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Research Article

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Productive Infection of Dendritic Cells by HIV-1 and Their Ability to Capture Virus Are Mediated through Separate Pathways

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Abstract

There is substantial evidence that dendritic cells (DC) residing within epithelial surfaces (e.g., Langerhans cells) are the initial cells infected with HIV after mucosal exposure to virus. To study DC–HIV interactions in detail, we propagated Langerhans cell–like DC from cord blood $CD34^+$ cells and from adult blood plastic-adherent PBMC in the presence of cytokines (GM-CSF, IL-4, and/or TNF-α). DC pulsed overnight with HIV_{BaL} or HIV_{IIIB} were infected productively with both viral subtypes (as assessed by PCR, supernatant p24 protein levels, electron microscopy, and antibody staining). Productive infection could be blocked by anti-CD4 mAbs, RANTES (regulated upon activation, normal T cell expressed and secreted) (for HIV_{BaL}), stromal cell–derived factor-1 (for HIV_{IIIB}), or azidothymidine added during the HIV pulse, as well as by blocking DC proliferation. However, pulsing DC with HIV under these blocking conditions had no effect on the ability of DC to capture virus and transmit infection to cocultured antigen-stimulated $CD4^+$ T cells. Thus, we show by several criteria that (*a*) productive infection of DC and (*b*) the ability of DC to capture virus are mediated through separate pathways. We suggest that strategies designed to block mucosal transmission of HIV should consider interfering with both virus infection and virus capture by DC. (*J. Clin. Invest.* 1997. 100:2043–2053.)

Key words: dendritic cells • HIV/AIDS • CCR-5 • CXCR-4 • immunopathogenesis

Introduction

The exact cellular and molecular mechanisms involved in the transmission of HIV after mucosal exposure to virus are unknown. However, there is substantial evidence that bone marrow–derived nonlymphoid dendritic cells (DC)[1] present within epithelial tissues (e.g., Langerhans cells) are involved in this process (for reviews, see references 1–3). The normal function of these DC is to survey epithelial surfaces for antigens (Ags), capture Ag by micropinocytosis or mannose receptor–mediated uptake, process captured proteins into immunogenic peptides, emigrate from tissue to paracortex of draining lymph nodes, and present peptides in the context of MHC molecules to T cells (TC), thereby initiating Ag-specific immune responses (4, 5). It is now generally believed that HIV subverts this normal trafficking process to gain entry into lymph nodes and access to $CD4^+$ TC. Recently, in vivo evidence for this theory has been provided using macaque models of simian immunodeficiency virus (SIV) primary infection (6, 7). Future research will focus most likely on further understanding DC–HIV interactions, with the hope that this knowledge could lead to strategies aimed at blocking early events in transmission.

Many studies have attempted to model primary HIV infection by exposing normal DC (derived from skin and/or blood) to HIV in vitro (8–23). Using skin-derived DC, most investigators have shown that HIV can enter these cells readily, but that infection is not productive when cultured in the absence of TC (10, 15, 16, 19, 20). This is not surprising, since Langerhans cells within epidermis and mucosal epithelium are $CD4^+$ (24) and have been shown recently to express the HIV coreceptors CCR-5 and CXCR-4 (reference 23, and our unpublished observations) (which would allow HIV entry), yet are nonreplicating cells in culture (25) (which would make productive infection difficult). However, this field is controversial, since other investigators have purported that Langerhans cells alone can be infected productively with HIV in vitro (21, 22). These studies are complicated because of the difficulty of isolating and purifying sufficient numbers of skin-derived DC to perform experiments, and because of the phenotypic changes in DC induced by the experimental isolation procedures. For example, trypsin required to make epidermal cell suspensions degrades CD4 (our unpublished observations), and isolating DC from skin explants by allowing them to migrate into culture activates DC, i.e., the cell surface phenotype changes from the Langerhans cells phenotype observed in situ to resemble the phenotype observed on lymphoid DC (15, 16, 23).

Similar controversy exists in the reported studies using blood-derived DC in HIV infection experiments. Several groups have reported productive infection of blood DC (8, 9, 17), whereas others have detected entry of HIV but little evidence for productive infection in the absence of TC (11–13, 17, 18). Again, these studies and the interpretation of their results are confounded by the following problems: (*a*) there are at least

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1. *Abbreviations used in this paper:* AB, adult blood–derived; Ag, antigen; APC, antigen-presenting cell; AZT, azidothymidine; CB, cord blood–derived; DC, dendritic cells; ICAM, intracellular adhesion molecule; LFA, leukocyte function–associated Ag; M/Mϕ, monocyte(s)/macrophage(s); RANTES, regulated upon activation, normal T cell expressed and secreted; rh, recombinant human; RT-PCR, reverse transcription–PCR; SDF, stromal cell–derived factor; SIV, simian immunodeficiency virus; TC, T cells; TCID, tissue culture infectious dose; TEM, transmission electron microscopy.

three distinct subtypes of DC in blood (17, 26), and the use of different experimental methods that favor the isolation of one particular subtype could lead to different results in HIV-infection studies; (*b*) small numbers of contaminating TC and monocytes/macrophages (M/Mϕ) (i.e., other known cellular targets for HIV infection) in DC populations may produce misleading results in studies of DC–HIV interactions (27); and (*c*) different methods have been used to define productive infection, with some investigators relying solely on transmission electron microscopy (TEM) findings.

Although there has been disagreement in the past, there is substantial evidence indicating that all types of DC pulsed with HIV in vitro can produce a vigorous infection when cocultured subsequently with TC (8, 10–13, 15–20, 22). The DC–TC microenvironment provides an explosive site for HIV replication, and the degree of infection appears to be related to the degree of TC activation (12, 13, 15). However, it is still unclear whether a similar scenario occurs in vivo in HIV-infected individuals. If it does, it is quite possible that TC activation, infection, and depletion of $CD4^+$ TC that occur in HIV^+ persons (28, 29) may depend primarily on DC-mediated activation and infection of TC. The exact cellular mechanisms involved in the transmission of HIV from DC to TC are unknown, although CD40–CD40L and CD28–CD80 interactions are thought to be important in this process (13). If documented to occur in vivo, and if the mechanisms of DC–HIV interactions could be defined more clearly, blocking transmission of virus from DC to TC might serve as a possible antiviral strategy for patients with HIV infection.

Recently, the ability to propagate DC from human blood and bone marrow in the presence of stimulating and differentiating cytokines (e.g., GM-CSF, IL-4, TNF-α) has provided an opportunity to perform detailed studies of DC biology using large numbers of relatively pure cells (30–32). In this study, we describe an in vitro model system, using Langerhans cell–like DC propagated from human adult or cord blood to study DC–HIV interactions in detail. We found that (*a*) productive infection of DC by HIV and (*b*) the ability of DC to capture virus (with subsequent infection of $CD4^+$ TC) are mediated through separate CD4- and HIV coreceptor–dependent and CD4- and HIV coreceptor–independent pathways, respectively. These findings help to clarify mechanisms involved in DC–HIV interactions, and may assist in the design of strategies aimed at blocking primary transmission of HIV and preserving $CD4^+$ TC in HIV^+ patients.

Methods

Preparation of human umbilical cord blood–derived (CB) and adult peripheral blood–derived (AB) DC. DC were propagated from human cord or adult peripheral blood, using protocols modified slightly from previous reports (30–33). For CB-DC, heparinized cord blood samples from $HIV\text{-}1^-$ mothers were collected, stored at room temperature, and transported to the laboratory within 18 h. Cord blood was pooled regularly from 2–5 donors to increase the yield of $CD34^+$ stem cells; there was no alloantigen-induced proliferation of stem cells in the pooled samples when compared with $CD34^+$ cell cultures isolated from single cord blood samples. The pooled blood was diluted 1:2 with PBS, layered over Ficoll/Hypaque (Organon Teknika Corp., Durham, NC), and centrifuged at 2,000 rpm for 20 min at 22°C. Buoyant cells were collected, and any remaining contaminating erythrocytes were removed with lysing buffer (ACK; Biofluids, Inc., Rockville, MD). CB mononuclear cells were then washed, resuspended at $0.8–1.0 \times 10^8$ cells/ml in 1% BSA containing biotinylated mouse anti–human CD34 mAbs (CellPro, Inc., Bothell, WA), and incubated for 30 min at 4°C, with gentle mixing every 10 min. The cells were then washed once in 1% BSA, resuspended in 5% BSA at $8–10 \times 10^6$ cells/ml, and layered over a continuous-flow column containing Streptavidin-coated beads (Ceprate® LC; CellPro, Inc.). Bound cells (i.e., $CD34^+$ cells) were eluted from the column according to manufacturer's guidelines, washed, resuspended in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Biofluids, Inc.), 100 U/ml penicillin (GIBCO BRL), 100 μg/ml streptomycin (GIBCO BRL), 2 mM L-glutamine (GIBCO BRL), 10 mM Hepes (GIBCO BRL), and 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO) (complete medium) at $2–5 \times 10^5$ cells/ml, and placed in 24- or 48-well tissue culture plates (Costar Corp., Cambridge, MA). Each well was supplemented with 1,000 U/ml recombinant human (rh)GM-CSF (Immunex Corp., Seattle, WA), 50 U/ml rhTNF-α (R & D Systems, Inc., Minneapolis, MN), and 1,000 U/ml rhIL-4 (R & D), and cells were then placed in a humidified 5% CO_2 environment at 37°C. Half of the total volume of medium was replaced with fresh complete medium and cytokines every other day, and cells were subcultured as necessary. At day 14, CB-DC were harvested, washed, and used for morphologic, phenotypic, functional, or HIV-infection studies.

For AB-DC, buffy coats were obtained from $HIV\text{-}1^-$ blood donors, diluted 1:4 with PBS, and PBMC were obtained as described for CB mononuclear cells above. PBMC were resuspended in FCS containing 10% DMSO (Pierce Chemical Co., Rockford, IL) and frozen in liquid nitrogen. When needed, aliquots of PBMC were thawed, washed, resuspended in complete medium at $5–8 \times 10^6$ cells/ml, and placed in 35-mm tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ) for 2 h at 37°C. After this incubation, nonadherent cells were drawn off gently, and fresh complete media were returned to culture wells supplemented with 1,000 U/ml rhGM-CSF and 1,000 U/ml rhIL-4. Cells were then cultured as described above for CB-DC.

At day 7, AB-DC were harvested, washed, and resuspended in a cocktail containing the following mouse anti–human mAbs (each at 5 μg/ml): anti-CD3, -CD14, -CD16, and -CD19 (see Table I for sources). Cells were incubated with cocktail for 30 min at 4°C, with gentle agitation every 10 min. During this incubation, magnetic beads (5 beads/cell) coated with sheep anti–mouse IgG antibodies (Dynal, Inc., Great Neck, NY) were washed in HBSS/10% FCS (wash medium) using a magnetic particle concentrator (MPC®-1; Dynal, Inc.) and resuspended in 1 ml of wash medium. After the 30-min AB-DC/cocktail incubation, cells were washed, mixed with the magnetic bead suspension, and incubated for an additional 30 min at 4°C, with gentle agitation. M/Mϕ, natural killer cells, and B cells were then separated from $CD3^-CD14^-CD16^-CD19^-$ cells (i.e., AB-DC) through a series of washes using the magnetic particle concentrator. AB-DC were derived from numerous individuals on separate occasions for morphologic, phenotypic, functional, and HIV-infection studies.

Phenotypic characterization of DC by flow cytometry. For single staining, $2–5 \times 10^5$ DC were resuspended in PBS/10% FCS/0.02% sodium azide (Fisher Scientific Co., Fair Lawn, NJ) (staining buffer) containing unconjugated primary mAbs (see Table I) diluted to 10 μg/ml and incubated in round-bottomed 96-well plates for 30 min at 4°C. Cells were then washed and incubated in staining buffer containing FITC-conjugated sheep $F(ab')_2$ anti–mouse IgG (Organon Teknika Corp.) for an additional 30 min at 4°C. Cells were then washed and examined by flow cytometry using a FACScan® (Becton Dickinson, Mountain View, CA) equipped with Lysis® II software (Becton Dickinson). Propidium iodide–permeable cells were excluded from all analyses.

For double-staining, DC were incubated first with primary mAbs as described above. Cells were then washed three times with staining buffer and incubated for 30 min with biotin-conjugated goat $F(ab')_2$ anti–mouse gamma and light chains (Biosource International, Camarillo, CA). The cells were then washed twice in staining buffer, once in buffer containing PBS, 0.2% BSA (Sigma Chemical Co.), and

0.02% sodium azide, and incubated for 10 min with mouse IgG (10 μg/ml) to block nonspecific binding. DC were then incubated for 30 min with a combination of FITC-conjugated mouse anti–human CD1a at 10 μg/ml (or FITC-conjugated isotype control antibodies) and phycoerythrin-conjugated Streptavidin at 1 μg/ml. The cells were then washed twice, resuspended in staining buffer, and transferred to polystyrene tubes for flow-cytometric analysis as described above.

Reverse transcription (RT)-PCR for detection of contaminating TC and M/Mϕ in DC preparations. To determine the degree of TC and M/Mϕ contamination in DC populations using a more sensitive method, mRNA was extracted from DC, reverse-transcribed to cDNA, and examined for the presence of CD3- and CD14-specific mRNA by PCR, as previously described (33, 34). Primer sets (Genosys Biotechnologies, Inc., The Woodlands, TX) were designed according to published GenBank sequences. Denaturation of DNA was performed for 1 min at 94°C, annealing for 1.5 min at 63°C, and extension for 2 min at 72°C for 18 (CD14) or 28 (CD3) cycles. These conditions were optimized using PBMC. To control for the amount and quality of mRNA, aliquots of mRNA were regularly transcribed to G3PDH-specific cDNA (primers from Clontech, Palo Alto, CA) and amplified by PCR (annealing at 55°C, 18 cycles; otherwise, conditions were as described above).

As described (33, 34), 5 μl of amplified PCR products were hybridized to an excess of ^{32}P–end-labeled probes, electrophoresed on 4% polyacrylamide gels, and dried, and signal intensity was determined by autoradiography (exposure time 2–24 h using BIO-MAX films and intensifying screens [Eastman Kodak Co., Rochester, NY]). Probes (Genosys Biotechnologies, Inc.) were designed to be complementary to internal sequences of the specific PCR products. CD14 and G3PDH primer and probe sequences were as published (33), and CD3 primers and probe sequences were as follows: 5′ primer, 5′-GACTTATGACTGTGCTGTCC (GenBank accession no. X06031); 3′ primer, 5′-ATCTCTGGGAACCTTGAGTC (no. X06032); probe, 5′-AGATTTAGGGCTGAAAGCTCTCTGCTA (no. X06032).

Morphologic analyses of DC by light microscopy and TEM. For light microscopy, 2×10^4 DC were cytocentrifuged onto clean glass slides and air-dried for 1 h at room temperature. Slides were fixed and stained using Diff-Quik® Stain Set (Baxter Healthcare Corp., McGaw Park, IL). For TEM, DC were fixed in 2.5% glutaraldehyde, minced, postfixed with osmium tetroxide, stained with uranyl acetate, embedded in plastic resin, poststained with lead citrate, and examined (all steps except glutaraldehyde fixation were performed by Advanced Biotechnologies, Inc., Columbia, MD).

Characterization of DC function. The phagocytic potential of DC was determined as described previously (35). Briefly, DC were incubated with 0.8-mm latex beads for 45 min at 37°C. The bead/cell mixture was then layered over FCS, centrifuged, washed in PBS, and examined by light microscopy. Cells found to have ingested ≥ 3 beads were scored positive for phagocytic activity. Plastic-adherent PBMC (i.e., M/Mϕ) were used as positive cell controls for these assays.

To assess antigen-presenting cell (APC) function, DC were tested for their ability to stimulate proliferation of allogeneic and recall Ag–stimulated autologous CD4$^+$ TC. Cryopreserved PBMC were washed, resuspended in HBSS supplemented with 10% FCS, and placed into 75-cm^2 plastic culture flasks (Costar Corp.) for 1 h at 37°C. Nonadherent cells were enriched for CD4$^+$ TC by negative selection using a commercially prepared mAb cocktail/complement reagent (Lympho-Kwik®, One Lambda, Inc., Los Angeles, CA) according to manufacturer's guidelines. 10^5 CD4$^+$ TC were resuspended in complete medium and cocultured with varying numbers of irradiated DC or M/Mϕ (2,000 rad, cesium-137 source). Tetanus toxoid (Connaught Laboratories Ltd., Willowdale, Ontario, Canada) at 2 Lf/ml or *Candida albicans* (Greer Laboratories, Inc., Lenoir, NC) at 20 μg/ml were used as recall Ags in assays containing APC and autologous TC. Cultures were performed in triplicate in 96-well flat-bottomed plates (Costar Corp.) and incubated in a humidified 5% CO_2 atmosphere at 37°C for 6 d. Cultures were pulsed with 1 mCi [^{3}H]thymidine at 5.5 d, harvested 16–18 h later, and thymidine incorporation was detected using a β-counter.

Viral strains and infection assays. HIV_{BaL}, a monocytotropic strain of HIV-1 (a kind gift of Drs. S. Gartner, M. Popovic, and R. Gallo, previously at The National Cancer Institute, Bethesda, MD, and purchased from Advanced Biotechnologies, Inc.), was used at a dose of ×200 tissue culture ID_{50} ($TCID_{50}$)/ml (moi = 0.0004) for all experiments. Infectivity of HIV_{BaL} in these preparations was determined in primary M/Mϕ cultures before use in experiments, as described previously (35). HIV_{IIIB}, a lymphocytotropic strain of HIV-1 (Advanced Biotechnologies, Inc.), was used at a dose of ×10,000 $TCID_{50}$/ml (moi = 0.02) for all experiments. Infectivity of HIV_{IIIB} in these preparations was determined using the H9 cell line before use in experiments, as described previously (35). These viral doses were selected initially based on infection experiments in our laboratories with M/Mϕ, and on the assumption that DC infection profiles would be similar to M/Mϕ infection profiles. We did not address specifically the question of whether DC were more or less susceptible to monocytotropic versus lymphocytotropic HIV-1. As controls, cells were pulsed with HIV_{BaL} or HIV_{IIIB} that had been heat-inactivated for 30 min at 56°C.

Irradiated (2,000 rad) or unirradiated DC were resuspended in complete medium with or without cytokines (i.e., GM-CSF, TNF-α, and IL-4 for CB-DC or GM-CSF, and IL-4 for AB-DC, doses as described above) at 5×10^5 cells/ml. HIV_{BaL} or HIV_{IIIB} was added to cells, and cultures were incubated overnight at 37°C. This culture period was assigned the designation of day −1 to day 0. In some experiments, DC were pulsed with HIV in the presence of mouse anti–human CD4 mAbs (Leu-3a [blocking], Becton Dickinson; or OKT4 [non-blocking], Ortho Diagnostic Systems [Raritan, NJ]) at 2 μg/ml, azidothymidine (AZT) (Sigma Chemical Co.) at 10 mM, or the chemokines rhRANTES (regulated upon activation, normal T cell expressed and secreted) or rh–stromal cell–derived factor (SDF)-1 (both from R & D Systems, Inc.) at 1 μg/ml. Each type of blocking experiment was performed at least three times.

On day 0 (after overnight incubation with HIV), DC were harvested, washed two times in wash medium and once in PBS, incubated in PBS containing 0.05% trypsin (SB Biologicals, Cleveland, OH) for 15 min at 37°C to remove extracellular bound HIV (36), washed an additional three times, and resuspended in complete medium. 2.5×10^5 DC were then cultured alone or cocultured with 5×10^5 allogeneic or autologous CD4$^+$ TC (with or without recall Ags, as described above). Cultures were performed in triplicate in 48-well tissue culture plates (Costar Corp.) and incubated in a humidified 5% CO_2 atmosphere at 37°C for 14 d. 80% of the total volume of cultures was collected, frozen at −20°C, and replaced with fresh complete media every other day. Of note, cultures containing DC alone were supplemented with cytokines (in doses as described above) to maintain viability of the cells, whereas DC-TC cocultures were always maintained in the absence of exogenous cytokines. In some experiments, TC were irradiated (5,000 rad) on day 0, immediately before coculture with DC.

Assessment of DC and DC/TC infection by HIV. HIV infection was assessed by PCR, measurement of HIV-1 p24 protein levels in culture supernatants, TEM, and HIV-specific antibody staining. For PCR, cells were harvested at days 1, 3, and 7 after infection. DNA was extracted from cells using a kit according to manufacturer's guidelines (Stratagene Inc., La Jolla, CA) and examined for the presence of HIV-1 proviral DNA by PCR using conserved *gag*-specific primers (SK462/SK431; Clontech). DNA extracted from chronically infected ACH-2 cells was used to establish PCR conditions for the amplification of *gag* and to estimate the number of HIV-1 DNA copies per cell in DC populations. Using a *gag*-specific probe (Clontech), PCR products were visualized as described above.

For the detection of HIV-1 p24 protein, culture supernatants (collected as described above) were thawed and examined by RIA (DuPont, Wilmington, DE), with a lower level of detection of 600 pg/ml, or by ELISA (DuPont), with a lower level of detection of 10 pg/ml, according to manufacturer's guidelines.

To identify specifically HIV-infected DC, cells were harvested on days 0, 7, or 10, processed for TEM as described above, and examined

for the presence and location of budding and mature HIV-1 virions. In addition, DC were harvested on day 7, washed three times in PBS, cytocentrifuged onto glass slides, air-dried for 1 h, and fixed in 2% paraformaldehyde (Sigma Chemical Co.). Infected DC or uninfected DC controls were then incubated with either HIV-1$^-$ normal human serum or serum from an AIDS patient containing high levels of anti–HIV-1 antibodies (each diluted at 1:50 in PBS containing 2% goat serum) for 1 h at room temperature. The slides were then washed three times for 5 min each in PBS and incubated for an additional 30 min at room temperature with a mixture of Texas red–conjugated goat F(ab′)$_2$ anti–human IgG (Accurate Chemical and Science Corp., Westbury, NY) at a final dilution of 1:200, and FITC-conjugated mouse anti–human CD1a (or FITC-conjugated isotype control) at a final dilution of 1:10. Slides were washed three times and examined by an immunofluorescence microscope.

To identify newly synthesized HIV-specific RNA in DC, we isolated mRNA as described above. As published previously (37), we chose the HIV-specific primers M667 and VPR2 to detect a 320-bp fragment of spliced HIV mRNA. For PCR, denaturation was performed at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 2 min, for a total of 18 cycles. An HIV-specific probe (5′-TTTCAGGTCCCTGTTCGGGCGCC [GenBank accession no. M27323]) was hybridized to the PCR products and visualized as described above.

Results

CB- and AB-DC resemble immature DC by phenotypic, morphologic, and functional criteria. Cultures of cells generated from cord blood CD34$^+$ stem cells in the presence of GM-CSF, TNF-α, and IL-4 were comprised of two cell types: large cells (20–40%) that exhibited numerous cell surface processes and veils (i.e., DC), and smaller eosinophil-like granulocytes (60–80%) that demonstrated prominent red granules upon modified Giemsa staining (not shown). Purifying DC from these cultures by negative selection was not feasible, since we were unable to identify cell surface Ags expressed specifically by the granulocytes, using the mAbs listed in Table I. Thus, the precise identity of the eosinophil-like cells was unclear; their presence, however, was not considered a major obstacle in the interpretation of HIV-infection studies using CB-DC because HIV was not localized to these cells (see below). 0.2–1.5 × 10^6 CD34$^+$ cells cultured on day 0 yielded 2–4 × 10^7 total cells by day 14. For AB-DC, ~ 2 × 10^8 total PBMC were plated on day 0, and plastic-adherent PBMC were cultured further, for 7 d in the presence of GM-CSF and IL-4. Before purification on day 7, 80–90% of cultured cells exhibited a dendritic morphology and phenotype, whereas remaining cells consisted of small numbers of TC and B cells, natural killer cells, and M/Mϕ (< 5% of each cell type). However, after purification of this population by immunomagnetic separation, AB-DC were consistently > 99% pure. Typically, 1–2 × 10^7 purified AB-DC could be obtained from a single buffy coat; however, there was considerable variation in this number with different blood donors. Importantly, contaminating TC and M/Mϕ were not detected in populations of CB-DC or purified AB-DC by flow-cytometric analysis or by RT-PCR for CD3 and CD14, respectively (not shown).

The phenotypic profile of CB- and AB-DC (summarized in Table I) resembled the phenotype of populations of immature DC isolated from peripheral blood and epidermis (i.e., Langerhans cells) (4, 5). CB- and AB-DC showed similar expression

Table I. Cell Surface Ag Expression by DC

High levels*
MHC class II molecules (HLA-DR[a], -DP[a], -DQ[b]), MHC class I molecules (HLA-A, -B, -C)[c], invariant chain (CD74)[d], CD1a[e], CD1b[d], CD1c[d], ICAM-1 (CD54)[a], ICAM-2 (CD50)[d], CD40[d], B7-2 (CD86)[b], CD11b[d], integrins β1 (CD29)[d] and β2 (CD18)[d], CD58[d], CD13[a], CD33[a], CD44[d], CD45RO[d]
Low levels*
CD4[a], CCR-5[j], CXCR-4[j], CD11a[d], CD11c[d], FcεRI[i], FcεRII (CD23)[d], FcγRII (CD32)[d], CD83[f], B7-1 (CD80)[a]
No expression*
CD3[b], CD5[b], CD8[d], CD25[d], CD26[g], CD28[a], CD14[a], CD36[d], FcγRI (CD64)[d], FcγRIII (CD16)[b], CD1d[d], CD19[a], CD24[b], CD34[a], CD56[b], CD57[a], E-cadherin‡[h]

**High levels*, mean fluorescence intensity (MFI) > 10 × MFI of isotype control mAb. *Low levels*, MFI 1.5–10 × MFI of isotype control mAb. *No expression*, MFI < 1.5 × MFI of isotype control mAb. ‡Low levels of expression on CB- but not on AB-DC. mAbs purchased from [a]Becton Dickinson, San Jose, CA; [b]PharMingen, San Diego, CA; [c]INCSTAR Corp., Stillwater, MN; [d]Biosource International; [e]Ortho Diagnostic Systems; [f]Immunotech, Marseilles, France; [g]Coulter Corp., Hialeah, FL; and [h]Zymed Laboratories, Inc., South San Francisco, CA; or [i]a kind gift from Dr. Jean-Pierre Kinet, National Institute of Allergy and Infectious Diseases, Rockville, MD. [j]Polyclonal Abs were a kind gift from Dr. Hana Golding, Food and Drug Administration, Bethesda, MD.

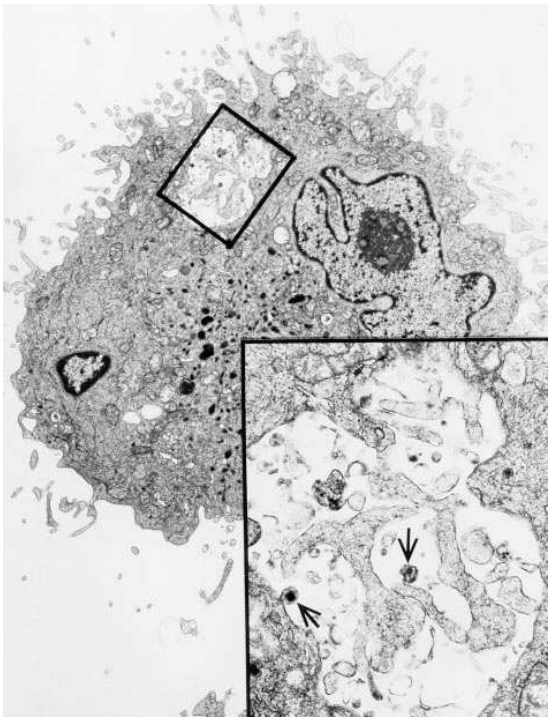

Figure 1. DC capture and retain HIV virions (*inset*, *arrows*) within trypsin-resistant compartments formed by cell surface dendritic processes. AB-DC were cultured overnight with HIV$_{IIIB}$, harvested, washed, trypsinized, and washed again. DC were then fixed in glutaraldehyde, and processed and examined by TEM. Cells pulsed with HIV, harvested, washed, and subjected to mock trypsinization in parallel showed similar findings, except that virions could also be observed attached to external cell membranes (not shown). Representative results from a single experiment are shown (×7,700; *inset*, ×27,500).

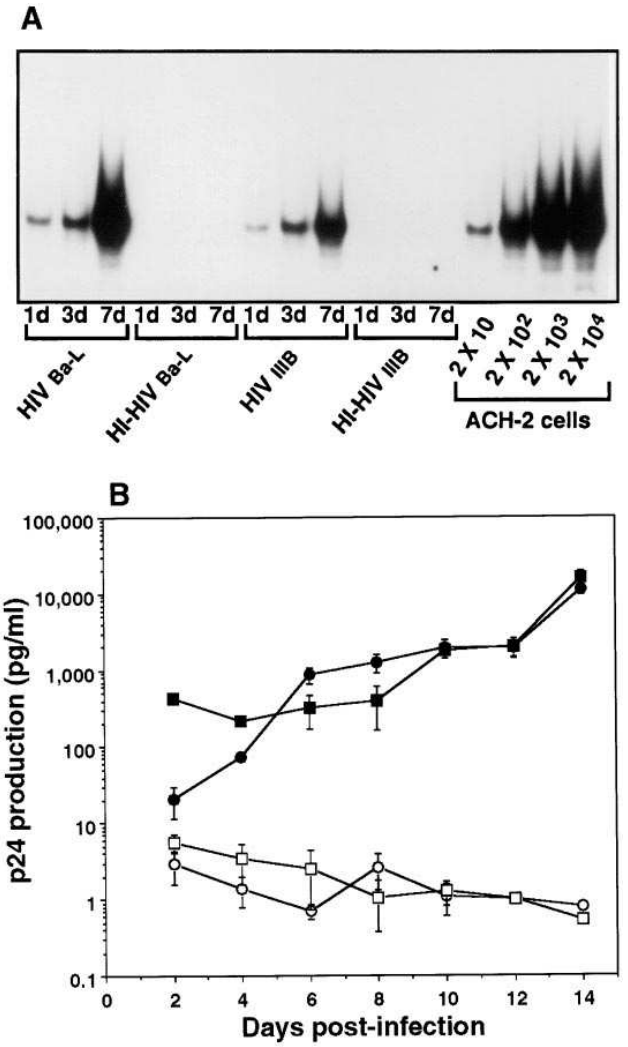

Figure 2. Cultures of DC are infected productively with HIV in vitro. CB-DC were pulsed overnight with HIV_{BaL} (●), HIV_{IIIB} (■), heat-inactivated (*HI*) HIV_{BaL} (○), or heat-inactivated HIV_{IIIB} (□). Cells were then washed, trypsinized, washed again, and placed back into culture in complete media supplemented with GM-CSF, TNF-α, and IL-4. Cells were harvested on days 1, 3, and 7, and DNA was extracted, amplified by PCR using HIV *gag*–specific primers, hybridized with an internal ^{32}P-labeled probe, electrophoresed, and visualized by autoradiography (*A*). Chronically infected ACH-2 cells (1 HIV copy/cell) were used as a positive control (*A*). In parallel, culture supernatants were collected every other day, replaced with fresh media and cytokines, and examined for HIV p24 protein by ELISA (*B*). p24 values shown represent means±SD of triplicate cultures. Results from a single representative experiment are shown. Similar results were observed in cultures containing purified AB-DC (not shown).

of all Ags tested, except for cell surface E-cadherin, which was expressed at low levels on CB- but absent on AB-DC. Of note, DC expressed low surface levels of the HIV-1 receptors CD4, CXCR-4, and CCR-5. On TEM analyses, both types of DC displayed large indented nuclei, dense cytoplasm, and numerous mitochondria, yet no evidence of Birbeck granules (Fig. 1, and see Fig. 3). These features are similar to those described for certain populations of DC (4). Functionally, both CB- and AB-DC were poorly phagocytic (< 10% of cells with detectable phagocytosed beads) compared with M/Mϕ studied in parallel (> 80% of cells with detectable phagocytosed beads) (not shown). In mixed lymphocyte reactions and recall Ag–stimulated assays, both irradiated and unirradiated CB- and AB-DC served as potent APC (see below). Specifically, DC stimulated resting allogeneic $CD4^+$ TC well (thymidine incorporation $> 2 \times 10^4$ cpm) when TC/DC ratios were ≤ 20. Fewer DC led to a dose-dependent decline in TC proliferation.

DC pulsed with HIV capture virus in trypsin-resistant compartments. Immediately after pulsing with HIV, washing, trypsinization, and further washing, DC fixed and examined by TEM showed mature virions encircled by cell surface dendritic processes (Fig. 1). When DC were not trypsinized but otherwise processed in an identical manner, virions were observed both within dendritic processes as described above and outside of dendrites attached to cell membranes (not shown). Thus, the unique surface morphology of DC allowed for capture of HIV and protection from degradation by trypsin.

Productive infection of CB- and AB-DC by HIV_{BaL} and HIV_{IIIB} is dependent on cytokines, CD4, and HIV coreceptors. Both CB- and AB-DC were infected productively by HIV_{BaL} and HIV_{IIIB} (Figs. 2–6). *gag*-Specific proviral DNA (Fig. 2 *A*), secreted HIV-1 p24 protein (Fig. 2 *B*, and see Fig. 5), and newly synthesized HIV-specific spliced mRNA (see Fig. 6) were detected in cultures containing DC exposed to HIV but not to heat-inactivated HIV (Fig. 2). For most experiments, the amount of proviral DNA correlated with p24 protein levels

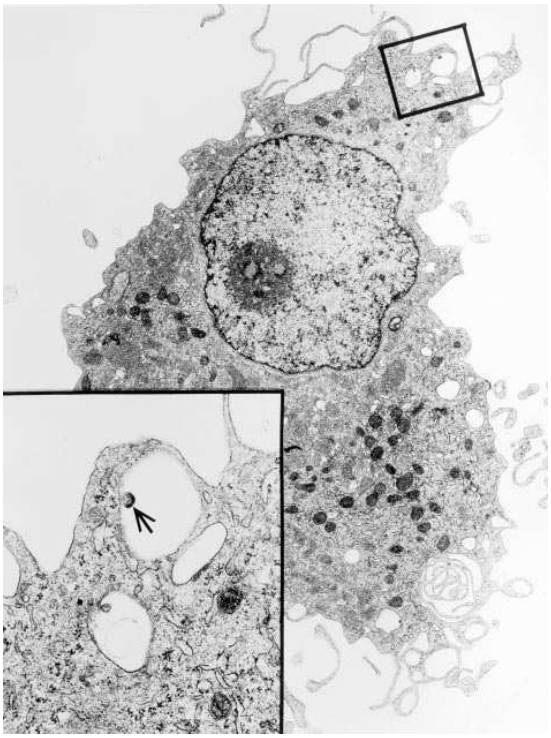

Figure 3. Localization of HIV infection to DC. TEM analysis of AB-DC harvested on day 7 after infection with HIV_{BaL}, demonstrating an HIV virion (*inset*, *arrow*) budding from the surface of a cell with typical DC ultrastructural features (×7,800; *inset*, ×28,500). Representative results from a single experiment are shown.

in infected culture supernatants. HIV_{BaL} and HIV_{IIIB} at the doses used induced comparable levels of productive infection in DC (Fig. 2 *B*, and see Fig. 5).

To rule out the possibility that proviral DNA and supernatant p24 were originating from a rare population of contaminating TC or M/Mϕ, we examined individually infected cells by TEM and by immunolabeling of cytospin preparations. By TEM, virions budding from the surface of cells exhibiting typical dendritic morphology were observed, although this was a rare finding (Fig. 3). No virions were observed within eosinophil-like granulocytes in CB-DC preparations, and cells with lymphocyte morphology were not observed in populations of either CB- or AB-DC during these analyses. In addition, $CD1a^+$ HIV^+ DC were observed clearly in the double-staining experiments of AB- and CB-DC harvested 7 d after initial infection (Fig. 4). No $CD1a^-$ eosinophil-like granulocytes from CB-DC preparations expressed HIV Ags in these experiments. Of note, cytopathic changes or decreased cell viability were not observed by light microscopy or by TEM in cultures of HIV-infected DC at the doses of HIV used for these experiments. APC function of HIV-infected DC also appeared normal, since we found that DC exposed overnight to HIV were able to stimulate naive allogeneic $CD4^+$ TC and recall Ag–specific autologous $CD4^+$ TC as well as DC pulsed with heat-inactivated HIV or untreated DC (see Fig. 7 *A*). However, in more recent experiments in our laboratory using much higher doses

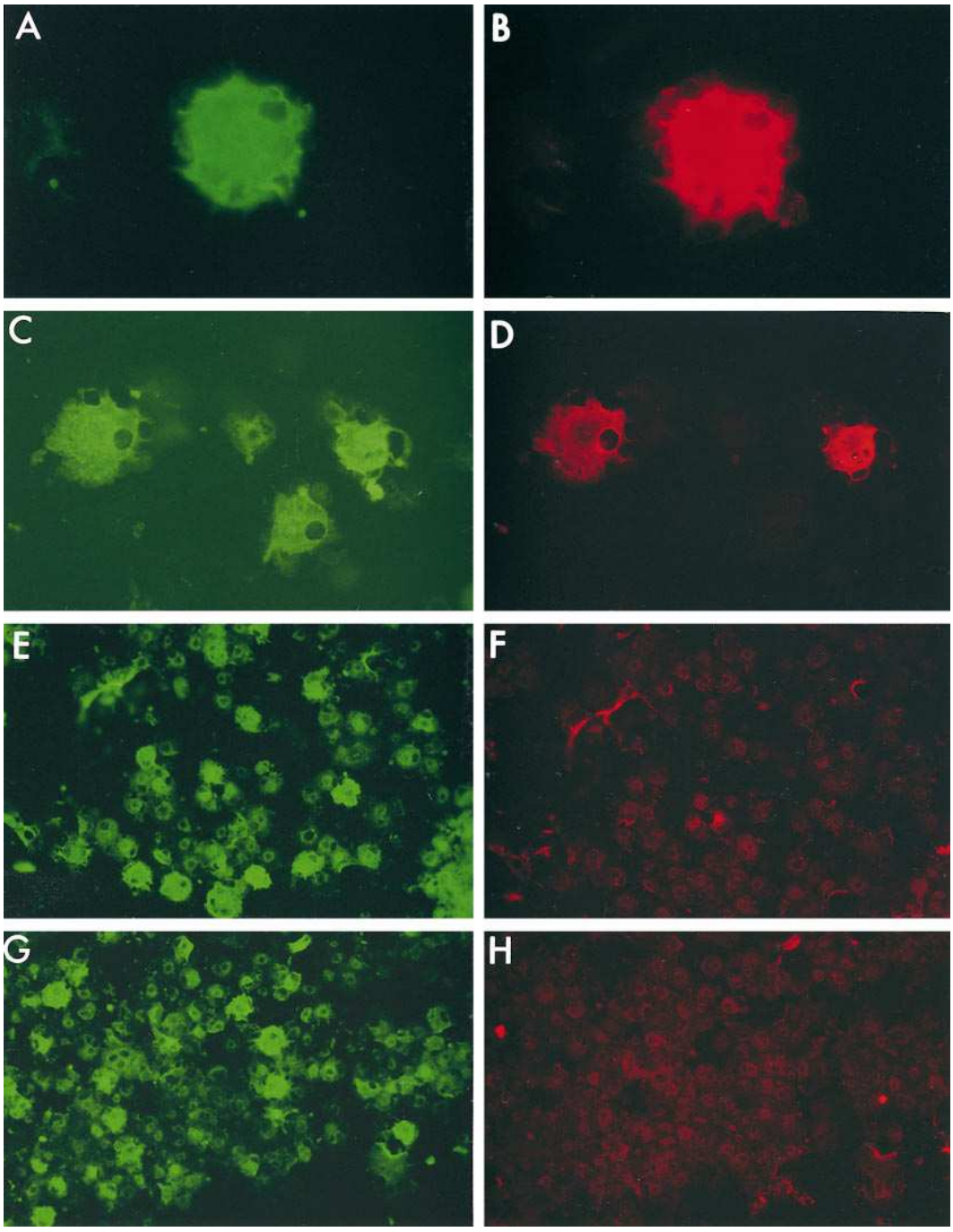

Figure 4. Localization of HIV infection to DC. AB-DC harvested on day 7 after infection with HIV_{BaL} were cytospun, fixed, and double-stained for CD1a and HIV proteins. Infected DC incubated with anti-CD1a mAbs (*A* and *C*) and anti-HIV serum (*B* and *D*). Infected DC incubated with anti-CD1a mAbs (*E*) and normal human serum (*F*). Uninfected DC incubated with anti-CD1a mAbs (*G*) and anti-HIV serum (*H*). Cells incubated with FITC-conjugated isotype control mAbs also served as negative controls (not shown). Representative results from a single experiment are shown.

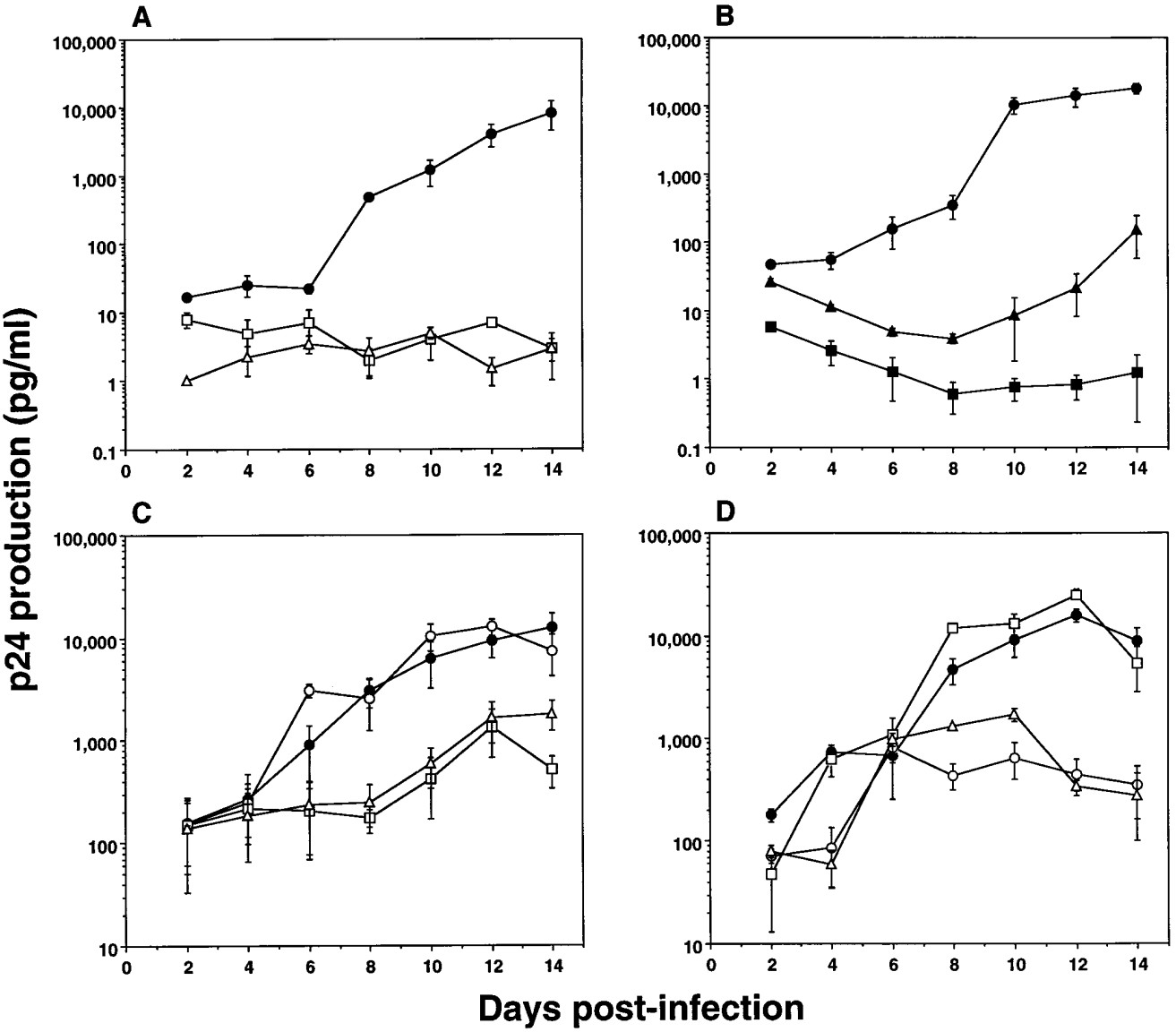

Figure 5. Productive infection of DC by HIV is dependent on DC proliferation, HIV reverse-transcription, and HIV binding to CD4 and HIV coreceptors. (*A* and *B*) CB-DC were pulsed overnight with HIV_{IIIB}, washed, trypsinized, washed again, and placed back into culture in complete media supplemented with GM-CSF, TNF-α, and IL-4. CB-DC were unirradiated (●) or were irradiated (2,000 rads) before (△) or after (□) HIV pulsing (*A*). (*B*) CB-DC were pulsed with HIV in the absence (●) or presence of anti-CD4 mAbs (■) (2 μg/ml) or AZT (▲) (10 mM). (*C* and *D*) Purified AB-DC were pulsed with either HIV_{IIIB} (*C*) or HIV_{BaL} (*D*) in the absence (●) or presence of RANTES (○), SDF-1(□), or both chemokines (△) (each at 1 μg/ml). Culture supernatants were collected every other day, replaced with fresh media and cytokines, and examined for p24 protein by ELISA or RIA. p24 values shown represent means±SD of triplicate cultures. Representative results from individual experiments are shown. Similar results as in *A* and *B* were obtained when DC were pulsed with HIV_{BaL} (not shown).

of HIV (e.g., moi > 0.1), infected AB-DC produce high levels of p24 (e.g., 100 ng/ml) and exhibit virus-induced syncytia formation and cell death (our unpublished observations). Productive infection of DC required the continuous presence of cytokines (i.e., GM-CSF, TNF-α, and IL-4 for CB-DC or GM-CSF, and IL-4 for AB-DC, added every other day with fresh media). Specifically, DC cultured in the absence of cytokines failed to proliferate, and died usually in less than 1 wk (see Fig. 7, *B* and *C*). Similarly, DC irradiated before or after HIV pulsing failed to become productively infected with HIV (Fig. 5 *A* and Fig. 6). When HIV pulsing was performed in the presence of anti-CD4 mAbs known to interfere with HIV-CD4 binding (e.g., Leu 3a), infection of DC by HIV was blocked (Fig. 5 *B* and Fig. 6); anti-CD4 mAbs which do not block HIV binding (e.g., OKT4) had no effect on DC infection (Fig. 6). As expected, the presence of AZT during HIV exposure also blocked infection of DC (Fig. 5 *B* and Fig. 6). In addition, HIV_{IIIB} was partially blocked by SDF-1 (Fig. 5 *C*), the natural ligand for CXCR-4 (the major coreceptor for TC line–tropic strains of HIV) (38), and HIV_{BaL} was partially blocked by RANTES (Fig. 5 *D*), a natural ligand for CCR-5 (the major coreceptor for monocytotropic strains of HIV) (39). No synergism in blocking was observed in cultures containing both chemokines (Fig. 5, *C* and *D*).

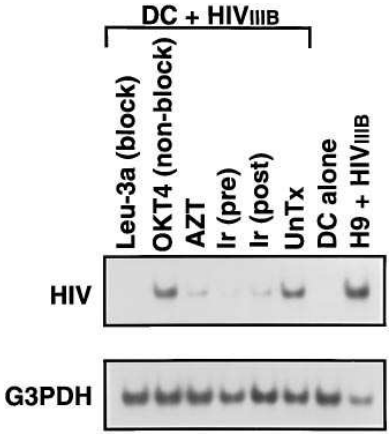

Figure 6. Intracellular spliced HIV mRNA production in DC pulsed with HIV correlates with extracellular p24 protein production. AB-DC were pulsed overnight with HIV_{IIIB}, washed, trypsinized, washed again, and placed back into culture in complete media supplemented with GM-CSF and IL-4. AB-DC were unirradiated (*UnTx*) or were irradiated (*Ir*; 2,000 rads) before (*pre*) or after (*post*) HIV pulsing. Unirradiated AB-DC were also pulsed with HIV in the presence of either blocking (*Leu-3a*) or nonblocking (*OKT4*) anti-CD4 mAbs (2 μg/ml), or AZT (10 mM). After 2 d in culture, DC were harvested, and mRNA was isolated. cDNA was made and amplified by PCR, using specific primers able to detect G3PDH or spliced HIV mRNA. mRNA isolated from H9 cells chronically infected with HIV was used as a positive control for RT-PCR. Representative results from an individual experiment are shown. Similar results were obtained when DC were pulsed with HIV_{BaL} (not shown).

Capture of HIV by DC (with subsequent transmission of virus to CD4⁺ TC) occurs independently of DC infection. As mentioned above, HIV-pulsed DC stimulated allogeneic $CD4^+$ TC and recall Ag–stimulated autologous TC as well as unexposed DC and DC exposed to heat-inactivated HIV (Fig. 7 *A*). As previously reported for AB-DC (40), we found that CB- and AB-DC pulsed with HIV induced high levels of infection in these Ag-stimulated DC/TC cocultures (Fig. 7, *B* and *C*).

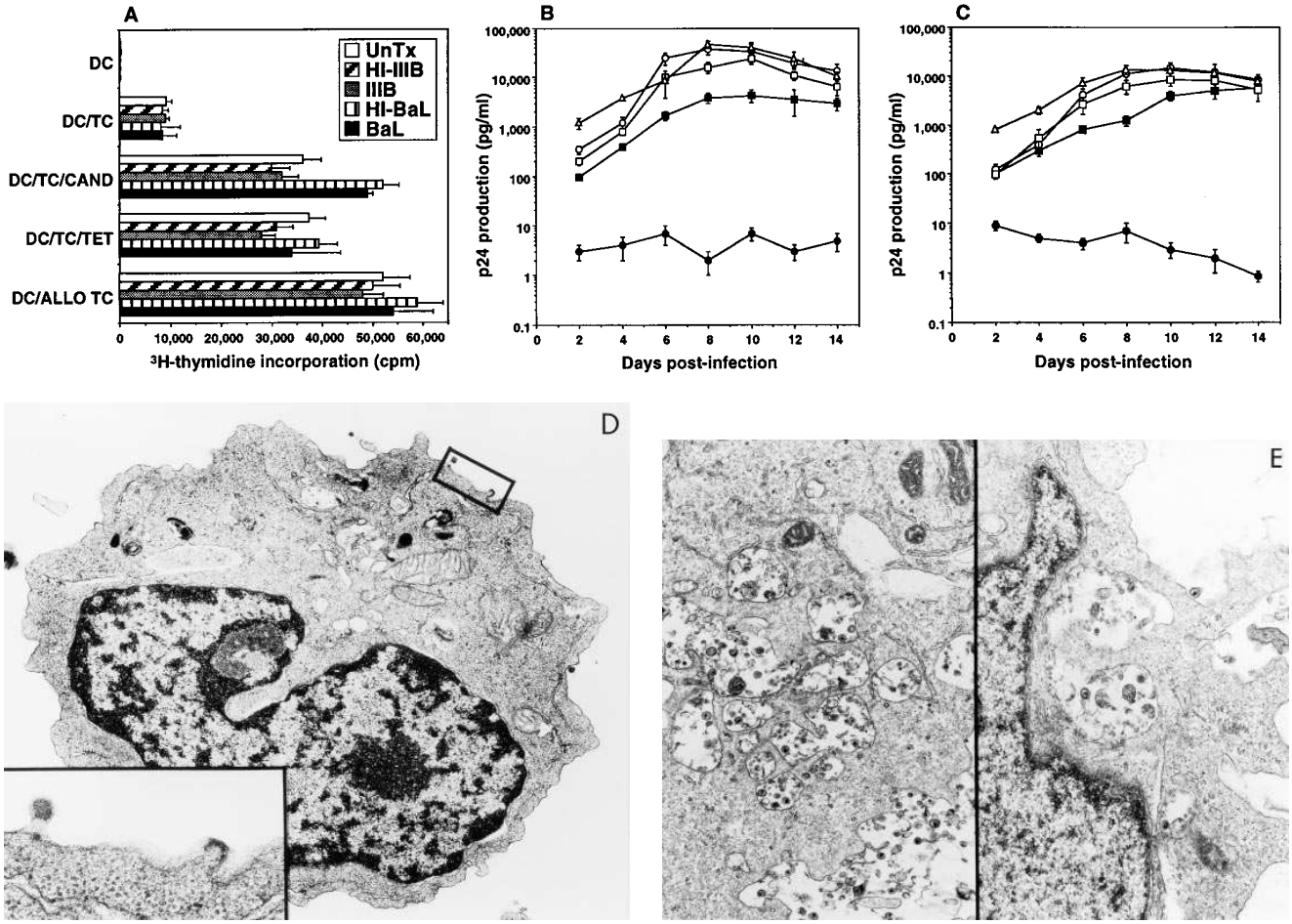

Figure 7. DC pulsed with HIV retain APC function and induce high levels of infection in cocultured $CD4^+$ TC, parallel to the degree of Ag-specific TC activation. AB-DC were untreated (*A*) or were pulsed with HIV_{IIIB} (*A*, *B*, *D*, and *E*), HIV_{BaL} (*A* and *C*), or heat-inactivated (*HI*) HIV (*A*). Cells were then washed, trypsinized, washed again, and cultured alone (●) (without exogenous cytokine supplementation) or with autologous (■) or allogeneic (*ALLO*) $CD4^+$ TC (△). Some cultures contained autologous $CD4^+$ TC and soluble protein Ags, *C. albicans* (○) (*CAND*), or tetanus toxoid (□) (*TET*). For functional studies, cultures were pulsed with [^{3}H]thymidine on day 5.5, harvested on day 6, and thymidine incorporation was measured (*A*). In parallel cultures, supernatants were collected every other day, replaced with fresh media, and examined for p24 protein by RIA (*B* and *C*). Thymidine incorporation and p24 values represent means±SD of triplicate cultures. When TC were irradiated before coculture with DC, p24 levels were comparable to those found in cultures of DC alone (not shown). In parallel, TEM analysis on day 10 after coculture of HIV-exposed DC and allogeneic $CD4^+$ TC showed several virions budding from TC membranes (*D*) (×15,500; *inset,* ×58,000), whereas DC demonstrated numerous mature viral particles encircled by dendritic processes (*E*) (*left*, ×19,300; *right*, ×25,500). Budding from cell membranes of DC was rarely observed (not shown). Representative results from single experiments are shown.

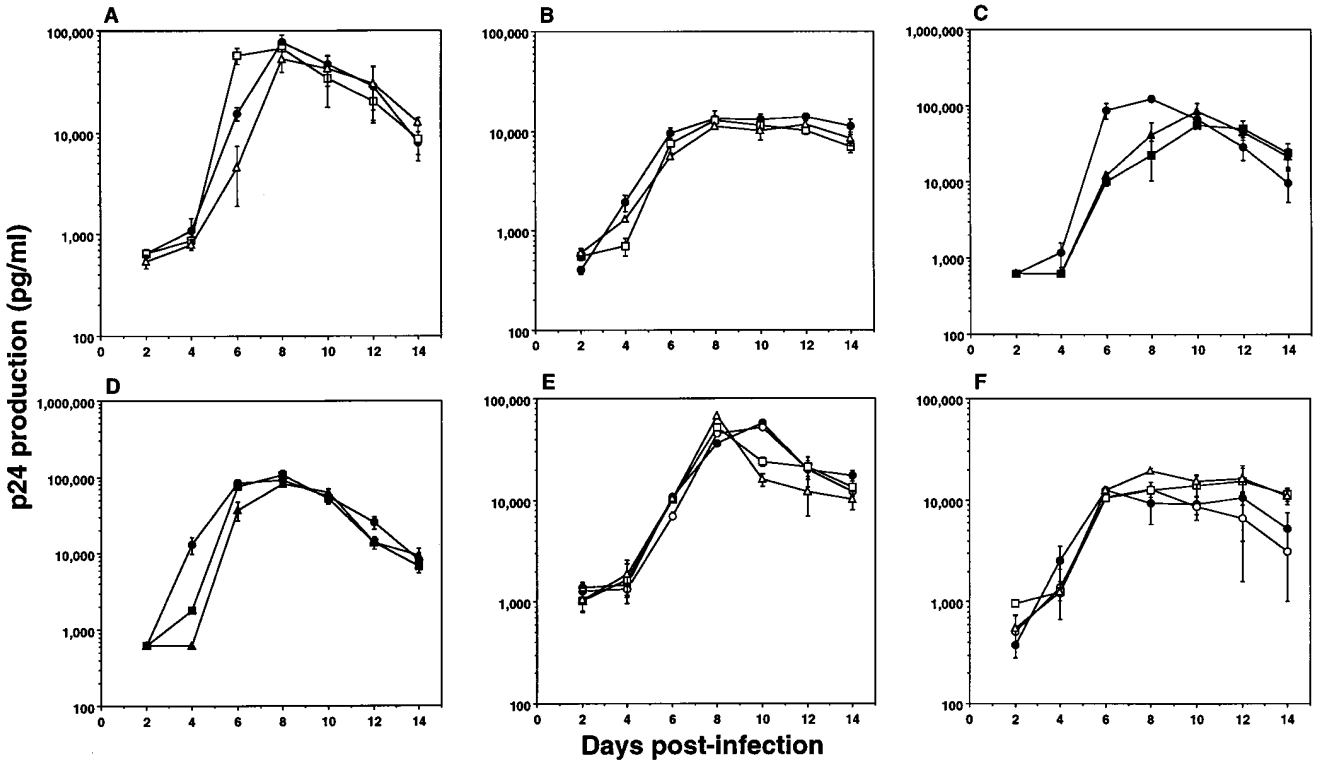

Figure 8. Capture of HIV by DC occurs independent of DC proliferation, HIV reverse-transcription, and HIV binding to CD4 and HIV coreceptors. CB-DC (*A–D*) or AB-DC (*E* and *F*) were pulsed with HIV_{IIIB} (*A*, *C*, and *E*) or HIV_{BaL} (*B*, *D*, and *F*), washed, trypsinized, washed again, and cocultured with allogeneic $CD4^+$ TC. (*A* and *B*) CB-DC were unirradiated (●) or were irradiated (2,000 rads) before (△) or after (□) HIV pulsing. (*C* and *D*) CB-DC were pulsed with HIV in the absence (●) or presence of anti-CD4 mAbs (■) (2 μg/ml) or AZT (▲) (10 mM). (*E* and *F*) Purified AB-DC were pulsed with HIV in the absence (●) or presence of RANTES (○), SDF-1(□), or both chemokines (△) (each at 1 μg/ml). Culture supernatants were collected every other day, replaced with fresh media, and examined for p24 protein by RIA. p24 values shown represent means±SD of triplicate cultures. Representative results from individual experiments are shown.

The extent of infection correlated directly with the degree of alloantigen- or recall Ag–stimulated TC proliferation (Fig. 7). Irradiation of TC before mixing with HIV-pulsed DC decreased markedly levels of infection in cocultures, to infection levels observed in cultures of DC alone, indicating that the predominant cells supporting HIV replication were $CD4^+$ TC (not shown). Furthermore, several virions were observed budding from the surface of TC by TEM (Fig. 7 *D*), while virions budding from the surface of DC were rarely detected (not shown). However, DC were remarkable due to the presence of numerous mature viral particles encircled (captured) by cell surface dendritic processes (Fig. 7 *E*). TC were infected by DC pulsed with either lymphocytotropic (i.e., HIV_{IIIB}) or monocytotropic (HIV_{BaL}) strains of HIV, although peak p24 levels in cocultures were three to fivefold greater when DC were pulsed with HIV_{IIIB}. No infection was observed in these cocultures when DC were pulsed with heat-inactivated HIV (not shown).

The ability of DC to capture HIV (with subsequent transmission of virus to cocultured TC) did not require infection of DC. Specifically, although irradiated DC did not support HIV replication (Fig. 5 *A* and Fig. 6), irradiation of DC before or after HIV pulsing did not impair the ability of DC to capture HIV and transmit infection to cocultured TC (Fig. 8, *A* and *B*). Similarly, DC pulsed with HIV in the presence of blocking anti-CD4 mAbs or AZT were not infected productively with HIV (Fig. 5 *B* and Fig. 6), yet these same DC were able to infect cocultured TC as well or nearly as well as DC pulsed in the absence of blocking anti-CD4 mAbs or AZT (Fig. 8, *C* and *D*). Chemokines could block DC infection partially (Fig. 5, *C* and *D*), yet could not block HIV capture by DC and transmission to TC (Fig. 8, *E* and *F*). Finally, unlike productive infection of DC, which required the addition of exogenous cytokines, productive infection of $CD4^+$ TC by HIV-pulsed DC was independent of exogenous cytokines.

Discussion

We have shown that there are at least two pathways by which HIV interacts with DC, both of which can occur simultaneously and independently of one another (summarized in Table II). Productive infection of DC at low levels was CD4- and HIV coreceptor–dependent, and required proliferation of DC, whereas the ability of DC to capture HIV was independent of HIV binding to CD4/HIV coreceptor, HIV reverse-transcription, and DC proliferation. During pulsing of DC with HIV, TEM analyses suggested that whole virions were taken up by cell surface dendritic processes into trypsin-resistant compartments. Subsequent transmission of virus to cocultured $CD4^+$ TC by HIV-pulsed DC resulted in high levels of infection that paralleled the degree of DC-dependent Ag-specific TC activation.

As discussed in the Introduction, in vitro infection of blood

Table II. Productive Infection of DC by HIV and Their Ability to Capture Virus Are Mediated through Separate Pathways

Productive infection of DC	Capture of HIV by DC
CD4-dependent	CD4-independent
CCR-5–dependent (for HIV_{BaL})	CCR-5–independent
CXCR-4–dependent (for HIV_{IIIB})	CXCR-4–independent
AZT-sensitive	AZT-insensitive
Exogenous cytokine–dependent	Exogenous cytokine–independent
Radiation-sensitive	Radiation-resistant

DC by HIV has been a controversial subject. In this study, we document CD4-, CCR-5– (for HIV_{BaL}), and CXCR-4–dependent (for HIV_{IIIB}) productive HIV infection of Langerhans cell–like DC (devoid of contaminating TC and M/Mϕ), but only in association with DC proliferation (Figs. 2–6). Others have also shown recently that DC propagated in vitro with cytokines were infectable with both lymphocytotropic and monocytotropic strains of HIV in a CD4- and HIV coreceptor–dependent manner (23, 40, 41). We suggest that the inability to infect highly purified blood DC productively in some previous studies may be explained by relatively low amounts of cell surface CD4/HIV coreceptors and/or by low levels of proliferation in these cell populations.

It has been clear that a variety of HIV-exposed DC can infect cocultured $CD4^+$ TC rigorously (8, 10–13, 15–20, 22). This ability of DC to infect TC efficiently appears to be unique to DC, since M/Mϕ, B cells, and TC pulsed in a similar manner fail to induce high levels of infection upon coculture with TC (12, 13, 15, 19). Not surprisingly, we have shown that the degree of TC infection parallels the degree of TC activation (Fig. 7), similar to previous reports (12, 13, 40). Also consistent with previous studies (11–13), we show that DC can infect TC even under conditions in which they do not replicate HIV themselves (Figs. 5, 6, and 8). However, our study is unique in showing that the processes of DC infection by HIV and their ability to capture virus can occur independently and simultaneously. In addition, we show for the first time that capture of HIV by DC is a process that occurs independent of HIV binding to CD4 or HIV coreceptors, DC proliferation, and HIV reverse-transcription (Fig. 8). However, it is still unclear whether capture and retention of HIV is mediated through a specific ligand–virus interaction (e.g., mannose receptor–mediated), or whether virus is taken up in a nonspecific manner by micropinocytosis. Further study is needed to elucidate this issue.

The in vivo relevance of this study is unclear at this time. It is possible that tissue DC (i.e., Langerhans cells) capture HIV after mucosal exposure to virus, migrate to local lymph nodes, and transmit infection to $CD4^+$ TC. Evidence to support this theory has been provided recently in macaque models of primary SIV infection (6, 7). Langerhans cell–mediated capture, which occurs independent of CCR-5 and CXCR-4, may explain in part why individuals with homozygous deletions in their CCR-5 genes may not be fully protected from HIV entry and infection (42–44). Our findings suggest that methods designed to block primary HIV infection should ideally address inhibition of both types of DC–HIV interactions (infection and capture pathways), as described here. Another possible implication of this work is that DC may be mediating HIV infection of activated TC. During the generation of immune responses, tissue DC (that are either infected with HIV or that capture virus en route to lymph nodes) may activate and infect TC concomitantly, and thus contribute to Ag-specific depletion of TC. Consistent with this theory, increases in viral replication have been documented after vaccination and acute illnesses in HIV-infected individuals (45–49). Thus, blocking DC-mediated infection of TC may be an important strategy for the treatment of HIV disease. In this regard, transmission of HIV from DC to TC can be blocked in vitro by interfering with costimulatory molecule–ligand interactions (e.g., with mAbs against CD4, intracellular adhesion molecule [ICAM]-1, leukocyte function–associated Ag [LFA]-1, LFA-3, CD40, and CD80) (13, 40); concentrations of inhibitors required, however, would likely be high, and such an approach would be likely also to inhibit normal immune function. Alternatively, we suggest that blocking capture of HIV by DC may be a more effective strategy for blocking DC-mediated HIV infection of TC. Additional studies are needed to identify agents that may selectively block this step in vitro, and to determine whether these compounds can be used for treatment of HIV^+ patients.

Taken together, we have shown for the first time that productive infection of DC and the ability of DC to capture virus (with subsequent transmission to cocultured TC) are mediated through separate pathways. This work helps clarify studies previously reported in the field of DC–HIV research by identifying molecules involved specifically in these two distinct pathways. In addition, the system we have described may serve as a model for further studies on mechanisms of DC–HIV interactions (e.g., identifying a possible HIV-specific ligand on DC which mediates capture). We suggest that strategies designed to block mucosal transmission of HIV should consider interfering with both virus infection and virus capture by DC. Finally, blocking DC-mediated HIV infection and death of activated TC may be a possible therapeutic approach for HIV disease.

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