A Nonneutralizing Anti-HIV Type 1 Antibody Turns into a Broad Neutralizing Antibody When Expressed on the Surface of HIV Type 1-Susceptible Cells. II. Inhibition of HIV Type 1 Captured and Transferred by DC-SIGN

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ABSTRACT

Previously, we demonstrated that the expression of a nonneutralizing human anti-HIV-1 gp41 scFv on the surface of HIV-1-susceptible cells markedly inhibits HIV-1 replication and HIV-1 envelope-mediated cell–cell fusion. The inhibition is at the level of viral entry, specific for the HIV-1 envelope, and independent of virus tropism. In the previous studies, cell-free viruses of laboratory-adapted HIV-1 strains from subtype B were used to infect human CD4 T cell lines. To further test the effectiveness of this membrane-bound scFv (m-scFv) on HIV-1 infection, in this study, we carried out experiments to determine whether the m-scFv can neutralize infection of primary isolates from various HIV-1 subtypes and whether the m-scFv can neutralize HIV-1 captured and transferred by DC-SIGN on the surface of monocyctic cell lines or DCs. We demonstrated that the m-scFv markedly inhibits primary isolates derived from various subtypes and significantly blocks HIV-1 captured and transferred by DC-SIGN on the surface of monocyctic cell lines or DCs. Therefore, a nonneutralizing antibody acts as a broad neutralizing antibody when expressed on the cell surface, which significantly inhibits infection of both cell-free and DC-SIGN-captured and transferred virus. Our studies further point out the potential use of m-scFv as a inhibitor against HIV-1 transmission as well as a tool to dissect the mechanism of HIV-1 entry via DC-SIGN capture and transfer to CD4 T cells.

INTRODUCTION

DENDRITIC CELLS (DCs) express Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that interact with pathogens.1,2 One of the CLRs, dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), is a type II transmembrane protein that recognizes a wide variety of pathogens such as viruses, bacteria, yeast, and parasites through binding of mannose or Lewis-x carbohydrate structures.3 DC-SIGN was originally characterized as a receptor for ICAM-3 to mediate DC-T cell interaction.4 Later, it was also found to bind to ICAM-2 on the surface of vascular endothelial cells to regulate transmigration of DC across vascular endothelia.5 In HIV-1 infection, DC-SIGN on the DCs binds to carbohydrates in gp120 of HIV-1 and functions as a trans-receptor that efficiently presents HIV-1 to CD4+ T cells.6 The captured HIV-1 particles are internalized by DCs and remain in an infectious form in an endocytic compartment for an extended period of time before transfer to target cells.5 Transfer HIV-1 to CD4+ T cells takes place through an infectious synapse, where internalized HIV-1 particles are concentrated at the interface between DCs and CD4+ T cells.7 DC-SIGN on the surface of mucosal tissue DCs acts as a sentinel molecule to capture HIV-1 on the mucosal surface and then efficiently to transfer HIV-1 to CD4+ T cells underneath the epithelial layer(s) of the mucosal surface or in draining...
lymph nodes. It thereby plays an important role in HIV-1 transmission and spread.

Previously, human B cell hybridomas were generated from an HIV-1 patient in Dr. Abner Notkins’ laboratory at the National Institutes of Health. The monoclonal antibody (TG15) derived from one of these hybridomas exhibits high affinity binding to the ectodomain of gp41 with a $K_d$ value of $4 \times 10^{-10}$ M. Using a competition assay by a panel of monoclonal antibodies with known specificity, it was found that only antibodies against cluster determinant II of gp41 can compete the binding of antibody (TG15) to HIV-1 envelope protein. This human monoclonal antibody and its recombinant Fab or scFv have been repeatedly shown to bind to HIV-1 gp160 or gp41, but fail to block viral entry. Thus, it is a true nonneutralizing antibody.

In a series of experiments, we targeted this nonneutralizing anti-HIV-1 scFv into the endoplasmic reticulum (ER) and trans-Golgi network (TGN) of HIV-1-susceptible cell lines and demonstrated that targeting the anti-HIV-1 gp41 scFv into ER and TGN compartments, but not secreted scFv, inhibited HIV-1 replication. Using a retroviral vector we also transduced anti-HIV-1 gp41 scFv-ER into rhesus macaque primary T cells and demonstrated that the scFv-ER markedly inhibits pathogenic SHIV replication in vitro (P. Zhou, unpublished observation). Thus, the nonneutralizing antibody, when expressed intracellularly, inhibits HIV-1. The epitope that the anti-HIV-1 gp41 antibody recognizes locates within the second heptad repeat (HR2) of the ectodomain of gp41 and is immediately N-terminal to two broad neutralization epitopes 2F5 and 4E10/Z13. The function of gp41 is to anchor the glycoprotein complex within the host-derived viral membrane and mediate membrane fusion.

Conformational changes in gp41 following the interaction of gp120 with CD4 and coreceptor expose a hydrophobic fusion peptide at its N-terminus. The latter then inserts into the target cell membrane. The bridged viral and target cell membranes are then brought together as two heptad repeats (HR1 and HR2) interact, which converts a prehairpin gp41 trimer into a fusogenic three-hairpin bundle. Short synthetic peptides, such as T20, that interact with sequences within HR1 or HR2, act as fusion inhibitors.

The intimate involvement of this epitope in the HIV-1 gp41-mediated fusion process leads us to wonder whether we can further explore the utility of this nonneutralizing human anti-HIV-1 gp41 scFv by expressing it on the surface of HIV-1-susceptible cells. We hypothesized that although when produced as a soluble protein this scFv does not neutralize HIV-1 entry, when expressed on the surface of HIV-1-susceptible cells, the membrane-bound scFv (m-scFv), due to its geographic proximity to the gp41-mediated fusion process, can act as a neutralizing antibody to block HIV-1 entry.

To test this hypothesis, we constructed an m-scFv in which the scFv was genetically linked to the human IgG3 hinge region and the transmembrane domain of type I interferon receptor subunit 1. The m-scFv was transduced into HIV-1-susceptible CEMss cells by an MMLV vector and expressed on the cell surface. The expression of m-scFv does not alter the surface expression of CD4, CXCR4, and CCR5. However, HIV-1 replication and HIV-1 envelope-mediated cell–cell fusion are markedly inhibited. The inhibition is at the level of viral entry, HIV-1 envelope specific, and was not affected by virus tropism. Therefore, this nonneutralizing antibody can be turned into a neutralizing antibody by expressing it on the surface of HIV-1-susceptible cells. In this recently published study, cell-free viruses of laboratory-adapted HIV-1 strains from subtype B were used to infect human CD4 T cell lines.

It has been known that the laboratory-adapted HIV-1 strains have a phenotype that does not represent the majority of viruses present in an infected individual. In general, laboratory-adapted strains replicate rapidly to a high titer and are more prone to be neutralized by antibodies than primary isolates. Many antibodies that neutralize laboratory-adapted strains very well are often much less effective in neutralizing primary isolates. Therefore, to further test the effectiveness of the m-scFv on HIV-1 infection, in this study, we carried out experiments to determine whether the m-scFv can block infection of primary isolates from various HIV-1 subtypes and whether the m-scFv can neutralize HIV-1 captured and transferred by DC-SIGN on the surface of monocyte cell lines or DCs.

**MATERIALS AND METHODS**

**Viruses and cell lines**

HIV-1 primary isolates Ug-92-029 (subtype A), 302056 (subtype B), 301960 (subtype C), Ug-92-005 (subtype D), and TH-93-054 (subtype E) were obtained from NIH AIDS Research and Reference Reagent Program (Germantown, MD) and propagated in the phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells (PBMCs). The R5-tropic primary HIV-1 isolate #A was isolated from subject A in a cohort of individuals who were recruited for a study of HIV-1 fitness and who were not receiving highly active antiretroviral therapy (HAART). Informed consent was obtained from the participants in accordance with the Institutional Review Boards of the University of Texas Health Science Center at Houston, Texas and the Baylor College of Medicine. The cell tropism of primary isolate #A was determined using a panel of GHOST coreceptor cell lines as previously described.

HIV-1 molecular clones MJ4 (R5 tropic, subtype C), NL4-3 (X4 tropic, subtype B), and Yu2 (R5 tropic, subtype B) were produced by transfecting proviral plasmids pMJ4, pNL4-3, or pYu2 (NIH AIDS Research and Reference Reagent Program) into 293T cells using a calcium phosphate precipitation or a Fugene-6 reagent method (Roche Diagnostics, Mannheim, Germany).

The packaging cell line 293T was maintained in complete Dulbecco’s modified essential medium (DMEM) [i.e., high glucose DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml); Invitrogen Life Technologies]. The packaging cell line PT67 was purchased from Clontech Laboratories (Palo Alto, CA) and maintained in complete DMEM. Cell line THP-1, that is Raji B cells according to the report by Wu et al., was maintained in complete RPMI 1640 medium [RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (2-ME)]. Stable transduced CEMss cell lines, CEMss-eGFP and CEMss-m-scFv, were generated before and maintained in complete DMEM supplemented with 1.5 mg/ml G418.
Generation of a stable THP-1 cell line expressing human DC-SIGN

Complementary DNA encoding human DC-SIGN was amplified by polymerase chain reaction (PCR) with a pair of oligos (DC1F: 5'-tgattagctcaccataggtactcaaggaacagtg-3' and DC1225R: 5'-ggtgaagttctgctacgcaggag-3') using plasmid pBlissTOPO p33-3 as a template (a generous gift from Dr. Sunil K. Ahuja at the University of Texas Health Science Center, San Antonio, TX) and cloned into a TA vector (Invitrogen Life Technologies) for sequencing analysis. cDNA containing a correct sequence of human DC-SIGN was cloned into HindIII and Hpal sites of the retroviral vector pLNCX-TCR, which is a modified version of pLNCX (Clontech Laboratories), into which we had inserted a TCR enhancer sequence upstream of the promoters, UG-92-029 (subtype A), 302056 (subtype B), MJ4 (subtype C), 901650 (subtype C), UG-92-005 (subtype D), and TH-93-054 (subtype E) (60,000 cpm reverse transcriptase activity) in a final volume of 0.5 ml. Cells were then washed three times with Hanks’ balanced salt solution (HBSS) and resuspended in 6 ml of complete DMEM and incubated at 37°C for 22–35 days dependent on the individual primary isolates tested. Every 3 or 4 days, 4.5 ml of cell suspensions was harvested and replaced with the fresh medium. The supernatants were then collected. HIV-1 p24 in the supernatants was measured by ELISA (Beckman Coulter) according to the manufacturer’s instruction.

HIV-1 infection and p24 assay

Pooled EGFP- and m-scFv-transduced CEM-ss cells lines (1 × 10⁶) were incubated overnight with HIV-1 primary isolates, UG-92-029 (subtype A), 302056 (subtype B), MJ4 (subtype C), 901650 (subtype C), UG-92-005 (subtype D), and TH-93-054 (subtype E) (60,000 cpm reverse transcriptase activity) in a final volume of 0.5 ml. Cells were then washed three times with Hanks’ balanced salt solution (HBSS) and resuspended in 6 ml of complete DMEM and incubated at 37°C for 22–35 days dependent on the individual primary isolates tested. Every 3 or 4 days, 4.5 ml of cell suspensions was harvested and replaced with the fresh medium. The supernatants were then collected. HIV-1 p24 in the supernatants was measured by ELISA (Beckman Coulter) according to the manufacturer’s instruction.

Generation of pseudotypes of HIV-1 vector and a single-cycle infectivity assay

To generate pseudotypes of HIV-1 vector, 4.5 × 10⁶ 293T packaging cells were cotransfected with 20 μg of HIV-1-luciferase transfer vector and 20 μg of DNA plasmid encoding either HIV-1 envelope pDOL or Q168 or VSV-G control using a calcium phosphate precipitation method. HIV-1 envelope pDOL is X4 tropic and HIV-1 envelope Q168 is R5 tropic. Because VSV-G envelope interacts with lipid moiety in the lipid bilayer of the cytoplasmic membrane, VSV-G pseudotypes bypass the requirement of the interaction between HIV-1 envelope and its receptor and coreceptor to enter cells. After overnight incubation, cells were washed once with HBSS and cultured in 10 ml of complete DMEM supplemented with 100 μM sodium butyrate. Six hours later, cells were washed once with HBSS and cultured in 10 ml of complete DMEM. The pseudotype-containing supernatants were harvested in 16–20 h and the amount HIV-1 p24 in the supernatants was measured by ELISA.

In a single-cycle assay to measure the infectivity of pseudotypes, 1 × 10⁴ CEMss-eGFP or CEMss-m-scFv cells were transduced with various amounts of pseudotype-containing supernatants overnight. Cells were then washed twice with HBSS and cultured in complete DMEM for 3 days. Cells were then harvested and washed once with HBSS (without phenol red) and resuspended in 200 μl of HBSS (without phenol red). Luciferase activity in 50 μl of cell suspensions was measured by a BrightGlo Luciferase assay according to the manufacturer’s instruction (Promega).
Capture and transfer assay with monocytic cell lines

THP-1 or THP-1 DC-SIGN cells (1 × 10⁶) were incubated with 2 ml of freshly harvested pDOL pseudotype-containing supernatants at 37°C and 5% CO₂ for 4 h. After the incubation, cells were washed extensively to remove free pseudotyes. Cells were resuspended in fresh complete DMEM at a concentration of 1 × 10⁶/ml. Pooled CEMss-eGFP and CEMss-m-scFv cells were also resuspended in fresh complete DMEM at a concentration of 1 × 10⁶/ml. Then 100 μl of THP-1 or THP-1-DC-SIGN (1 × 10⁵ cells) and 100 μl of CEMss-eGFP and CEMss-m-scFv cells (1 × 10⁵ cells) were seeded in triplicate onto a 96-well round-bottom plate. As controls, we also seeded THP-1/pDOL, or THP-1-DC-SIGN/pDOL, alone without CEMss-eGFP or CEMss-m-scFv. After a brief centrifugation, cells were incubated at 37°C and 5% CO₂ for 3 days. Then 50 μl of cell suspension was collected from each well and luciferase activity was measured by a Bright-Glo Luciferase assay (Promega). In some capture and transfer experiments THP-1 or THP-1-DC-SIGN cells were pretreated with or without 20 μg/ml of mannan (Sigma) for 30 min. After the pretreatment, cells were washed to remove free mannan and resuspended with freshly harvested pDOL pseudotype-containing supernatants as described above. After 4 h incubation, cells were extensively washed to remove free pseudotyes. Subsequent coculture of pDOL pseudotype-captured THP-1 or THP-1-DC-SIGN cells with CEMss-eGFP or CEMss-m-scFv cells and the measurement of relative luciferase activity in cell suspension were the same as described above.

Ex vivo generation of human DCs

Peripheral blood was obtained from healthy donors at the Gulf Coast Regional Blood Center, Houston, TX. PBMCs were isolated using Ficoll density gradient centrifugation as described before. To isolate CD14⁺ monocytes, the anti-CD14 microbeads and miniMACS system were used according to the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). Briefly, 10⁸ PBMCs were resuspended in 800 μl of binding buffer (PBS containing 0.5% BSA and 2 mM EDTA). Then 200 μl of MACS CD14 microbeads were added, mixed, and incubated at 4°C for 15 min. The cell–bead mixture was then washed once with binding buffer and resuspended in 1 ml of fresh binding buffer. CD14⁺ cells were positively selected using an MS⁺/RS⁺ column tip. The isolated cells were washed once with complete RPMI and cultured in complete RPMI supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (1000 U/ml) and interleukin (IL)-4 (1000 U/ml) (R & D System, Minneapolis, MN) for 7 days to generate immature DCs as previously described. The expression of DC-SIGN on the cell surface of immature DCs was measured by anti-DC-SIGN antibody staining followed by FACS analysis as described above.

Capture and transfer assay with human DCs

DCs (10⁵ per well) were seeded in triplicate in a 96-well U-bottom plate and incubated with 25 μl of NL4-3, 50 μl of YU-2, or 100 TCID₅₀ of primary isolate #A in a total volume of 200 μl at 37°C and 5% CO₂ for 4 h. Cells were then washed three times with PBS to remove free viruses and resuspended in 2 ml of complete RPMI into wells of a 24-well plate with or without 1.5 × 10⁵ CEMss-eGFP or CEMss-m-scFv cells added. Cells continued to be cultured for 30 days. Every 3 days, medium was removed from each well and replaced with fresh complete medium. HIV-1 p24 in the supernatants was measured by ELISA as described above.

RESULTS

Cell surface expression and stability of m-scFv in transduced CEMss cells

To determine the stability of the m-scFv in transduced cells, pulse-chase experiments were performed in stable m-scFv- and eGFP-transduced CEMss cells. Figure 1A shows that m-scFv was relatively stable, with a t₁/₂ about 12 h. Figure 1B shows the cell surface expression of m-scFv in a representative CEMss-m-scFv cell line as compared to parental CEMss cells. Cells were stained with anti-human κ chain antiserum followed by PE-conjugated goat antihuman IgG Fc portion antibody.

FIG. 1. (A) Pulse-chase analysis of m-scFv proteins in cell lysates of CEMss-eGFP and CEMss-m-scFv cell lines. Chase time in hours. (B) Cell surface expression of m-scFv in a representative CEMss-m-scFv cell line as compared to parental CEMss cells. Cells were stained with anti-human κ chain antiserum followed by PE-conjugated goat antihuman IgG Fc portion antibody.
As reported in our previous studies, no significant difference in the cell surface expression of CD4, CXCR4, and CCR5 among parental or eGFP- or m-scFv-transduced CEMss was found (data not shown).

Inhibition of viral replication of primary HIV-1 isolates by m-scFv

To test whether the m-scFv has broad neutralizing activity, pooled CEMss-m-scFv lines along with pooled CEMss-eGFP controls were incubated overnight with HIV-1 primary isolates UG-92-029 (subtype A), 302056 (subtype B), 301960 (subtype C), Mj4 (subtype C), UG-92-005 (subtype D), and TH-93-054 (subtype E). Pooled cell lines were used for the infection to minimize potential variation among individual cell lines. Among six primary isolates tested, two isolates (302056, and TH-93-054) infected poorly. During 35 days of culture, we were not able to detect p24 in the supernatants of both CEMss-m-scFv and CEMss-eGFP controls, indicating that these viruses may not infect CEMss cell lines (data not shown). Four other isolates (UG-92-029, 301960, Mj4, and UG-92-005) infect and replicate quite well in CEMss-eGFP cells; while in CEMss-m-scFv cells HIV-1 replication was markedly inhibited (Fig. 2). For cells infected with UG-92-029, inhibition was apparent at day 5 and continued throughout the 22-day experiment. Supernatants collected on days 12, 15, 19, and 22 showed 98–99.9% reduction (Fig. 2A). For cells infected with Mj4, inhibition was apparent at day 6 and continued throughout the 32-day experiment. At the peak of viral production a more than 4-log reduction was observed (Fig. 2D). These results indicated that the m-scFv not only can inhibit laboratory-adapted HIV-1 strains as shown in a previous report, but it also can effectively inhibit primary isolates. Moreover, although the monoclonal antibody (TG15) (from which the m-scFv was derived) was originally derived from a human B cell hybridoma generated from a subtype B HIV-1-infected individual, it not only neutralizes HIV-1 from B subtypes (shown in the previous studies as well as studies with

<table>
<thead>
<tr>
<th>Pseudotypes</th>
<th>CEMss-eGFP</th>
<th>CEMss-m-scFv</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDOL</td>
<td>41,279 ± 301</td>
<td>1,037 ± 45</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Q168</td>
<td>30,705 ± 210</td>
<td>496 ± 30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VSV-G</td>
<td>211,326 ± 2,342</td>
<td>242,440 ± 3,255</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Mock</td>
<td>250 ± 21</td>
<td>270 ± 25</td>
<td></td>
</tr>
</tbody>
</table>

*Mean value ± standard deviation.
HIV-1 envelope pseudotypes, see below), but also HIV-1 from subtypes A, C, and D as well. Thus, on the cell surface, the m-scFv acts as a broad and potent neutralizing antibody.

**Inhibit free HIV-1 pseudotype infection in a single-cycle infectivity assay by m-scFv**

To test whether the m-scFv can neutralize HIV-1 captured and transferred by DC-SIGN in a monocytic cell line or dendritic cells, we used a single-cycle infectivity assay. To accomplish this, we generated pseudotypes by cotransfecting 293T packaging cells with an HIV-1-luciferase transfer vector and a DNA plasmid encoding either HIV-1 envelope pDOL or Q168 or VSV-G control using a calcium phosphate precipitation method (see Materials and Methods for details). After the pseudotype-containing supernatants were harvested, the amount of HIV-1 p24 in the supernatants was measured by ELISA and the infectivity of pseudotypes was first measured using free viral particles. We found that the amount of p24 in the supernatants varies among different batches, ranging from 30 to 930 ng/ml for Q168 pseudotypes, from 200 to 9000 ng/ml for pDOL pseudotypes, and from 20 to 932 ng/ml for VSV-G pseudotypes. For both Q168 and pDOL pseudotypes, supernatants equivalent to or more than 100 ng of p24 were required to obtain measurable infectivity in this single-cycle infectivity assay. However, for VSV-G pseudotypes, supernatants equivalent to 0.01 ng of p24 already yielded measurable infectivity (data not shown).

Table 1 shows the results of relative luciferase activity of CEMss-eGFP and CEMss-m-scFv transfected with or without pDOL, Q168, or VSV-G pseudotypes in a representative experiment. While both pDOL and Q168 pseudotypes infected well in CEMss-eGFP cells, significant reduction in infectivity was observed in CEMss-m-scFv cells. For CEMss-m-scFv cells infected with pDOL pseudotypes, close to a 40-fold reduction was observed as compared to CEMss-eGFP cells. For CEMss-m-scFv cells infected with Q168 pseudotypes, about a 60-fold reduction was observed as compared to CEMss-eGFP cells. In contrast, similar infectivity to CEMss-eGFP and CEMss-m-scFv cells was observed with VSV-G pseudotype. The experiment has been repeated three more times with similar results. Therefore, similar to what was found previously, these results indicated that the m-scFv specifically targets the HIV-1 envelope.

**Inhibits the infection of HIV-1 captured and transferred by DC-SIGN on a monocytic cell line by m-scFv**

Having demonstrated that m-scFv specifically neutralizes the infectivity of free HIV-1 envelope pseudotypes, we next determined whether it can also neutralize HIV-1 envelope pseudotypes captured and transferred by DC-SIGN. We transduced THP-1 (Raji B cell line) with a gene encoding human DC-SIGN using a retroviral vector. Stable THP-1-DC-SIGN cell lines were established by G418 selection (see Materials and Methods for details). Figure 3A shows the cell surface expression of DC-SIGN in human DC-SIGN-transduced THP-1 cells as compared to parental THP-1 cells stained with antihuman DC-SIGN antibody and followed by FACS analysis.

In capture and transfer experiments, THP-1 or THP-1-DC-SIGN cells were incubated with freshly harvested pDOL pseudotype-containing supernatants for 4 h. After the incubation, cells were washed extensively to remove free pseudotypes. The THP-1 or THP-1-DC-SIGN cells and pooled CEMss-eGFP and CEMss-m-scFv cells were seeded in triplicate onto a 96-well U-bottom plate. As controls, we also seeded THP-1/pDOL or THP-1-DC-SIGN/pDOL alone without CEMss-eGFP or CEMss-m-scFv (see Materials and Methods for details). Cells were incubated at 37°C and 5% CO₂ for 3 days and luciferase activity was measured. Table 2 shows the results of relative luciferase activity of CEMss-eGFP and CEMss-m-scFv infected with pDOL pseudotypes captured and transferred by THP-1 or THP-1-DC-SIGN in a representative experiment. THP-1 cells alone did not capture HIV-1 pseudotypes, resulting in minimum infectivity in CEMss-eGFP and CEMss-m-scFv cells. In contrast, THP-1-DC-SIGN cells captured both pDOL pseudotypes, resulting in the expression of luciferase in THP-1-DC-SIGN cells themselves (Table 2, without target cells) and the transfer of pseudotypes to target cells (Table 2, CEMss-eGFP). The luciferase activity in CEMss-eGFP cells cocultured with pDOL pseudotype-captured THP-1-DC-SIGN cells increased 14.5-fold as compared to pDOL pseudotype-captured THP-1-DC-SIGN cells alone. In contrast, the luciferase activity in CEMss-m-scFv cells cocultured with pDOL pseudotype-captured THP-1-DC-SIGN cells increased only slightly as compared to pDOL pseudotype-captured THP-

**FIG. 3.** (A) Cell surface expression of DC-SIGN of representative THP-1 and THP-1-DC-SIGN cell lines stained with FITC-conjugated antihuman CD209 (DC-SIGN) antibody. (B) Cell surface expression of DC-SIGN on human monocyte-derived DCs stained with either FITC-conjugated antihuman CD209 antibody or FITC-conjugated isotype-matched control antibody.
The experiment has been repeated several times with similar results. Therefore, our results clearly demonstrated that the m-scFv can also neutralize pseudotypes captured and transferred by the DC-SIGN molecules in monocytic cell line.

To further test whether the capture and transfer of pseudotypes to target cells were DC-SIGN specific, in subsequent experiments THP-1 or THP-1-DC-SIGN cells were pretreated with or without 20 μg/ml of soluble mannan. The DC-SIGN molecules are known to contain a single carbohydrate recognition domain (CRD) consisting of a distinctive triplet of amino acids (Glu-Pro-Asn) that recognizes mannose-type ligands.31 Mannan binds to the CRD of DC-SIGN and blocks DC-SIGN to capture mannose-type ligands such as those on HIV-1 envelope proteins.31 After the pretreatment, cells were washed to remove free mannan and the capture and transfer experiments with freshly harvested pDOL pseudotype-containing supernatants are the same as described above. Table 3 shows the results of relative luciferase activity of CEMss-eGFP and CEMss-m-scFv infected with pDOL pseudotype captured and transferred by THP-1 or THP-1-DC-SIGN with or without the pretreatment of mannan in a representative experiment. As shown above, THP-1 cells alone did not capture HIV-1 pseudotypes, whereas THP-1-DC-SIGN cells captured pseudotypes, resulting in the expression of luciferase in THP-1-DC-SIGN cells themselves (Table 3, without target cells) and the transfer of pseudotypes to target cells (Table 3, CEMss-eGFP). In contrast, the luciferase activity in CEMss-m-scFv cells cocultured with pseudotype-captured THP-1-DC-SIGN cells only slightly increased as compared to pseudotype-captured THP-1-DC-SIGN cells alone. Moreover, the pretreatment of mannan significantly reduced pseudotypes captured by THP-1-DC-SIGN cells, which in turn significantly reduced pseudotypes transferred to target cells. The experiment has been repeated several times with similar results. Taken together, in these single-cycle infectivity studies with HIV-1 envelope pseudotypes, we clearly demonstrated that m-scFv neutralizes free HIV-1 as well as HIV-1 captured and transferred by the DC-SIGN molecules on the cell surface of monocytic cell lines.

**m-scFv inhibits the infection of HIV-1 captured and transferred by human DCs**

To test the effect of m-scFv on the infection of HIV-1 captured and transferred by human DCs, monocyte-derived human DCs were first tested using freshly harvested pDOL or Q168 pseudotype-containing supernatants in a single cycle infection assay as described above. Due to the small number of DCs available in the assay, we did not obtain measurable luciferase activity in this single cycle infection assay (data not shown). Therefore, in subsequent capture and transfer experiments monocyte-derived human DCs were incubated with HIV-1 NL4-3, Yu-2, or primary isolate #A instead. After the incubation, cells were washed extensively to remove free viruses. Infected DCs and pooled CEMss-eGFP and CEMss-m-scFv cells were seeded in triplicate onto a 24-well plate. As controls, we also seeded DCs infected with NL4-3, Yu-2, or primary isolate #A alone (see Materials and Methods for details). Cells were cultured for 30 days. Every 3 days, medium was removed from each well and replaced with fresh complete medium. HIV-1 replication was measured by HIV-1 p24 assay. Figure 3B shows the expression of DC-SIGN in monocyte-derived human DCs. As reported before,30 DC-SIGN molecules are well expressed on the cell surface of monocyte-derived human DCs. Table 4 shows the amount of HIV-1 gag p24 in supernatants of CEMss-eGFP and CEMss-m-scFv cocultured with NL4-3, Yu-2, or primary isolate #A alone and #A captured and transferred by human DCs in a representative experiment. DCs alone infected with NL4-3, Yu-

### Table 2. Relative Luciferase Activity in CEMss-eGFP or CEMss-m-scFv Cells Cocultured with pDOL Pseudotype-Captured and Transferred by THP-1 or THP-1 DC-SIGN Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>CEMss-eGFP</th>
<th>CEMss-m-scFv</th>
<th>Without target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1/pDOL</td>
<td>147 ± 20</td>
<td>233 ± 25</td>
<td>158 ± 26</td>
</tr>
<tr>
<td>THP-1-DC-SIGN/pDOL</td>
<td>37,873 ± 551</td>
<td>2,861 ± 230</td>
<td>2,621 ± 224</td>
</tr>
<tr>
<td></td>
<td>(&lt;0.01)a</td>
<td>(&lt;0.01)b</td>
<td>(&gt;0.05)c</td>
</tr>
<tr>
<td>Without</td>
<td>114 ± 10</td>
<td>127 ± 12</td>
<td></td>
</tr>
</tbody>
</table>

*p value between CEMss-eGFP and CEMss-m-scFv.

**Table 3. Relative Luciferase Activity in CEMss-eGFP or CEMss-m-scFv Cells Cocultured with pDOL Pseudotype with or without Pretreatment of 20 μg/ml of Mannan**

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<thead>
<tr>
<th>Condition</th>
<th>CEMss-eGFP</th>
<th>CEMss-m-scFv</th>
<th>Without target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1/pDOL</td>
<td>90 ± 10</td>
<td>171 ± 15</td>
<td>129 ± 15</td>
</tr>
<tr>
<td>THP-1-DC-SIGN/pDOL</td>
<td>105 ± 9</td>
<td>112 ± 10</td>
<td>170 ± 20</td>
</tr>
<tr>
<td>THP-1-DC-SIGN/pDOL/mannan</td>
<td>33,407 ± 270</td>
<td>2,514 ± 112</td>
<td>2,021 ± 89</td>
</tr>
<tr>
<td>THP-1-DC-SIGN/pDOL/mannan</td>
<td>306 ± 15</td>
<td>223 ± 15</td>
<td>647 ± 35</td>
</tr>
</tbody>
</table>

*p value between CEMss-eGFP and CEMss-m-scFv.

**p value between CEMss-eGFP and without target cells.

**p value between CEMss-m-scFv and without target cells.
INHIBITION OF HIV-1 BY NONNEUTRALIZING Ab

The lower level of HIV-1 replication in DCs found in this study is consistent to the recent report by Nobile et al. that HIV-1 replicates covertly and slowly in monocyte-derived human DCs. However, 6 days after coculturing CEMss-eGFP with NL4-3-infected DCs the amount of p24 in the supernatant increased 336-fold as compared to NL4-3-infected DCs alone (1244 ng/ml vs. 3.7 ng/ml). In contrast, 6 days after coculturing CEMss-m-scFv with NL4-3-infected DCs the amount of p24 in the supernatant increased only 24-fold as compared to NL4-3-infected DCs alone (87.6 ng/ml vs. 3.7 ng/ml). Therefore, although both CEMss-eGFP and CEMss-m-scFv can be infected with DC-captured NL4-3, CEMss-m-scFv is much less susceptible to the infection than CEMss-eGFP. Moreover, as reported previously with cell-free HIV-1 IIB infection, the inhibition of NL4-3 infection by m-scFv is also transient. Fifteen days after coculturing with DC-captured NL4-3, there is no difference in the amount of HIV-1 gag p24 in the supernatants between CEMss-eGFP and CEMss-m-scFv (data not shown). Coculturing CEMss-eGFP and CEMss-m-scFv with DC-captured R5 viruses (Yu-2 and #A) resulted in much slower HIV-1 replication kinetics. Only after 3 weeks or later could measurable HIV-1 gag 24 be detected in the supernatants (data not shown). Thirty days after coculturing CEMss-eGFP with primary isolate #A-infected DCs the amount of p24 in the supernatant increased 70-fold as compared to primary isolate #A-infected DCs alone (21.2 ng/ml vs. 0.3 ng/ml). In contrast, 30 days after coculturing CEMss-m-scFv with primary isolate #A-infected DCs the amount of p24 in the supernatant increased only 2.7-fold as compared to primary isolate #A-infected DCs alone (0.8 ng/ml vs. 0.3 ng/ml), indicating that CEMss-m-scFv is much less susceptible to the infection of DC-captured primary isolate #A than CEMss-eGFP. Thirty days after coculturing CEMss-eGFP with Yu-2-infected DCs the amount of p24 in the supernatant increased 2-fold as compared to Yu-2-infected DCs alone (72.3 ng/ml vs. 35.6 ng/ml). Intriguingly, 30 days after coculturing CEMss-m-scFv with Yu-2-infected DCs the amount of p24 in the supernatant was only 2.6 ng/ml, which was much lower than Yu-2-infected DCs alone (35.6 ng/ml). The experiment has been repeated once with similar results. A much lower amount of p24 in the supernatants of CEMss-m-scFv cocultured with Yu-infected DCs than in Yu-2-infected DCs alone suggests that m-scFv may also act in trans to block HIV-1 replication in DCs. Taken together, our results demonstrated that m-scFv can neutralize HIV-1 captured and transferred by human DCs.

**DISCUSSION**

In our previous studies, we demonstrated that although the expression of m-scFv on the cell surface does not alter the surface expression of CD4, CXCR4, and CCR5, it markedly inhibits HIV-1 replication of laboratory adapted strains of subtype B and HIV-1 envelope-mediated cell–cell fusion. The inhibition is at the level of viral entry, HIV-1 envelope specific, and was not affected by virus tropism. In this study, we further demonstrated that the m-scFv not only inhibits laboratory-adapted HIV-1 strains, but it also effectively inhibits primary isolates. It not only neutralizes HIV-1 from B subtypes, but also HIV-1 from subtypes A, C, and D as well. Thus, taken together, it is likely that on the cell surface, the m-scFv acts as a broad neutralizing antibody.

In this study, we also demonstrated that the m-scFv not only inhibits the infection of CD4 T cells by cell-free viruses, but also significantly blocks HIV-1 captured and transferred by DC-SIGN on monocytic cell lines and on human DCs. In a time-lapse experiment McDonald et al. have shown that before DCs contact target cells the HIV-1 captured and internalized by DCs were evenly distributed throughout cells. However, soon after DCs contact target CD4 cells the majority of the HIV-1 relocated to the initial site of contact to form an infectious synapse. Within the infectious synapse local concentration of HIV-1 as well as receptor and coreceptor are greatly increased, which facilitates transmission of HIV-1 from DCs to target cells. Since the m-scFv markedly inhibits HIV-1 captured and transferred by DCs, it is likely that the m-scFv on the target cell surface may also migrate to the infectious synapse. By so doing, it effectively blocks transmission of HIV-1 from DCs to target CD4 T cells. In addition, we unexpectedly found that the amount of p24 in the supernatants of CEMss-m-scFv cocultured with Yu-2-infected DCs is significantly lower than in Yu-2-infected DCs alone (see Table 4). Therefore, the m-scFv in the infectious synapse not only blocks HIV-1 transmission from DCs to CD4 target cells, but also acts in trans to block HIV-1 replication in DCs. It will be interesting to determine how the m-scFv works in trans.

It has been postulated that DC-SIGN on the surface of mucosal tissue DCs acts as a sentinel to capture HIV-1 on the mucosal surface and then efficiently transfer HIV-1 to CD4 T cells underneath the epithelial layer (s) of mucosal surface or in draining lymph nodes. It thereby plays an important role in HIV-1 transmission and spread. Significant inhibition of HIV-1 transmission from DCs to target CD4 T cells by m-scFv leads us to postulate that if m-scFv can be delivered to the surface of cells in mucosal tissues either through viral vectors or through

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**Table 4. Amount of HIV-1 gag p24 (ng/ml) Detected in Supernatants of CEMss-eGFP or CEMss-m-scFv Cells Cocultured with Human DC-Captured HIV-1 NL4-3, Yu-2, or #A**

<table>
<thead>
<tr>
<th></th>
<th>CEMss-eGFP</th>
<th>CEMss-m-scFv</th>
<th>Without target cells&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human DCs/NL4-3</td>
<td>1244 ± 410</td>
<td>87.6 ± 1.5</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Human DCs/Yu-2</td>
<td>72.3 ± 9.6</td>
<td>2.6 ± 0.3</td>
<td>35.6 ± 1.8</td>
</tr>
<tr>
<td>Human DCs/#A</td>
<td>21.2 ± 6.6</td>
<td>0.8 ± 1.1</td>
<td>0.3 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Without means the amount of HIV-1 gag p24 detected in supernatants of NL4-3, Yu-2, or #A infected DCs without cocultured with CEMss-eGFP or CEMss-m-scFv.
chemical means, it may act as a blocking agent to prevent HIV-1 transmission between individuals. However, given the relatively short half-life of m-scFv (about 12 h, see Fig. 1A), increasing the half-life of m-scFv on the cell surface will be an important consideration for future usage of this m-scFv as a blocking agent to prevent HIV transmission. Experiments are currently underway to test the half-life and the level of expression of several new modified m-scFv.

Finally, besides cell-free virus, HIV-1 transmission can take place in cell-associated forms, such as an infectious synapse between DCs and target CD4 T cells or exosome exchange. Conventional soluble neutralizing antibodies are known to be effective in blocking infection by cell-free viruses. But whether they are also effective in blocking infection by cell-associated viruses is questionable. Neutralizing activity was demonstrated only when antibody and virus were preincubated before being added to DCs. However, after virus was captured by DCs, antibody no longer prevented the transmission of virus from DCs to CD4 T cells. Thus, DCs may facilitate escape from neutralizing antibodies. Infectious synapse could be too tight to be accessible to these antibodies and viruses carried by exosomes could be invisible to detection by these antibodies. However, membrane-bound antibody, due to its presence at the infectious synapse and on the target cell surface, may be able to block both cell-free and cell-associated viruses. Therefore, significant inhibition of transmission of DC-captured and transferred HIV-1 to CD4 T cells by the m-scFv in this study strongly argues that membrane-bound antibody can block virus transmission under conditions where conventional soluble neutralizing antibody fails to do so. Because of this, the m-scFv may also be used as a tool to dissect the mechanism of HIV-1 entry via DC-SIGN capture and transfer to CD4 T cells.

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