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Research Article

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Priming of Human CD4$^+$ Antigen-specific T Cells to Undergo Apoptosis by HIV-infected Monocytes

A Two-step Mechanism Involving the gp120 Molecule

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Abstract

The study of the pathology of HIV-1 infection in chimpanzees supports the idea of the crucial role of HIV-infected monocytes in the pathogenesis of AIDS, although viral mechanisms that lead to T cell dysfunction and deletion during HIV infection are still unclear. We show here that HIV-1–infected antigen-presenting monocytes (APCs) are able to prime in vitro non–HIV-infected antigen-specific CD4$^+$ T cell lines or peripheral blood CD4$^+$ T cells to undergo apoptosis after antigen-specific restimulation. The priming of T cells for apoptosis occurs in the absence of HIV replication in the T cells. Priming for apoptosis required two concomitant signals present on the same APC, an antigenic stimulus and a second signal provided by the HIV gp120 protein as demonstrated by the use as APCs of EBV-LCLs infected with different recombinant deleted proviruses or transfected with different HIV proteins. These results provide a mechanism for the priming for apoptosis of T cells in HIV-infected patients, implicating a role for HIV-infected APCs in the induction of T cell dysfunction and depletion in AIDS. (*J. Clin. Invest.* 1997. 99:257–266.) Key words: human immunodeficiency virus • monocytes • apoptosis • gp120 • T cells

Introduction

Human infection with HIV is characterized by a progressive decline in the number of CD4$^+$ T cells leading to AIDS (1). However, immune dysfunction of CD4$^+$ T cells is seen early after infection (2–4), and before any significant reduction in the absolute number of CD4$^+$ T cells (5, 6). We (7) and others (8, 9) have shown that a high proportion of CD4$^+$ T cells from HIV-1–infected individuals are abnormally primed in vivo to undergo cell death by apoptosis upon mobilization of their T cell receptor for antigen (TCR)[1], a mechanism that may involve either HIV envelope interactions with the CD4 molecule (10, 11) or a defect in the accessory cell function of HIV-infected antigen presenting cells (APCs) (12).

These observations prompted us to speculate that the mechanism that renders T cells from HIV-infected patients susceptible to activation-induced cell death (AICD) in vivo probably involves two steps. First, a priming step in a region with a high burden of infection where many CD4$^+$ T cells circulate, leading to interactions between uninfected T cells and infected APCs, following by an activation step, which occurs later when T cells recirculate in the periphery, where they actually are induced to undergo apoptosis. However, in the different models that tried to mimic this mechanism in vitro, the priming and activation of T cells were always carried out concomitantly and have always involved high doses of recombinant HIV proteins (10, 11, 13). In addition, stimulation was often performed in the absence of APCs (10, 11, 14–16), a condition that even induced apoptosis in normal activated T cells (17). Finally, in some studies only spontaneous cell death was analyzed (13) even though it has been shown that spontaneous cell death affecting mostly CD8$^+$ T cells is not correlated with AIDS (18, 19).

It has been reported that lymphoid organs represent the major reservoir of HIV in asymptomatic infected people (20, 21). Follicular dendritic cells (22), monocytes, and macrophages are target cells for HIV infection and reservoirs for infectious virus. Both infected and uninfected CD4$^+$ T cells migrate to the lymph nodes during generation of an immune response. In this setting, activated uninfected CD4$^+$ T cells are in close proximity to infected antigen-presenting cells. Thus, it is likely that these infected accessory cells may play an important role in the pathogenesis of HIV–1–induced immunosuppression (23). The hypothesis that HIV-infected monocytes may be crucial for the development of AIDS is also supported by studies on the pathology of HIV-1 in chimpanzees. The absence of clinical symptoms despite many similarities in the course of HIV-1 infection compared with humans may be explained by the complete insusceptibility of chimpanzee monocytes to HIV-1 infection (24).

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1. *Abbreviations used in this paper:* AICD, activation-induced cell death; APC, antigen presenting cell; AZT, azidothymidine; LTR, long terminal repeat; PPD, purified protein derivative; PWM, pokeweed mitogen; RT, reverse transcription; SEB, *Staphylococcal* Enterotoxin B; $TCID_{50}$, titrated as culture infective dose; TCR, T cell receptor; TT, Tetanus toxoid.

We decided to analyze the role of HIV-infected monocytes in a physiological model using infected cells, rather than purified recombinant proteins. We have previously reported that in vitro incubation of antigen-specific T cell lines with antigen-presenting monocytes pulsed with a lymphotropic isolate (III_B-LAI) of HIV does not impair antigen-mediated T cell proliferation and expansion after IL-2 addition, but results in T cell unresponsiveness to further antigen restimulation (25, 26). We show here that in fact, HIV-infected monocytes prime antigen-specific $CD4^+$ T cells in vitro for death by apoptosis upon subsequent restimulation, a mechanism that closely resembles the proliferative defect and apoptosis induction observed in peripheral blood T cells from HIV-infected individuals. Finally, we show that the gp120 molecule of HIV-1 plays a critical role in this mechanism.

Methods

Monoclonal antibodies and chemicals. Culture supernatant fluids were assayed for HIV-1 p24 core antigen with a commercial enzyme immunoassay (Abbott Laboratories Diagnostic Division, Chicago, IL).

Recombinant cytokine IL-2 was purchased from Boehringer Mannheim (Meylan, France). Cyclosporin A (CsA) was purchased from Sandoz Ltd. (Paris, France); cycloheximide, geneticin, and hygromycin B from Sigma Chemical Co. (L'Isle d'Abeau Chesnes, France), and azidothymidine (AZT) from Burroughs Wellcome (Paris, France).

Purified protein derivative (PPD) was purchased from Pasteur Diagnostic (Paris, France) and Tetanus toxoid (TT) from Rhone-Merieux (Lyon, France).

Cell preparation. Antigen-specific $CD4^+$ T cell lines rather than T cell clones were used to represent in vitro the polyclonal situation in vivo (26). PBL were purified from normal donors on Ficoll-Hypaque. The cells were resuspended at 2×10^6 cells/ml in RPMI 1640 containing 10 mmol/liter glutamine, 10 mmol/liter sodium pyruvate, and 10% fetal calf serum. The cell suspension was dispensed in 24-well cluster plates (Costar Corp., Cambridge, MA) at 1.5 ml per well. PPD and TT were added at 25 and 10 μg/ml final concentration, respectively. After 5 d, the cultures were split at 4×10^5 cells per well and recombinant IL-2 was added at 30 U/ml final concentration. 6 d later, 2×10^5 blast cells were stimulated with antigen plus irradiated autologous PBL. After 3 d of stimulation with antigen, the cells were expanded with rIL-2 for 4–7 d. Weekly cycles of antigen stimulation and IL-2 expansion were repeated to maintain the lines. The lines used in these experiments had been maintained in culture for over a year.

Monocytes were purified from PBMC by adherence to plastic dishes and further depleting adherent cells with two cycles of magnetic beads coated with CD2 and CD19 mAb to remove residual T and B cells.

$CD4^+$ T cells were purified by negative purification. Negative purification was performed using a cocktail of antibodies directed against non–$CD4^+$ T cells: CD8, CD14, CD16, CD19, CD20, CD56, and HLA-DR. Cells were incubated with saturating amounts of antibodies for 20 min at 4°C. After washing, Dynabeads (Dynal, Norway) were added at a ratio of 10 beads/target cell and incubated for 2 h at 4°C. Beads and contaminating cells were removed by magnetic field. The remaining cells were resuspended with the same amount of beads and a second incubation period for 1 h at 4°C was performed. After removing contaminating cells, $CD4^+$ T cells were analyzed by FACScan® (Becton Dickinson & Co. Mountain View, CA) and revealed to be > 90–95% positive.

Pulsing with HIV and/or antigen. HIV-1 was obtained from the HIV-1–producing megacaryocyte cell line DAMI (27). It was used because, after several passages on the DAMI cell line, that substrain of HIV-LAI was able to efficiently infect both T cells and monocytes in the absence of any cytopathic changes in monocytes. Virus-containing supernatants were harvested, purified by ultracentrifugation, titrated as culture infective dose ($TCID_{50}$) on MT4 indicator cells in a syncytial assay and kept at −70°C.

Monocytes were dispensed at 10^6 cells/ml into polypropylene conical tubes and HIV was added at 100 $TCID_{50}$; antigens TT and PPD were added at, respectively, 50 and 20 μg/ml. After 4 h at 37°C, cells were washed three times in culture medium to remove free virus and excess antigen, and used in proliferative assays. Preliminary experiments performed by stimulating T cells with monocytes pulsed first with HIV, and then with antigen or vice versa showed no difference in the T cell response. A sample of HIV-1–infected monocytes was tested for syncytium-forming capacity on MT4 cells to monitor for residual infectious virus or virus produced by monocytes.

Cell proliferation assay. In proliferative assays, 5×10^4 monocytes were added to an equal number of T cells, and [^{3}H]TdR was added in the last 12 h of day 2. In priming experiments, 5×10^4 monocytes and T cells for each relevant antigen specificity were cultured in 96-well plates. After 2 d, the cultures were expanded in 24-well cluster plates in the presence of rIL-2 (10 U/ml). 10 d later, cells were collected and purified with a Ficoll gradient to remove residual dead cells and, in some experiments, by depleting contaminating monocytes with anti–CD14 mAb and magnetic beads. T cell proliferative response to soluble antigen presented by uninfected autologous monocytes or to various mitogens was then tested.

Evaluation of cell death. Cells were incubated as for proliferative assays. Nonadherent cells were harvested by gentle pipetting and diluted 1:2 with 0.1% trypan blue in PBS. Live and dead cells were counted in a hemocytometer. Blue cells as well as cells with condensed nuclei were considered as dead cells. The evaluation of chromatin condensation was performed as described (28).

DNA fragmentation assay. DNA fragmentation was determined according to the method described previously (7). Briefly, 10^6 cells were collected by centrifugation at 200 *g* for 10 min and lysed in 500 μl hypotonic lysing buffer (5 mM Tris, pH 7.4, 5 mM EDTA, 0.5% Triton X 100). Lysates were treated with proteinase K (5 μg/ml) for 2 hr at 50°C, and centrifuged at 13,000 *g* for 15 min. Supernatants were deproteinized by extraction once in phenol/chloroform and twice in chloroform/isoamyl alcohol (24:1), and precipitated at −20°C in 50% isopropanol/130 mM NaCl. After electrophoresis on 2% agarose slab gels, DNA was stained by ethidium bromide.

Reverse transcription and PCR analysis. Total RNA was purified using RNAzol according to the manufacturer's instructions (Bioprobe, Montreil sous Bois, France). Before reverse transcription (RT), RNA samples were treated with DNAse I (GIBCO BRL, Gaithersburg, MD) to remove any cellular or viral DNA contaminant. 2 μg of RNA were treated with 2 U of DNAse I RNAse free for 15 min at room temperature. The DNAse was then inactivated by the addition of 1 μl of 25 mM EDTA, and samples were heated for 10 min at 65°C. Reverse transcription was performed as described previously (29). Briefly, 2 μg of total RNA in 9 μl were mixed with 4 μl buffer (5× RT buffer: 250 mM Tris-HCl, pH 8.3, 300 mM KCl, 15 mM $MgCl_2$), 1 μl primer, 0.5 μg oligo dT; 4 μl dNTP, 10 mM; 0.1 μl dithiothreitol, 1 M; 0.1 μl RNAsin, 40 U/μl; 0.1 μl bovine serum albumine, 1 μg; 1 μl *Escherichia coli* rRNA, 4 μg; 0.8 μl Moloney murine leukemia virus reverse transcriptase (80 U). This mixture was centrifuged and incubated at 42°C for 1 h. Samples were heat denatured at 95°C for 3 min and, after rapid cooling on ice, 50 U in 2 μl of Moloney murine leukemia virus–RT was added and a second cycle of reverse transcription performed.

For analysis of the presence of proviral DNA, PCR analysis was performed on purified genomic DNA (27).

For PCR amplification, 1 μl of the reverse transcriptase reaction or purified DNA was added to a total reaction volume of 25 μl of the PCR mix (1× PCR buffer, 200 μM of each dNTP, 0.5 μM of each primer, 2 mM $MgCl_2$, and 2.5 U/100 μl Taq polymerase). After an initial denaturation step at 94°C for 2 min, 35 cycles of PCR were performed (1 min at 55°C, 1 min at 72°C, 1 min at 94°C). A 20-μl aliquot was run on a 2% agarose gel containing ethidium bromide. We used

three different pairs of primers, all of which have been described: one specific for HIV-*env* (SK68, SK69) (30) amplifying a 134-bp fragment, one specific for HIV-*tat* (31) amplifying a 345-bp fragment, and one specific for the TCR Cα gene (29) amplifying a 129-bp fragment.

Quantitative PCR for HIV-infected cells. Quantitative PCR analysis was performed according to the method already described (32). For the preparation of cell lysates, cells were diluted in PBS at different concentrations from 10^7 to 10^3. 1 μl (containing 10,000 to 1 cells) was added to 9 μl of distilled water into a 96-well plate, and then 20 μl of PCR buffer was added (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 1 mg/ml gelatin, 0.05 mg/ml proteinase K, 20 mM dithiothreitol, and 0.7 μM sodium dodecyl sulfate). Cell number was determined by counting cells before lysis and complete lysis was observed under phase contrast microscopy. Lysates were then transferred to a 500-μl tube, vortexed for 30 s, incubated for 1 h at 37°C, incubated for 10 min at 86°C, and stored at −20°C. Because this technique analyzes total cell lysates, it detects all retrotranscribed HIV DNA, even the nonintegrated forms, giving an overestimation of the productive infection.

We used the two different pairs of primers specific for *env* and *tat* described above. These two sets of primers gave identical results and only results obtained with SK68/69 are shown. 20 μl of cell lysate containing the genomic DNA was introduced in a 500-μl tube and 80 μl of PCR buffer was added. After an initial denaturation step at 94°C for 10 min, 30 cycles of PCR were performed (1 min at 55°C, 1 min at 72°C, 1 min at 94°C). A 10-μl aliquot was removed from the tube and made up to 100 μl as above, and another 30 PCR cycles were performed.

End point dilution assays for evaluation of HIV infection. As previously described (33), decreasing numbers of T cells (10^6, 10^5, 10^4, 10^3, 800, 600, 400, 200, 100, 50, 10, 5, 2, 1) monocytes or diluted monocyte supernatant that represent the same number of cells were cocultured with 10^5 MT4 cells in 200 μl. Cocultures were subsequently monitored for the presence of syncytia twice weekly for up to 28 d, and p24 antigen levels measured at days 10 and 28. After the first week, MT4 cells were diluted 1:2 twice weekly.

Preparation of plasmids containing nef, tat, and env genes. The Nef expressing vector has already been described elsewhere (34). The *tat* gene, kindly provided by Dr. A. Meyerhans (31) was cloned using the HindIII, KpnI sites into the pREP10 (Invitrogen Corp., San Diego, CA). This expression vector, that drives the expression of the transgene with the RSV long terminal repeat (LTR) was chosen because it possesses a hygromycin resistance gene and the EBV origin of replication that allows high-copy episomal replication especially in EBV-LCLs. The *env* gene was cloned by PCR with the following primers 5′: GATCCTCGAGGAGCAGAAGACAGTGGCA, 3′: ATATGCGGCCGCTGGCTCAGCTCGTCTCAT, using the pNL4.3 plasmid as a template. The PCR product of 2,985 bp was subcloned into pBluescript SK^+ (Stratagene Inc., La Jolla, CA) and then excised by the XhoI, NotI sites encoded by the primers and cloned into the pREP10 vector. All plasmids were transfected into both J-Jahn or autologous EBV-LCLs. The *nef* and *tat* genes were stably transfected into both cell types selected with the appropriate selection drug. The control of the expression of the *nef* gene has been previously described elsewhere (34). The *tat* gene expression has been monitored into each cell type by transitory transfection with an LTR-luciferase construct as previously described (27) or by coculture experiments using DAMI cell lines that constitutively contained an LTR-luciferase construct (27). As we were unsuccessful obtaining EBV-LCLs that stably expressed the *env* gene, we used transient expression, 48 h after electroporation. gp120-expressing EBV-LCLs were positively selected by a monoclonal antibody (27) and magnetic beads. The expression was further controlled by FACS® analysis and purified cells revealed to be > 95% positive.

Preparation of deleted provirus. J-Jahn cells or autologous EBV-LCLs were stably cotransfected with pLN4.3 (32, 35) (10 μg) (referred to as HIV or HIV-LAI) and pSV2neo (2 μg) and selected with geneticin (G418). Quantitative PCR as well as p24 staining by immunofluorescence showed that after selection virtually all cells were infected. J-Jahn cells as well as autologous EBV-LCLs were also stably transfected with HIVΔ*nef* and HIVΔ*env*. HIVΔ*nef* was constructed

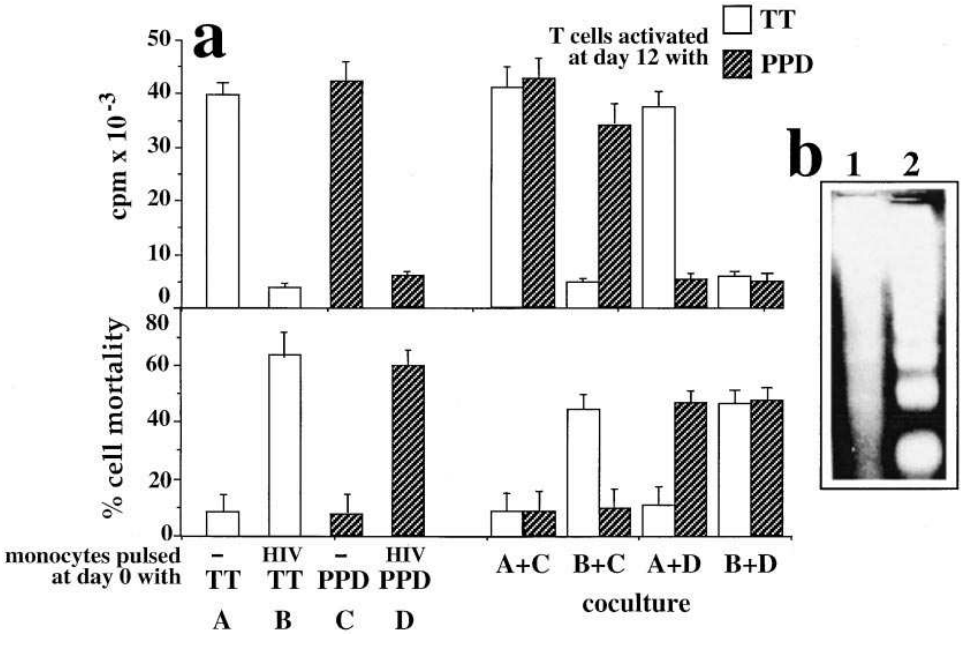

Figure 1. Priming of T cells for apoptosis by HIV-pulsed monocytes. (*a*) Human TT-specific (*A* and *B*) or PPD-specific (*C* and *D*) T cells were stimulated with monocytes pulsed with a combination of HIV and their respective antigens (*A* = TT alone, *B* = TT + HIV; *C* = PPD alone, *D* = PPD + HIV). In coculture experiments, the two cell lines cultured in the same well were stimulated by mixtures of monocytes pulsed in the different combinations as indicated. After expansion by addition of rIL-2 at day 2, T cells were purified and restimulated at day 12 using noninfected autologous monocytes in the presence of soluble antigens, TT, white bars and PPD, crosshatched bars, as indicated. In the upper panel, the proliferative response was measured by thymidine incorporation and the results are expressed in counts per minute and represent the mean of triplicate cultures±SD of one representative experiment out of six. In addition, cell death was determined on nonadherent cells by trypan blue exclusion at day 13, 24 h after stimulation. Results in the lower panel are expressed as percentage of dead cells and represent the mean±SD of five different experiments. (*b*) A TT-dependent cell line was primed with HIV-1–infected monocytes (lane *2*) or uninfected monocytes (lane *1*), expanded with rIL-2, and restimulated at day 12 with uninfected monocytes in the presence of TT antigen. After 18 h, 10^6 nonadherent cells were collected and their DNA was purified, loaded on an agarose electrophoresis gel, and stained by ethidium bromide. Lane *1* shows some necrosis in the control cells, whereas in lane *2* there is a clear pattern of cell death by apoptosis.

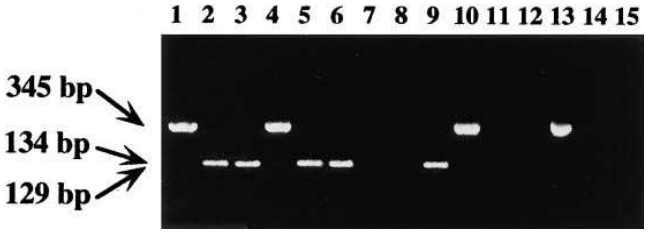

Figure 2. PCR detection and RT-PCR analysis of *tat* and *env* gene expression in HIV-infected monocytes. PCR fragments generated after PCR on DNA or RT-PCR on RNA isolated from monocytes infected with HIV-1. In all cases, DNA or RNA were collected from monocytes pulsed with HIV as indicated in Methods, except for lanes *7–9* where DNA was collected from monocytes pulsed with HIV in the presence of AZT (10^{-5} M). (Lanes *1*, *4*, and *7*) *tat*-specific primers using DNA as template. (Lanes *2*, *5*, and *8*) *env*-specific primers using DNA as template. (Lanes *3*, *6*, and *9*) Cα-specific primers using DNA as template. (Lanes *10* and *13*) RT-PCR with *tat*-specific primers. (Lanes *11* and *14*) RT-PCR with *env*-specific primers. (Lanes *12* and *15*) RT-PCR with Cα-specific primers to exclude any T cell contamination in monocytes preparation (T cells being more easily infected by HIV than monocytes).

by inserting a neo^r cassette into the ClaI site of pNL4.3. HIVΔ*env* is derived from HIVΔ*nef* and was constructed by deleting a 1,200-bp fragment of the *env* gene. Cells infected with both viruses were also selected with geneticin.

HIVΔ*tat* was kindly provided by Dr. A. Meyerhans (31). It is derived from pNL4.3; however, it contains a stop codon in the first exon of the *tat* gene. Since this provirus cannot be expressed after transfection in J-Jahn cells, it was cotransfected with pSV2neo into J-Jahn cells stably transfected with the *tat* expression vector described above. All stably transfected J-Jahn cells were cultured in selective medium for > 2 mo, washed three times, cultured 1 wk in nonselective medium, washed three times, cultured for 2 additional d, and supernatants were collected for viral stocks. Supernatants were ultracentrifuged at 10,000 *g* for 10 min, the pellets washed with PBS, recentrifuged, and resuspended in 10 ml of fresh medium. The $TCID_{50}$ of HIV and HIVΔ*nef* were monitored on Molt4 cells. Since HIVΔ*env* and purified HIVΔ*tat* were not able to induce syncytia, the p24 core antigen level was assayed and the $TCID_{50}$ of each preparation was estimated according to the $TCID_{50}$ and the p24 antigen assay in HIV and HIVΔ *nef.* The inability of HIVΔ*tat* to induce syncytia was not due to an absence of Env protein on the viral particle since addition of exogenous Tat to HIVΔ*tat* restored its ability to induce syncytia formation.

Results

HIV-infected monocytes did not impair antigen-specific T cell proliferation, but specifically primed T cells to unresponsiveness. Antigen-pulsed, HIV-pulsed monocytes stimulate proliferation of antigen-specific $CD4^+$ T cell lines as efficiently as noninfected monocytes (25, 26) and no cell death is detected after activation of T cells with HIV-infected monocytes (not shown). However, 12 d later, after expansion of the T cell lines with IL-2, when the proliferative response of T cell lines to the same antigen presented by uninfected monocytes was tested again, no proliferation was detected in T cell lines primed with HIV-pulsed monocytes (Fig. 1 *a*). The failure to respond to antigen-specific stimulation was not due to expansion of these cells in IL-2 because nonresponsiveness was also detected in T cells that had been kept in the absence of IL-2 for 8 d. This nonresponsiveness induced by Ag/HIV-pulsed monocytes was even maintained after a second round of expansion with IL-2, 20 d after the priming (not shown). In contrast, the antigen-specific proliferative response of T cell lines that have been primed with non–HIV-infected monocytes pulsed with antigen was normal (Fig. 1 *a*).

To monitor whether monocytes pulsed with HIV were infected, we performed PCR analysis and coculture experiments. PCR analysis showed that monocytes incubated in the presence of 100 $TCID_{50}$ of HIV-1 contained retrotranscribed HIV-1 DNA (Fig. 2) and expressed the *tat* gene as detected by RT-PCR analysis at 18 h (Fig. 2); *env* transcripts were not detected after 18 h but after 3 d (not shown). Moreover, coculture analysis using MT4 cells as indicator reveals that monocytes are productively infected and that the virus can also be detected in the supernatants of HIV-infected monocytes (Table I).

Table I. P24 Antigen Measurement of End-point Dilution Coculture of HIV-infected Monocytes or Primed T Cells with MT4 Cells

Number of cells	10^6	10^5	10^4	10^3	800	600	400	200	100	50	5	1
Monocytes												
Monocytes (day 5)	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	820	51	65	15
Monocytes supernatants	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	781	314	39	47	49	91
Monocytes + AZT (day 5)	62	67	41	37	68	70	67	48	24	59	36	48
T cells												
T cells (day 12)	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	23	31	29	24	29	27	72
T cells + AZT (day 12)	200	42	31	22	31	27	39	17	91	17	64	31
T cells (day 13)	> 1,000	> 1,000	> 1,000	> 1,000	27	32	48	75	54	26	38	57
Infected J-Jahn	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	> 1,000	850

Monocytes were infected with HIV in the presence or absence of AZT (10^{-5} M) as explained above and after 5 d, cells were collected and centrifuged, and the pellet of cells was washed twice. The $CD4^+$ TT-specific T cell line was primed with HIV-infected monocytes in the presence or absence of AZT (10^{-5} M) as indicated and was collected and purified to remove any contaminant-infected monocytes at either day 12 after priming with infected APC or at day 13 after reactivation. Decreasing number (as indicated) of HIV-infected monocytes, or the quantity of supernatant corresponding to the same number of cells or primed T cells or HIVΔ*nef*-infected J-Jahn (used as a positive control) were cocultured with 10^5 MT4 cells as reporter cells. Culture supernatant fluids were collected after 18 d of culture and were assayed for HIV-1 p24 core antigen with a commerical enzyme immunoassay. Results are expressed in picograms per milliliter. Cultures were also monitored for syncytia formation that gave similar results; i.e., p24 antigen was detected in samples after syncytia formation.

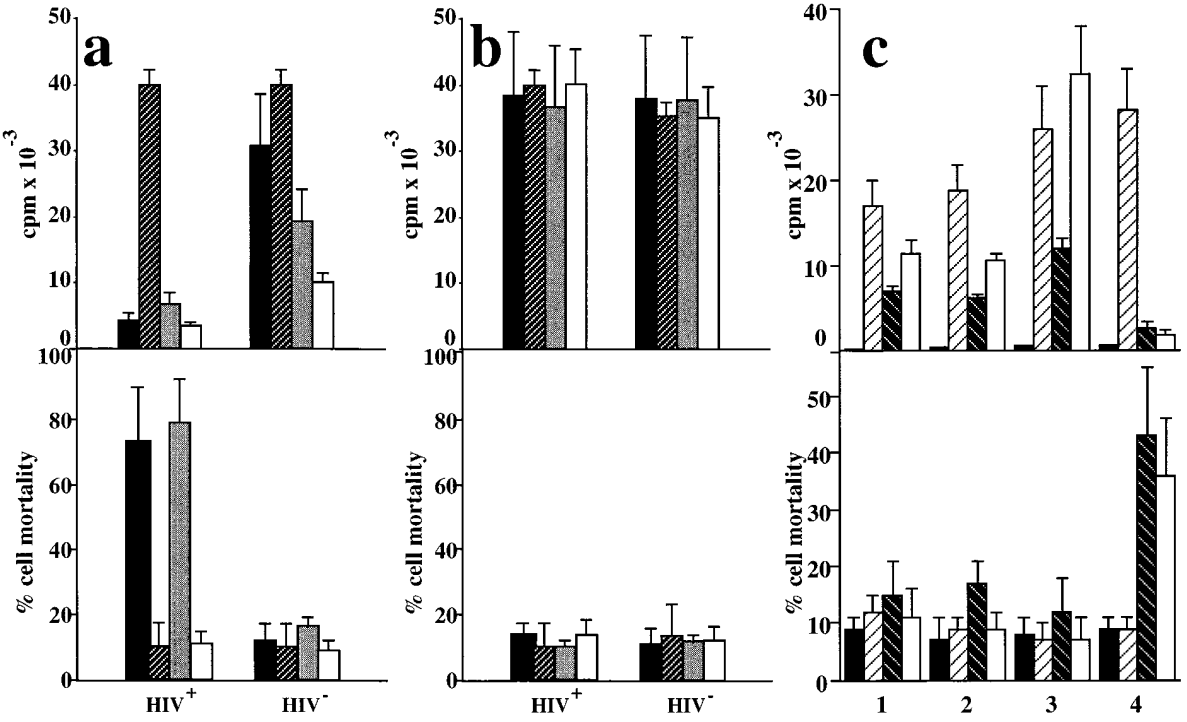

Figure 3. Peripheral blood T cells are primed for apoptosis by HIV-infected monocytes, the effect of restimulation with different mitogens. (*a*) A TT-dependent T cell line was primed with HIV-1–infected or noninfected monocytes (as indicated) and stimulated 12 d later with noninfected monocytes in the presence of TT antigen (*black bars*), PHA (10 μg/ml, *black crosshatched bars*), PWM (10 μg/ml, *dotted bars*), or with TT antigen in the presence of cyclosporin A (200 ng/ml, *white bars*). Cell proliferation (*top*) and cell death (*bottom*) were measured as described above. Results represent one experiment out of three. (*b*) A TT-dependent cell line was incubated with either noninfected or HIV-infected monocytes (as indicated) in the absence of antigen. After different times of incubation (3 d, *black bars*; 6 d, *black crosshatched bars*; 9 d, *dotted bars*; and 12 d, *white bars*) cells were collected, purified, and restimulated with TT presented by noninfected monocytes. Cell proliferation (*top*) and cell death (*bottom*) were monitored as described above. Results represent one experiment out of three. (*c*) Purified $CD4^+$ T cells isolated from peripheral blood lymphocytes were cultured for 12 d with noninfected monocytes (*1*), HIV-infected monocytes (*2*), noninfected monocytes + SEB (10 ng/ml, *3*), or HIV-infected monocytes + SEB (*4*). After 12 d, T cells were repurified and restimulated with medium alone (*black bars*), PHA (10 μg/ml, *white crosshatched bars*), PWM (10 μg/ml, *black crosshatched bars*), or SEB (10 ng/ml, *white bars*). Cell death (*bottom*) was measured at day 13 by trypan blue exclusion, and cell proliferation at day 15 by thymidine incorporation. Results represent one representative experiment out of three.

HIV-infected monocytes prime antigen-specific T cells for apoptosis. The failure of T cells that have been primed by HIV-infected APCs to proliferate in response to a second antigenic stimulation is due to the induction of cell death by apoptosis in up to 60 to 70% of the T cells (Fig. 1 *a*). Apoptosis was demonstrated by visualization of nucleosome-sized DNA multimers of 200 bp that form the characteristic "step-ladder" appearance after size separation on agarose gels (Fig. 1 *b*, lane *2*) and by examination for chromatin condensation after nuclear staining with Hoechst dye number 33342 (not shown). Cell death by apoptosis was an active process, which could be inhibited by cycloheximide (not shown) and cyclosporin A (Fig. 3 *a*), and which had similar features to AICD induced in T cells from individuals infected with HIV (7): it could be induced by antigen, and also by pokeweed mitogen; whereas activation with phytohemmaglutinin did not induce cell death, but led to T cell proliferation (Fig. 3 *a*).

The priming mechanism requires antigen-mediated interaction between HIV-pulsed monocytes and T cells. To determine whether priming of T cells for apoptosis induced by HIV-pulsed APCs required a direct interaction between monocytes, antigen, and antigen-specific T cells, we performed coculture experiments. T cells could only be primed for subsequent AICD by stimulation with APCs that had been pulsed with HIV and antigen (Fig. 1 *a*). T cell lines stimulated by antigen-pulsed uninfected APCs, and by HIV-infected monocytes pulsed with a different antigen, were not primed for AICD, since these cells proliferated normally after restimulation (Fig. 1 *a*). These results show that monocytes must provide two concommitant signals to prime T cells for AICD, the specific antigen and an HIV-specific signal. Furthermore, they show that these two signals must be present on the same cell. This notion was confirmed by control experiments showing that T cell lines that were incubated with HIV-infected monocytes in the absence of antigen for different time periods proliferated normally after restimulation with antigen and noninfected APCs (Fig. 3 *b*).

Peripheral blood T cells are primed for apoptosis by HIV-infected monocytes. To analyze whether priming for apoptosis by HIV-infected monocytes was a general mechanism, purified peripheral blood $CD4^+$ T cells from normal controls were incubated with HIV-infected or noninfected autologous monocytes in the presence or absence of the superantigen *Staphylococcal* Enterotoxin B (SEB, 10 ng/ml). IL-2 was added in the

Table II. Infection and Transcription of HIV in Monocytes are Necessary for the Apoptosis-priming Mechanism

Assays Days	AZT 0	p24 2	Proliferation 2	p24 13	Cell death 13	Proliferation 14
APC infected with						
HIV	−	458.99	31,455±3,657	134.17	63	5,697±961
HIV	+	6.67	29,117±2,987	8.75	58	4,110±867
HIVΔ*nef*	−	359.71	34,125±2,589	110.91	60	4,021±1,235
HIVΔ*env*	−	22.50	32,354±3,512	17.27	13	36,982±2,036
HIVΔ*tat*	−	15.45	33,568±2,548	12.73	16	33,546±3,579
HIV + AZT	−	14.55	34,512±3,241	10.91	18	34,587±2,014

APC were pulsed with 50 μg/ml of TT antigen, and with an equivalent of 100 $TCID_{50}$ of HIV collected from J-Jahn infected with either HIV, HIVΔ*nef*, HIVΔ*env*, or HIVΔ*tat*, in the presence or absence of AZT (10^{-5} M). After 4 h at 37°C, monocytes were washed three times and APCs were used to stimulate a TT-specific cell line in the presence or absence of AZT (10^{-5} M) as indicated. P-24 antigen (in picograms per milliliter) was monitored in supernatants by using an ELISA kit at days 2 and 13. Cell proliferation was monitored at day 2 on an aliquot of cells. The remaining T cells were kept in culture and expanded by addition of IL-2. After 12 d, nonadherent cells were collected, purified, and restimulated with syngeneic noninfected monocytes pulsed with the antigen. Cell death was monitored 24 h after restimulation and cell proliferation was measured at the end of day 2 after restimulation. For proliferative assays, results are in counts per minute and represent mean of triplicate culture±SD. For cell death assays, results are indicated in percentage of dead cells and represent the mean of duplicate measurements. Results show one representative experiment out of three.

culture at day 5. 6 d later, cells were recovered, purified, and restimulated with PHA, pokeweed mitogen (PWM), or SEB. Similar to antigen-specific T cell lines, activation of normal $CD4^+$ T cells by SEB in the presence of HIV-infected monocytes primed peripheral blood $CD4^+$ T cells for apoptosis. Strong reduction in their proliferative response was observed after a subsequent activation step with either SEB or PWM (Fig. 3 *c*). Furthermore, as observed in peripheral blood T cells from HIV-infected individuals, normal proliferative response and no induction of cell death were observed when the T cells were restimulated with PHA (Fig. 3 *c*). Finally, no priming for apoptosis was observed when T cells were incubated with HIV-infected monocytes for 12 d, in the absence of antigen-specific activation (Fig. 3 *c*). These results indicate that antigen-specific priming for apoptosis is a general property of HIV-infected monocytes that is induced in $CD4^+$ T cells of healthy donors.

Apoptosis is not due to T cell infection. It has been previously reported that APCs infected with a monocytotropic strain of HIV-1 are able to transmit HIV to T cells (36). It has also been shown that the cytopathic effect of HIV-1 in T cells is associated with apoptosis (15, 37). However, several lines of evidence indicate that AICD detected in T cells primed in vitro by HIV-pulsed APCs is not related to the cytopathic effect of replicating virus in activated HIV-infected T cells. First of all, no syncytia formation was observed in our cultures and no evidence for or a very low level of infection, as detected by p24 antigen assay, was observed during priming and subsequent restimulation of the primed T cells (Table II). Secondly, the addition of the reverse transcriptase inhibitor AZT, which prevents T cell infection as shown by coculture experiments using MT4 cells as indicator (Table I), did not affect the ability of HIV-pulsed monocytes to prime T cell lines for apoptosis (Table II). Finally, quantitative coculture analysis (33) (Table I) using MT4 cells as reporter cells and quantitative PCR analysis (32) (Fig. 4) showed that < 2% of T cells (a maximum detected in only one experiment by PCR) were infected, whereas 60 to 70% of cells died by apoptosis.

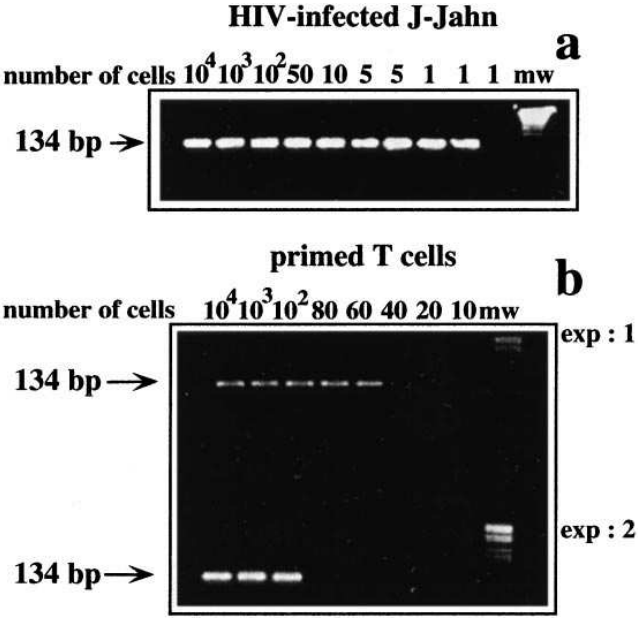

Figure 4. Quantitation of HIV-1 in infected T cells 12 d after coculture with infected monocytes. (*a*) PCR products generated from J-Jahn cells infected with HIVΔ*nef* and cultured in the presence of geneticin (G418). Our quantitative PCR method was standardized using this infected cell line. Immunofluorescence experiments using an anti–p24 antigen on permeabilized cells as previously described (32) showed that all cells were infected (not shown). According to the procedure described in Methods, 10^4, 10^3, 10^2, 10, 5, and 1 infected J-Jahn cell(s) were lysed. PCR was performed directly on the cell lysate using env primers (SK 68/69), and showed that amplification of the env product occurred when even one infected cell was present. (*b*) PCR products generated from different dilutions (as indicated) of antigen-specific T cells collected at day 12 before restimulation with noninfected monocytes. Cell lysates from non adherent purified T cells were prepared as described above and PCR was performed using the oligonucleotide primer pair SK 68/69. Results show two representative experiments out of three.

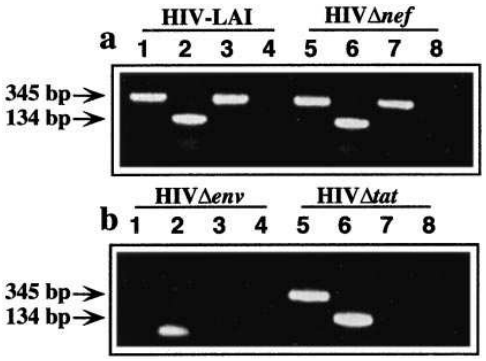

Figure 5. PCR detection and RT-PCR analysis of *tat* and *env* gene expression in monocytes incubated with different deleted proviruses. PCR fragment generated after PCR of DNA or RT-PCR of RNA from monocytes infected with HIV-LAI and HIVΔ*nef* (*a*), and HIVΔ*env* and HIVΔ*tat* (*b*), respectively. (Lanes *1* and *5*) *tat*-specific primers using DNA as template. (Lanes *2* and *6*) *env*-specific primers using DNA as template. (Lanes *3* and *7*) RT-PCR with *tat*-specific primers. (Lanes *4* and *8*) RT-PCR with *env*-specific primers.

Priming of T cells for apoptosis was associated with infection and transcription of viral genes in APCs. To test whether viral gene expression in infected APCs is required to prime T cells for AICD, we pulsed APCs with deleted retroviruses. The HIV-LAI and the HIVΔ*nef* viruses were both able to infect monocytes (Fig. 5 *a*) and were transcribed in these cells as detected by RT-PCR using primers specific for the *tat* gene (Fig. 5 *a*). APCs infected with either of these two viruses were able to prime T cells for AICD (Table II), showing that the expression of the *nef* gene is not required in this process. The HIVΔ*env* virus did not infect monocytes (Fig. 5 *b*) and consequently, HIVΔ*env*-pulsed monocytes were not able to prime T cells for apoptosis (Table II). Similarily, although HIVΔ*tat* virus infects monocytes (Fig. 5 *b*), HIVΔ*tat*-pulsed monocytes were not able to prime T cells for apoptosis (Table II) since Tat mutants do not replicate (38, 39), and the synthesis of all viral proteins is affected by Tat. These experiments using deleted provirus indicate that both infection and expression of viral proteins in APCs are necessary for APCs to prime T cells for apoptosis. This was further confirmed by pulsing APCs with HIV in the presence of AZT in order to prevent monocyte infection (Fig. 3 and Table I). Under these conditions, pulsed APCs were not able to prime antigen-specific T cell lines for apoptosis (Table II).

The gp120 molecule is necessary and sufficient for the priming for apoptosis. To further analyze the respective role of *nef*, *tat*, and *env* gene products in the mechanism that leads to priming for apoptosis, it was necessary to use as antigen-presenting cells EBV-LCLs, because in contrast with monocytes, these cells can be easily transfected. In Table III, it is shown that HIV-infected EBV-LCLs, like HIV-infected monocytes, were able to prime antigen-specific T cells for apoptosis, since nearly 50% of T cells activated with HIV-infected EBV-LCLs undergo apoptosis after a subsequent activation (Table III), whereas only 13% cell death was detected in the control cells activated in the absence of HIV.

The role of the different HIV proteins was analyzed by using EBV-LCLs transfected with the different deleted proviruses (Table III) or with vectors encoding for the three different proteins (Table III). As observed with infected monocytes, EBV-LCLs infected with the HIVΔ*nef* provirus were able to prime T cells for apoptosis (Table III). Moreover, EBV-LCLs expressing the Nef protein of HIV-1 failed to prime antigen-specific T cells for apoptosis (Table III), ruling out a role of this molecule in that process.

Table III. EBV-LCLs Infected by HIV or Expressing gp120 Prime Antigen-specific T Cells to Undergo Apoptosis

Autologous EBV-LCLs-APCs transfected with	Proliferation Day 2	Cell death Day 13	Proliferation Day 14
Proviruses			
Nothing	74,587±1,025	13±5	74,123±549
HIV	77,894±1,234	42±8	4,215±687
HIVΔ*nef*	75,489±1,025	45±9	3,654±578
HIVΔ*env*	78,947±1,543	14±5	78,459±2,054
HIVΔ*tat*	74,578±1,367	15±4	75,487±1,598
Proteins			
Control plasmid	66,897±2,147	12±4	68,214±2,547
nef	70,214±1,875	11±5	69,874±1,875
env	65,487±2,014	36±12	22,457±1,054
tat	66,472±1,420	13±3	63,987±2,014
env + *tat*	64,348±2,049	41±8	18,976±1,567
env + *nef*	68,974±2,894	35±10	21,478±2,367

Autologous EBV-LCLs were transfected with either pNL4.3 (HIV + psv2 neo^R, in a cotransfection), HIVΔ*nef*, HIVΔ*env*, or HIVΔ*tat* + pSV2 neo^R and selected with G418 for a month. 100% infected cells were pulsed with 50 μg/ml of Tetanus toxoid antigen. Alternatively, EBV-LCLs were transfected with pREP-10 plasmid containing either no recombinant gene (control plasmid) or *env*, *tat*, or *nef* genes and selected with Hygromycin as described above. Stably transfected cells for *nef* or *tat* or transitory transfected cells 48 h before for *env* were pulsed with 50 μg/ml of Tetanus toxoid antigen. After 4 h at 37°C, cells were washed three times and cultured in the presence of T cells as described above. For proliferative assays, results are in counts per minute and represent mean of triplicate culture±SD. For cell death assays, results are indicated in percentage of dead cells and represent the mean of triplicate measurements±SD. Results show one representative experiment out of three.

The HIVΔ*tat*-infected EBV-LCLs were not able to prime antigen-specific T cells for apoptosis (Table III). However, no priming for apoptosis of T cells incubated in the presence of *tat*-expressing EBV-LCLs was detected (Table III), showing that this molecule was not sufficient by itself. In addition, as for HIVΔ*tat*-infected monocytes, no mRNA encoding for HIV-1 proteins was detected by RT-PCR in these infected EBV-LCLs (not shown) suggesting that, as explained above, Tat acts primarily by inducing HIV-1 protein synthesis.

The HIVΔ*env*-infected EBV-LCLs failed to prime antigen-specific T cells to undergo apoptosis (Table III), suggesting a role of gp120 in this process. Indeed, *env*-transfected EBV-LCLs primed antigen-specific T cells for apoptosis. Table III shows that after restimulation > 35% of T cells undergo apoptosis, confirming the notion that the gp120 molecule of HIV plays a major role in this mechanism. Finally, no cooperation between HIV proteins was noticed in cotransfection experiments using EBV-LCLs transfected with the different proteins (Table III), in contrast with a previous report showing that tat and gp120 cooperate to sensitize T cells to Fas-mediated apoptosis in a different system (40).

Discussion

We have previously shown that $CD4^+$ T cells from asymptomatic patients infected with HIV-1 undergo cell death by apoptosis upon activation through their T cell receptor (7). We show here that HIV-infected APCs prime in vitro $CD4^+$ T cells to undergo apoptosis. After the stimulation of antigen-specific $CD4^+$ T cells with HIV-infected APCs, T cells were able to proliferate in response to IL-2 and could be maintained up to 1 mo in culture (data not shown). However, when these cells were restimulated, either with specific antigens or superantigens like SEB, they died by apoptosis. These $CD4^+$ T cells primed in vitro for apoptosis by HIV-infected monocytes share the same feature as $CD4^+$ T cells isolated from HIV-infected individuals. In both cases, apoptosis is induced by antigens, PWM, or superantigens, whereas activation with PHA does not induce cell death by apoptosis but leads to cell proliferation (Fig. 3). Coculture experiments or culture of T cells in the presence of HIV-infected monocytes in the absence of antigen have shown that this apoptotic priming mechanism required two signals: an antigenic stimulus and a second signal provided by the gp120 molecule of HIV. Moreover, coculture experiments have shown that the two signals must be present on the same APC. Finally, the specificity for $CD4^+$ T cells was addressed by using purified $CD8^+$ T cells in which no priming for apoptosis was observed with HIV-infected monocytes after stimulation with SEB (not shown).

The requirement for monocyte infection and expression of HIV-proteins were illustrated by experiments in which monocyte infection was blocked by AZT addition (Table II), and by the use of HIVΔ*tat* viruses, in which no HIV proteins are produced. In these conditions, no priming for apoptosis occurs (Tables II and III).

We have excluded the possibility of the transfer of infectious virus particles from infected APCs to specific T cells as a likely explanation of our observations. It has been previously shown that apoptosis can be due to the cytopathogenic effect of the replicative virus (15, 37) on the T cells after activation through their TCR. In fact, by using different techniques to measure the percentage of T cell infection after stimulation with HIV-infected monocytes, we have shown that $< 2\%$ of cells were infected after the priming step, whereas 60 to 70% of cells died by apoptosis. Moreover, the quantitative PCR used to analyze HIV infection overestimates the productive infection of T cells (as explained in Methods). Furthermore, no viral replication was detected after the induction of apoptosis in T cells (Table I) and, moreover, T cells were primed for apoptosis even in the presence of AZT, which completely blocked T cell infection (Tables I and II). Finally, the fact that gp120-transfected EBV-LCLs can effectively prime T cells for apoptosis in the absence of any virus (Table III) completely excludes any role for T cell infection in this mechanism.

The infection of monocytes or EBV-LCLs by a *nef*-deleted provirus has clearly shown that this molecule is not involved in the priming of T cells for apoptosis since HIVΔ*nef*-infected APCs are as efficient as HIV-infected APCs to prime T cells for apoptosis (Tables II and III). This result was confirmed by the absence of priming capability of EBV-LCLs that constitutively expressed the Nef molecule.

The direct effect of the Tat protein was analyzed by using Tat-expressing EBV-LCLs that failed to prime antigen-specific $CD4^+$ T cells to undergo apoptosis (Table III). These results were not due to the lack of activity of the Tat molecule since it was able to complement a *tat*-defective provirus after cotransfection in J-Jahn or EBV-LCL cell lines and to enhance the transcription of an LTR-luciferase construct transfected into these cells (not shown). Moreover, the secretion and activity of the Tat molecule was tested by measuring the luciferase activity of DAMI cell lines stably transfected with an LTR-luciferase construct (27) cocultured with *tat*-expressing J-Jahn or EBV-LCLs cell lines (not shown). Several investigators have demonstrated that T cells treated with soluble HIV-transactivating protein, Tat, and *tat*-transfected T cells are inhibited in their ability to respond to TCR-induced stimulation in vitro (41, 42). However, the inhibitory effect of Tat on antigen-specific proliferation was not confirmed by all investigators (43) and the precise mechanism has yet to be clearly elucidated. More recently, soluble Tat protein added on PBMC or *tat*-expressing Jurkat cells have been shown to induce apoptosis in serum-deprived conditions (13). However, a Hela-Tat cell line or even another Jurkat-Tat cell line did not undergo apoptosis when cultured in serum-free conditions and it has also been reported that *tat* expression protects against apoptosis (44, 45). Accordingly, we did not observe any enhancement of apoptosis in *tat*-expressing J-Jahn cell lines or *tat*-expressing EBV-LCLs and no enhancement of apoptosis was observed after coculture of antigen-specific T cell lines with *tat*-expressing EBV-LCLs.

The viral envelope glycoprotein, gp120, binds with high affinity to the $CD4^+$ T cell surface glycoprotein that serves as a receptor for HIV. As the deletion of T cells observed in HIV-infected individuals is restricted to $CD4^+$ T cells, it is likely that the gp120 molecule might be implicated in any direct or indirect mechanisms that lead to cell deletion. In this study, by using APCs that expressed the gp120 at their cell surface, we have shown that the crosslinking of CD4 molecules by gp120, which occurs concomitantly with antigen presentation, primes T cells to undergo apoptosis after restimulation (Tables II and III). The necessity of coexpression on the same APCs of the peptide/MHC complex and the gp120 molecule was demonstrated by different experiments (Figs. 1 and 2). When using HIV-infected monocytes to activate antigen-specific $CD4^+$ T cells, the defect of the costimulatory potential of monocytes (36, 46), along with the concomitant crosslinking of the CD4 molecules that induces a sequestration of p56lck and a partial desensitization of the TCR (47, 48), primes $CD4^+$ T cells to a profound unresponsive state that leads to cell death after restimulation.

The study of the pathology of HIV-1 infection in chimpanzees supports the idea of the crucial role of HIV-infected monocytes in the pathogenesis of AIDS (24). Persistently HIV-infected chimpanzees do not develop clinical symptoms (49) despite many similarities to the course of HIV infection in humans, such as persistence of virus (50) and emergence of mutants that escape neutralization (51). Comparison of immunological and virological parameters between chimpanzees and humans infected with HIV has revealed that T cells isolated from chimpanzees did not present any immunological defect. Peripheral blood T cells have normal proliferative response and no AICD was detected in contrast with the human situation (8, 18, 24). In these chimpanzees, comparable with the observations performed on T cells from HIV-infected asymptomatic individuals, the frequencies of cells harboring proviral DNA is low. However, only lymphotropic variants

were isolated, no macrophage-tropic HIV variants were detected (24). This observation is explained by the complete insusceptibility of chimpanzee macrophages for in vitro inoculation with a panel of HIV variants (52, 53). These observations suggest that, due to the inability to HIV-1 to infect chimpanzees' monocytes, systemic immune dysfunction will not occur. The importance of infected monocytes in the cascade of AIDS pathogenesis is further supported by the observation that, in macaques experimentally infected with SIV that develop disease, monocytes infection is observed and macrophage-tropic variants can be recovered from the blood (54). The presence of monocytes infection is again correlated with a selective proliferative defect of $CD4^+$ T cells due to induction of cell death by apoptosis (8, 18).

The proliferative defect observed in $CD4^+$ T cells from asymptomatic HIV-infected individuals can be explained by the priming for apoptosis induced by HIV-infected monocytes. However, as priming for apoptosis requires a concomitant cross-link of the TCR and the sequestration of CD4 molecules, our results suggest that priming for apoptosis is restricted to antigen-specific activated $CD4^+$ T cells in vivo. Thus, $CD4^+$ T cell depletion should occur predominantly in memory cells (as previously reported in reference 55) by deleting antigen-specific T cells. This mechanism explains the gradual proliferative defect in response to recall antigens observed in asymptomatic HIV-infected individuals during the course of the disease where T cells specific for frequently encountered antigens are the first deleted (6, 56). After a while, the progressive destruction of the lymph node architecture, the destruction of dendritic cells by the virus, the successive deletion of antigen-specific $CD4^+$ T cells, and the chronic and continuous activation of the immune system culminate in a collapse of the whole machinery, leading to the accelerated final deletion of $CD4^+$ T cells characteristic of AIDS. Since apoptosis is an active death process that can be potentially prevented (7, 57), the understanding of mechanisms that are able to prime T cells from HIV-infected individuals to undergo apoptosis may have implications for the delineation of specific therapeutic strategies aimed at the prevention of AIDS.

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