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DC-SIGN Mediates Cell-Free Infection and Transmission of Human T-Cell Lymphotropic Virus Type 1 by Dendritic Cells[▽]

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Despite the susceptibility of dendritic cells (DCs) to human T-cell lymphotropic virus type 1 (HTLV-1) infection and the defined role of these cells in disease pathogenesis, the mechanisms of viral binding to DCs have not been fully delineated. Recently, a glucose transporter, GLUT-1, heparan sulfate proteoglycans (HSPGs), and neuropilin-1 (NRP-1) were demonstrated to facilitate HTLV-1 entry into T cells. DCs express their own array of antigen receptors, the most important being the DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) with respect to retrovirus binding. Consequently, the role of DC-SIGN and other HTLV-1 attachment factors was analyzed in viral binding, transmission, and productive infection using monocyte-derived DCs (MDDCs), blood myeloid DCs, and B-cell lines expressing DC-SIGN. The relative expression of DC-SIGN, GLUT-1, HSPGs, and NRP-1 first was examined on both DCs and B-cell lines. Although the inhibition of these molecules reduced viral binding, HTLV-1 transmission from DCs to T cells was mediated primarily by DC-SIGN. DC-SIGN also was shown to play a role in the infection of MDDCs as well as model B-cell lines. The HTLV-1 infection of MDDCs also was achieved in blood myeloid DCs following the enhancement of virus-induced interleukin-4 production and subsequent DC-SIGN expression in this cell population. This study represents the first comprehensive analysis of potential HTLV-1 receptors on DCs and strongly suggests that DC-SIGN plays a critical role in HTLV-1 binding, transmission, and infection, thereby providing an attractive target for the development of antiretroviral therapeutics and microbicides.

Human T-cell lymphotropic virus type 1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Dendritic cells (DCs) are of particular significance in HTLV-1 pathogenesis, because progression to HAM/TSP is associated with the rapid maturation of DCs (25), whereas ATL is associated with defects in their maturation (29). We previously demonstrated that DCs exhibited a greater degree of binding to HTLV-1 virions than to other cell types examined, including T cells, the primary target cell population during the course of disease (20). DCs from HAM/TSP patients were found to be infected with HTLV-1 (25) and were shown to be infected in vitro by both cell-free (19, 27) and cell-associated (5) virus. Infected DCs were able to transmit virus to $CD4^+$ T cells, leading to their transformation (19), as seen in ATL patients. Moreover, DCs cocultured with an HTLV-1-producing T-cell line were able to stimulate high levels of proliferation in autologous lymphocytes (28), as observed in HAM/TSP patients. HTLV-1 Tax protein also was shown to modulate DC maturation and function (1, 2, 15). Therefore, the importance of DCs in HTLV-1 pathogenesis is evident with respect to both ATL and HAM/TSP. Thus, it is critical to understand the mechanism of viral binding and entry into DCs. In this respect, a glucose transporter (GLUT-1) was identified as a receptor for HTLV-1 (30). Further investigations, however, established that GLUT-1 was not a universal receptor for HTLV-1 but was associated with cell-to-cell transmission (40). Two additional molecules, heparan sulfate proteoglycans (HSPGs) (18) and neuropilin-1 (NRP-1) (12), also were shown to mediate interactions of HTLV-1 with T cells. Most HTLV-1 receptor studies, however, have been performed with established cell lines (often non-T cells) or on $CD4^+$ T cells exposed to viral receptor binding domains or the soluble surface unit (SU) of the viral envelope, because primary T cells are difficult to infect with HTLV-1 in vitro, whereas DCs can be infected easily with HTLV-1 and express their own set of receptors for pathogen recognition. Of these receptors, C-type lectins recognize carbohydrate structures present on viral envelopes. One of the most well-characterized C-type lectins is DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin) (9), which is known to function as a pathogen receptor with broad specificity (11). With respect to HTLV-1, DC-SIGN has been shown to facilitate the fusion of HTLV-1-infected T cells with DCs (5). In this study, we investigated the role of DC-SIGN and other HTLV-1 receptor molecules in the binding, entry, and transmission of HTLV-1 by DCs.

DC-SIGN is found in high levels on monocyte-derived DCs (MDDCs), some macrophages (39), and activated B cells (36). In vivo, DC-SIGN-positive cells were demonstrated in lymph nodes (8, 13, 23), tonsils (39), skin (7), and the subepithelial region of the cervix (16, 38). Of the major DC subsets in vivo, dermal DCs but not myeloid, plasmacytoid, or Langerhans cells express DC-SIGN (42, 43). The high-level expression of DC-SIGN on MDDCs is attributed to interleukin-4 (IL-4) (37), which also has been shown to induce DC-SIGN expression on blood myeloid DCs (mDCs) (13). The results pre-

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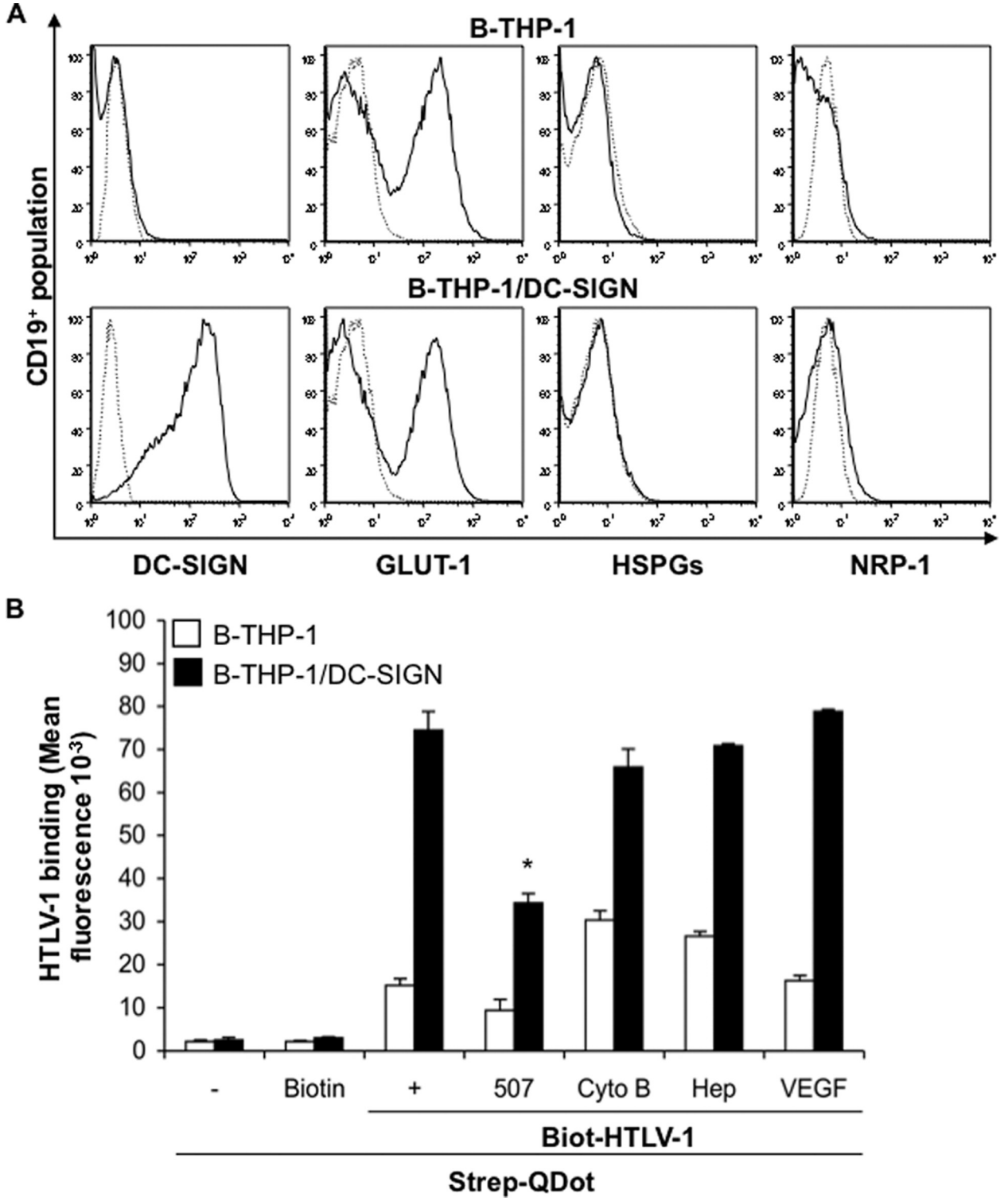


FIG. 1. Analysis of HTLV-1 binding to B-THP-1 cells. (A) Parental B-THP-1 cells or cells transduced to stably express DC-SIGN (B-THP-1/DC-SIGN) were incubated with an FITC-labeled anti-CD19 Ab in combination with APC-labeled anti-DC-SIGN, Alexa Fluor 647-labeled anti-GLUT-1, or anti-HSPGs, and PE-labeled anti-NRP-1 Abs and were analyzed by flow cytometry. A total of 50,000 events collected for each sample were gated to include the $CD19^+$ population. Isotype controls are represented by dotted-line histograms, whereas solid-line histograms represent Ab reactivity with respect to the indicated receptor molecules. (B) A QDot-based binding assay was employed to determine the effect of blocking DC-SIGN and other receptor molecules on HTLV-1 binding to B-THP-1 cells. Target cells (1×10^6) either were left untreated or were treated (30 min, room temperature) with a blocking Ab against DC-SIGN (clone 507; 20 μg/ml), the GLUT-1 inhibitor Cyto B (20 μM), the HSPG inhibitor Hep (20 mU), or NRP-1 ligand (VEGF; 50 ng/ml). Cells subsequently were incubated with Biot-HTLV-1 (125 ng/10^6 cells) for 45 min on ice, and the binding was quantified as described in Materials and Methods. Cells incubated with QDots or biotin alone were used as negative controls. HTLV-1 binding was estimated as the fluorescence measured at 400 or 605 nm. The results shown represent the mean fluorescence ± standard deviations (T bars) from three independent experiments each performed in duplicate. An asterisk denotes a statistically significant decrease in fluorescence compared to maximum binding without any inhibitor ($P \leq 0.05$).

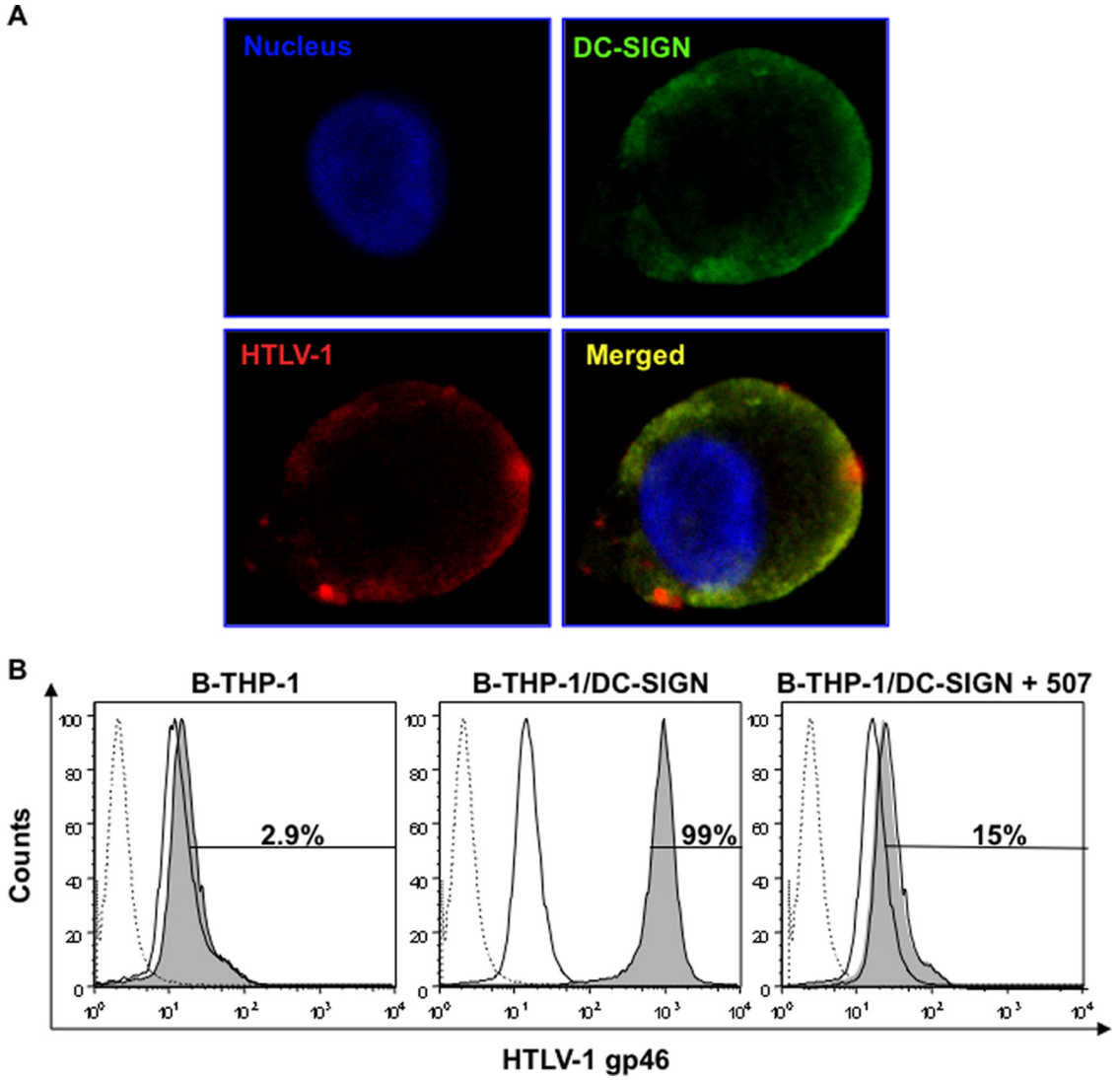


FIG. 2. HTLV-1 colocalizes with DC-SIGN on B-THP-1 cells. (A) A fraction of cells from the QDot binding assay were analyzed by confocal microscopy. Biot-HTLV-1/Strep-QDot-bound DCs were plated on poly(D)-lysine-coated slides, fixed, and incubated with an anti-DC-SIGN MAb (1:80; clone 507) and then by an Alexa Fluor 488 (1:200)-conjugated secondary Ab. Confocal microscopy at 100× magnification demonstrated that HTLV-1 (red) colocalized specifically with DC-SIGN (green) in a uniformly but occasionally patchy configuration. Hoechst 33342 (blue) was used to demarcate the nucleus. (B) FACS analyses were performed to reconfirm the DC-SIGN-mediated binding of HTLV-1 to B-THP-1 cells. Parental and DC-SIGN-transduced B-THP-1 cells were incubated with HTLV-1 in the absence or presence of a blocking Ab against DC-SIGN (clone 507; 20 μg/ml). Unbound virus particles were removed by washing, and cells were incubated with an Alexa Fluor 647-labeled MAb against HTLV-1 gp46. A total of 50,000 collected events were gated to include the $CD19^+$ population. The dotted-line histogram represents the isotype Ab; a solid-line histogram indicates the results obtained with untreated cells, and the filled histogram represents B-THP-1 cells positive for HTLV-1 gp46. The results shown are representative of one of the two independent experiments.

sented here demonstrate that HTLV-1 enhances DC-SIGN expression on mDCs by the induction of IL-4, leading to their enhanced susceptibility to infection. DC-SIGN also has been suggested as a potential target for retroviral therapeutic and preventive strategies. Both RNA interference (RNAi) (3, 32) and carbohydrate-binding agents (4) have been shown as potential means to inhibit the DC-SIGN-mediated transmission of human immunodeficiency virus type 1 (HIV-1). We have previously demonstrated that monoclonal antibodies (MAbs) to DC-SIGN inhibit the binding of HTLV-1 to DCs (20). In this study, we have shown that the silencing of DC-SIGN inhibits the HTLV-1 infection of DCs. Additionally, the blocking of DC-SIGN was shown to prevent HTLV-1 transmission from DCs to T cells, indicating the suitability of DC-SIGN as an antiretroviral drug target. Overall, this study establishes a clear involvement of DC-SIGN in HTLV-1 pathogenesis and provides further insight into retrovirus infection and transmission.

(This work was presented during the 13th International Conference on Human Retrovirology: HTLV and Related Viruses, Hakone, Japan, 21 to 25 May 2007.)

MATERIALS AND METHODS

Generation of MDDCs. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using Ficoll-Paque (GE Healthcare, Piscataway,

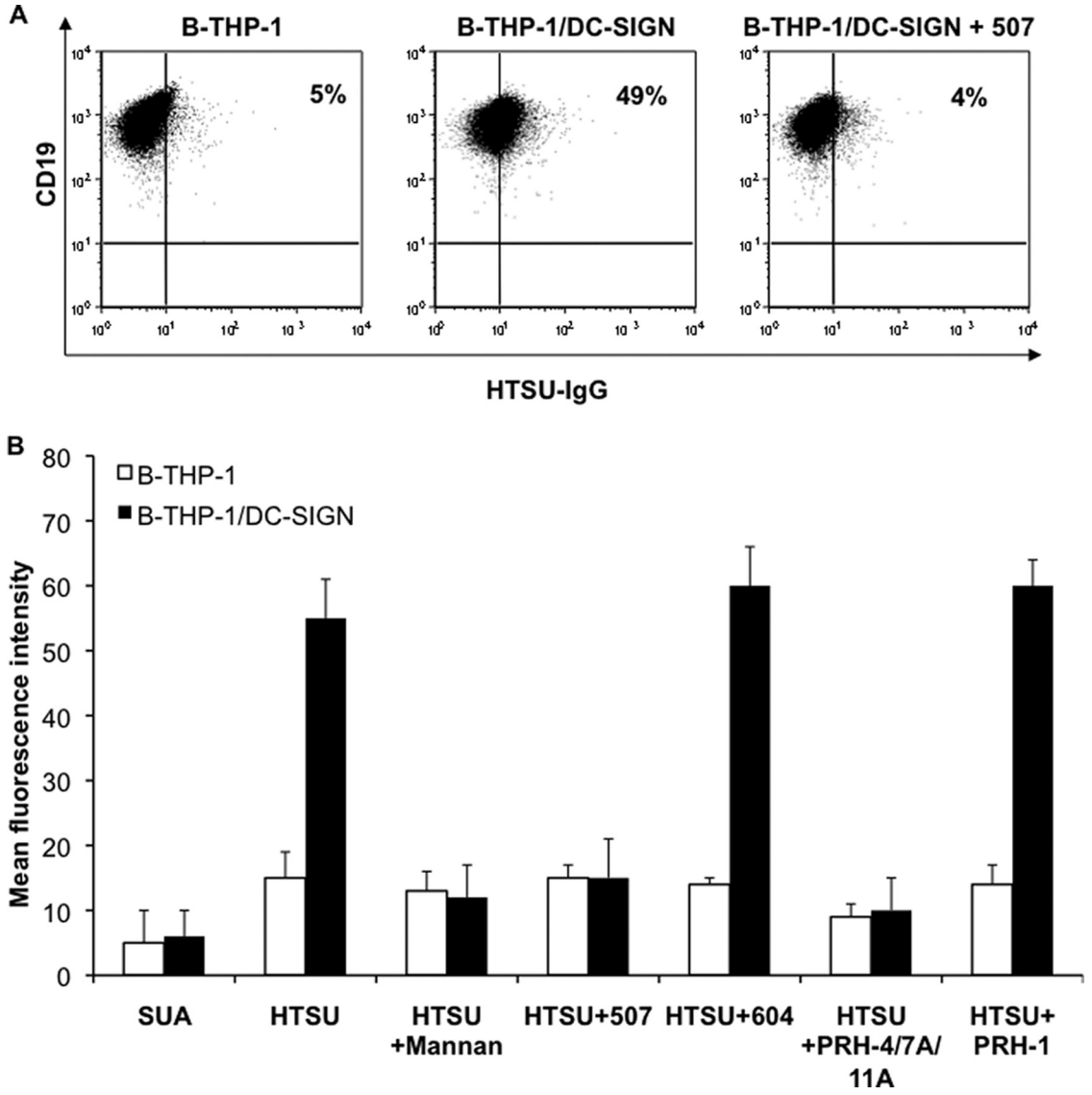


FIG. 3. DC-SIGN interacts with HTLV-1 envelope glycoprotein. (A) To examine the role of the HTLV-1 envelope glycoprotein gp46 in the DC-SIGN-mediated binding of HTLV-1, target cells (1×10^6) were left untreated or were treated (30 min, room temperature) with carbohydrate mannan (20 μg/ml; Sigma), a MAb specific for DC-SIGN (clone 507; 20 μg/ml), or a MAb directed against L-SIGN (clone 604; 20 μg/ml) and then incubated with HTSU-IgG (200 ng/ml) or SUA-IgG (negative control) for 30 min on ice. In a parallel experiment, HTSU was preincubated either with a pool of three anti-HTSU neutralizing MAbs (PRH-4, PRH-7A, and PRH-11; 10 μg/ml) or with the nonneutralizing MAb PRH-1. A total of 50,000 events collected for each sample were gated to include live cells. (A) The numbers shown indicate the percentages of $CD19^+$/HTSU-IgG^+ cells. (B) The results shown represent the MFI values ± standard deviations (T bars) from the triplicate samples.

NJ) density gradient centrifugation. Immature MDDCs were generated from purified monocytes in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (100 IU/ml) and IL-4 (300 IU/ml) (both from Peprotech, Inc., Rocky Hill, NJ) as previously described (15). Differentiation to DCs was confirmed by multicolor fluorescence-activated cell sorting (FACS) analyses for MDDC surface markers (HLA-ABC, HLA-DR, CD40, CD80, and CD86) using fluorochrome-conjugated Abs (eBioscience, San Diego, CA) along with a lineage cocktail containing Ab (Lin-1; BD Biosciences, San Jose, CA) to exclude all non-MDDC types.

Cell lines and virus. Parental and DC-SIGN-expressing B-cell lines B-THP-1 and B-THP-1/DC-SIGN (catalog numbers 9940 and 9941, respectively; NIH AIDS Research and Reference Reagent Program) were maintained in RPMI medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), HEPES (10 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Sucrose density gradient-purified HTLV-1 was purchased from Advanced Biotechnologies, Inc. (Columbia, MD). Virus preparation included 4.62×10^{11} virus particles/ml in 1.2 mg/ml total protein as determined by the Pierce protein estimation method using bovine serum albumin as the standard. We have determined the equivalent value for HTLV-1 Gag protein p19 using a p19-specific enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (ZeptoMetrix Corp., Buffalo, NY). As determined in this assay system, total viral protein (1.2 mg/ml) was found to be equivalent to approximately 0.5 mg/ml of p19.

Phenotyping of surface receptors on MDDCs and B-THP-1 cells. The expression of the HTLV-1 receptors on MDDCs and B-THP-1 cells was analyzed by flow cytometry using Abs against DC-SIGN (eBioscience), GLUT-1 (MAB1418; R&D Systems, Minneapolis, MN), HSPGs (clones F58-10E4 and F69-3G10; Seikagaku Corp., Tokyo, Japan), and NRP-1 (Miltenyi Biotec, Inc., Auburn, CA). Of the two utilized MAb clones specific for HSPGs, F58-10E4 recognizes the intact molecule on the cell surface while F69-3G10 recognizes an epitope that is revealed after the cleavage of HSPGs. Abs for both GLUT-1 and HSPGs were labeled with Alexa Fluor 647 using a Microscale Protein Labeling kit (Invitrogen, Carlsbad, CA). A total of 50,000 events collected for each sample were gated to

include the Lin-1$^-$/HLA-DR$^+$ population for DCs and the CD19$^+$ population for B-THP-1 cells. Samples were analyzed for the percentage of positive cells and the corresponding mean fluorescence intensity (MFI) values.

Analysis of virus binding by flow cytometry and quantum dot (QDot)-based binding assay. For the analysis of virus binding by flow cytometry, 1×10^6 target cells (B-THP-1 cells or MDDCs) either were left untreated or were treated (for 30 min at room temperature) with specific inhibitors of receptors, including a blocking Ab against DC-SIGN (clone 120507; 20 μg/ml; R&D Systems), the GLUT-1 inhibitor cytochalasin B (Cyto B; 20 μM; Sigma, St. Louis, MO), the HSPG inhibitor heparinase III (Hep; 20 mU; Sigma), or NRP-1 ligand vascular endothelial growth factor-A165 (VEGF; 50 ng/ml; R&D Systems). HTLV-1 binding then was assessed by incubating cells with the pretitrated amount of purified virus (125 ng/10^6 cells) for 45 min at 4°C. Unbound virus particles were washed off, and cells were incubated for another 30 min with 1.0 μg of Alexa Fluor 647-labeled MAb directed against the HTLV-1 envelope glycoprotein (anti-gp46; clone 65/6C2.2.34; ZeptoMetrix). Cells were gated for the lineage markers and analyzed by flow cytometry to determine the percentage of Lin-1$^-$/gp46$^+$ cells.

HTLV-1 binding also was examined using a QDot-based fluorescence binding assay as previously described (20). Briefly, target cells (1×10^6) were pretreated with inhibitors as described above and then incubated with biotinylated HTLV-1 (Biot-HTLV-1) (125 ng/10^6 cells) for 45 min on ice. Cells were washed, incubated with streptavidin-conjugated QD605 (10 nM; Invitrogen) for 30 min at 4°C, and fixed with 2% paraformaldehyde (PFA). Fluorescence subsequently was measured at 400 and 605 nm as an estimate of HTLV-1 binding. Duplicate values were averaged and normalized with basal fluorescence (QDots alone and biotin alone).

Confocal microscopy. An aliquot of cells from the binding assay was plated onto poly(D)-lysine-coated 8-well tissue culture slides (BD Labware, Bedford, MA) at 5×10^4 cells per well. Cells were allowed to adhere for 2 h and fixed with two concentrations of PFA (2% for 10 min and 4% for 20 min) before incubation with an anti-DC-SIGN MAb (1:80; clone 507; R&D Systems) and then an Alexa Fluor 488-conjugated secondary Ab (1:200, Invitrogen). Hoechst 33342 (0.6 μl/ml) was used for nuclear identification. Images were obtained with a dual laser confocal microscope (Leica DMRE) connected to a TCS SP2 camera.

Binding assay of envelope glycoprotein immunoadhesins (HTSU-IgG). The soluble form of the HTLV-1 SU (gp46) protein fused to rabbit immunoglobulin G (HTSU-IgG) was generated by transfecting 293 cells with 8 μg of HTSU-IgG/pSK100 plasmid (provided by Kathryn Jones, National Cancer Institute, Frederick, MD) as previously described (17). Immunoadhesins obtained as culture supernatants of transfected cells were quantitated by ELISA using paired Abs to measure rabbit IgG-Fc and rabbit IgG (Research Diagnostics, Inc., Flanders, NJ). Immunoadhesins (SUA-rIgG) obtained by transfecting 293 cells with a control plasmid encoding the SU protein from the avian leukosis/sarcoma virus served as the negative control. To examine the binding of HTSU-IgG, 1×10^6 target cells (B-THP-1/DC-SIGN) either were left untreated or were treated (for 30 min at room temperature) with carbohydrate mannan (20 μg/ml; Sigma), a MAb specific for DC-SIGN (clone 507; 20 μg/ml), or a MAb specific for L-SIGN (clone 604; 20 μg/ml) and then incubated with HTSU-IgG (200 ng/ml) or SUA-IgG (negative control) for 30 min on ice. In a parallel experiment, HTSU was preincubated either with a pool of three anti-HTSU neutralizing MAbs (PRH-4, PRH-7A, and PRH-11; 10 μg/ml) or with nonneutralizing MAb PRH-1 (provided by Steven Foung, Stanford University School of Medicine, Palo Alto, CA). After being washed, cells were incubated with a fluorescein isothiocyanate (FITC)-labeled Ab against rabbit IgG (Sigma) and analyzed by flow cytometry. A total of 50,000 events collected for each sample were gated to include live CD19$^+$ B-THP-1 cells exhibiting reactivity to HTSU-IgG. Data were analyzed to determine the percentage and MFI of the CD19$^+$/HTSU-IgG$^+$ population.

HTLV-1 gene expression and virus production subsequent to infection. B-THP-1 and B-THP-1/DC-SIGN cells were infected with cell-free HTLV-1 (3 μg/10^6 cells). At increasing times (0, 2, 4, 8, 16, and 24 h) postinfection, cells were harvested for the isolation of total RNA (Qiagen, Valencia, CA) followed by treatment with DNase (rDNase I; Ambion, Inc., Austin, TX). The reverse transcription reaction was performed with 1 μg of total RNA using Omniscript reverse transcriptase (4 U; Qiagen) in the presence of deoxynucleoside triphosphates (0.5 mM each deoxynucleoside triphosphate; Qiagen), oligo(dT)$_{15}$ primer (1 μM; Promega, Madison, WI), and RNase inhibitor (10 U; Ambion). The kinetics of viral gene expression was examined by real-time PCR (RT-PCR) (ABI Prism 7700; Applied Biosystems, Foster City, CA). Each data point was set up in triplicate. Dissociation or melting curve analysis was performed to ensure the presence of a single peak at the correct melting temperature. Gene expression was measured by the quantitation of cDNA converted from the mRNA present in infected samples relative to that present in uninfected samples using the $\Delta\Delta C_T$ method. To analyze productive infection, cells were infected with HTLV-1 for 24 to 36 h, washed, and incubated with fresh medium. At the indicated time points, supernatants were assayed to measure HTLV-1 p19 protein (pg/ml) by ELISA (ZeptoMetrix).

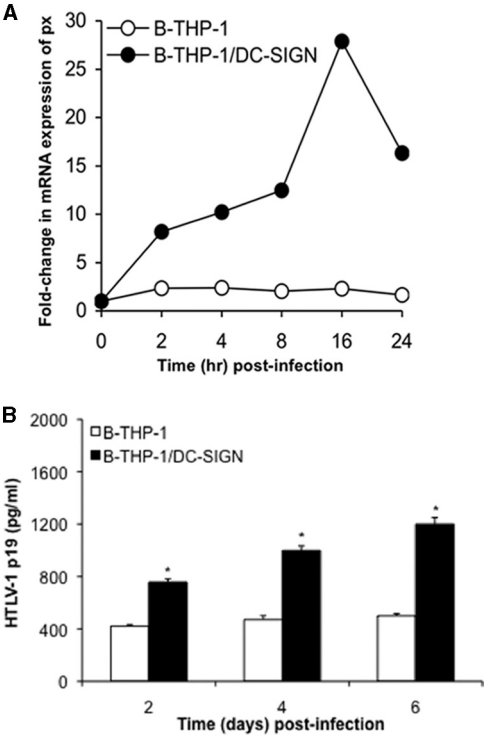


FIG. 4. Expression of DC-SIGN on B-THP-1 cells enhances HTLV-1 infection. Target cells were incubated with purified HTLV-1 (3 μg/10^6 cells) at the indicated time periods. (A) At increasing times after treatment, total RNA was isolated, converted to cDNA, and subjected to real-time PCR using pX-specific primers. The threshold cycle values obtained for duplicate samples were averaged and normalized to levels of β-actin, and the change (*n*-fold) in mRNA expression with respect to the control was calculated as described in Materials and Methods. (B) Productive infection was analyzed in supernatants of infected cells collected on days 2, 4, and 6 after infection using an HTLV-1 p19 (gag)-specific ELISA. A standard curve generated for p19 antigen was used to determine p19 levels (in picograms/milliliter) and is represented as the means ± standard deviations from triplicate samples. An asterisk denotes a statistically significant increase in the amount (in picograms/milliliter) of p19 in the presence of DC-SIGN ($P \leq 0.05$).

Transmission of HTLV-1 from DCs to T cells. MDDCs were pretreated with inhibitors as described above and then incubated with cell-free HTLV-1 (3 μg/10^6 cells) for 2 h at 37°C, trypsinized, and washed in serum-free medium. Virus-pulsed DCs were mixed with equal numbers of autologous peripheral blood leukocytes and cultured for 6 days. Toward the end of coculture, cells were harvested and stained for the T-cell marker using phycoerythrin (PE)-Cy5-anti-CD3 (eBioscience). Following fixation and permeabilization, cells were stained intracellularly with anti-HTLV p19 mouse MAb (4 μg; ZeptoMetrix) labeled with Alexa Fluor 647 (Invitrogen) or a mouse IgG1 isotype control Ab (eBioscience). Cells subsequently were washed in permeabilization buffer and analyzed by flow cytometry to determine the percentage of the CD3$^+$/p19$^+$ population.

Silencing of DC-SIGN expression in MDDCs and its effects on HTLV-1 infection of DCs. MDDCs were transfected with small interfering RNAs (siRNAs) using a SMARTPool containing four DC-SIGN-specific RNA duplexes (Dharmacon RNAi Technologies, Lafayette, CO). A nontargeting siRNA was used in parallel with siDC-SIGN as a control for the off-target activity of the silencing procedure. The transfection of DCs with siDC-SIGN (100 nM) or control siRNA (100 nM) was performed using the GeneSilencer transfection reagent (Gene

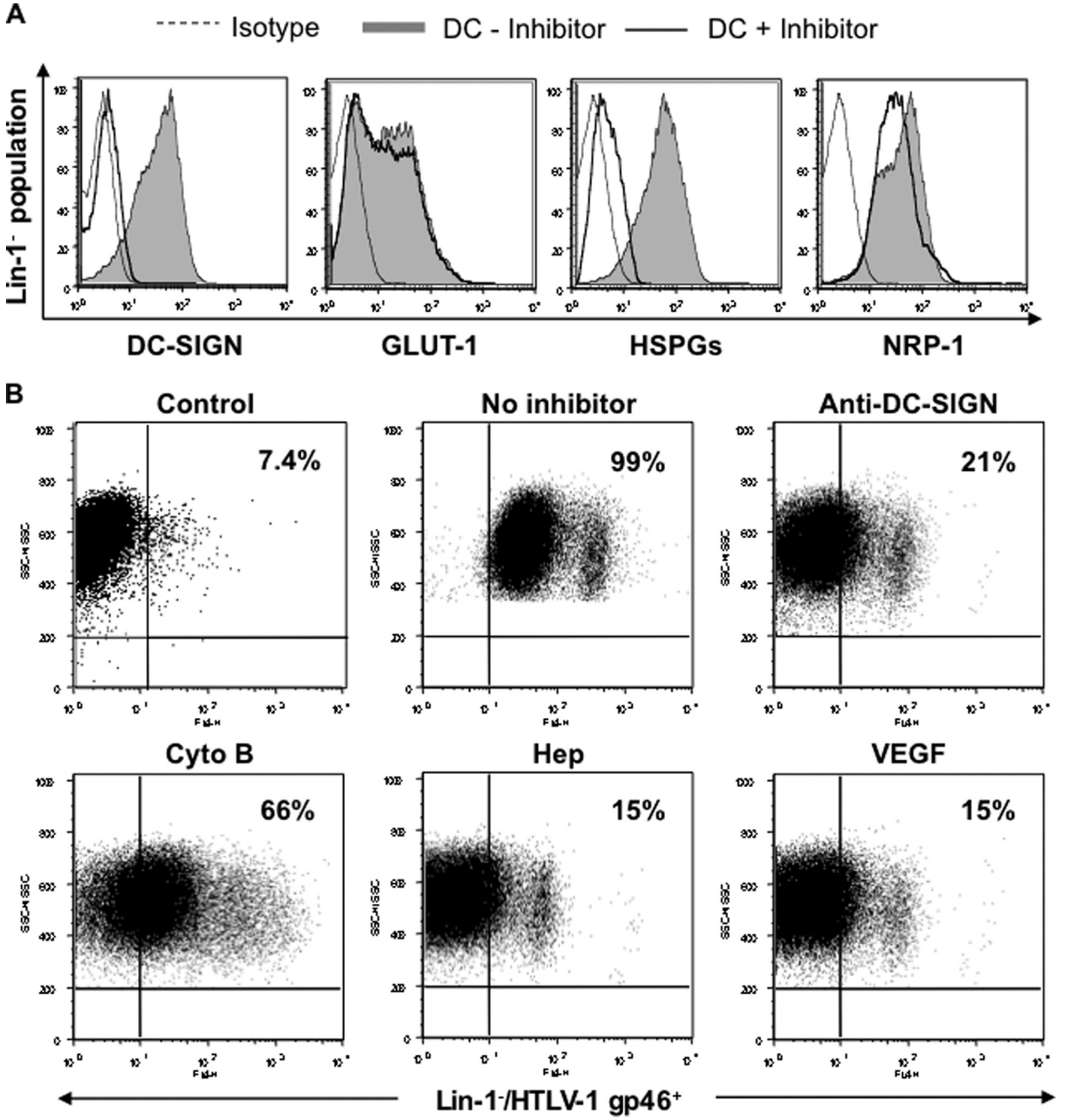
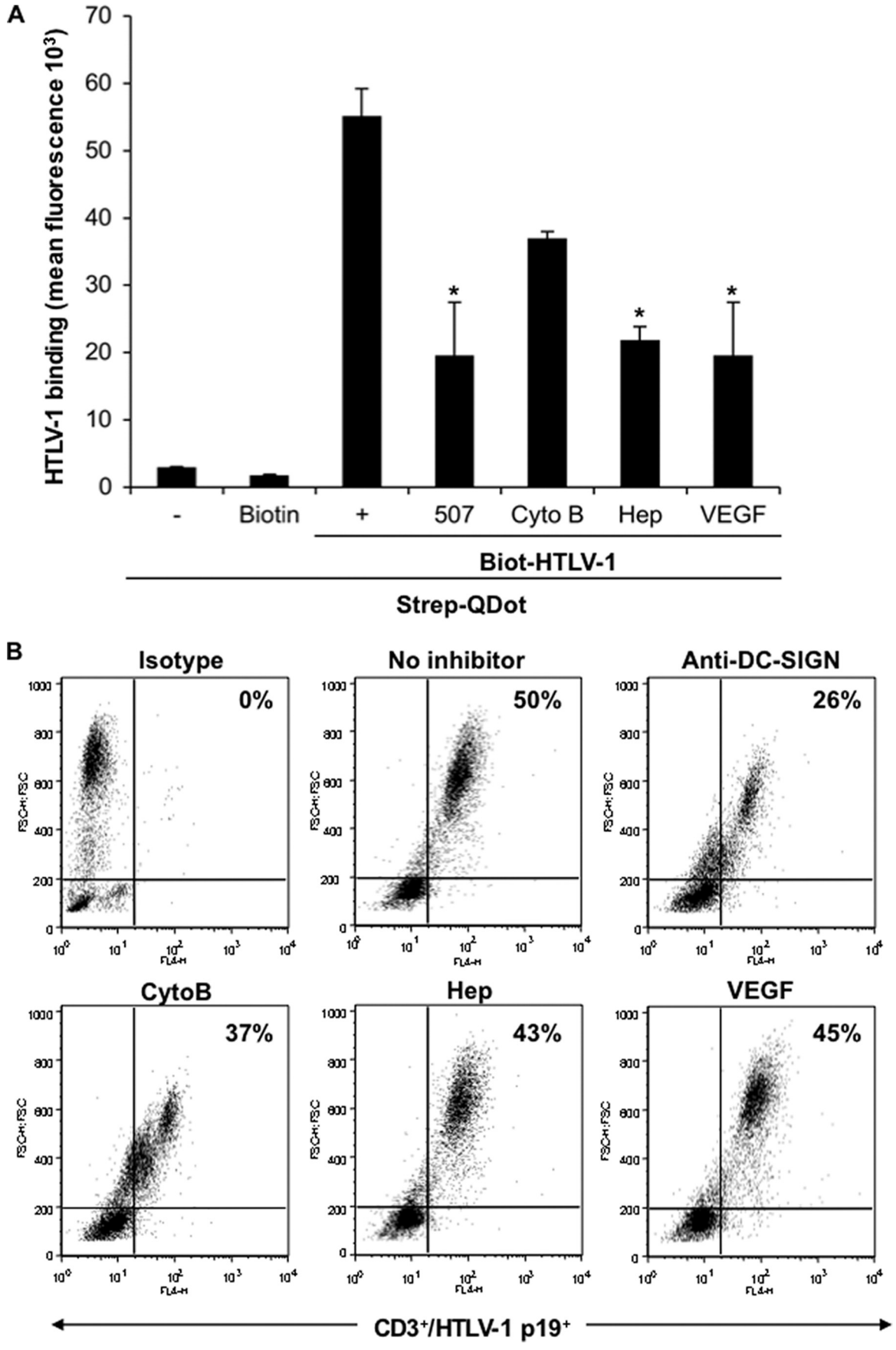


FIG. 5. Analyses of HTLV-1 binding to MDDCs. Immature DCs were differentiated from highly purified monocytes, and their phenotype was determined using a FITC-labeled lineage cocktail (Lin-1) to determine the absence of other leukocytes. (A) To detect the presence of various HTLV-1-binding receptors on DCs, immature DCs were incubated with a FITC-labeled Lin-1 Ab in combination with APC-labeled anti-DC-SIGN, Alexa Fluor 647-labeled anti-GLUT-1, or anti-HSPGs and PE-labeled anti-NRP-1 Abs and was analyzed by flow cytometry. A total of 50,000 events collected for each sample were gated to include the Lin-1$^-$ population. Isotype controls are represented by dotted-line histograms, whereas filled histograms represent the corresponding Ab binding on untreated MDDCs. Solid-line histograms represent receptor expression postincubation (30 min) with the respective inhibitor. The results are representative of MDDCs differentiated from six different donors. (B) Immature DCs (1×10^6) either were left untreated or were treated (30 min, room temperature) with a DC-SIGN-blocking Ab (clone 507; 20 μg/ml), Cyto B (20 μM), Hep (20 mU), or VEGF (50 ng/ml). Cells subsequently were incubated with cell-free HTLV-1 (125 ng/10^6 cells) for 45 min on ice. Unbound virus particles were removed by excessive washing, and viral binding was examined by flow cytometry using an Alexa Fluor 647-labeled MAb with the HLTV-1 envelope glycoprotein gp46. Following appropriate color compensation, live cells collected for each sample were gated to include the Lin-1$^-$/HTLV-1 gp46$^+$ population. The control sample represents non-virus-pulsed DCs to exclude the nonspecific effects of the anti-gp46 Ab. The results are representative of one of four independent experiments.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26%
CytoB
37%
Hep
43%
VEGF
45%
FSC-H:FSC
FL4-H
CD3⁺/HTLV-1 p19⁺

Therapy Systems, San Diego, CA). DC-SIGN silencing was confirmed both by RT-PCR using primers spanning the silenced region (370 bp) and by FACS analysis for the corresponding surface protein expression. The effect of DC-SIGN silencing on the HTLV-1 infection of DCs was examined by infecting DCs with cell-free HTLV-1 virions (3 μg/10^6 cells) for 3 days. Infected DCs subsequently were cultured in fresh medium for an additional 3 days, and HTLV-1 p19 protein levels were determined in culture supernatants by ELISA (ZeptoMetrix).

Effect of HTLV-1 infection on the expression of IL-4. PBMCs were incubated in the absence or presence of cell-free HTLV-1 (3 μg/10^6 cells) for 6 days. During the last 4 h of incubation, cells were treated with 1 μl/ml Brefeldin A (GolgiPlug; BD Biosciences), and the intracellular levels of IL-4 were monitored using an allophycocyanin (APC)-conjugated MAb (MP4-25D2; eBioscience). A total of 50,000 collected events were analyzed to determine the percentage of IL-4-secreting cells.

Analysis of HLTV-1 infection of PBMCs and mDCs. PBMCs first were examined for the expression of DC-SIGN following HTLV-1 infection and IL-4 stimulation. Cells (1×10^6) either were left untreated, incubated with cell-free HTLV-1 (3 μg/10^6 cells), or treated with IL-4 (10 ng/ml; R&D Systems). After 6 days, cells were assayed for DC-SIGN expression by determining the percentage of positive cells and MFI for total PBMCs as well as mDCs gated on the Lin-1^-/CD11c^+ population. When DC-SIGN expression in the presence of virus and IL-4 was confirmed, cells were processed for the analysis of virus entry. PBMCs were infected with cell-free HTLV-1 (3 μg/10^6 cells) in either the absence or the presence of the DC-SIGN-blocking Ab. After 6 days, cells were surface stained with FITC–Lin-1 and PE–anti-CD11c Ab and stained intracellularly with anti-HTLV p19 mouse MAb (4 μg; ZeptoMetrix) labeled with Alexa Fluor 647 (Invitrogen) and analyzed by flow cytometry.

RESULTS

DC-SIGN expression enhances binding of HTLV-1 to B-THP-1 cells. Studies were initiated with a well-characterized B-cell line stably transduced to express DC-SIGN, designated B-THP-1/DC-SIGN (47), in parallel with its parental cell line, B-THP-1. Besides DC-SIGN, the participation of other cell surface molecules previously shown to facilitate HTLV-1 entry into T cells was examined. As expected, B-THP-1 cells were uniformly negative for the endogenous expression of DC-SIGN, whereas B-THP-1/DC-SIGN expressed high levels of this receptor (MFI = 100), which is consistent with the previous report (47). GLUT-1 was expressed equally on both cell lines (MFI = 108 and 91 for B-THP-1 and B-THP-1/DC-SIGN, respectively), whereas HSPGs and NRP-1 were not expressed on these cells (Fig. 1A). Consistent with these observations, the expression of HSPGs (44) and NRP-1 (34) previously was reported on activated, but not on naïve, B cells. HTLV-1 binding to B-THP-1 cells was examined by using cell-free infectious virus to circumvent the problem of improper conformation associated with isolated envelope components as used in previous studies (30). The quantitative estimation of binding was determined by the QDot-based viral binding assay in which Biot-HTLV-1 was used in conjunction with streptavidin-QDots (Strep-QDots) (20). To confirm that the labeled virus was structurally intact and functional, the immunoreactivity of the viral envelope was tested with an anti-HTLV-1 Env Ab (data not shown). Cells were pretreated with a known blocking MAb against DC-SIGN (clone 507), the GLUT-1 inhibitor Cyto B, Hep (an enzyme known to specifically remove the heparan sulfate chains from the core proteoglycan), or the NRP-1 ligand, VEGF. B-THP-1 cells exhibited a low level of binding to HTLV-1 (15,164 fluorescence units [FU]), which increased fivefold in the presence of DC-SIGN (74,508 FU) and was reduced in the presence of the DC-SIGN-blocking Ab but not by Cyto B, Hep, and/or VEGF (Fig. 1B). Although it was expected for HSPGs and NRP-1, given their absence from these cells, GLUT-1, a known HTLV-1 receptor, did not seem to participate in HTLV-1 binding despite being highly expressed on B-THP-1 cells. This observation is consistent with a recent report demonstrating that GLUT-1 did not mediate the binding of HTLV-1 to T cells but was associated with T-cell-to-cell transmission (40). Overall, these observations suggest that the expression of DC-SIGN on B-THP-1 cells facilitates HTLV-1 binding, whereas GLUT-1 does not participate in this process.

To further examine the role of DC-SIGN in HTLV-1 binding, an aliquot of cells from the binding assay was subjected to confocal microscopy. A distinct colocalization of the Biot-HTLV-1/Strep-QDot complex (red) with DC-SIGN (green) in a uniform but patchy configuration (Fig. 2A) was observed, demonstrating that bound HTLV-1 specifically colocalized in the same area as DC-SIGN at the cell surface within a short period and suggesting that HTLV-1 directly interacts with DC-SIGN during the initial stages of viral entry. As an alternative to the QDot-based binding assay, flow-cytometric analysis was used to detect surface-bound HTLV-1 using an anti-gp46 MAb. As indicated in Fig. 2B, while there were 2.9% HTLV-1-bound CD19$^+$ B-THP-1 cells, 99% of B-THP-1/DC-SIGN cells bound to the virus. In contrast, in the presence of DC-SIGN-blocking Ab, binding was reduced to 15% positive cells. These results confirm that the heterologous overexpression of DC-SIGN on B-THP-1 cells enhanced HTLV-1 binding, substantiating the importance of this C-type lectin in viral binding to DC-SIGN-expressing cells.

HTLV-1 envelope glycoprotein gp46 interacts with DC-SIGN during viral binding. To further characterize the interaction of HTLV-1 with DC-SIGN and to determine the involvement of the viral envelope in this process, a soluble form of the surface glycoprotein gp46 (HTSU-IgG) was used that

FIG. 6. Transmission of HTLV-1 from DCs to T cells. The flow-cytometric observations shown in Fig. 4B were further confirmed by a QDot-based binding assay as described in Materials and Methods. (A) MDDCs were pretreated with the specific inhibitors of the receptor and subsequently were incubated with biotin–HTLV-1 (125 ng/10^6 cells) for 45 min on ice, washed three times, and incubated with Strep-QDot for 30 min. After being washed, cells were fixed and analyzed for HTLV-1 binding as determined by fluorescence measured at 400 or 605 nm. Cells exposed to QDot or biotin alone were used as negative controls. The results shown represent mean fluorescence ± standard deviations from three independent experiments, each performed in duplicate. The asterisk denotes a statistically significant decrease in fluorescence compared to that of maximum binding without any inhibitor ($P \leq 0.05$). (B) To examine the role of the cellular receptor molecules in the cell-free transmission of HTLV-1 from DCs to T cells, immature DCs were pretreated with inhibitors as described for panel A and incubated with HTLV-1 (3 μg/10^6 cells) for 2 h at 37°C, washed, and mixed with equal numbers of autologous T cells. Following 6 days of coculture, cells were incubated with PE-Cy5-labeled anti-CD3 Ab, fixed, permeabilized, and incubated with the Alexa Fluor 647-labeled anti-p19 Ab. A total of 50,000 events collected for each sample were gated to include live CD3$^+$/HLTV-1 p19$^+$ T cells. The numbers shown indicate the percentage of cells positive for both CD3 and HLTV-1 p19$^+$. The results are representative of one of the three independent experiments.

previously has been shown to bind to the activated $CD4^+$ T cells (17, 33). The binding of HTSU-IgG to B-THP-1 cells was minimal (5% positive cells) but was enhanced to 49% positive cells in the presence of DC-SIGN and returned to basal levels in the presence of DC-SIGN-blocking Ab (Fig. 3A). To confirm the specificity of the interaction between HTSU and DC-SIGN, a number of controls were used, and the results are presented as the MFI of the positive cell population in Fig. 3B. First, the binding of an unrelated retrovirus immunoadhesin (SUA) was negligible, whereas a 3.6-fold increase was observed in HTSU binding to B-THP-1/DC-SIGN cells compared to that with parental B-THP-1 cells. This binding was inhibited by the carbohydrate mannan and DC-SIGN-blocking Ab (clone 507) but not by an L-SIGN-specific Ab (clone 604), confirming the specificity of the interaction with DC-SIGN. Moreover, the preincubation of HTSU with a pool of three human MAbs (PRH-4, PRH-7A, and PRH-11) directed against the conformational epitopes of HTSU abrogated the binding. These Abs previously were shown to block HTLV-1-induced syncytium formation, while another, MAb PRH-1, was unable to block syncytium formation (14) and did not inhibit HTSU binding in the studies shown (Fig. 3B), thereby confirming the specificity of the interaction with HTSU. These results clearly demonstrate that the interaction of HTLV-1 with DC-SIGN involves the viral envelope glycoprotein and further suggests a specific interaction between these two proteins.

Expression of DC-SIGN on B-THP-1 cells leads to productive replication. Having demonstrated the DC-SIGN-mediated enhanced binding of HTLV-1 to B-THP-1/DC-SIGN cells, we proceeded to determine whether viral entry was followed by productive replication. Viral gene expression in infected B-THP-1 and B-THP-1/DC-SIGN cells was analyzed quantitatively by RT-PCR using primers specific for the pX region. As indicated in Fig. 4A, B-THP-1 cells infected with HTLV-1 demonstrated a constant twofold increase in viral gene expression during the entire interval examined. In sharp contrast, levels of viral gene expression for B-THP-1/DC-SIGN cells increased to 8-, 10-, and 12-fold at 2, 4, and 8 h postinfection, respectively, and peaked dramatically at 28-fold at the 16-h time point, finally tapering down to 16-fold at 24 h postinfection. These results demonstrate that DC-SIGN played a significant role in enhancing viral entry and subsequent viral gene expression. To analyze productive infection, cells first were infected with HTLV-1 for 24 h, washed to remove cell-free virus, and then supplied with new medium for an additional 2, 4, and 6 days. At the end of the incubation period, supernatants were harvested at the times indicated and analyzed using an HTLV-1 p19-specific ELISA. A quantitative estimation of HTLV-1 p19 protein (in picograms/milliliter) was extrapolated from a standard curve generated using purified p19. As shown in Fig. 4B, an increase in supernatant HTLV-1 p19 was detected in HTLV-1-infected B-THP-1/DC-SIGN cells compared to that with the supernatants derived from B-THP-1 cells. Despite an observed 10-fold difference in pX gene expression between parental and DC-SIGN-transduced cells, supernatant p19 differs by only 2.0- to 2.5-fold. All observations made with B-THP-1 cells (Fig. 1 to 4) were reproducible in two other B-cell line systems, Raji and Ramos, which are stably transduced with DC-SIGN (data not shown).

HTLV-1 binding to MDDCs is multifactorial. The promising observations made with the cell line system prompted further investigations concerning the interaction of HTLV-1 with MDDCs that endogenously expressed high levels of DC-SIGN and provide a suitable model of primary DC-SIGN-expressing cells. MDDCs first were phenotyped to confirm their purity (~99%) and the expression of relevant surface molecules. Immature MDDCs prepared from multiple donors expressed DC-SIGN (MFI = 27) as well as GLUT-1 (MFI = 12), HSPGs (MFI = 45), and NRP-1 (MFI = 30). GLUT-1 expression on MDDCs has not been reported to date, whereas the expression of both HSPGs (6, 46) and NRP-1 (41) has been reported previously for MDDCs, and the observations reported herein are consistent with these reports. Due to the presence of all four receptor molecules on the surface of MDDCs, it was necessary to determine the effectiveness of their respective inhibitors in controlling surface expression. The expression of DC-SIGN and HSPGs was not detected on the MDDC surface after incubation with inhibitor, whereas GLUT-1 and NRP-1 remained detectable (Fig. 5A). These observations were expected, because all of these inhibitors are known to operate by reducing the accessibility of the receptor rather than by directly affecting their expression (12, 18, 20, 21, 24, 30, 35, 48); therefore, the detection of their respective receptors by flow cytometry depends on the binding site for the corresponding Ab versus the site occupied by the inhibitor. The functionality of inhibitors was evident with respect to their impact on HTLV-1 binding to MDDCs, however. In the absence of any inhibitor, 99% of $Lin\text{-}1^-$ MDDCs were found to be positive for the viral glycoprotein gp46 (Fig. 5B). Within the HTLV-1-bound cell population, a small portion of cells (approximately 20%) exhibited a much higher retention of bound virus than the other fraction. Blocking DC-SIGN reduced HTLV-1 binding to MDDC by nearly fivefold (21% bound population and 99% in the absence of any inhibitor), whereas GLUT-1 inhibitor exhibited only a 1.5-fold reduction (66% bound population and 99% in the absence of any inhibitor). Inhibitors of both HSPGs and NRP-1 reduced binding by 6.6-fold (15% bound population and 99% in the absence of any inhibitor), indicating that these attachment factors help to concentrate the virus on MDDCs. These observations were strengthened by QDot-based binding assays (Fig. 6A). The efficient binding of HTLV-1 to MDDCs was significantly inhibited by the blocking of DC-SIGN, HSPGs, and NRP-1 and, to a small extent, by the GLUT-1 inhibitor Cyto B. Overall, results obtained from both systems have indicated that HTLV-1 binding to DCs is multifactorial and that DC-SIGN clearly plays a major role in this process. The results shown here were obtained with the optimal titrated doses of the inhibitors; however, each inhibitor was tested at three different concentrations, with relatively similar observations (data not shown).

DC-SIGN is involved in DC-mediated transmission of HTLV-1 to T cells. Having demonstrated that HTLV-1 binding to DCs may occur via DC-SIGN or several other known HTLV-1 cell surface binding proteins, the role of these receptor molecules in the transmission of HTLV-1 from DCs to uninfected T cells was examined. Pretreated DCs were pulsed with cell-free HTLV-1 for 2 h and mixed with equal numbers of autologous peripheral blood lymphocytes. Following 6 days of coculture, cells were examined for intracellular levels of

HTLV-1 p19 core protein. As shown in Fig. 6B, in the absence of any inhibitors, 50% of the $CD3^+$ T cells in the DC/T-cell coculture were positive for the HTLV-1 p19. The inhibition of DC-SIGN and GLUT-1 resulted in decreased percentages (26 and 37%, respectively) of $CD3^+/p19^+$ T cells, whereas the inhibition of HSPGs or NRP-1 did not demonstrate any effect on DC-mediated HTLV-1 transmission. Collectively, these results suggest that despite viral binding to multiple surface molecules, the transmission of HTLV-1 from infected DCs to T cells is mediated primarily by DC-SIGN, which is consistent with its known role for other viruses, including HIV-1 (10). GLUT-1 also seems to be involved in DC–T-cell transmission, as has been shown previously for its role in T-cell–to–T-cell transmission (40).

RNAi-mediated silencing of DC-SIGN expression inhibits HTLV-1 infection of DCs. RNAi-mediated inhibition of DC-SIGN expression has been postulated as a potential therapy for HIV-1 (3, 32). Therefore, it was of interest to determine whether the silencing of DC-SIGN expression affects the HTLV-1 infection of MDDCs. To this end, the expression of DC-SIGN was effectively inhibited using DC-SIGN-specific siRNA, and the impact of the resultant gene silencing on HTLV-1 infection of MDDCs was observed. The silencing of DC-SIGN was confirmed both at the mRNA level (Fig. 7A) and at the cell surface level (Fig. 7B). Mock-transfected and nontargeting siRNA-transfected DCs demonstrated no changes in DC-SIGN expression. The expression of the internal control β-actin also was not affected by DC-SIGN silencing. MDDCs silenced for DC-SIGN then were infected with cell-free HTLV-1. The silencing of DC-SIGN resulted in a significant reduction (approximately 4.5-fold) in the level of productive infection in MDDCs (Fig. 7C).

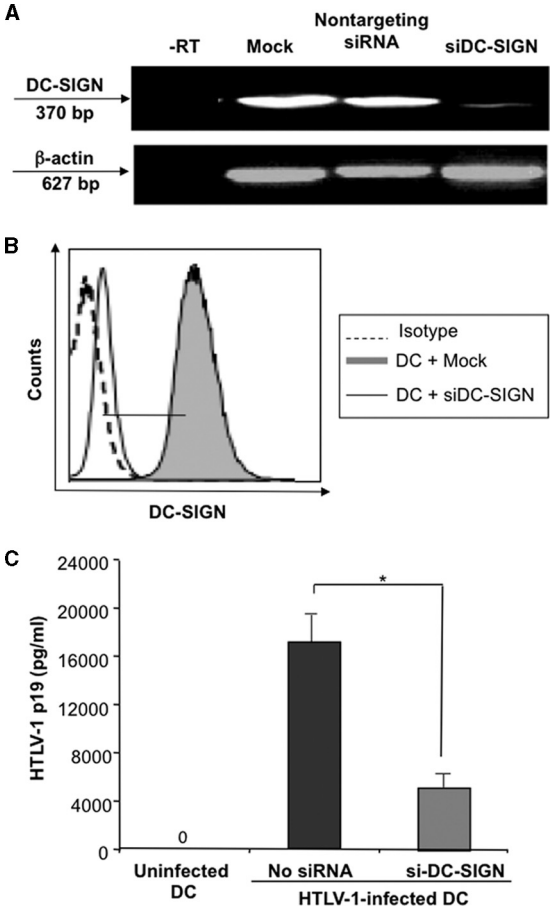
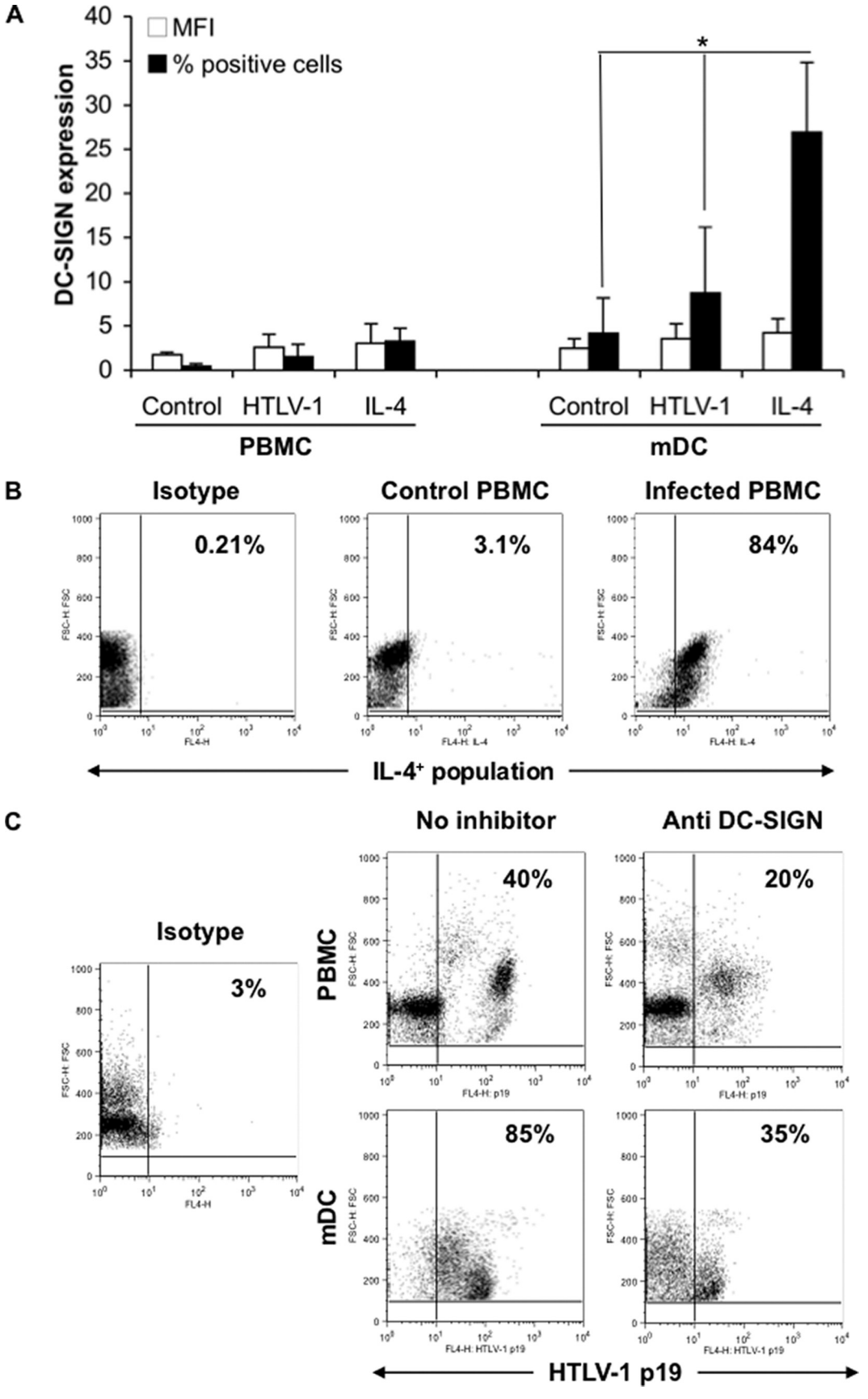


FIG. 7. Silencing of DC-SIGN expression inhibits HTLV-1 infection of DCs. DC-SIGN was silenced by transfecting DCs with siRNA targeted against DC-SIGN. Nontargeting siRNA was used as a negative control, whereas DCs only with transfection reagent were considered the mock control. (A) To confirm gene silencing, DC-SIGN mRNA levels were determined in GeneSilencer (mock)-transfected cells and cells transfected with nontargeting siRNA or siDC-SIGN. Real-time PCR analysis using DC-SIGN-specific primers to amplify a 370-bp product spanning the silenced region confirmed DC-SIGN silencing in transfected cells. β-Actin was used as an internal control. (B) The effect of silencing also was monitored by the cell surface expression of DC-SIGN. A total of 50,000 events collected for each sample were gated to include the $Lin\text{-}1^-/DC\text{-}SIGN^+$ population. The dotted-line histogram represents the isotype control, while filled and solid-line histograms represent DC-SIGN expression on the mock-transfected and siDC-SIGN-transfected DCs. (C) DCs silenced for DC-SIGN were infected with cell-free HTLV-1 for 3 days, washed, and cultured in fresh medium for an additional 3 days. The level of HTLV-1 gag protein (p19) in the supernatant of infected DCs was determined by ELISA. Supernatant from uninfected DCs was used as a negative control. A standard curve generated for HTLV-1 p19 antigen was used to determine productive infection in each sample. p19 levels (in picograms/milliliter) are represented as the means ± standard deviations from triplicate samples. The experiment was repeated twice. An asterisk denotes a statistically significant decrease in HTLV-1 p19 levels in siDC-SIGN-transfected DCs compared to that of mock-transfected DCs ($P \leq 0.05$).

HTLV-1 induces DC-SIGN expression on mDCs via IL-4 production, leading to infection in *cis* and in *trans*. Results obtained so far with the DC-SIGN-expressing B-cell lines and MDDCs prompted further studies concerning the role of DC-SIGN in freshly isolated mDCs. The expression of DC-SIGN has been difficult to detect on mDCs (26). Additional studies, however, have shown that IL-4 can induce mDCs to express elevated levels of DC-SIGN (13). Therefore, studies were performed to determine whether HTLV-1 infection could induce the expression of DC-SIGN on total PBMCs as well as on mDCs (gated ex vivo for $Lin\text{-}1/CD11c^+$). Cells stimulated with purified IL-4 were used as the positive control. Total PBMCs (from multiple donors) exhibited a small increase in DC-SIGN expression both at the level of percent positive cells as well as the MFI, whereas the mDC population showed a significant induction that was similar to the levels observed with IL-4 exposure (Fig. 8A). Based on these observations, studies were designed to determine whether the induction of DC-SIGN expression was due to IL-4 secretion in the presence of HTLV-1. Because IL-4 could be released by T cells and/or DCs after HTLV-1 infection, total PBMCs were analyzed for IL-4 production. As shown in Fig. 8B, 84% of the cells were found to be positive for intracellular IL-4 expression after HTLV-1 infection, indicating that HTLV-1-induced IL-4 expression leads to the enhanced expression of DC-SIGN on mDCs.

Having confirmed that HTLV-1 was capable of directly and/or indirectly inducing DC-SIGN expression on mDCs, we proceeded to determine whether the increased DC-SIGN expression could facilitate HTLV-1 entry into and infection of mDCs. The experiment again was performed with the total PBMCs to circumvent the need for supplying extracellular IL-4. Freshly isolated PBMCs were infected with HTLV-1 in


A
MFI
% positive cells
DC-SIGN expression
40
35
30
25
20
15
10
5
0
*
Control
HTLV-1
IL-4
Control
HTLV-1
IL-4
PBMC
mDC
B
Isotype
Control PBMC
Infected PBMC
0.21%
3.1%
84%
FSC-H: FSC
FL4-H
FL4-H: IL-4
IL-4+ population
C
No inhibitor
Anti DC-SIGN
Isotype
PBMC
mDC
3%
40%
20%
85%
35%
FSC-H: FSC
FL4-H
FL4-H: p19
FL4-H: HTLV-1 p19
HTLV-1 p19

the absence or presence of DC-SIGN-blocking Ab, and viral infection was quantitated by the assessment of intracellular HTLV-1 core antigen p19 in total PBMCs as well as mDCs 6 days after culture. Results demonstrated that 40% of the PBMCs were infected with HTLV-1, possibly by means involving T cells, monocytes, and mDCs. The frequency of infected cells was reduced by 50% in the presence of DC-SIGN-blocking Ab. With respect to mDCs, 85% of the cells showed positivity for the intracellular HTLV-1 antigen, a value that was reduced to 35% by the inhibition of DC-SIGN (Fig. 8C). These results demonstrate that during an ongoing viral infection, the blocking of DC-SIGN could prevent HTLV-1 infection in *cis* as well as in *trans*. Within the total PBMC population, T cells, B cells, mDCs, and monocytes are susceptible to HTLV-1 infection and are capable of transmitting virus to other neighboring cells. This process most likely is facilitated by the enhanced expression of DC-SIGN on different cells within the PBMC compartment. Collectively, the results from all three systems confirmed that DC-SIGN facilitates HTLV-1 binding to DCs, leading to DC infection as well as viral transmission; therefore, the inhibition of this molecule can prevent not only initial viral infection but also subsequent transmission to T cells.

DISCUSSION

The role of DC-SIGN and other known receptors first was investigated using a B-cell line system stably transduced to express DC-SIGN. The B-THP-1 cell line is a subclone of the Raji B-cell line that has been used extensively to study the DC-SIGN-mediated transmission of HIV-1 and other viral pathogens; however, in previous publications this B-cell line was misidentified as a THP-1 monocytic cell line, while it bears no similarity to THP-1 cells (47). B-THP-1 cells were found to be negative for the endogenous expression of DC-SIGN, HSPGs, and NRP-1 but positive for GLUT-1. The exogenous expression of DC-SIGN in these cells facilitated the binding of HTLV-1, which was inhibited in the presence of DC-SIGN-blocking Ab. The observed increase in binding might be due to a shift in affinity in the presence of DC-SIGN. Conversely, virus may bind to B-THP-1 cells with the same affinity as that seen with the B-THP-1/DC-SIGN cells, but DC-SIGN cells may simply have more binding sites. The incomplete inhibition observed with the DC-SIGN-blocking Ab could be due to Ab/inhibitor efficiency, epitope recognition, or Ab/inhibitor concentration. An alternative DC-SIGN-independent pathway of viral entry also may be in operation, because despite being negative for DC-SIGN expression, parental B-THP-1 cells always exhibited some degree of HTLV-1 binding. Moreover, neither of the DC-SIGN-reactive MAb or polyclonal Ab, as reported previously (20), demonstrated the complete abrogation of HTLV-1 binding to B-THP-1/DC-SIGN cells, further supporting the involvement of other molecules in the viral entry process. In this respect, GLUT-1 did not seem to have a role in HTLV-1 binding to B-THP-1 cells. As expected, the inhibition of HSPGs and NRP-1 has no effect on viral binding, because the expression of these molecules was not detected on B-THP-1 cells. The choice of inhibitors was based on our own experimental observations and/or available information in the literature. For DC-SIGN, MAb clone 507 has been used in several previous studies as a blocking Ab and did inhibit HTLV-1 binding to DC-SIGN-expressing cells in our previous studies. Conversely, L-SIGN-specific MAb 604 (specificity control) did not exhibit any effect in similar studies (20), giving us confidence with respect to the use of this clone in the studies reported here. Similarly, with respect to GLUT-1 inhibition, an extensive body of literature on glucose transport indicates that Cyto B directly binds to GLUT-1 (21, 24). Additionally, previous studies related to HTLV-1 and GLUT-1 confirmed that the effect of Cyto B on HTLV-1 binding was due largely to its physical interaction with the receptor and not to the indirect effects on actin microfilaments; as a related molecule, Cyto D, did not affect viral binding to GLUT-1 (30). Along the same line, Hep has been shown to specifically recognize intact HSPGs on the cell surface and specifically removes the heparan sulfate chains from the core proteoglycan and, thus, eliminates the epitope recognized by F58-10E4, an Ab that recognizes native HSPGs (18). To further ensure that Hep worked in our assay, we used another Ab, F69-3G10, that recognizes an epitope revealed after the cleavage of HSPGs (data not shown). Finally, it has been reported that the incubation of cells with VEGF165 induces a rapid upregulation of NRP-1 (a VEGF receptor) and enhances the binding of VEGF (35). Therefore, VEGF treatment reduces the accessibility of NRP-1 to Ab rather than reducing the expression of this receptor (12). The same fact could be applied to the other inhibitors (such as Cyto B) that primarily operate at the cell surface level and reduce the receptor accessibility (21, 24, 30).

FIG. 8. Analysis of DC-SIGN expression following HTLV-1 infection and the role of DC-SIGN in HTLV-1 infection of PBMCs and mDCs. (A) PBMCs obtained from healthy donors either were left untreated, incubated in the absence or presence of cell-free HTLV-1 (3 μg/10^6 cells) for 6 days at 37°C, or pulsed with recombinant IL-4 (10 ng/ml) for 24 h. Following incubation with the indicated stimuli, cells were examined to determine the effect of HTLV-1 infection and IL-4 pulsing on the expression of DC-SIGN on PMBCs and mDCs (gated on the Lin-1^-/CD11c^+ population). A total of 300,000 events were analyzed to determine the geometric MFI and the percentage of cells positive for DC-SIGN. The values of MFI and percent positive cells are shown as means ± standard deviations from six different donors. An asterisk denotes a statistically significant increase in DC-SIGN expression in the presence of HTLV-1 or IL-4 compared to that of untreated control mDCs ($P \leq 0.05$). (B) PBMCs also were analyzed for the expression of IL-4 following HTLV-1 infection. Following the fixation and permeabilization of infected cells, the intracellular expression of IL-4 was detected using an APC-labeled MAb. A total of 300,000 events collected for each sample were gated to include live PBMCs shown to be positive for IL-4. The numbers shown indicate the percentage of cells shown to be positive for IL-4 in uninfected and HTLV-1-infected PBMCs, respectively. (C) The role of DC-SIGN in HTLV-1 entry into PBMCs and mDCs was examined by infecting cells with HTLV-1 (3 μg/10^6 cells) in the absence or presence of the DC-SIGN-blocking Ab (20 μg/ml) for 6 days. The level of intracellular HTLV-1 p19 was determined with Alexa Fluor 647-labeled anti-p19. A total of 300,000 events collected for each sample were gated to include live cells for PBMCs and the Lin-1^-/CD11c^+ population for mDCs. The numbers shown indicate the percentage of HLTV-1 p19$^+$ cells. The results are representative of three independent experiments.

Therefore, the detection of their respective receptor expression by flow cytometry depends on the binding site of the staining Ab versus the site occupied by the inhibitor, which explains the observations provided in Fig. 5A with respect to the expression of GLUT-1 and NRP-1 after inhibitor treatment.

Further confirming the specificity of inhibitor activity is the fact that both Hep and VEGF did not show any effect on viral binding in nonexpressing B-THP-1 cells but did work in the case of DCs that express these molecules.

HTLV-1 binding to MDDCs appeared to be multifactorial; however, not all binding receptors examined necessarily facilitated the transmission of virus from DCs to T cells. The transmission of HTLV-1 was mediated primarily by DC-SIGN, with some indication of GLUT-1 involvement but not HSPGs or NRP-1, which is contrary to a recently published study (19) that suggested the involvement of these molecules in the DC/T-cell transmission of HTLV-1. A careful comparison of the present studies with those previously published revealed significant differences. Studies by Jones et al. were performed by preincubating SupT1/18x21-EGFP cells with inhibitors before mixing them with infected DCs and were analyzed after 3 h; in our studies, DCs were pretreated with inhibitors, exposed to HTLV-1, and then mixed with uninfected T cells for 6 days. Therefore, these results suggest a sustained long-term effect of DC-SIGN inhibition on HTLV-1 transmission. After we established DC-SIGN as being critical to the transmission of HTLV-1, we examined its role in the productive infection of DCs. We observed a significant effect of the RNAi-mediated silencing of DC-SIGN expression on the productive HTLV-1 infection of DCs. The same strategy was shown to inhibit the HIV-1 infection of DCs and has been proposed as a potential therapeutic strategy to block the binding of HIV-1 to the target cells (32). Moreover, compared with HIV-1, HTLV-1 is less likely to generate siRNA escape mutants, making this type of intervention more favorable for this viral pathogen.

Because MDDCs may express several attachment factors not present in mDCs (28), critical observations were recapitulated with mDCs. The results demonstrated the expression of DC-SIGN on mDCs following HTLV-1-induced IL-4 expression, leading to the entry of virus within the mDC compartment and perhaps in other DC-SIGN-bearing monocytes and B cells within the PBMC compartment; therefore, blocking DC-SIGN expression during an ongoing infection provides a potential therapeutic strategy. Overall, these studies provide important insights into the participation of DCs in HTLV-1 pathogenesis and establish a role for DC-SIGN in this pathogenic process. During early infection, it is possible that following viral binding, DCs bearing infectious HTLV-1 particles migrate to the lymph nodes, where viral replication can be amplified to facilitate efficient viral transmission to the surrounding HTLV-1 target cell populations. Once infection is established in T cells, HTLV-1 again can exploit DC-SIGN to infect DCs. In both cases, HTLV-1 is capable of using DC-SIGN to enhance replication and pathogenesis. Other studies (10, 22, 31) have shown that HIV can enter DCs and avoid degradation for several days and subsequently can be transferred to other target cell populations and/or released as infectious virus (45). Therefore, if HTLV undergoes a similar process, an additional role is implicated for DC-SIGN in HTLV-1 pathogenesis, allowing this virus to evade the immune system temporarily. Our studies suggest that DC-SIGN provides a point of entry for HTLV-1 into DCs, T cells, or other target cell populations; therefore, therapeutic approaches should be directed toward interrupting viral infection and transmission mediated by this receptor molecule. Collectively, these studies potentially serve as the foundation for the development of therapeutic strategies to treat HTLV-1-associated disease. In preliminary studies, carbohydrate targeting agents as well as a novel microbicidal compound prevented the interaction of HTLV-1 and HIV-1 envelope glycoprotein gp120 with DC-SIGN (data not shown). Future studies will use a large library of small-molecule inhibitors to identify compounds that prevent the interaction of DC-SIGN with the HLTV-1 gp46 and HIV-1 gp120 proteins, with the goal of finding a novel antiretroviral therapy.

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