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High Level of Coreceptor-independent HIV Transfer Induced by Contacts between Primary CD4 T Cells*

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Cell-to-cell virus transmission is one of the most efficient mechanisms of human immunodeficiency virus (HIV) spread, requires CD4 and coreceptor expression in target cells, and may also lead to syncytium formation and cell death. Here, we show that in addition to this classical coreceptor-mediated transmission, the contact between HIV-producing cells and primary CD4 T cells lacking the appropriate coreceptor induced the uptake of HIV particles by target cells in the absence of membrane fusion or productive HIV replication. HIV uptake by CD4 T cells required cellular contacts mediated by the binding of gp120 to CD4 and intact actin cytoskeleton. HIV antigens taken up by CD4 T cells were rapidly endocytosed to trypsin-resistant compartments inducing a partial disappearance of CD4 molecules from the cell surface. Once the cellular contact was stopped, captured HIV were released as infectious particles. Electron microscopy revealed that HIV particles attached to the surface of target cells and accumulated in large (0.5–1.0 μm) intracellular vesicles containing 1–14 virions, without any evidence for massive clathrin-mediated HIV endocytosis. The capture of HIV particles into trypsin-resistant compartments required the availability of the gp120 binding site of CD4 but was independent of the intracytoplasmic tail of CD4. In conclusion, we describe a novel mechanism of HIV transmission, activated by the contact of infected and uninfected primary CD4 T cells, by which HIV could exploit CD4 T cells lacking the appropriate coreceptor as an itinerant virus reservoir.

For many viruses, cell-to-cell virus transmission is the most efficient mechanism of viral spread because of the extremely low infectivity of free viral particles (1). In the case of human immunodeficiency virus (HIV),[1] free viral particles are infectious but show a short life span at 37 °C (2) and lower infective capacity than cell-to-cell transmission (3). This latter phenomenon is favored by the polarization of viral production in the infected cell (4) and the viral receptors and coreceptors in the target cell leading to the formation of a functional (infectious) virological synapse (5, 6). Cell-to-cell virus transmission is probably involved in the spread of HIV among different populations of $CD4^+$ cells *in vivo* and seems to play an essential role in sexual or vertical transmission through epithelia (7, 8).

The process of membrane fusion induced by the envelope glycoprotein complex of HIV is independent of pH, and therefore endocytic internalization and endosomal acidification are not required to activate HIV entry into the cytoplasm (9–12). Instead, viral entry involves direct fusion of viral and plasma cell membrane that allows for the delivery of the viral core into the cytoplasm of target cells (13). First, HIV envelope (gp120/gp41, Env) binds to CD4 and then to a chemokine receptor (CXCR4 for X4 strains and CCR5 for R5 strains), which is used by HIV particles to activate the gp41-mediated membrane fusion.

A variety of cell types such as macrophages, endothelial and epithelial cells, and also lymphoid cells are able to bind and to internalize HIV particles into vesicular structures coordinately or independently of HIV receptors (7, 14–18). Once internalized, HIV particles may follow different pathways: they can be secreted (19), as in the case of transcytosis (7), degraded (20), or they can fuse with vesicular membranes to inject the viral core into the cytoplasm and initiate the infectious viral cycle (16, 17). Consistently, pharmacological stabilization of the endosomal compartment increases HIV infection (18, 20). Nevertheless, the exact contribution of endocytic pathways to the infection of CD4 T lymphocytes and to HIV pathogenesis *in vivo* is mostly unknown, and the mechanisms involved in endocytosis are unclear. The term endocytosis includes at least four mechanisms: phagocytosis, macropinocytosis, clathrin-mediated and caveolin-mediated endocytosis (21), showing different properties such as vesicular size, markers, and regulation. Macropinocytosis, which is characterized by big sized vesicles, and clathrin-coated pit-mediated endocytosis seem to be the major nonfusogenic HIV entry pathway in most cell types (15, 16).

HIV transmission during cell-to-cell contacts follows the same mechanism as cell-free HIV particles for entry into target cells. However, the potential implications of synaptic structures in the mechanism of transmission are still unclear. We have used previously developed experimental models to study cell-to-cell contacts during HIV infection (22–24). We observed that during cellular contacts primary cells underwent massive cell-to-cell viral transmission. The characterization of this phe-

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[1] The abbreviations used are: HIV, human immunodeficiency virus; AZT, 3′-azido-3′deoxythymidine; Env, envelope; IL, interleukin; mAb, monoclonal antibody; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; RFI, relative fluorescence intensity.

nomenon showed that in addition to fusion-dependent HIV transmission, high amounts of HIV were reversibly transferred from infected cells to trypsin-resistant compartments of target cells by a mechanism that required gp120 binding to CD4 but not to coreceptor function. CD4 T cells lacking the appropriate coreceptor may therefore become an itinerant virus reservoir that may contribute to the spread of HIV infection.

EXPERIMENTAL PROCEDURES

Cells—Peripheral blood mononuclear cells (PBMC) from healthy donors were purified by Ficoll-Hypaque sedimentation. When necessary, CD4 T cells were immediately purified (>95%) from PBMC by negative selection using the CD4 T cells enrichment kit (StemCell Technologies, Vancouver, Canada). Unless indicated, primary cells were used without previous stimulation. The CEM cell line derivative A201 lacking CD4 expression and several clones expressing either wild type CD4 (A301), a truncated form of CD4 at the position 403 which lacks the cytoplasmic tail (A201/403), and a chimeric CD4/CD8 protein (A201/CD8) that contains the gp120 binding site of CD4 were described by Dr. D. Littman (11) and obtained through Dr. M. Biard (Montpellier, France). Primary cells and CEM derivatives were cultured in RPMI. HeLa P4R5 and U87.CD4/CCR5 (NIBSC and NIH, AIDS Reagent Programs) were cultured in Dulbecco's modified Eagle's medium. Media were supplemented with 10% heat-inactivated fetal calf serum (Invitrogen) and selection antibiotics when required.

Viruses and Chronically Infected Cells—Recombinant viruses carrying envelope (Env) sequences corresponding to the X4 HIV-1 strain NL4-3 or the R5 HIV-1 strain BaL were constructed in a HIV_{HXB2} backbone as described previously (25) and used to infect MOLT-4/CCR5. After the infection peak, persistently infected cultures were grown and characterized for Env expression and virus production (24). Uninfected MOLT-4/CCR5 cells were used as negative controls in all experiments. 8E5 cells, which carry one copy of the HIV genome defective for reverse transcriptase activity and produce noninfectious viral particles (26), were cultured in RPMI.

Cocultures of Infected and Uninfected Cells—Primary cells (PBMC or purified CD4 T cells, 2×10^5 cells) were usually cultured in 96-well plates with effector (uninfected or infected) MOLT-4/CCR5 cells at a ratio of 1:1 in the absence or the presence of the following HIV inhibitors: 10 μg/ml murine IgG (Santa Cruz Biotechnologies), 0.25–5 μg/ml anti-CD4 mAb Leu3a or 10 μg/ml L120.3 (Becton & Dickinson), 10 μg/ml neutralizing anti-gp120 mAb IgGb12 or 2G12, 1 μM CCR5 antagonist TAK779 (all reagents from NIH AIDS Reagent Program and NIBSC AIDS Reagent Project), 1 μM gp41 inhibitor C34 (Service of Peptide Synthesis, University of Barcelona), and 2 μM reverse transcriptase inhibitor AZT (Sigma). Other drugs used (all from Sigma) were 100 nM bafilomycin A1, 1 nM concanamycin A, 30–100 μM amiloride, and 1–3 μM cytochalasin D.

The A201 cell line and its derivatives were cultured for 6 h in conic bottom tubes with effector (uninfected or BaL-infected) MOLT-4/CCR5 cells at a ratio of 1:1 in the absence or the presence of 0.25 μg/ml of the anti-CD4 mAb Leu3a or 1 μM gp41 inhibitor C34. Target cells were labeled with 1 μM CMFDA (green cell tracker, Molecular Probes) and washed exhaustively before being cocultured with effector cells.

Evaluation of HIV Transfer—After coculture, cells were stained with anti-CD8-PerCP and anti-CD3-FITC to identify cellular populations, or with Leu3a or L120.3 anti-CD4 mAbs to evaluate CD4 internalization. Binding of gp120 to CD4 masks the Leu3a but not the L120.3 epitope (27). After surface staining, cells were fixed, permeabilized (Fix & Perm, Caltag, Burlingame, CA), and stained with KC57 anti-HIV-p24 antigen (p24) mAb (Coulter, Barcelona, Spain) and analyzed in a FACScalibur flow cytometer (Becton & Dickinson) or visualized in a Nikon eclipse TE-200 microscope coupled to a CCD Kappa camera. In cocultures involving PMBC, primary CD4 T cells were identified after simultaneous staining of HIV antigen p24, CD3, and CD8. When CD4 T cells were purified prior to coculture, target cells were gated by morphological parameters. Morphologically gated cells were 98% single CD4 T cells (24). In some experiments, in which morphological identification was not feasible, target cells were labeled with the CMFDA green cell tracker. For trypsin treatment, prior to staining, cells were washed and were treated (5′–20′, reverse transcriptase) with 0.25% trypsin solution (Invitrogen). The action of trypsin was controlled by the disappearance of the Leu3a epitope of CD4 in trypsin-treated cells (28). Trypsin action was stopped by the addition of fetal calf serum. Cells were then washed and stained as indicated above. Quantification of HIV transfer was either assessed by the percentage of $p24^+$ cells (using uninfected cells as control) or by the relative fluorescence intensity (RFI), which reflects the ratio of mean fluorescence intensity (MFI) values obtained from infected and uninfected samples.

In some experiments, up to 1 mg/ml Alexa 488-labeled dextran (Molecular Probes) was added to cells 30 min prior to mixing and maintained during the coculture period (6–24 h). Analysis of dextran uptake was performed by flow cytometry and fluorescence microscopy.

Fusion Assays—HIV-mediated fusion was determined in cocultures of uninfected or infected MOLT-4/CCR5 cells with reporter HeLa P4R5 cells (ratio 1:1) in the absence or presence of the above-mentioned drugs. Reporter cells express β-galactosidase under the control of the HIV-1 long terminal repeat, which is activated after cell-to-cell or virus-to-cell fusion. After 24 h of coculture, syncytium formation was scored by visualizing cultures in a Nikon Eclipse TE-200 microscope, and long terminal repeat transactivation was measured by determining β-galactosidase activity in lysed (0.5% Nonidet P-40) cells as described previously (24).

Electron Microscopy—Cocultured cells were washed in phosphate-buffered saline, fixed in 2.5% glutaraldehyde for 1 h, and washed twice in cacodylate buffer before postfixation in 1% osmium (1 h) and final wash in cacodylate buffer. Hot (50 °C) agar was then added to the cells. After solidification, small (1 mm^3) blocks were cut, dehydrated in increasing concentrations of ethanol, included in araldite, and incubated at 60 °C for 48 h. Thin sections were cut to select areas of the block to be processed for analysis of ultrathin sections in a Jeol JEM 1010 electron microscope.

Infectivity of Captured Virions—To analyze the fate of captured virions, we purified CD4 T cells after coculture with BaL-infected MOLT-4/CCR5 cells. Briefly, 10×10^6 CD4 T cells were cultured with 10×10^6 BaL-infected MOLT-4/CCR5 in 15-ml tubes in a final volume of 1 ml in the absence or presence of 10 μM TAK779 or 10 μg/ml mAb IgGb12. After 6 h of coculture, cells were recovered, and CD4 T cells were purified again by negative selection using the CD4 T cells enrichment kit. Purified cells were assayed for the percentage of contaminating MOLT-4/CCR5 cells by both morphological parameters and p24 staining and treated with trypsin for 10 min to remove extracellular attached viruses and antibodies. After extensive washes in phosphate-buffered saline, cells were cultured in RPMI supplemented with 10% fetal calf serum in the absence or presence of 1 μM AZT to prevent productive infection. The content of p24 antigen was evaluated at 12, 24, 48, and 72 h in the supernatant (Innogenetics enzyme-linked immunosorbent assay kit, Barcelona, Spain) and in the purified cells by flow cytometry as indicated above. Supernatants were recovered at 12 h of culture, and their infective titer was evaluated in U87.CD4/CCR5 cells. Syncytium formation were evaluated at day 6 postinfection by visualizing cultures in a Nikon Eclipse TE-200 fluorescence microscope after staining with 1 μM Hoechst 33324. Wells showing syncytia with more than four nuclei were scored as positive to calculate infectious titers.

Analysis of HIV Transfer in Primary Cells—PBMC were stimulated for 48 h with 1 μg/ml phytohemagglutinin and 10 IU/ml interleukin (IL)-2 and then infected with the R5 HIV-1 BaL strain (100 ng of p24 antigen/10^7 cells) for 4 h at 37 °C. After removal of unbound virus, cells were cultured at 10^6 cells/ml in RPMI containing 20% fetal calf serum and 10 IU/ml IL-2. In the following days, the expression of p24 antigen, CD3, CD4, and CD8 was monitored by flow cytometry as described above. Usually at day 4 after infection, the percentage of $CD8^-/CD4^-/p24^+$ cells reached a plateau (4–6% of total lymphocytes), and $CD8^+$ and $CD4^+$ cells were depleted from the culture using a combination of CD8 and CD4 T cells enrichment kits. Only $CD8^-/CD4^-/p24^+$ cells were considered to be productively infected because of the complete disappearance of CD4 from the surface of HIV-infected cells. Selected cells were reanalyzed for p24 antigen expression and were cocultured for 24 h with unstimulated uninfected fresh CMFDA-labeled CD4 T cells (ratio 1:1) in the presence of IL-2 (10 IU/ml) to maintain HIV expression by infected cells. Transfer of p24 antigen from infected to uninfected cells labeled with CMFDA was assayed as described above.

RESULTS

Transfer of HIV Antigens during Cell-to-cell Contacts—We studied cell-to-cell virus transmission in cocultures of unstimulated PBMC (target cells) with several MOLT-4/CCR5 cell lines (24) either uninfected or infected with the CXCR4-using HIV isolate NL4-3 or the CCR5-using HIV isolate BaL (effector cells). After 24 h of coculture, staining for HIV antigen p24 (p24) revealed high levels of HIV antigens in PBMC (21 and 55% of primary cells stained positive for p24 after contacting HIV-1 NL4-3- and BaL-infected cells, respectively, Fig. 1*A*).

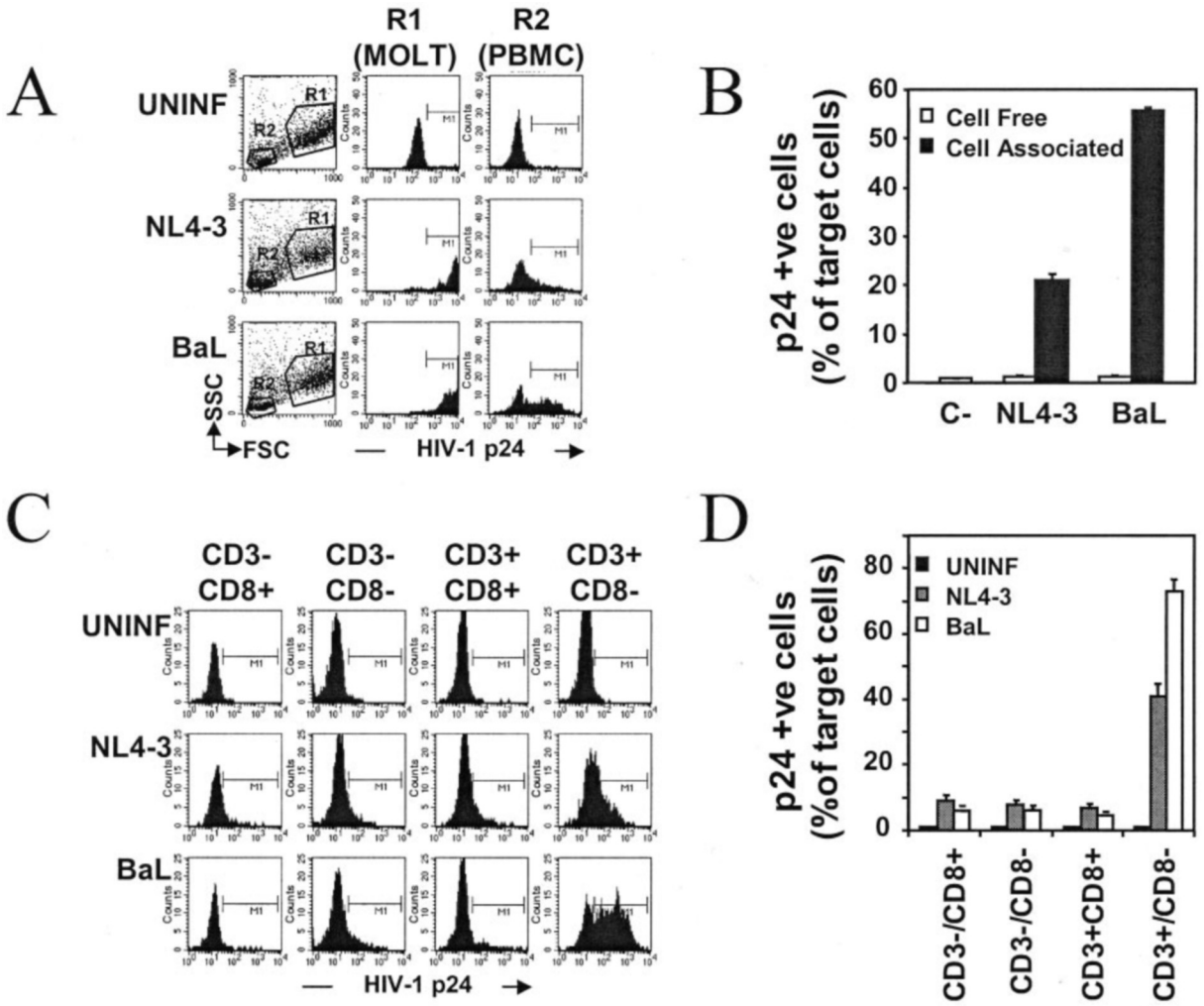

FIG. 1. **Enhanced transfer of HIV antigens to CD4 T cells during cell-to-cell contacts.** *A*, uninfected or HIV-infected (NL4-3 or BaL) MOLT-4/CCR5 cells (R1) were cultured for 24 h with freshly isolated unstimulated PBMC (R2) and stained for HIV p24 antigen. Effector cells (MOLT-4/CCR5) and target cells (primary lymphocytes) were gated according to forward and side scatter values prior to analysis. *B*, equivalent amounts of p24 antigen (73 and 35 ng of p24 antigen/well for NL4-3 and BaL viruses, respectively) were presented to PBMC as cell-free virus preparations (*empty bars*) or infected cells (*solid bars*). Analysis of p24 in target cells was performed after 24 h of culture as in *A*. *C*, in these cocultures, lymphocyte subsets were gated according to CD3 and CD8 expression and then analyzed for p24 antigen staining. *D*, quantification of the percentage of positive cells in each subset showed the highest levels of HIV antigens in $CD3^+/CD8^-$ cells, identified as $CD4^+$ T cells (mean ± S.D. of two independent experiments).

Cell-free virus preparations containing amounts of p24 equivalent to infected cells failed to transfer HIV antigens to unstimulated primary lymphocytes 24 h postinfection (<3% of $p24^+$ cells, Fig. 1*B*), indicating that p24 antigen uptake occurred during cell-to-cell contacts. Combined CD3 and CD8 staining showed a low level of transfer of p24 (less than 10% of $p24^+$ cells) to $CD4^-$ lymphocyte subsets ($CD3^+/CD8^+$, $CD3^-/CD8^+$, or $CD3^-/CD8^-$, Fig. 1*C*), whereas a high percentage of $CD3^+/CD8^-$ cells (identified as $CD4^+$ cells, Fig. 1*D*) were positive for p24 antigen staining.

In all experiments performed, BaL-infected cells induced the highest level of HIV antigen transfer, albeit of low CCR5 and high CXCR4 expression in target cells (10 ± 2% and 96 ± 4% of positive cells, respectively; data not shown). Moreover, the transfer of BaL antigens from infected to target cells was blocked by the neutralizing anti-CD4 mAb Leu3a (Fig. 2*A*) but was unaffected by a coreceptor antagonist, a gp41 peptide or a reverse transcriptase inhibitor (TAK779, C34, or AZT, respectively) (Fig. 2*A*). Similar results were observed when NL4-3-infected MOLT-4/CCR5 cells were used. In this case, the blockade of CXCR4 with AMD3100 or gp41 with C34 induced a significant increase in the amount of transferred NL4-3 antigens (Fig. 2*B*), concomitant to a total inhibition of cell-to-cell fusion and cell death (22). The activity of anti-HIV drugs was evaluated in cocultures of MOLT-4/CCR5 cells with HeLa P4R5 cells and contrasted with the lack of inhibition or enhancing effect on virus transfer (Table I). To confirm that p24 content in CD4 T cells was the result of direct antigen transfer, we cocultured PBMC with 8E5 cells, which produce replication-defective HIV particles (26). Coculture resulted in the transfer of high amounts of HIV antigens to CD4 T cells (Fig. 2*C*) which was inhibited by Leu3a, unaffected by the reverse transcriptase inhibitor AZT, and increased by the fusion inhibitor C34 (Fig. 2*D*). Taken together these results suggested that accumulation of p24 in CD4 T cells occurred by fusion-independent transfer of HIV antigens from infected to target cells in the absence of *de novo* viral production in CD4 T cells.

CD4 T Cells Internalized HIV Particles and Cell Surface CD4 during Cell-to-cell Contacts—To evaluate the role of HIV binding to CD4 in viral transfer, we measured the occupancy of the gp120 binding site of CD4 using the Leu3a mAb, directed against this site. Cell surface CD4 expression was monitored using the gp120-insensitive mAb L120.3. Cocultures of HIV BaL-infected cells with CD4 T cells reduced the levels of CD4

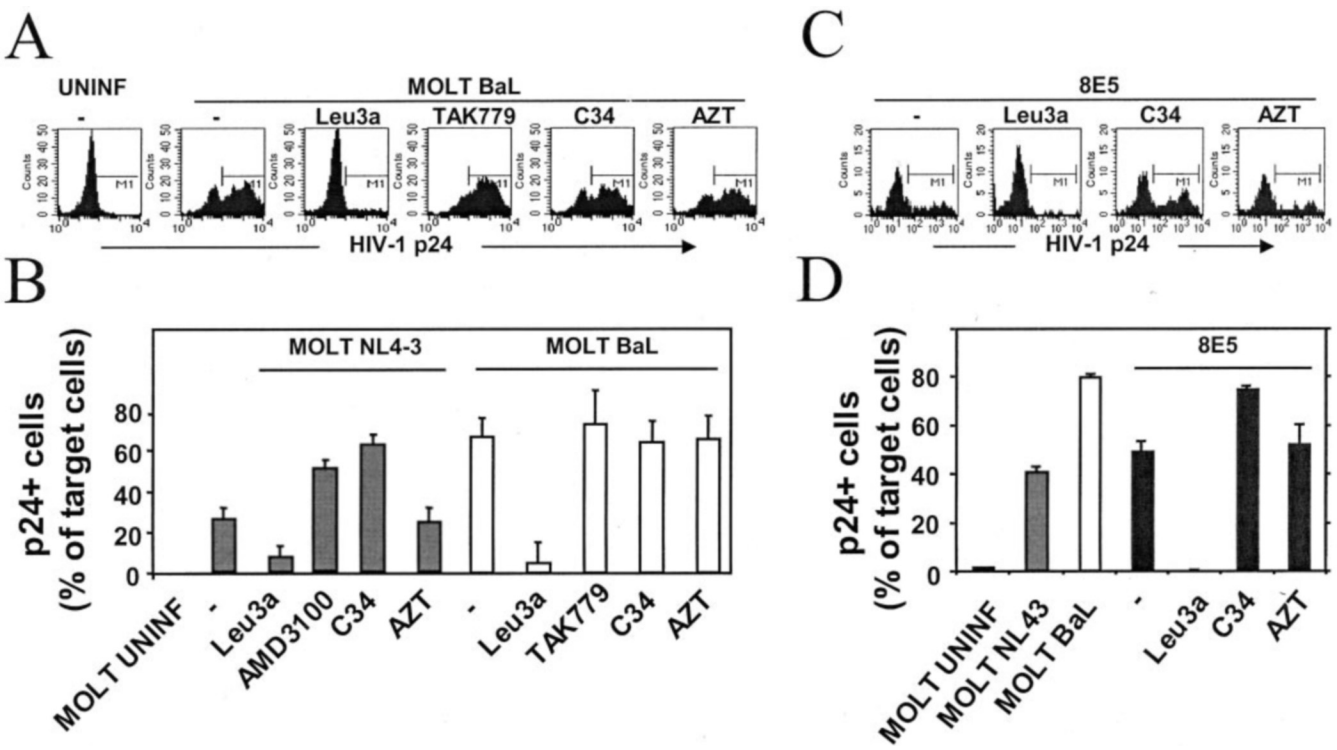

FIG. 2. **Transfer of HIV did not require a fusion-dependent process.** *A*, HIV BaL-infected MOLT-4/CCR5 were cocultured for 24 h with purified CD4 T cells. Transfer of HIV antigens was assayed as in Fig. 1. The content of p24 antigen in CD4 T cells was inhibited by the CD4 mAb Leu3a (0.25 μg/ml) but was insensitive to coreceptor (1 μM TAK779), fusion (1 μM C34), and reverse transcriptase (2 μM AZT) inhibitors. *B*, quantification of transferred p24 antigen in CD4 T cells cocultured with NL4-3- or BaL-infected MOLT-4/CCR5 cells in the presence or the absence of the indicated drugs (concentrations as in *A*, AMD3100 was used at 10 μg/ml, mean ± S.D. of three independent experiments). *C*, transfer of p24 antigen occurring in 24-h cocultures of 8E5 cells (producing reverse transcriptase-defective noninfectious HIV particles) and PBMC in the presence of the indicated drugs. *D*, quantification of HIV transfer induced by 8E5 cells. MOLT-4/CCR5 cells were used as controls. The percentage of $p24^+$ cells in CD4 T cells was analyzed by flow cytometry after 24 h of coculture. Results shown are the mean ± S.D. of two independent experiments.

TABLE I
Inhibition of HIV Env-mediated fusion and transfer by HIV entry inhibitors

	HIV Env-mediated cell-to-cell fusion[a]		HIV-Env mediated cell-to-cell transfer[b]	
	NL43	BaL	NL43	BaL
	%		%	
Leu3a	94 ± 8	96 ± 5	96 ± 2	97 ± 3
AMD3100	94 ± 7	<20	330 ± 27(enh)[c]	<20
TAK779	<20	91 ± 2	<20	<20
C34	85 ± 5	96 ± 5	310 ± 22 (enh)	<20
AZT	<20	<20	<20	<20

[a] Percent inhibition assayed after 24 h of coculture of MOLT-4/CCR5 cells with HeLa P4R5 cells by determining ß-galactosidase activity.
[b] Percent inhibition assayed after 24 h of coculture of MOLT-4/CCR5 cells with CD4 T cells by p24 staining.
[c] (enh) indicates significant enhancing effect compared with untreated cocultures.

expression in target cells as measured by both Leu3a and L120.3 mAbs (Fig. 3*A*). Reduction of MFI was higher for the Leu3a mAb but was also significant for the L120.3 epitope. Interestingly, the extent of CD4 down-modulation correlated with the extent of HIV uptake (35 ± 14% and 69 ± 4% reduction induced by NL4-3 and BaL-infected cells, respectively, Fig. 3*B*), suggesting that a fraction of CD4 molecules disappears from the cell surface during cell-to-cell contacts.

Because HIV binding has been associated with endocytosis in different cell lines including primary cells (29), we investigated the role of HIV internalization in virus transfer. Trypsin treatment of CD4 T cells cocultured for 24 h with uninfected or BaL-infected MOLT-4/CCR5 cells removed almost completely (more than 90%) cell surface CD4. However, trypsin had a low impact in the percentage of $p24^+$ cells (89% *versus* 86%, Fig. 3*C*), although the MFI of p24 antigen staining was lowered by 58 ± 4%. Increasing the time of trypsin treatment failed to increase p24 removal (Fig. 3*D*). Moreover, similar amounts of trypsin-resistant p24 antigen were observed in the presence of the inhibitors TAK779 and C34 (Fig. 3*E*). Kinetic analysis of trypsinized or untreated cocultures of MOLT-4/CCR5 cells with CD4 T cells showed HIV transfer at short incubation times (2–4 h) that rapidly directed p24 antigen to trypsin-resistant compartments. Longer incubation times (up to 24 h) allowed for the progressive accumulation of extracellular HIV antigens from BaL-infected cells. In contrast, NL4-3-infected cells transferred p24 antigen with a different profile, showing a stable and low level of trypsin-resistant antigen transfer (Fig. 3*F*).

In fluorescence microscopy analysis of trypsinized cocultures, HIV BaL-infected cells showed homogeneous p24 antigen staining, whereas the smaller target cells showed punctuated trypsin-resistant staining (Fig. 4*A*, *i–iii*). We further confirmed HIV internalization by electron microscopy. Effector and target cells were clearly identified by size, heterochromatin structure, and cytoplasm opacity. Ultrastructural analysis revealed strong cell-to-cell contacts between HIV-infected MOLT-4/CCR5 and CD4 T cells in which viruses accumulate. HIV particles were readily identified on the surface of CD4 T cells at the sites of cell-to-cell contacts (Fig. 4*B*, *i*) and at sites of membrane appendage (Fig. 4*B*, *ii*), inducing lamellipodia that seemed to engulf HIV particles. Moreover, 14% of cells showed large intracellular vesicles ranging from 0.2 to 1.2 μm, with an average diameter 0.7 ± 0.2 μm (Fig. 4*B*, *iii–vi*). Most positive cells displayed at least one vesicle that contained 1–14 (8 ± 4) viral particles, confirming that primary CD4 T cells internalize HIV by endocytic mechanisms after viral presentation by infected cells.

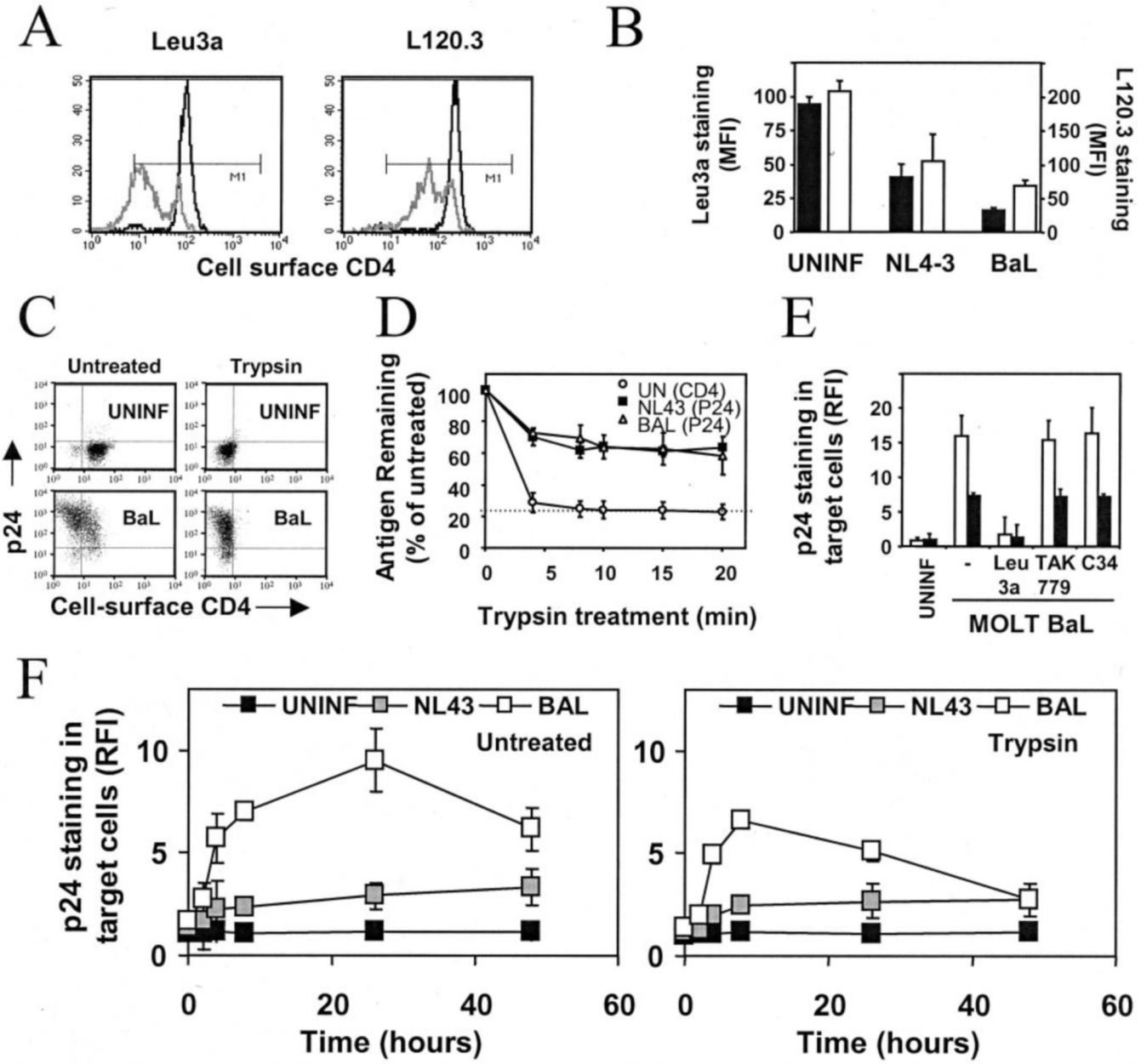

FIG. 3. **CD4 T cells showed trypsin-resistant p24 antigen and CD4 internalization.** *A*, the availability of cell surface CD4 epitopes Leu3a (*left*) and L120.3 (*right*) was measured in negatively selected CD4 T cells cultured for 24 h with uninfected (*black peaks*) or HIV BaL-infected (*gray peaks*) MOLT-4/CCR5 cells. *B*, the disappearance of cell surface CD4 in cocultures of CD4 T cells with uninfected and NL4-3- or BaL-infected MOLT-4/CCR5 cells was calculated from MFI data (mean ± S.D. of three experiments) of Leu3a (*black bars*) or L120.3 (*white bars*) labeling. *C*, dot plots of combined cell surface staining of CD4 and total p24 content in CD4 T cells cocultured for 24 h with uninfected or Bal-infected MOLT-4/CCR5 cells. Trypsin-resistant p24 antigen was observed after staining of trypsin-treated (*right*) cells. Trypsin action was controlled by the elimination of the cell surface Leu3a epitope of CD4 T cells (*right panels*). *D*, kinetics of trypsin treatment. CD4 T cells were cultured for 24 h with uninfected and NL4-3- or BaL-infected MOLT-4/CCR5 cells, washed, and treated with trypsin for the indicated times. Uninfected cocultures were analyzed for Leu3a epitope disappearance (*circles*). Cocultures of NL4-3- (*squares*) and BaL- (*triangles*) infected cells were analyzed for the disappearance of p24 staining in gated CD4 T cells. The *dotted line* indicates the level of background fluorescence. Data are the percentage of MFI values of untrypsinized cells (mean ± S.D. of three experiments). *E*, the RFI of p24 antigen staining was calculated in untreated (*empty bars*) or trypsin-treated (*solid bars*) CD4 T cells after coculture with MOLT-4/CCR5 BaL cells in the presence of the indicated drugs (mean ± S.D. of two independent experiments). *F*, kinetic analysis of p24 staining (RFI) in gated CD4 T cells. Cocultures of purified CD4 T cells with uninfected or HIV-infected (NL4-3 or BaL) MOLT-4/CCR5 cells were incubated for the indicated times and analyzed by p24 staining either untreated (*left*) or after trypsin treatment (*right*). Data are the mean ± S.D. of four experiments. In all *panels*, CD4 T cells were gated according to morphological parameters.

HIV Uptake Required Intact Actin Cytoskeleton and Binding of gp120 to CD4 but Not the Cytoplasmic Tail of CD4—To characterize the requirements for the activation of HIV uptake induced by cellular contacts, we investigated the effect of several mAbs directed against different epitopes of CD4 or gp120. The anti-CD4 mAb Leu3a and the anti-gp120 mAb IgGb12 that block gp120-CD4 interaction inhibited HIV uptake by CD4 T cells either measured as the percentage of $p24^+$ cells (Fig. 5) or as the MFI of p24 staining in CD4 T cells (data not shown). This effect was concomitant with a near total inhibition (80–100%) of HIV envelope-induced fusion (Fig. 5). In contrast, anti-gp120 or anti-CD4 antibodies (2G12 and L.120.3, respectively) shown not to block the binding of gp120 to CD4 (27, 31) showed little or no inhibitory effect on HIV transfer, irrespective of their effect on Env-mediated fusion. A 32% reduction in the transfer of BaL antigens was observed in the presence of 2G12. How-

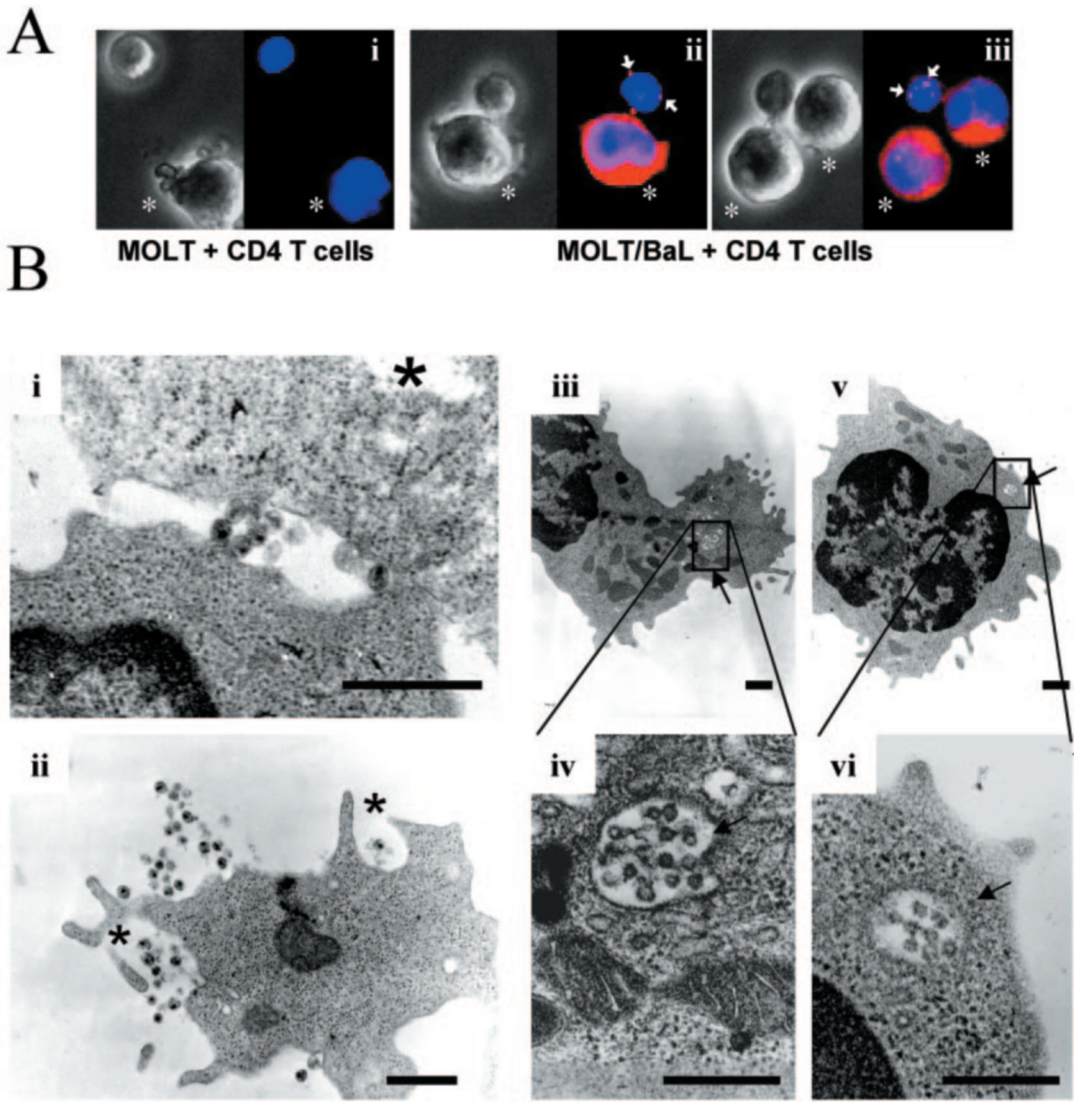

FIG. 4. **CD4 T cells internalized HIV.** *A*, CD4 T cells cocultured for 24 h with uninfected (*i*) or BaL-infected MOLT-4/CCR5 cells (*ii* and *iii*), treated with trypsin, and examined by fluorescence microscopy after p24 antigen (*red*) and nuclear (*blue*) staining. Effector cells were identified by size and intense homogeneous p24 antigen staining (*asterisks*). Images show the phase contrast and the overlay of nuclear and p24 antigen staining. *B*, untreated cocultures of BaL-infected MOLT-4/CCR5 cells and primary CD4 T cells were also analyzed by electron microscopy. *i*, cellular contacts showing trapped viruses between MOLT-4/CCR5/BaL cells (*asterisk*) and a CD4 T cell. *ii*, viral particles bound to the surface of CD4 T cells at sites of membrane invaginations (*asterisks*). *iii–vi*, CD4 T cells showing large intracellular vesicles containing several HIV particles, indicated by *arrows*. *Scale bars* are 1 μm.

ever, as noted for C34 and AMD3100 (Table I), a 2-fold increasing effect of 2G12 was observed in NL4-3 transfer, which was associated to the inhibition (71%) of fusion.

Disruption of actin function by cytochalasin D treatment reduced by near 80% the amount of transferred NL4-3 or BaL antigens, either total (not shown) or trypsin-resistant (Fig. 5), suggesting that cytoskeleton rearrangements are required not only for viral internalization but also for the massive HIV binding to CD4 induced by cellular contacts. Conversely, cytochalasin D increased the infection of HeLa P4R5 cells induced by NL4-3 and BaL-infected MOLT-4/CCR5 cells as described previously (32), highlighting the different mechanisms operating cell-to-cell HIV transmission in primary cells. A similar effect was found when different inhibitors of vesicular trafficking were used (bafilomycin A1 and concanamycin A). Both drugs failed to increase HIV transfer to CD4 T cells at 24 h (Fig. 5) or shorter time points (not shown). In contrast, both drugs enhanced HIV infectivity in HeLa P4R5 cells (Fig. 5) as described previously (14, 20). Amiloride, a classical inhibitor of macropinocytic entry, failed to modify either transfer or fusion at the highest nontoxic concentration tested (Fig. 5).

The key role of CD4 in HIV binding and internalization may suggest a mechanism of CD4-dependent endocytosis of HIV particles. However, this mechanism is known to be mediated by clathrin-coated pits (33, 34), a phenomenon seen only sporadically in the electron microscopy analyses of HIV-loaded CD4 T cells (data not shown); in our cocultures, most HIV particles appeared to be endocytosed by membrane invaginations (Fig. 4*B*). To clarify the role of CD4 in the endocytosis process, we used several cell clones either lacking CD4 and CCR5 expression (A201), expressing CCR5 (A201/CCR5), or expressing wild type CD4 (A301), a truncated form of CD4 at position 403 (A201/403) or a chimera CD4-CD8 (A201/CD8). The inability of truncated or chimeric form of CD4 to be endocytosed was analyzed after treatment of cells with phorbol 12-myristate 13-acetate, which resulted in the exclusive down-regulation of wild type CD4 (data not shown). Conversely, all three clones were able to bind HIV during cell-to-cell contacts, as measured

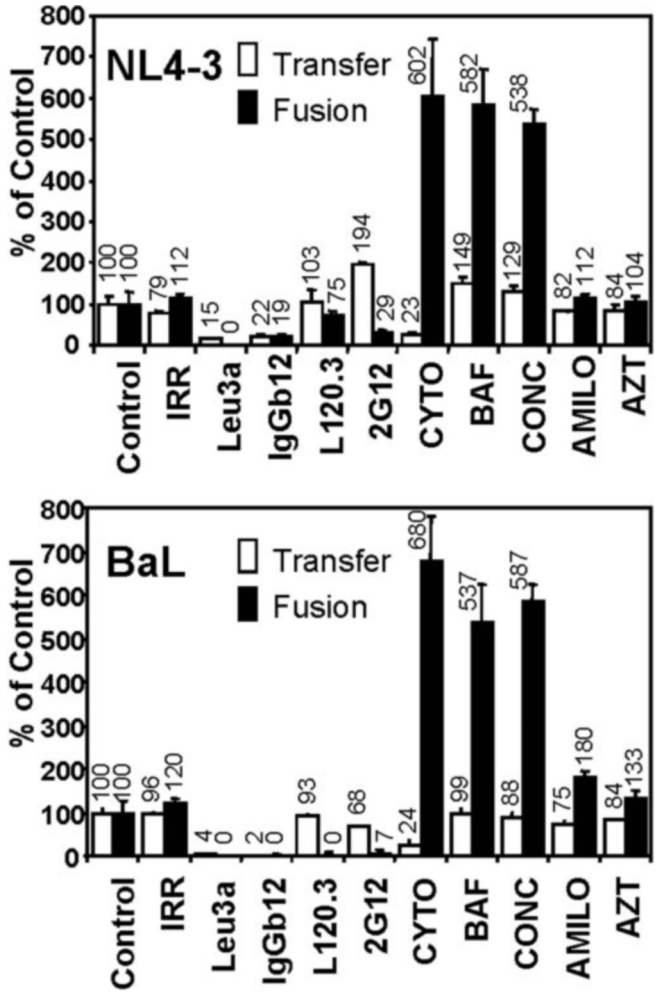

FIG. 5. **Coreceptor-independent HIV transfer requires binding of gp120 to CD4 and an intact actin cytoskeleton.** Purified CD4 T cells or HeLa P4R5 cells were cocultured for 24 h with uninfected, NL4-3- or BaL-infected MOLT-4/CCR5 cells in the absence (*Control*) or the presence of the following inhibitors: irrelevant IgG (*IRR*), anti-CD4 mAbs Leu3a and L120.3, anti-gp120 mAbs IgGb12 and 2G12, cytochalasin D (*CYTO*), bafilomycin A1 (*BAF*), concanamycin A (*CONC*), amiloride (*AMILO*), and AZT. The amount of p24 transferred to primary cells was calculated after trypsinization of cells and p24 staining as the percentage of $p24^+$ cells (using uninfected cells as a control). The extent of HIV envelope-mediated cell-to-cell fusion in HeLa P4R5 cells was evaluated in cell lysates by determining β-galactosidase activity induced by HIV transactivation. The effect of the different drugs on HIV transfer (*empty columns*) and fusion (*filled columns*) was calculated for NL4-3-infected (*upper*) and BaL-infected (*lower*) cocultures, as the percent ratio between measures in treated and untreated (*Control*) cultures. Data shown correspond to one representative experiment of three performed and are the mean ± S.D. of duplicate wells.

by total p24 associated with CEM cells after 6 h of coculture with MOLT-4/CCR5 BaL-infected cells (data not shown). Moreover, all CEM clones expressing CD4 forms were able to internalize p24 antigen to trypsin-resistant compartments by a mechanism requiring gp120 binding to CD4 but not gp41-mediated fusion (Fig. 6*A*). Very low levels of attachment and endocytosis were observed in A201 control cells and in A201/CCR5 cells (Fig. 6*B*). Analysis of cells by fluorescence microscopy showed that $p24^+$ CEM cells were not the result of cellular aggregates but were individual cells displaying punctuated trypsin-resistant p24 staining. These data confirmed that HIV endocytosis required extracellular but not intracellular moiety of CD4.

HIV Capture by CD4 T Cells Was Reversible and Yielded Infectious HIV Particles—Many cell types lacking CD4 or coreceptor expression may capture HIV particles and transfer them to permissive cells (2, 7). To test whether CD4 T cells lacking coreceptor expression may act in a similar way, we purified CD4 T cells after being cocultured with uninfected or BaL-infected MOLT-4/CCR5 cells. Purification rendered preparations of CD4 T cells contaminated with less than 10% of MOLT-4/CCR5 cells (Fig. 7*A*). To evaluate the infectivity of remaining MOLT-4/CCR5 cells, we performed cocultures in the presence of the anti-gp120 mAb IgGb12. In addition, TAK779 was used to block fusion-mediated transfer completely. Once purified, CD4 T cells were trypsinized to remove extracellular virions and bound IgGb12 (not shown), cultured in the absence or presence of AZT, and analyzed for intracellular and extracellular p24 content. CD4 T cells released p24 to the supernatant concomitant with a reduction in its intracellular p24 content (Fig. 7*B*). This release was not modified by the addition of TAK779 (which failed to inhibit HIV transfer) or by the presence of AZT during the culture of purified CD4 T cells (not shown), thus suggesting that it was not the consequence of productive viral infection but of the release of captured HIV particles. Consistently, there was a significant reduction in p24 antigen released by CD4 T cells purified from cocultures that were treated with mAb IgGb12, which blocked HIV transfer

Supernatants from purified CD4 T cells were collected after 12 h of culture and assayed for infectivity in U87.CD4/CCR5 cells (Fig. 7*C*). Supernatants from control or TAK779-treated cocultures showed productive virus replication as measured by p24 production and syncytium formation. Conversely, IgGb12-treated cocultures (which contained background levels of infectious particles coming from MOLT-4/CCR5 cells) showed significantly ($p < 0.05$) lower infectivity.

HIV Transfer during Infection of Primary Cells—To evaluate the ability of primary CD4 T cells to transfer HIV virions as observed for cell lines, we purified HIV-producing cells from IL-2-stimulated PBMC that were infected with HIV BaL. Analysis of infected cultures revealed two different populations of $p24^+$ cells, both $CD3^+$ and $CD8^-$ (data not shown): a productively infected $p24^{bright}$ population (MFI = 460 for p24 antigen staining) showing complete CD4 down-regulation (Fig. 8*A*) and a $CD4^+/p24^{dim}$ population showing a lower level of p24 antigen staining (MFI = 198) and 27% reduction of cell surface CD4 molecules compared with $CD4^+/p24^-$ cells (Fig. 8*A*). To evaluate whether endocytic HIV transfer contributed to the appearance of the $p24^+/CD4^{dim}$ population, we enriched $CD8^-/CD4^-/p24^+$ cells (considered to be productively infected cells) by depleting $CD4^+$ and $CD8^+$ cells. Final preparation had 63 ± 3% of putatively infected cells (Fig. 8*A*) that were cocultured with uninfected unstimulated CMFDA-labeled CD4 T cells for 24 h. Enriched infected primary cells transferred HIV particles to target cells (Fig. 8*B*). As seen for HIV BaL-infected MOLT-4/CCR5 cells, the anti-CD4 mAb Leu3a but not the fusion inhibitor C34 or the reverse transcriptase inhibitor AZT blocked the transfer of HIV to CMFDA-labeled CD4 T cells (Fig. 8*C*). These data indicate that transfer of virus may occur from infected to uninfected primary CD4 T cells.

DISCUSSION

Our initial observation on the high level of p24 antigen content in CD4 T cells after contacting infected cells (Fig. 1) was puzzling for several reasons. First, we used unstimulated target cells; therefore only low levels of p24 antigen production should be expected. Second, HIV binding to primary lymphocytes appeared to be mostly independent of CD4 (35) and could not explain the specificity of transfer to CD4 T cells. Third, the higher transfer of HIV by BaL-infected cells compared with NL4-3-infected cells was inconsistent with the expression of HIV coreceptors by PBMC, which were 98% $CXCR4^+$, but less

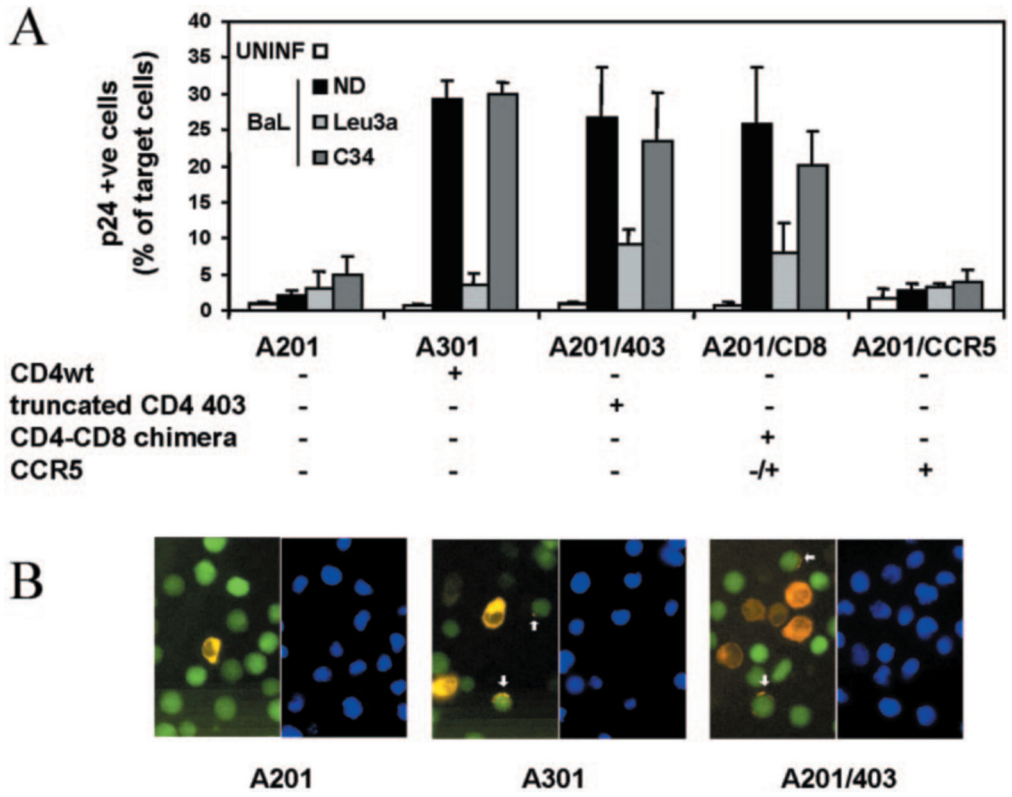

FIG. 6. **The cytoplasmic tail of CD4 is not required for coreceptor-independent endocytosis of HIV particles by CD4$^+$ cells.** Several CEM clones either lacking CD4 expression (A201) or expressing wild type CD4 (A301), a truncated form of CD4 at position 403 (A201/403), or a chimera CD4-CD8 (A201/CD8) were cocultured for 6 h with uninfected (*UNINF*) or BaL-infected MOLT-4/CCR5 cells in the absence (*ND*) or the presence of the anti-CD4 mAb Leu3a (10 μg/ml) or the fusion inhibitor C34 (1 μg/ml). An additional control of A201 cells expressing CCR5 (A201/CCR5) was included in this set of experiments. All A201 clones were labeled with the cell tracker CMFDA before coculture to facilitate identification of target cells after p24 staining. *A* shows the percentage of p24$^+$ cells in gated *green* target cells after trypsin treatment. The expression of the different forms of CD4 and the coreceptor CCR5 is indicated. *B* shows micrographs of p24-stained cocultures. Cells expressing wild type (A301, *middle*) or a truncated form (A201/403) of CD4 showed p24 trypsin-resistant punctuated p24 staining. The CD4$^-$ cell line A201 was used as control. Target cells can be identified by CMFDA green staining. Nuclear counterstain is also shown.

than 10% CCR5$^+$ (data not shown). After characterizing the mechanisms of HIV capture by CD4 T cells, we concluded that infected cells rapidly transferred HIV antigens to CD4 T cells during cellular contacts. Transfer could be observed at short times (Fig. 3), in the absence of functional coreceptors (Fig. 2) but showed an absolute dependence of CD4 engagement by gp120. In turn, transfer was dependent of actin polymerization (cytochalasin D-sensitive, Fig. 5) and was reversible because HIV particles were rapidly released by CD4 T cells (Fig. 7). Although these data may suggest a massive HIV binding to CD4, HIV particles did not seem to be just bound to CD4 T cells but appeared to reside transiently in trypsin-resistant compartments (Fig. 3*F*). Therefore, we postulate that cell-to-cell contacts between HIV-infected (Env-expressing) and target (CD4-expressing) cells concentrate viral particles in contact areas, increasing the binding of virus to CD4 and, in the absence of coreceptor, inducing the endocytosis of bound particles (Figs. 1–4).

Several cell types such as macrophages or endothelial cells use macropinocytic mechanisms to internalize cell-free viral particles (15, 16). A compensatory mechanism for HIV fusion and endocytosis has been described recently after presentation of cell-free virus particles to CD4$^+$ cell lines (18). The presence of HIV antigens in CD4 T cells after treatment with trypsin, the concomitant disappearance of CD4 molecules from the cell surface (Fig. 3), and the fluorescence and electron microscopy data (Fig. 4) might suggest the existence of a similar compensatory CD4-dependent endocytic mechanism in primary cells. Further characterization of the endocytic process showed that bafilomycin A1 and concanamycin A, two classical inhibitors of vesicular trafficking, did not modify HIV transfer and that the cytoplasmic tail of CD4 was dispensable for HIV uptake (Fig. 6). Moreover, based on ultrastructural data (Fig. 4*B*) and in search of a clathrin-independent mechanism, we explored the possible involvement of macropinocytosis in the endocytic process. The macropinocytosis inhibitor amiloride failed to block HIV capture by CD4 T cells (Fig. 5), and, importantly, HIV capture was not associated with fluid phase uptake by CD4 T cells, as assessed with Alexa 488-labeled dextran (data not shown), ruling out a classical macropinocytic mechanism.

The mechanism of HIV transfer during cellular contacts in the absence of coreceptors clearly differed from that governing infectious HIV transfer to HeLa cells (Fig. 5) and was not mediated by clathrin or macropinocytic pathways. In contrast, our observations are in agreement with the intercellular transfer of antigens described at immune synapses. These synapses are built around a central active zone of exocytosis and endocytosis encircled by adhesion domains. Surface molecules may be incorporated into and around the active synaptic zones and modulate the functional state of the synapse (36) activating a process, recently called trogocytosis (37), which allows one of the cells to transfer up to 20% of specific synaptic surface antigens to its partner (38, 39). Trogocytosis has been suggested to act as a mechanism of control of the length of synaptic contacts and might be used by infected cells to increase viral transfer. Indeed, synaptic structures involving HIV envelope, CD4, and coreceptors have been described for the contact of dendritic cells and CD4 T cells and for infected/uninfected CD4 T cell contacts (6, 40). Consistent with this hypothesis, disruption of actin cytoskeleton by cytochalasin D, which prevents the

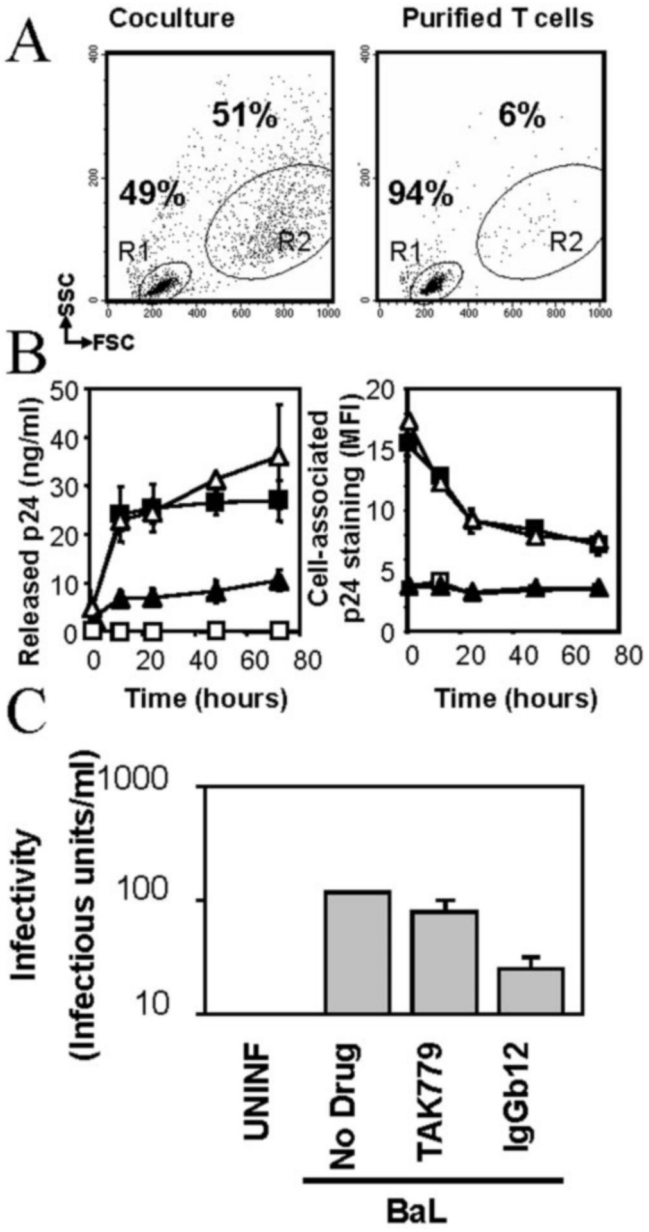

FIG. 7. **Release of infectious HIV particles from CD4 T cells.** Purified CD4 T cells were cultured for 6 h (ratio 1:1) with uninfected (*UNINF*) or BaL-infected MOLT-4/CCR5 cells. After coculture, CD4 T cells were repurified by negative selection and cultured for 72 h without stimulation. *A* shows the morphological analysis of CD4 (R1 gate) and MOLT-4/CCR5 cells (R2 gate) before and after purification. *B*, time course of HIV release (*left*) and p24 content of purified trypsinized CD4 T cells as assessed by enzyme-linked immunosorbent assay and flow cytometry, respectively. Prior to purification, CD4 T cells had been cultured with uninfected (*empty squares*) or BaL-infected MOLT-4/CCR5 cells in the absence (*No Drug*, *filled squares*) or the presence of TAK779 (*empty triangles*) or the neutralizing mAb IgGb12 (*filled triangles*). *C*, supernatants from purified CD4 T cells cocultured in these conditions were recovered at 12 h after purification and used to infect U87.CD4/CCR5 cells. The *panel* shows the infectious titer of these preparations. In *B* and *C* the data are the mean ± S.D. of two experiments performed with CD4 T cells obtained from different healthy donors.

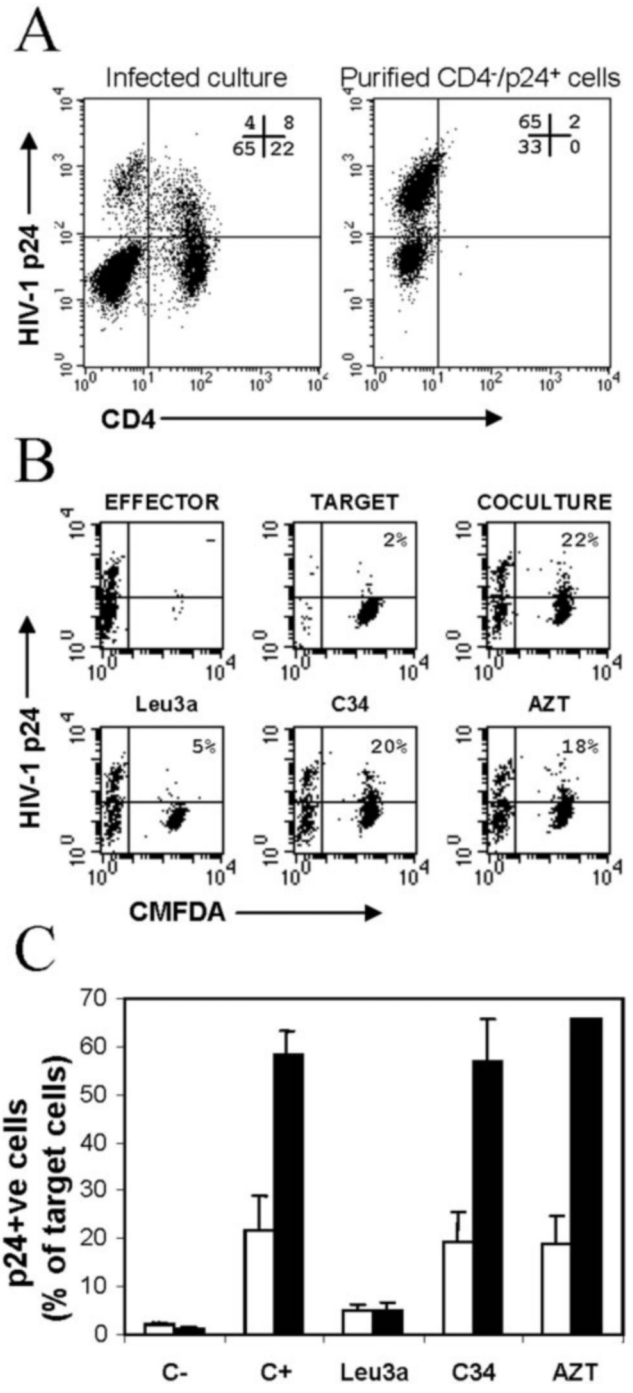

FIG. 8. **HIV-infected primary cells transferred HIV particles to CD4 T cells.** *A*, PHA/IL-2-activated PBMC were infected with HIV BaL. The percentage of $p24^+$ cells was evaluated at day 4 after infection by combining extracellular CD4 and intracellular p24 staining. The figure shows dot plots of a whole infected culture before (*left*) and after depletion of $CD4^+$ and $CD8^+$ cells. $CD4^-$ cells were enriched to 65% of $p24^+$ cells (*right*). Values indicate the percentage of cell in each quadrant. *B*, dot plots of p24 antigen and CMFDA staining of whole cultures and cocultures of enriched $p24^+$ cells (*EFFECTOR*) and CMFDA-labeled unstimulated CD4 T cells (target). Drugs were used as in Fig. 2. Values indicate the percentage of $p24^+$ cells in the $CMFDA^+$ (*TARGET*) cell gate. *C*, purified HIV BaL-infected primary $CD4^-$ cells (*empty bars*) or HIV BaL-infected MOLT-4/CCR5 cells (*solid bars*) were cultured with unstimulated CD4 T cells for 24 h in medium containing IL-2 in the presence of the indicated drugs, and the percentage of gated CD4 T cells staining for p24 antigen was examined (mean ± S.D. of three experiments).

formation of synaptic structures between infected and target cells (6), strongly inhibited HIV transfer and endocytosis (Fig. 5).

Our results suggest that for CD4 T cells, the interaction of HIV envelope with CD4 appears to be sufficient for the establishment of cellular contacts that allow for a massive fusion-independent transfer of virus, adding complexity to the process of cell-to-cell virus transmission. This process begins in the infected partner of the cellular contact, in which cytoskeleton rearrangements polarize cellular adhesion molecules and HIV

budding to the sites of cell-to-cell contact (4, 5, 41). Then, infectious viral entry during cell-to-cell transmission requires expression of viral receptors on the surface of target cells (42), and this may result not only in viral entry, but also in profound cytopathic events, as cell-to-cell fusion and cell death (22, 43). Although HIV endocytosis may also occur in coreceptor-expressing cells, it is more evident in cells lacking the appropriate coreceptor (Fig. 1). These cells are protected from cytopathic events and from fusion-mediated viral entry to the cytoplasm (24). Survival of target cells probably increases the effective time of cell-to-cell contact allowing for the accumulation of viral particles on the cell surface and vesicular structures.

The possible *in vivo* relevance of the mechanism of cell-to-cell viral transmission described herein is highlighted by early ultrastructural studies of lymphoid tissue of homosexual men with HIV infection (44) describing viral structures (similar to those shown in Fig. 4) inside vesicular bodies of tissular lymphocytes (44). However, to infect the carrier cell (infection *in cis*), virions require fusion (coreceptor)-mediated injection of the viral core into the cytoplasm (29). The endocytosed HIV (which can be found in CD4 T cells even after 3 days after the end of the synaptic contact, Fig. 7) could reach the cytoplasm solely after changes in chemokine receptor expression, as a consequence of changes in the surrounding chemokine concentrations or in cellular activation that would affect HIV coreceptor expression such as IL-2 (45) or IL-7 (30).

Alternatively, as shown in Fig. 7, the reversibility of the uptake of HIV by CD4 T cells makes possible the release of captured viruses and the transmission *in trans* to a third cell, providing a novel mechanism of cell-to-cell HIV transmission, by which HIV may exploit CD4 T cells lacking the appropriate coreceptor as an itinerant viral reservoir. This mechanism of viral transmission should be taken into account when developing pharmacological and immunological anti-HIV strategies.

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