.005$), indicating that the effect is HIV-1 Ab specific (Fig. 1, *D* and *E*). Furthermore, blocking DC-SIGN reduced the number of captured HIV-1-GFP by iMDDCs up to 70% for virus

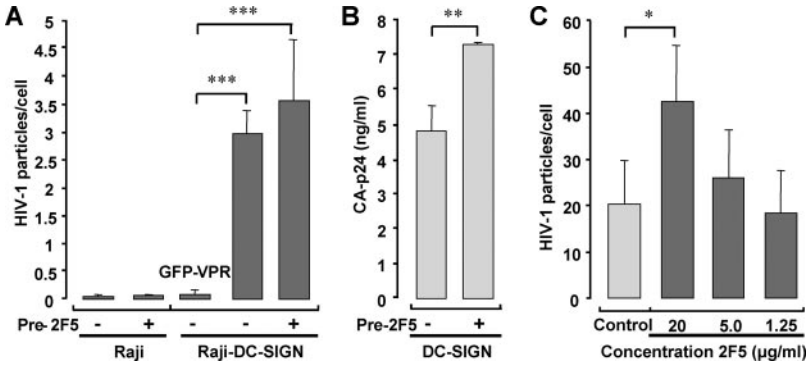

FIGURE 2. DC-SIGN-mediated capture of 2F5-neutralized virus. *A*, Raji or Raji-DC-SIGN cells were inoculated with 2F5-preneutralized or nonneutralized HIV-1-GFP and capture was measured with confocal microscopy. GFP-VPR particles were counted in a single plane on different fields on the coverslip. The number of particles in each field divided by the number of cells was determined. In total, four different fields were measured containing ~25 cells. Background fluorescence is indicated with GFP-VPR on Raji-DC-SIGN cells. *B*, Capture of (2F5-neutralized) HIV-1 by DC-SIGN was analyzed with intracellular CA-p24 ELISA. Capture of Raji cells was subtracted from Raji-DC-SIGN cells. *C*, Capture of HIV-1 by Raji-DC-SIGN cells was measured with confocal microscopy. Three-dimensional pictures were made of different fields on the coverslip and the number of GFP-VPR virions per positive Raji-DC-SIGN cell was counted with a computer program (Qwin). Statistical analyses were performed with the Student *t* test, and the data represent three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

treated with nonspecific human IgG1 Ab and 75% for 2F5-treated virus, indicating that DC-SIGN is important for capture of HIV-1 and HIV-1 ICs ($p < 0.0005$) (Fig. 1, *D* and *E*).

DC-SIGN enhances capture of 2F5-neutralized virus

To further analyze the role of DC-SIGN in the observed capture of neutralized virus by iMDDCs, we used the Raji-DC-SIGN line, which is derived from a B cell lineage. As an APC, this line is able to capture and transfer HIV-1 to $CD4^+$ T lymphocytes in a similar manner as iMDDCs (32). The advantage of using this cell line is the availability of the Raji control cell not expressing DC-SIGN. A capture experiment was performed and the number of GFP particles in one plane divided by the number of cells was calculated to quantify binding. The background GFP fluorescence of Raji-DC-SIGN cells (mean particles/cell, 0.07 ± 0.10 SD) was determined by using the supernatant from C33A cells transfected with GFP-VPR DNA, but without HIV-1. Raji-DC-SIGN cells efficiently captured nonneutralized HIV-1-GFP (mean particles/cell, 2.96 ± 0.41 SD) as well as 2F5-neutralized virus (mean particles/cell, 3.54 ± 1.09 SD), whereas Raji cells did not capture 2F5-neutralized virus (mean particles/cell, 0.051 ± 0.04 SD) or nonneutralized virus (mean particles/cell, 0.04 ± 0.03 SD; $p < 0.0005$) (Fig. 2*A*). Due to the low number of HIV-1 GFP-particles in a plane per cell, the capture of neutralized HIV-1 by DC-SIGN was measured with an intracellular CA-p24 ELISA. The capture of 2F5-neutralized HIV-1 by DC-SIGN (mean CA-p24, 7.26 ng/ml ± 0.07 SD) was significantly higher ($p < 0.005$) than the nonneutralized virus (mean CA-p24, 4.79 ng/ml ± 0.74 SD), showing that the uptake of neutralized virus was stimulated by mAb 2F5 (Fig. 2*B*). To investigate 2F5-neutralized HIV-1 dependent capture by DC-SIGN, HIV-1-GFP was neutralized with a serial dilution of the 2F5 mAb. Instead of one plane (Fig. 2*A*) the whole cell was scanned and the number of particles/cell in all layers was counted. Neutralizing HIV-1-GFP with a 2F5 concentration of 20 μg/ml enhanced capture compared with control virus ($p < 0.05$) (Fig. 2*C*) in a dose-dependent manner. This demonstrates that virus capture is stimulated by 2F5 mAb and that capture is dependent on DC-SIGN.

Neutralization of HIV-1 can be reversed by coculture of DC-SIGN-expressing cells with $CD4^+$ T lymphocytes

iMDDCs are known to enhance the infection of $CD4^+$ T lymphocytes by HIV-1 (32). To determine the role of DC-SIGN in enhancing $CD4^+$ T lymphocyte infection by HIV-1, iMDDCs were treated with the DC-SIGN-blocking mAb AZN-D1. Additionally, Raji-DC-SIGN and the control cell line Raji were used to exclusively investigate the role of DC-SIGN on enhancing HIV-1 infection. The infection of $CD4^+$ T lymphocytes was determined by counting the number of CD3 and CA-p24 double-positive cells. The coculture of $CD4^+$ T lymphocytes with iMDDCs or Raji-DC-SIGN cells significantly increased the infection of the lymphocytes by HIV-1 compared with infection on $CD4^+$ T lymphocytes alone, 32-fold for iMDDCs and 47-fold for Raji-DC-SIGN cells, respectively ($p < 0.0005$) (Fig. 3*A*). The blocking of DC-SIGN on iMDDCs with AZN-D1 resulted in a 71% decreased HIV-1 infection compared with untreated iMDDCs. The infection of $CD4^+$ T lymphocytes cocultured with Raji cells was 92% lower than Raji-DC-SIGN cells, although Raji cells also appeared to increase infection by 3.2-fold ($p < 0.05$) (Fig. 3*A*). This could be the result of DC-SIGN expression, because Raji cells are a B lymphocyte cell lineage that are capable of up-regulating DC-SIGN expression upon the induction by IL-4 together and CD40L (33). These results illustrate the importance of DC-SIGN on APCs in accelerating HIV-1 infection.

As previously shown, 100% virus neutralization by the mAb 2F5 was observed on $CD4^+$ T lymphocytes alone (31). When $CD4^+$ T lymphocytes were cocultured with iDCs or Raji-DC-SIGN cells in the presence of 2F5, infection was observed (Fig. 3*B*) although reduced compared with that seen for coculture in the absence of the Ab (reduction of 92% for iMDDCs and 89% for Raji-DC-SIGN cells) (Fig. 3, *A* and *B*). However, when iMDDCs were treated with AZN-D1 a full block to infection was observed, again indicating the involvement of DC-SIGN. The Raji cell line also showed no infection, providing further evidence that DC-SIGN is required for infection of $CD4^+$ T lymphocytes in the presence of a neutralizing mAb.

2F5-neutralized HIV-1 regains infectivity upon transfer by DC-SIGN-expressing cells

Because we identified an enhanced capture of a neutralized virus by DC-SIGN-expressing cells and an increased infection of $CD4^+$ T lymphocytes with a neutralized virus in the presence of DC-SIGN-expressing cells compared with $CD4^+$ T lymphocyte infection alone, we addressed whether the neutralized virus captured by

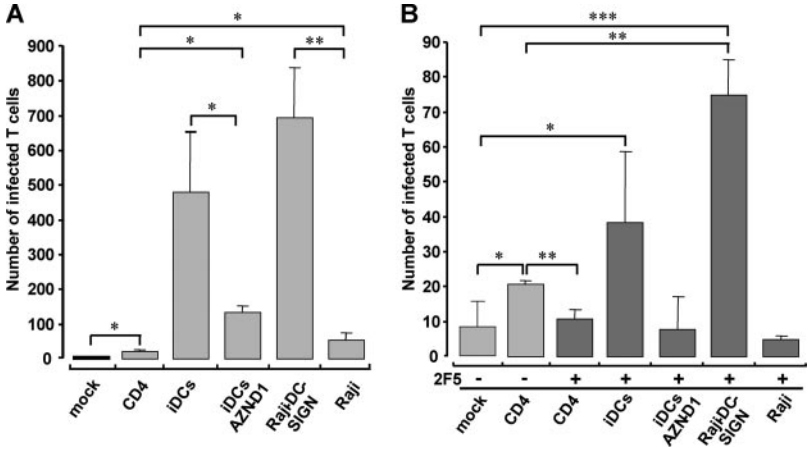

FIGURE 3. Neutralization capacity of 2F5 on HIV-1 $CD4^+$ T lymphocytes infection cocultured with DC-SIGN-expressing cells. HIV-1 was neutralized with 2F5 before incubation with $CD4^+$ T lymphocytes mixed with iMDDCs, iMDDCs pretreated with the DC-SIGN-blocking mAb AZN-D1, Raji-DC-SIGN cells, or Raji cells. As the control a nonneutralized virus was used. The infection of $CD4^+$ T lymphocytes was measured with FACS flow cytometry. *A*, HIV-1 infection of $CD4^+$ T lymphocytes cocultured with DC-SIGN-expressing cells inoculated with nonneutralized HIV-1 ($n = 3$). *B*, HIV-1 infection of $CD4^+$ T lymphocytes cocultured with DC-SIGN-expressing cells inoculated with preneutralized HIV-1 in the presence of mAb compared with the infection of $CD4^+$ T lymphocytes inoculated with nonneutralized virus ($n = 3$). Mock is determined as the number of double-positive cells (8.2 ± 7.6) ($n = 3$) without the addition of HIV-1. Data represent three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

DC-SIGN-expressing cells could be transferred to the lymphocytes. The transfer of captured virus by DC-SIGN-expressing cells was measured by washing out unbound virus together with the neutralizing mAb before incubation with activated $CD4^+$ T lymphocytes.

Surprisingly, the 2F5-neutralized virus captured by Raji-DC-SIGN cells or iMDDCs could efficiently infect $CD4^+$ T lymphocytes, indicating that neutralized HIV-1 is released as infectious virus during transmission (Fig. 4). The readdition of Ab during transmission resulted in a ~90% reneutralization of the released infectious particles by either Raji-DC-SIGN cells or iMDDCs, indicating that the Ab can efficiently retarget HIV-1 in the infectious synapse formed between $CD4^+$ T lymphocytes and DC-SIGN-expressing cells during transmission. This would also explain the rather low number of infected $CD4^+$ T lymphocytes in cocultures with either iMDDCs or Raji-DC-SIGN cells in the presence of 2F5 (Fig. 3*B*).

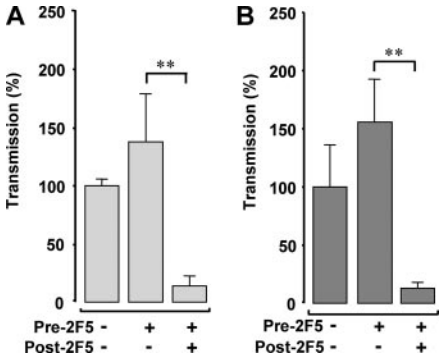

FIGURE 4. DC-SIGN-expressing cells release infectious virus after the capture of HIV-1 ICs. HIV-1 was neutralized with 2F5 before incubation with iMDDCs (*A*) or Raji-DC-SIGN cells (*B*) with a nonneutralized virus used as a control. The release of infectious HIV-1 captured by DC-SIGN-expressing cells was measured by the infection of $CD4^+$ T lymphocytes with FACS flow cytometry ($n = 3$). To determine reneutralization after virus release by DC-SIGN-expressing cells during transfer, fresh 2F5 mAb was added to the coculture. *A* and *B* represent two and four independent experiments, respectively. **, $p \leq 0.005$.

Envelope-directed HIV-1 ICs are captured by DC-expressing cells and released as infectious virus

To determine whether DC-SIGN-mediated uptake and propagation on $CD4^+$ T lymphocytes of neutralized HIV-1 was dependent on

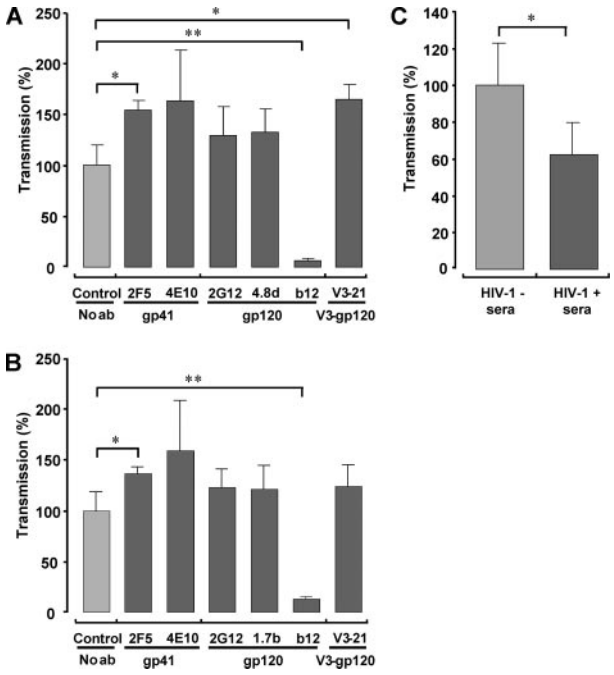

FIGURE 5. DC-SIGN mediated capture of HIV-1, neutralized with gp41, gp120, and V3-directed envelope Abs, and the release of infectious virions. HIV-1 was preneutralized with Abs and incubated with Raji-DC-SIGN cells (*A*) or iMDDCs (*B*). *C*, HIV-1 was inoculated with heat inactivated patient sera from HIV-1 negative and positive donors for 2 h before addition of iMDDCs. DC-SIGN expressing cells were washed three times and cultured with $CD4^+$ T lymphocytes and infection measured by FACS. Transmission was plotted as a percentage of infection when compared to non-neutralized HIV-1 ($n = 3$). Transmission of virus from iMDDCs incubated with sera from HIV-1 negative donors compared to HIV-1 positive donors was plotted as percentage of four sera in Fig. 3*C*. Data represents three independent experiments. *, $p < 0.05$; **, $p < 0.005$.

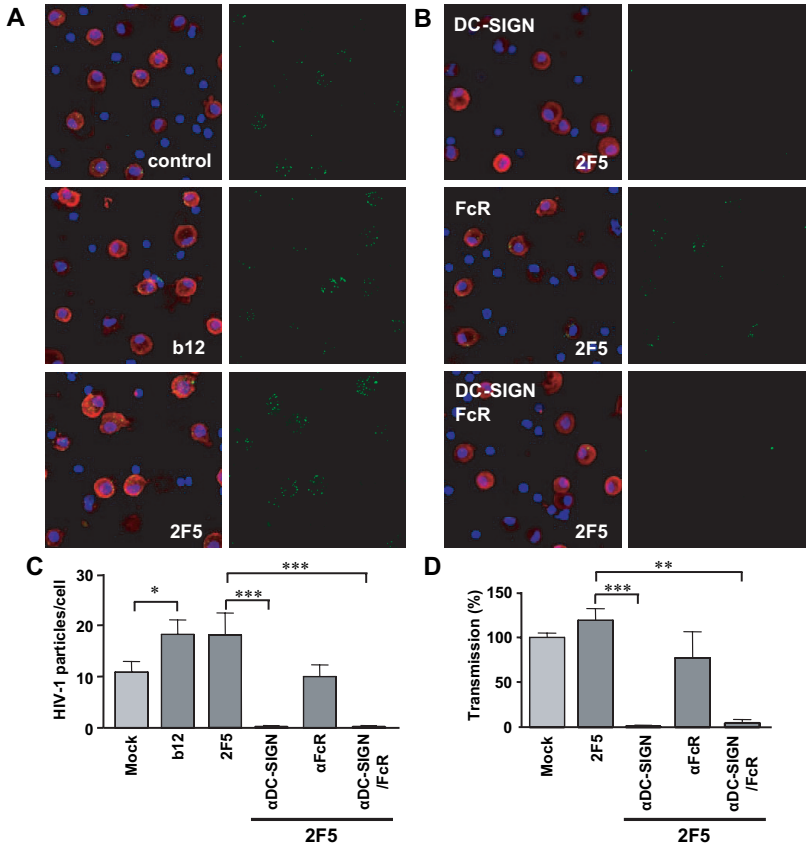

FIGURE 6. Fc receptors (FcR) enhance the capture of neutralized HIV-1 by DC-SIGN. *A*, HIV-1-GFP (green) was neutralized with either mAb 2F5 or b12 and captured by iMDDCs at 4°C for 1 h. *B*, iMDDCs, pretreated with the AZN-D1 mAb to block DC-SIGN and or Abs against Fc receptors I, II, and III, were used to capture 2F5-neutralized HIV-1-GFP at 4°C for 1 h. Cells in *A* and *B* were stained for DC-SIGN (red) and nuclear DNA (blue). *A* and *B* represent four and two independent experiments, respectively. *C*, Analyses of the number of HIV-1-GFP particles captured per cell from panels *A* and *B* ($n = 10$). The dashed line indicates that the virus was preneutralized with 2F5. *D*, HIV-1 was preneutralized and captured by iMDDCs, pretreated with AZN-D1 or Abs against FcγRI, II, and III at 4°C. Washed iMDDCs were cocultured with $CD4^+$ T lymphocytes at 37°C. The level of infected $CD4^+$ T lymphocytes, measured with FACS flow cytometry, indicates the release of infectious virus by iMDDCs ($n = 3$). The dashed line indicates that virus was preneutralized with 2F5 before capture. Data represents three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

the type of Ab, different envelope-directed Abs were tested. Two gp41-directed mAbs (2F5 and 4E10), three gp120-directed mAbs, (2G12, 4.8D, and b12) and one V3 (V3-21) mAb was used to form HIV-1 ICs before capture by Raji-DC-SIGN cells (Fig. 5*A*) or iMDDCs (Fig. 5*B*). The transfer of a 2F5-treated virus compared with a nonneutralized virus was 1.5-fold higher ($p < 0.05$) for Raji-DC-SIGN cells and 1.36-fold higher ($p < 0.05$) for iMDDCs, which was consistent with the previous results (Fig. 4, *A* and *B*). The mean transfer of 4E10-treated HIV-1 by Raji-DC-SIGN cells as well as iMDDCs was 1.5-fold higher, but not statistically significant, than that of the nonneutralized virus. 2G12 also did not show any neutralization after the transfer from Raji-DC-SIGN cells or iMDDCs, and the same was observed for the gp120 1.7b, and 4.8d mAbs. Virus treatment with the V3 binding gp120 mAb "V3-21" increased infection after transfer from Raji-DC-SIGN cells ($p < 0.05$), although this was not seen for iMDDCs. Interestingly, transfer of the virus preneutralized with the b12 mAb from Raji-DC-SIGN cells or iMDDCs completely blocked $CD4^+$ T lymphocyte infection ($p < 0.0005$). These results indicate that several mAb can be released from the virus during capture and transfer by DC-SIGN-expressing cells. We subsequently tested the transmission of HIV-1 captured by iMDDCs that had been treated with patient serum from HIV-1-negative and -positive donors. Although the levels or Ab types (nonneutralizing vs neutralizing; b12-like vs 2F5-like) in sera from positive donors were not known, infection after transmission was reduced by 37% ($p < 0.05$) (Fig. 5*C*) although a relatively efficient transfer of virus was observed.

Fc receptors enhance DC-SIGN-mediated capture of 2F5-neutralized virus by iMDDCs

Recently it was shown that blocking Fc receptors on iMDDCs increased the infection of iMDDCs with a 2F5-neutralized virus (16), indicating that HIV-1 ICs can interact with Fc receptors. Although we could not observe the capture of a 2F5-neutralized virus by Fc receptors on Raji cells, we were interested in the role of Fc receptors on DC-SIGN-mediated virus capture by iMDDCs. To minimize HIV-1 capture by iMDDCs via phagocytosis, pinocytosis, or other processes, we performed the capture assay at 4°C. Fc receptors on iMDDCs were blocked with Abs against Fc receptors I, II, and III and DC-SIGN was blocked with AZN-D1. Virus capture was assayed with confocal microscopy and analyzed (Fig. 6, *A–C*). On average, more 2F5-neutralized (mean particles/cell, 18.11 ± 4.3 SEM) or b12-neutralized virus (mean particles/cell, 18.25 ± 2.8 SEM; $p < 0.05$) was captured by iMDDCs compared with the nonneutralized virus (mean particles/cell, 10.85 ± 2.0 SEM) at 4°C (Fig. 6*C*). Interestingly, we had shown that b12-neutralized HIV-1 could not establish infection upon transfer from Raji-DC-SIGN cells or iMDDCs (Fig. 5, *A* and *B*). This suggests that b12-neutralized HIV-1 is not released or is released during transmission as an IC and is therefore noninfectious.

Blocking DC-SIGN with AZN-D1 completely abrogated the uptake of 2F5-neutralized HIV-1-GFP (Fig. 6*B*), indicating that the interaction of HIV-1 ICs with the Fc receptor is weak and that the capture is highly dependent on DC-SIGN (Fig. 1*C*). Furthermore, blocking the Fc receptor on iMDDCs reduced the capture of the 2F5-neutralized virus, indicating that Fc receptors increase the capture of HIV-1 ICs via DC-SIGN (Fig. 6*C*).

In a transmission assay determined by FACS flow cytometry, we addressed the role of Fc receptors on the capture of a neutralized virus by DC-SIGN on iMDDCs at 4°C and its transmission to $CD4^+$ T lymphocytes at 37°C (Fig. 6*D*). The transmission of a 2F5-neutralized virus captured by iMDDCs was reduced to levels similar to those of a nonneutralized virus upon blocking of the Fc receptors (Fig. 6*D*). No infection was observed when DC-SIGN was blocked on iMDDCs at 4°C, as expected (Fig. 6*B*). Taken

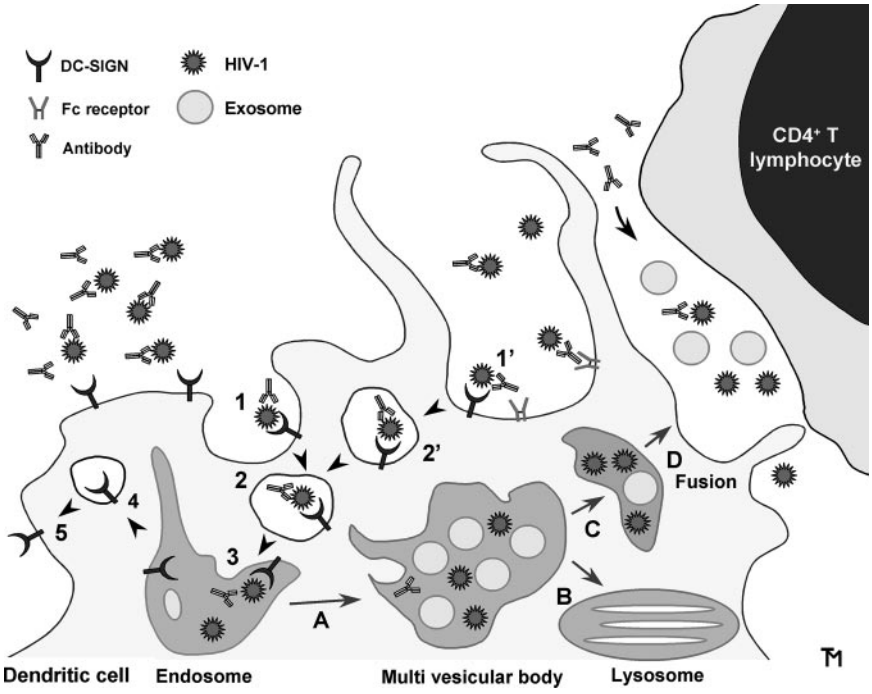

FIGURE 7. Model of DC-SIGN-mediated capture of HIV-1 in an IC with neutralizing mAb by DCs and the release of infectious HIV-1 upon transfer to $CD4^+$ T lymphocytes. Neutralized HIV-1 can either be captured by DC-SIGN directly (*location 1*) or via Fc receptor-mediated transfer (*location 1′*). At *location 2* the captured virus is internalized via receptor-mediated endocytosis and enters an early endocytic compartment (*location 3*), where HIV-1 IC is released from DC-SIGN. DC-SIGN recycles back to the cell surface (*locations 4 and 5*). *Arrow A*, The neutralizing Ab is released from HIV-1 due to a gradual decrease of the pH while traveling deeper in the endocytic pathway. *Arrow B*, HIV-1 is degraded in the lysosome or is released as infectious virus by fusion of endocytic compartments with the plasma membrane (*arrows C* and *D*), which can occur spontaneously (43) or is mediated via DC-T signaling. HIV-1 particles in the infectious synapse can infect the $CD4^+$ T lymphocytes or be retargeted by neutralizing Abs.

together, our results demonstrate that Fc receptors are partially responsible for Ab-enhanced capture of HIV-1 ICs by DC-SIGN.

Discussion

We specifically studied the role of DC-SIGN as a potential receptor for HIV-1 in the capture and transfer of neutralized HIV-1 to $CD4^+$ T lymphocytes. Both Raji-DC-SIGN and iMDDCs, with high DC-SIGN expression, were efficiently able to capture neutralized HIV-1 with different Abs and transfer the virus to activated $CD4^+$ T lymphocytes.

HIV-1 capture by DCs occurs via a number of processes such as phagocytosis, pinocytosis, or, specifically, via capture by receptors resulting in a receptor-mediated endocytosis (34, 35). C-type lectins such as DC-SIGN have been shown to be potent receptors for HIV-1 and are believed to be involved in the transmission of HIV-1 (6, 28). However, the role of DC-SIGN in participating in HIV-1 capture and its subsequent transfer to $CD4^+$ T lymphocytes is still controversial. Bovine lactoferrin, AZN-D1, or other DC-SIGN-blocking Abs reduced the capture by DC-SIGN of Lai (36), Ba-L (37), or pseudotyped HIV-1 with an HXB2 envelope (38) on DCs by at least 50%. Similar results were obtained in ELISA studies where the gp120 from Ba-L or CMV235 was used to bind DC-SIGN (39). However, other studies reported that blocking DC-SIGN on DCs with Abs only moderately reduced capture of the HIV-1 Ba-L strain (13). They demonstrated that when DC-SIGN expression on iMDDCs was reduced with the use of siRNA there was little influence on either the capture or the replication of HIV-1 Ba-L (13). In another study, HXB2 binding was reduced by 50% with siRNA silencing of DC-SIGN expression (11), suggesting some involvement of this receptor in capturing the virus. We show here that the capture of neutralized HIV-1 by iMDDCs can be largely dependent on DC-SIGN. Furthermore, our inhibition results for HIV-1 capture or transfer to $CD4^+$ T lymphocytes at 37°C vs 4°C (Figs. 1*E* and 6*C*) with the DC-SIGN mAb suggests that the DC-SIGN receptor is recycling at 37°C to the cell surface with the Ab removed, enabling its further interaction with HIV-1. This does not rule out the possibility that other C-type lectins or receptors expressed on DCs can perform similar functions that may become evident with higher viral inputs. The differences between the studies may also arise from the variant viruses used. We have previously shown that altered V1V2 length, V3 charge, and N-linked glycosylation patterns can modulate the association of HIV-1 with DC-SIGN expressing cells (40). Because envelope modifications can modulate the interaction of HIV-1 with its coreceptors (CCR5 and CXCR4) as well as DC-SIGN, it will therefore be of interest to identify whether viruses of different coreceptor phenotypes or genotypes in ICs are differentially transferred by DCs (13, 40).

The role of neutralizing Abs in an HIV immune response is to limit cellular infection and virus replication, which partially occurs through the blocking of HIV-1 binding to the host receptors required for infection (39). We demonstrate here that HIV-1 ICs can be efficiently captured by DC-SIGN on DCs and undergo sufficient transfer to $CD4^+$ T lymphocytes. We have shown that Fc receptors facilitate the enhanced capture of HIV-1 ICs by DC-SIGN on iMDDCs, which is in line with the function of iMDDCs to capture as many Ags as possible. We suggest that the Ab Fc tail of HIV-1 ICs is captured by Fc receptors with low affinity, resulting in an efficient transfer of the complex to DC-SIGN, which has a higher affinity for HIV-1 ICs. The heightened capture of HIV-1 by iMDDCs via this mechanism would therefore normally result in the degradation of more HIV-1 particles and, thus, an increased loading of Ags into MHC class II complexes. However, if the iMDDC encounters $CD4^+$ T lymphocytes during the degradation process, then the HIV-1 captured in endocytic compartments can fuse with the plasma membrane (10, 41) and thereby expose the Ab-dissociated infectious virus to the $CD4^+$ T lymphocytes in the synapse.

HIV-1 captured by DC-SIGN can reside on the plasma membrane or can be internalized by the DC. Confocal microscopy images demonstrated that captured, neutralized, or nonneutralized HIV-1 was clustered underneath the cell membrane and occasionally colocalized with DC-SIGN. Our results, together with published data, indicate that internalized HIV-1 is released from DC-SIGN for degradation in lysosomes in the endocytic pathway, whereas DC-SIGN is redistributed to the cell surface (34, 35).

However, a proportion of the internalized HIV-1 manages to survive degradation and can be found in CD81 and CD63-enriched compartments (10, 41). These compartments can fuse with the plasma membrane upon lymphocyte stimulation, causing the release of HIV-1 (42, 43). We speculate that neutralizing Abs dissociate from HIV-1 ICs due to an increase in acidification while passing through the different endocytic compartments and that infectious HIV-1 is released during DC-$CD4^+$ T lymphocyte contact. We have observed differences in the transmission of Ab-neutralized HIV-1 captured by iMDDCs; the b12 mAb provided low transmission or a high degree of neutralization, whereas 2F5 provided no neutralization after DC-mediated transfer to $CD4^+$ T lymphocytes. The differences observed between 2F5 and b12 could not be explained by differences in the capture of ICs. One hypothesis is that the affinity of the b12 mAb is much higher than the 2F5 mAb to HIV-1 at a lower pH. We have incorporated our hypothesis and what is currently known in the literature into a schematic model (Fig. 7).

Our results imply that in the continued presence of a neutralizing Ab the infection of $CD4^+$ T lymphocytes is still reduced to low levels, suggesting that the virus emerging from iDCs and within the infectious synapse can be targeted. This has implications for the in vivo significance of our findings and a proposed immune evasion mechanism. It can be envisaged that HIV-1 ICs can be captured at sites of high Ab concentration and infect $CD4^+$ T lymphocytes at a different site where Ab concentration levels are lower and less effective. This also has implications for HIV-1 transmission where HIV-1 coated with a neutralizing Ab can be captured and cleared of the Ab in the host, thereby allowing for the establishment of infection. The evasion mechanism we propose may also have implications for vaccines designed to induce strong mucosal neutralizing Ab responses that could be rendered useless or less effective through DC capture and the subsequent infection of $CD4^+$ T lymphocytes in lymph nodes. A previous study has demonstrated that for myeloid DCs infected with HIV-1 the transmission of a virus to $CD4^+$ T lymphocytes, although reduced, still occurs in the presence of an Ab (44). It therefore remains to be determined whether myeloid DCs can perform a similar capture and transfer of HIV-1 ICs as is observed for iDCs.

DC-SIGN expression on iDCs has important implications regarding the design of HIV-1 vaccines, because viruses can bind DC-SIGN and undergo efficient transfer to $CD4^+$ T lymphocytes. This can be envisaged as an effective means of evasion from neutralizing mAb responses in vivo. A vaccine designed to block transmission and induce the effective control of infection through Abs will have to induce Abs with high affinity, such as that observed with the b12 mAb, to prevent DC-SIGN-mediated evasion at the mucosal surface. It is possible that enhanced viral replication observed in animals vaccinated against the feline immunodeficiency virus or SIV is mediated by the process described here (45–47).

In a study where chronically and acutely infected HIV-1 participants were passively immunized with a mixture of 2F5, 4E10, and 2G12 Abs during antiretroviral therapy interruption, some individuals demonstrated a delay in viral rebound after the cessation of treatment (26). No escape variants against the gp41-directed 2F5 or 4E10 Abs could be identified, whereas variants insensitive to 2G12 neutralization were found, suggesting that 2G12 is the effective component of the mixture. The lack of evasion to the effects of 2F5 and 4E10 could possibly be due, or partly due, to immune evasion by DC capture and transfer. Other studies have also shown that escape variants against 2G12 can be selected in vivo (26, 48, 49). Interestingly, no participants demonstrated heightened viral loads after rebound compared with before treatment, suggesting that there is no enhancement to infection in the presence of extremely high concentrations of the neutralizing Abs administered.

Collectively, our results suggest that the induction of Abs able to block HIV-1 from interacting with DC-SIGN or other C-type lectins will be desirable in future vaccines, either prophylactic or therapeutic. How this can be achieved given the interaction of gp120 with DC-SIGN remains to be determined. Alternatively, small molecule inhibitors aimed at preventing HIV-1 from stably interacting with DC-SIGN should be considered in the future design of HIV-1 microbicidal or therapeutic agents.

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Disclosures

The authors have no financial conflict of interest.

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