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Dendritic Cells Exposed to Human Immunodeficiency Virus Type–1 Transmit a Vigorous Cytopathic Infection to CD4+ T Cells

Paul U. Cameron, Peter S. Freudenthal, Jeanne M. Barker, Stuart Gezelter, Kayo Inaba, Ralph M. Steinman*

The paucity of virus-laden CD4+ cells in individuals infected with human immunodeficiency virus type–1 (HIV-1) contrasts with the greatly reduced numbers and function of these lymphocytes. A pathway is described whereby dendritic cells carry HIV-1 to uninfected T cells, amplifying the cytopathic effects of small amounts of virus. After exposure to HIV-1, dendritic cells continue to present superantigens and antigens, forming clusters with T cells that are driven to replicate. Infection of the dendritic cells cannot be detected, but the clustered T cells form syncytia, release virions, and die. Carriage of HIV-1 by dendritic cells may facilitate the lysis and loss of antigen specific CD4+ T cells in acquired immunodeficiency syndrome.

The critical loss of CD4+ T lymphocytes that follows infection with human HIV-1 depletes cells that defend against opportunistic infections (*1*). Whereas HIV-1 is cytopathic for CD4+ T cells in culture (*2*, *3*), the number of HIV-1–infected lymphocytes in patients is small, 1% of the total or much less (*4–9*). The paucity of infected T cells has stimulated experiments to look for autoimmune and other mechanisms that eliminate CD4+ lymphocytes. An alternative possibility is that antigen-presenting cells (APCs) that have been exposed to HIV-1 mediate the lysis of uninfected–CD4+ T cells that are responding to antigens in the presence of small amounts of virus. Here we show that the trace population of antigen-presenting dendritic cells represents such a catalyst. The APCs are exposed to HIV-1, washed, and added to T cells that are then stimulated with a superantigen or an antigen. An explosive cytopathic infection in the responding T cells ensues even though the dendritic cells themselves are not productively infected.

Dendritic cells are a widely distributed system of APCs [reviewed in (*10*)]. Although few in number, these can be enriched from several human tissues including blood, inflammatory exudates, thymus, lung, skin, gut, and tonsil. In each instance, the dendritic cells have a characteristic stellate shape and motility, high levels of adhesion molecules and antigen-presenting major histocompatibility complex (MHC) class II products, and potent accessory function for a variety of T cell–dependent responses. For example, in the primary mixed leukocyte reaction, or in the primary response to superantigens, small numbers of dendritic cells stimulate T cells to undergo extensive proliferation (*11–13*). During these events, the dendritic cells and responding T cells form discrete aggregates that represent the microenvironment for the immune response (*11*, *14*). Because stimulated rather than resting T cells are the principal targets for a cytopathic infection with HIV-1 (*3*, *15*), the microenvironment that is created by dendritic cells would in the presence of virus seem ominous for those T cells that are attempting to respond.

An improved enrichment procedure for human blood dendritic cells was recently described (*12*). A combination of markers that selectively identify other cell types (for example, CD3, CD14, and CD19 for T cells, monocytes, and B cells, respectively) was used to negatively select the trace dendritic cell subset in a fluorescence-activated cell sorter (*12*, *16*). In addition to typical morphology and motility, the enriched dendritic cells had high levels of class II MHC products and low levels of CD4. The absence of CD2, αβ T cell receptor (TCR), CD3, CD4, and CD8 indicated a virtual absence of contaminating T cells.

To test whether dendritic cells were highly susceptible to HIV-1 infection as suggested (*17–19*), we used polymerase chain reaction (PCR) to quantitate reverse transcripts in APCs relative to positive controls, which were stimulated CD4+ lymphocytes or "T blasts" (*20–23*). After exposure to HIV-1 (*24*), T blasts rapidly transcribed proviral DNA (Fig. 1A). Within 6 hours, high concentrations of full-length provirus were evident with gag-specific primers [SK68 and SK69 (*21*)]. In contrast, the purified dendritic cells did not contain provirus at 6 or 12 hours (Fig. 1A), nor were early transcripts detected with long terminal repeat (LTR) primers (see below).

In experiments, which are illustrated with the HTLV-III$_B$ isolate in Fig. 1B, the

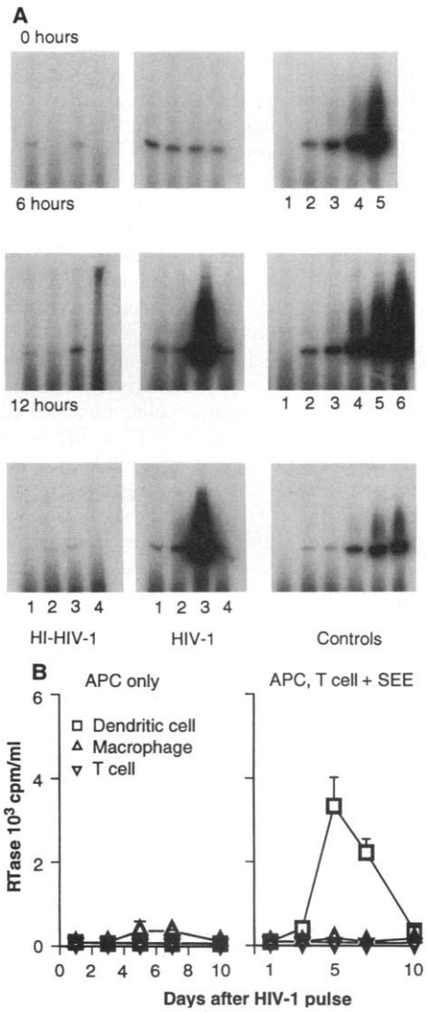

Fig. 1. Dendritic cells, purified by fluorescence-activated cell sorting and pulsed with HIV-1, do not become infected but can transmit an infection to T cells activated by superantigens. (**A**) Different cell populations (legend) were pulsed with HTLV-III$_B$ at a multiplicity of infection of 0.5 for 1.5 hours at 37°C. Control cells were pulsed with heat-inactivated HIV-1 (HI-HIV-1). The cells were washed and recultured in complete medium. The cells were harvested 0, 6, and 12 hours later, and DNA was prepared for PCR of gag-specific sequences (primers SK38 and SK39). Lane 1, sorted dendritic cells; lane 2, B + NK cells [metrizamide high-density cells]; lane 3, T blasts; and lane 4, CD4+ T cells. The controls were ACH-2 cells diluted in uninfected Er+ T cells; 6, 10^4; 5, 10^3; 4, 10^2; 3, 10^1; 2, 1; and 1, 0 ACH-2 cells per milliliter. (**B**) Dendritic cells (10^4), monocytes, or T cells that had been pulsed with HTLV-III$_B$ (or several other isolates) (*24*) were cultured in round-bottom 96-well plates without (left panel) or with (right panel) 10^5 T cells plus superantigen (SEE, 5 ng/ml). Culture supernatants were harvested every other day and assayed for RT (*25*). In the APC-only cultures (left), only small numbers of cells were monitored and the APCs were not exposed to cytokines or other stimuli.

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HIV-1–pulsed dendritic cells that were incapable of producing reverse transcriptase (RT) (*25*, *26*) did transmit an infection to stimulated T cells. As stimulant, we used the superantigen staphylococcal enterotoxin E (SEE), which acts by binding to MHC class II molecules on the APC and specific V_β segments of the T cell receptor for antigen (*27*). Several different isolates of HIV-1 including monocytotropic strains were tested with similar results. In the cultures that were stimulated by HIV-1–pulsed dendritic cells, numerous syncytia and trypan blue–positive dead cells were noted by days 3 and 5 of the dendritic cell–T cell coculture, respectively. At similar doses to dendritic cells, virus-pulsed monocytes and T cells (Er^+ cells) were ineffective in transmitting a productive infection (Fig. 1B), indicating the importance of presenting the HIV-1 in association with dendritic cell APCs. If resting T cells were pulsed with virus and then added to dendritic cells, RT activity was weak or absent in spite of strong T cell proliferation. It was necessary to pulse the dendritic cells at 37°C versus 4°C for these APCs to transmit HIV-1. No RT was produced, and T cells did not proliferate if SEE was added in the absence of APCs. Therefore, it seems necessary to pulse the dendritic cells with HIV-1 to initiate proliferation and productive infection in resting T cells.

The rapid course of the infection in Fig. 1B (RT detectable between days 3 and 5 and peaking between days 5 and 7) suggested that infection of dendritic cells was not a prerequisite for transmission to T cells. Nonetheless, it was conceivable that dendritic cells would become permissive to HIV-1 in the same way that $CD4^+$ T cells become permissive after lymphocyte activation (*3*, *15*). Because it is known that murine $CD4^+$ T cells do not permit entry of HIV-1 (*28*), we developed a strategy with xenogeneic coculture to substantiate that virus-pulsed dendritic cells did not become infected during their interaction with $CD4^+$ cells. At appropriate concentrations of SEE, we found that dendritic cells induced comparable proliferation of homologous and xenogeneic $CD4^+$ T cells. Therefore various mixtures (human or mouse dendritic cells with either mouse or human $CD4^+$ T cells) were exposed to virus at 0, 24, and 48 hours after initiation of coculture. Six hours after adding HIV-1, DNA was extracted for PCR analysis of late (gag) and early LTR reverse transcripts. Few gag-specific sequences occurred in any of the cultures pulsed at time 0 (Fig. 2A). When the $CD4^+$ T cells were of human origin, there were increasing numbers of gag target sequences in cultures pulsed after 24 or 48 hours. In contrast, murine or human dendritic cells in culture with murine $CD4^+$ T cells showed little if any such increase in PCR signal (Fig. 2A, lanes 2 and 4).

These data on gag transcripts did not exclude the possibility that viral entry into dendritic cells may have occurred but without appropriate signals for efficient transcription of gag-containing retrovirus. Therefore, cocultures were also examined for early transcripts with LTR-specific primers. Quiescent human $CD4^+$ T cells (Fig. 2B, time 0, lanes 1 and 3) contained many copies of early transcripts, confirming previous work (*15*). During activation (48-hour time point) there was no further increase in LTR transcripts. No early or late transcripts were noted in the human dendritic cell–containing cultures (Fig. 2, A and B).

The experiments were carried out in another way. HIV-1–pulsed murine or human dendritic cells were added to murine or human $CD4^+$ T cells in all four possible combinations. Virus production was assessed by RT and p24 (Fig. 2, C and D). Virus was produced only in cultures containing T cells of human origin. Both murine and human dendritic cells transmitted infection to human $CD4^+$ T cells. To substantiate that dendritic cell infection with HIV-1 was not necessary for transmission, we added azidothymidine (AZT) to block any infection of dendritic cells that might occur during exposure to HIV-1. In control experiments, AZT (10 μM) did block the infection of HIV-1–pulsed T blasts. However, AZT-treated virus-pulsed dendritic cells transmitted the infection to superantigen-stimulated $CD4^+$ T cells.

We then used the mixed leukocyte reaction (MLR) to test if virus-pulsed dendritic cells would transmit HIV-1 to antigen-responsive T cells. In the MLR, antigens on

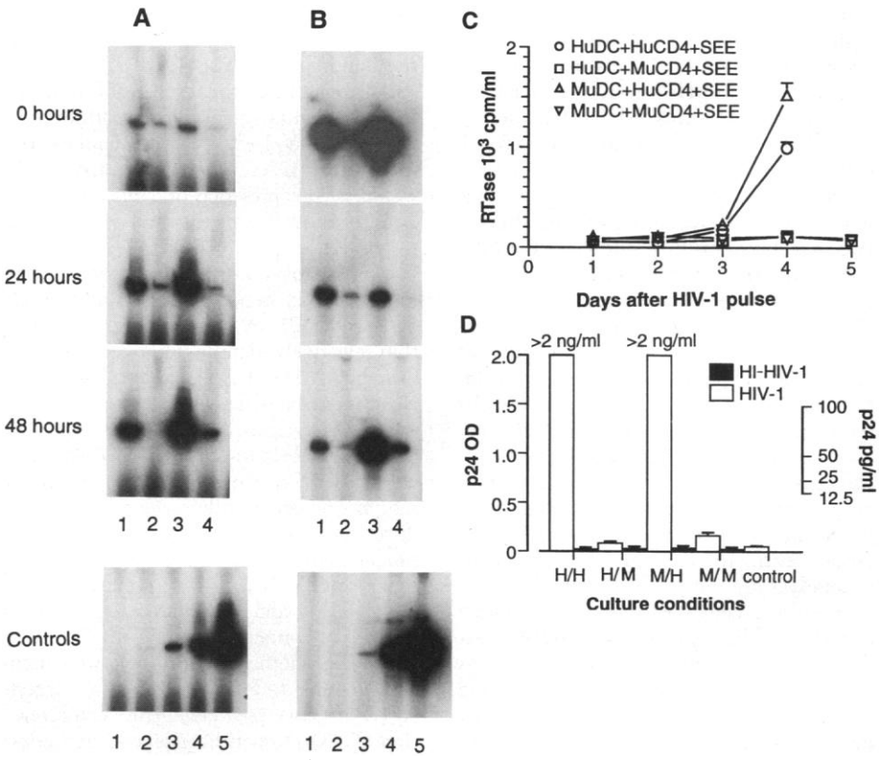

Fig. 2. Xenogeneic mixtures of dendritic cells and $CD4^+$ T cells replicate HIV-1 only when human $CD4^+$ T cells are present. In (**A**) and (**B**), the cultures contained 3×10^4 dendritic cells and 5×10^4 (human) or 10^5 (murine) $CD4^+$ cells in round-bottom microtiter plates. The controls were graded doses of ACH-2 cells (lane 1, 0 ACH-2 cells per milliliter; lane 2, 10 cells; lane 3, 10^2; lane 4, 10^3; and lane 5, 10^4). In (**C**) and (**D**), 10^4 human or mouse dendritic cells were pulsed with HIV-1 or heat-inactivated HIV-1 (HI-HIV-1) and added to triplicate cultures of xenogeneic or allogeneic $CD4^+$ cells (10^5 human or 2×10^5 murine; flat-bottom microtiter plates), and superantigen was added (final SEE of 100 pg/ml human/human; 1 ng/ml mouse/mouse and mouse/human; and 5 ng/ml human/mouse). (A) Cultures were pulsed with HIV-1 at the indicated times, and 6 hours later cellular DNA was isolated and amplified for full length reverse transcripts (gag primers). Lane 1, human dendritic cells (DC)/human CD4; lane 2, human DC/mouse CD4; lane 3, mouse DC/human CD4; and lane 4, mouse DC/mouse CD4. (B) As in (A), but the DNA was amplified for early transcripts (LTR primers). (C) RT activity in culture supernatants measured daily for 5 days. Hu, human; Mu, murine. (D) HIV p24 antigen quantitated by enzyme-linked immunoabsorbant assay (ELISA) at 5 days. OD, optical density; H, human; M, mouse.

dendritic cells from one individual vigorously stimulate T cells from another individual to proliferate, produce lymphokines, and form killer T cells (*11, 12, 14, 29*). We noted that HIV-1–pulsed dendritic cells were fully active as MLR stimulators at least at the early time points, days 2 to 4. At later time points, when T cell cytotoxicity was manifest (below), DNA synthesis in response to HIV-1–pulsed dendritic cells was reduced 50% or more relative to controls. When HIV-1 infection was monitored with standard assays for RT, the use of virus-pulsed dendritic cells led to a vigorous infection that was dependent on the dose of APCs (Fig. 3A). RT was detectable at day 3 of the MLR with a dendritic–T cell ratio of 1:30, and at day 5 with a ratio of 1:100. Syngeneic APC–T cell mixtures exhibited little or no DNA synthesis and no release of RT (Fig. 3A).

In any one individual, only a minor fraction of T cells respond to antigens on another individual's dendritic cells. To monitor the development of a cytopathic infection more directly, we isolated the clusters of interacting dendritic cells and T cells that characteristically develop in an MLR (*11*). The clustered cells represented <5% of the cells in the culture and proliferated vigorously (Fig. 3B). If the dendritic cells had been pulsed with live HIV-1, the clusters generated a cytopathic infection with release of RT and cell death (Fig. 3, C and D). Nonclustered cells did not proliferate or show evidence of infection (Fig. 3, B, C, and D).

By electron microscopy (Fig. 4A, arrows), the clusters contained T blasts and dendritic cells, the latter extending numerous and characteristic sheet-like processes. Budding of HIV-1 was evident only on lymphocytes, but not on the extensive sheets of dendritic cell surface (Fig. 4B).

To assess whether dendritic cells were themselves becoming infected during the MLR, we used an assay described by Lifson *et al.* (*30*). Cells that are productively infected with HIV-1, if labeled with a fluorescent dye, transfer the dye to uninfected $CD4^+$ T cells. Dye transfer is the result of the syncytium formation that occurs when HIV envelope proteins on one cell fuse with CD4 on another. If dendritic cells were productively infected with HIV-1, the expressed gp 120/41 should result in their fusion with the many closely apposed $CD4^+$ T blasts and syncytia in the MLR clusters. In control studies, we found that dendritic cells that were tagged with two different fluorescent dyes stimulated the MLR in an identical fashion to untreated dendritic cells although now the discrete dye-labeled APCs in the clusters could be observed. When pulsed with HIV-1, the dye-labeled dendritic cells did not fuse with the syncytia (Fig. 4, C and D). As a positive control we noted that dye-labeled, HIV-1–infected T blasts delivered the dye to the syncytia that were numerous in our MLR cultures.

Our results differ from a report that the APC function of dendritic cells is ablated after exposure to the $HTLV\text{-}III_B$ strain (*31*). Perhaps the HIV-1 preparation used in previous studies contained other suppressants or APC function was measured at later time points after T cell cytopathicity had begun. The capacity of HIV-1–pulsed dendritic cells to function normally as APCs must underlie our findings. The ability of these APCs to create long-lived aggregates of responding T blasts creates an environment in which one would expect an infection of even one T cell to be rapidly transmitted through syncytium formation or virus production to the other activated cells in the cluster. Indeed, syncytia can be observed in most of the clusters that develop when lymphocytes respond to antigen or superantigen on dendritic cells.

Our results also differ from reports sug-

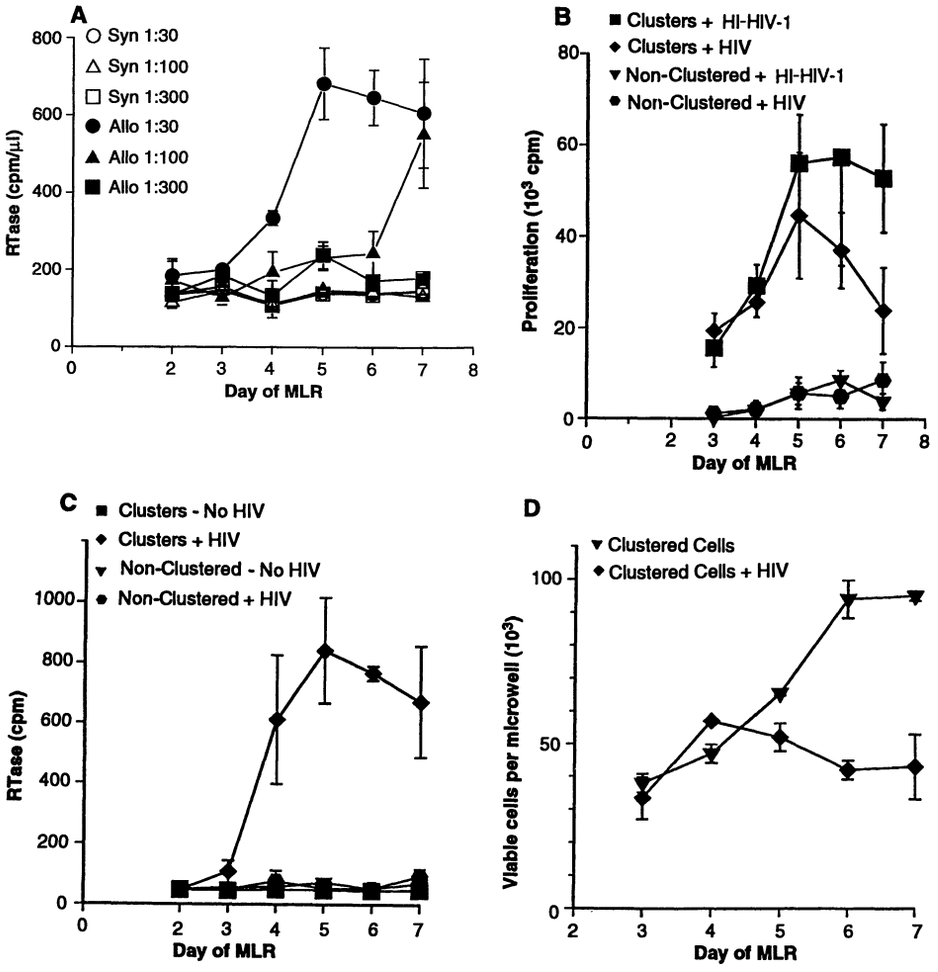

Fig. 3. Transmission of HIV-1 from virus-pulsed dendritic cells to $CD4^+$ T cells responding in the MLR. (**A**) Time course of RT activity in MLR cultures in which graded doses of dendritic cells were added to a constant dose of 10^5 $CD4^+$ allogeneic (Allo, closed symbols) or syngeneic (Syn, open symbols) lymphocytes. The data are mean counts per minute per microliter of culture medium (triplicates). No RT was detected in cultures where the dendritic cells were pulsed with HI-HIV-1. (**B**) Dendritic cell–$CD4^+$ T lymphocyte clusters are the focus of the immune response in the MLR. Clusters of interacting cells were separated from 24-hour MLR cultures that had been set up in 24-well plates with 5×10^4 dendritic cells and 1.5×10^6 $CD4^+$ T cells in 1 ml of medium. Clusters were isolated by applying six to ten cultures to 1-g columns made of 5 ml of RPMI-1640 supplemented with 40% fetal calf serum in 15-ml conical tubes. The clusters were allowed to settle to the bottom of the tube for 1 to 1.5 hours on ice and the nonclustered cells were removed from the top. The fractions were cultured separately in microtiter wells, clusters at 3×10^4 per well and nonclusters at 1.5×10^5 per well. We measured DNA synthesis by the uptake of ^{3}H-thymidine (added for 8 hours at 1 μCi per well; 6 Ci/mM where 1 Ci = 37 GBq). (**C**) RT (assays on 1 μl of culture supernatant) is generated from clustered T cells in the MLR. (**D**) Loss of cell viability (trypan blue–positive and –negative cells) in the clustered fraction in the MLR. Nonclustered cells from infected and control cultures showed no significant change in cell numbers.

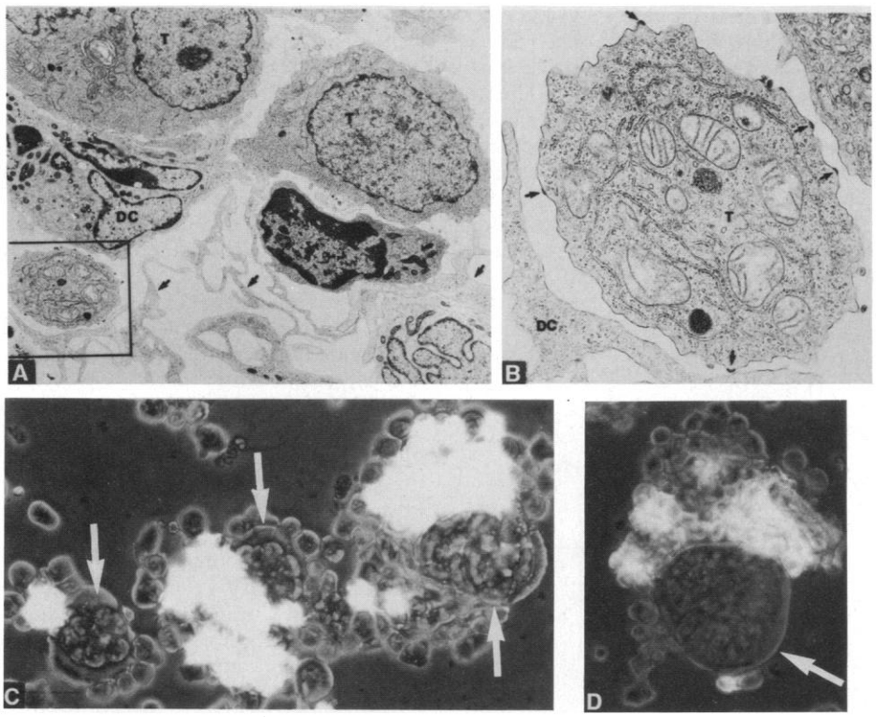

Fig. 4. Morphologic assays demonstrate that dendritic cells are not productively infected with HIV-1 during their interaction with responding CD4+ T cells. (**A**) Low-power electron microscopic view showing T blasts (T) and many dendritic cell processes (DC, arrows) in cell clusters isolated at day 3 of a primary MLR, ×9750. (**B**) Higher magnification of the boxed area in (A) to demonstrate viral budding (arrows) from lymphocytes but not from dendritic cells whose processes are abundant, ×32,500. (**C** and **D**) Dendritic cells labeled with a fluorescent dye do not fuse with HIV-1–induced syncytia. The dendritic cells were labeled with the fluorescent PKH2 dye [(C); Zynaxis (Malvern, Pennsylvania) 5 μg/ml for 3 to 4 min in Hanks basic salt solution (HBSS) at room temperature] or the dill carbocyanine dye [(D); Molecular Probes, Eugene, Oregon; 20 μg/ml of HBSS for 30 min at 37°C]. The labeling was stopped by adding an equal volume of fetal calf serum and washing twice with complete medium. The MLR between dye-labeled dendritic cells and unlabeled allogeneic CD4+ T cells was observed daily in a Nikon inverted stage microscope equipped with epifluorescence. For the higher power observations shown here, the cells were spun at 900 rpm for 2 min onto glass slides in a Shandon cytocentrifuge and photographed immediately without fixation. The fluorescence micrograph is superimposed on the phase contrast micrograph of a dendritic–T cell cluster. Many brightly fluorescent (white) dendritic cells are juxtaposed with large syncytia (white arrows), but there is no fusion or transfer of dye from the APCs to the T cells.

gesting that blood dendritic cells are readily infected by HTLV-III$_B$ (*17–19*). The lack of infection may be attributed to the paucity of CD4 molecules on dendritic cells, so that other populations with higher concentrations of CD4 such as the epidermal Langerhans cells are in need of study. Nonetheless, our findings emphasize another feature of dendritic cell function: to act as a catalyst in the transmission of a cytopathic infection. The efficiency of virus transmission from virus-pulsed dendritic cells to T cells suggests a synthesis between our report and earlier publications. We enriched human dendritic cells by cell sorting and monitored the extent of T cell depletion by cytofluorography, whereas the populations that were used in other reports may have contained contaminating T cells that were further stimulated with exogenous lymphokines (*18*). Also the PCR and dye-labeling approaches that we used (Fig. 4) provide direct and quantitative means for showing that efficient reverse transcription of HIV-1 and virus budding did not occur in either resting or stimulated human blood dendritic cells. Transmission through dendritic cells is more efficient than through other APCs (Fig. 1B). This may be attributable to the potency of dendritic cells in activating large numbers of CD4+ T cells (*11, 32*).

In situ, mucosal and airway dendritic cells may carry and present virus to responding T cells at the same time as the T cells are activated by other environmental antigens carried by the dendritic cells. An analogous mechanism may operate in the germinal centers of lymphoid organs. Here B cells may be presenting antigens to CD4+ T cells in the presence of a reservoir of HIV-1–bearing follicular dendritic cells (*9, 10, 33*). The latter differ from the bone marrow–derived dendritic cells that we have studied, but in both instances extensive losses of antigen-specific, previously uninfected T cells result from the transmission of HIV-1 that is carried by APCs.

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16. We isolated mononuclear cells from buffy coats of HIV-1 seronegative donors (New York Blood Center) by flotation over Ficoll-Hypaque gradients. We separated the cells into T cell–enriched (erythrocyte rosette–positive, Er+) and depleted (Er−) fractions by rosetting with neuraminidase-treated sheep erythrocytes at 4°C. These populations were incubated 1 to 2 days in complete medium containing 10% AB+ normal human serum in RPMI 1640 with glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (10 mM), and 2-mercaptoethanol (0.05 mM). We negatively selected CD4+ lymphocytes by coating Er+ cells with anti–HLA-DR (clone 9.3C9, HB180, ATCC) and anti-CD8 (OKT8, ATCC) hybridoma supernatants and then panning them on bacteriologic petri dishes coated with goat anti-mouse immunoglobulin G (IgG) (Cappel, West Chester, PA) (10 μg/ml). Dendritic cells were prepared as described (*12*). Er− cells were depleted of monocytes by sequential plastic adherence and panning on human IgG–coated plates (Cappel). Partially enriched dendritic cells were prepared by gradient centrifugation in 14.5% by weight metrizamide columns (900*g*, 10 min). This population typically contained 20 to 40% large, irregularly shaped cells with the phenotype of dendritic cells on cytofluorographic analysis (*12*). Dendritic cells were then further purified by cell sorting with a cocktail of fluorochrome-labeled monoclonals to contaminating B, NK, monocytes, and T cells. Monocytes were plastic adherent cells that had been incubated for 3 to 4 hours at 37°C in complete medium and allowed to detach. B and NK cells were plastic nonadherent, Er− cells that were recovered from the pellet of the metrizamide column used to enrich dendritic cells (above). Murine dendritic cells and CD4+ T cells were prepared from mouse spleen as described (*14*).
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20. Quantitative PCR for gag sequences (nucleotides 1543 to 1657) used the primers SK38 and SK39 (*21*); for LTR sequences, primers SK29 and SK30;

and for controls, HLA-DQ primers GH26 and GH27 (*22*). A modified quantitative PCR (*23*) protocol was used. One of each of the HIV primer pairs was end-labeled with $^{32}P\gamma ATP$; 10^6 cpm (5 to 10 pM) of labeled and 10 to 20 pM of the unlabeled complementary primer were included in each reaction. DNA for PCR was prepared (*5*) by washing the cells in phosphate-buffered saline (PBS) and suspending in hypotonic lysis buffer with proteinase K (600 μg/ml at 56°C for 1 hour followed by 95°C for 10 min). Samples were stored at −20°C until amplified in a Perkin-Elmer thermal cycler (5 min at 94°C, 30 s at 94°C, 30 s at 60°C, 1 min at 72°C, 5 min at 72°C extension after 28 cycles). As a control, 25 pM of each of the unlabeled DQ primers were included in the gag reactions and the reaction product resolved and visualized on a 2% agarose stained with ethidium bromide.

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24. HIV-1 was grown in blood mononuclear cells, except for $HTLV\text{-}III_B$, which was also grown in the CEM T cell line. Patient isolates were obtained by coculturing patient cells with phytohemagglutinin (PHA)-induced blasts and passaged twice in PHA blasts. Virus was harvested at the peak of RT activity, passed through a 0.2 μM filter, and stored at −70°C. HIV with T and macrophage tropism was titered on primary T blasts, whereas $HTLV\text{-}III_B$ was titered on CEM. Viral supernatants used in PCR experiments were first incubated for 0.5 hours with deoxyribonuclease I (30 to 50 U/ml) (Boehringer Mannheim, Indianapolis, IN) and filtered. We prepared inactivated HIV-1 by heating virus supernatant to 56°C for 30 min. To infect target cells, virus supernatant at multiplicities of 0.05 to 0.5 was added for 1.5 hours at 37°C. Cells were washed three times in medium and resuspended.
25. RT was assayed by a microtiter method as modified for HIV-1 (*26*). Supernatant (10 μl) was harvested into microtitre plates and stored at −70°C. The reaction mix contained 50 mM tris (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 0.05% Nonidet NP-40, 0.1 Å units (about 6 μg/ml) $pA_n \cdot dT_{12-18}$ template primer (Pharmacia, Piscataway, NJ) and 20 μCi/ml ^{32}P dTTP (Amersham, Arlington Heights, IL). Reaction mix (50 μl) was added to each well and the plate incubated at 37°C for 1.5 hours. The 6 μl of solution was spotted onto DEAE-cellulose paper, air-dried, washed five times in 2× standard saline citrate (SSC) twice in 95% ethanol, dried, and counted with scintillant.
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34. P.U.C. is a Commonwealth AIDS Research Grants postdoctoral fellow, and P.S.F. is a Rockefeller University graduate fellow. Support was provided by the NIH (grant AI 24775), a Clinical Research Center grant (MOI-RR00102), the Aaron Diamond Foundation, the Irvington Institute for Medical Research, the Kaskel fund, and a Grant-in-Aid from the Ministry of Education, Science, and Culture of Japan, program 03044086.

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Negative Inotropic Effects of Cytokines on the Heart Mediated by Nitric Oxide

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The direct effects of pro-inflammatory cytokines on the contractility of mammalian heart were studied. Tumor necrosis factor α, interleukin-6, and interleukin-2 inhibited contractility of isolated hamster papillary muscles in a concentration-dependent, reversible manner. The nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) blocked these negative inotropic effects. L-Arginine reversed the inhibition by L-NMMA. Removal of the endocardial endothelium did not alter these responses. These findings demonstrate that the direct negative inotropic effect of cytokines is mediated through a myocardial nitric oxide synthase. The regulation of pro-inflammatory cytokines and myocardial nitric oxide synthase may provide new therapeutic strategies for the treatment of cardiac disease.

Pro-inflammatory cytokines are a class of secretory polypeptides that are synthesized and released locally by macrophages, leukocytes, and endothelial cells in response to injury (*1–4*). Reperfusion of ischemic myocardium is associated with the infiltration by leukocytes and macrophages that may be responsible for a transient depression of myocardial contractility ("stunned myocardium") (*5–9*). This report attempts to determine if pro-inflammatory cytokines produce direct reversible inotropic effects in isolated papillary muscle preparations.

The addition of recombinant human tumor necrosis factor α (TNF-α) and interleukin-6 and interleukin-2 (IL-6 and IL-2) to the medium bathing isolated papillary muscles resulted in a concentration-dependent, reversible negative inotropic effect (Fig. 1). The negative inotropic effects were observed within 2 to 3 min, were maximal after 5 min, remained constant for at least 20 min, and were completely reversed within 40 min after the cytokines were removed from the bath. Recombinant human IL-1α had little inotropic effect.

Concentration response curves were generated for each of the cytokines (Fig. 2). TNF-α demonstrated a negative inotropic effect in a concentration range from 150 to 3200 U/ml. The majority of the effect was observed at concentrations below 900 U/ml (59 ± 7% and 44 ± 3% of baseline tension

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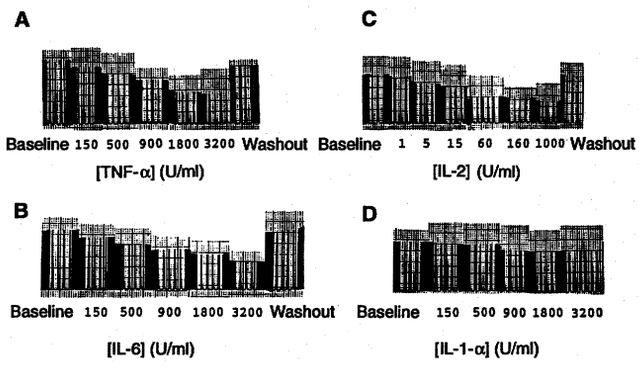

Fig. 1. Photographs of representative chart recordings illustrating the effects of adding increasing concentrations of cytokines on tension generated at 1 Hz by isolated papillary muscles prepared from 4-month-old F_1B control Syrian hamsters (Biobreeders Inc., Fitchburg, Massachusetts) (*19*). Vigorously beating hearts were removed and placed in oxygenated Tyrode's solution containing 130 mM NaCl, 4.7 mM KCl, 20 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 5.6 mM glucose oxygenated with 95% O_2 and 5% CO_2 (pH 7.4 at 25°C). Left ventricular papillary muscles were excised and attached at one end to a pressure transducer in a bath (5 ml) containing the same Tyrode's solution with bovine serum albumin (1%) oxygenated with 95% O_2 and 5% CO_2 warmed to 37°C (pH 7.4). Papillary muscles were stimulated at twice the threshold voltage with bipolar platinum electrodes and the tension generated was recorded with a Gould model 3400 recorder with built-in dc bridge preamplifier and transducer. We administered recombinant human cytokines (Genzyme, Cambridge, Masschusetts) by pipetting portions (50 μl) directly into the tissue bath, and then the medium was replaced with continuous perfusions of Tyrode's solution alone at 7.5 ml/min for 30 min; 150 to 3200 U/ml of TNF-α (**A**), 150 to 3200 U/ml of IL-6 (**B**), 1 to 1000 U/ml of IL-2 (**C**), 150 to 3200 U/ml of IL-1-α (**D**).