

HIV-1 Directly Kills CD4⁺ T Cells by a Fas-independent Mechanism

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Summary

The mechanism by which HIV-1 induces CD4⁺ T cell death is not known. A fundamental issue is whether HIV-1 primarily induces direct killing of infected cells or indirectly causes death of uninfected bystander cells. This question was studied using a reporter virus system in which infected cells are marked with the cell surface protein placental alkaline phosphatase (PLAP). Infection by HIV-PLAP of peripheral blood mononuclear cells (PBMCs) and T cell lines leads to rapid depletion of CD4⁺ T cells and induction of apoptosis. The great majority of HIV-induced T cell death in vitro involves direct loss of infected cells rather than indirect effects on uninfected bystander cells. Because of its proposed role in HIV-induced cell death, we also examined the Fas (CD95/Apo1) pathway in killing of T cells by HIV-1. Infected PBMCs or CEM cells display no increase in surface Fas relative to uninfected cells. In addition, HIV-1 kills CEM and Jurkat T cells in the presence of a caspase inhibitor that completely blocks Fas-mediated apoptosis. HIV-1 also depletes CD4⁺ T cells in PBMCs from patients who have a genetically defective Fas pathway. These results suggest that HIV-1 induces direct apoptosis of infected cells and kills T cells by a Fas-independent mechanism.

Despite significant advances in our understanding of the pathogenesis of AIDS, the mechanism by which human immunodeficiency virus 1 (HIV-1) infection induces CD4⁺ T lymphocyte depletion is not known. Recent studies indicate that turnover of both HIV-1 and CD4⁺ T cells is extremely rapid (1–3) and that the viral load soon after infection predicts the rate of CD4⁺ T cell loss and the development of AIDS (4). These observations suggest that active HIV-1 replication drives the loss of CD4⁺ T lymphocytes. However, there are important gaps in our knowledge of how HIV-1 causes this loss of T cells.

The fundamental issue of whether HIV-1 predominantly kills infected cells or induces death of uninfected cells remains controversial. This debate was initially prompted by the low frequency of HIV-infected cells detected in vivo, which led to a search for indirect causes of T cell depletion (5). Indirect mechanisms of bystander T cell death could include the following: syncytium formation between infected and uninfected cells; aberrant T cell signaling due to binding of free gp120 to CD4 and cross-linking by anti-gp120 antibodies; triggering of apoptotic pathways in uninfected cells by soluble HIV-1 gene products or by infected macrophages expressing Fas ligand; or cytokine dysregula-

tion, such as overproduction of TNF- α , leading to T cell death (5–7). However, given the rapid turnover of CD4⁺ T cells, it is possible that direct killing by HIV-1 leads to depletion without requiring that a high percentage of cells be productively infected at any given point in time. Therefore, to understand the pathogenesis of AIDS, it is important to know the extent to which HIV-induced T cell death involves direct loss of infected cells versus indirect killing of uninfected bystander cells.

The process by which cells die has generally been divided into apoptosis and necrosis based upon morphologic and biochemical criteria. There is accumulating evidence that T cell apoptosis is increased in patients with HIV-1 infection. PBMCs from HIV-infected patients undergo apoptosis in culture or after activation at a higher rate than PBMCs from uninfected controls (8–10). Increased apoptosis is also seen in lymph nodes from patients with HIV-1 infection (11, 12). In animal models, increased T cell apoptosis is seen in SIV-infected macaques, which develop an AIDS-like syndrome, but not in HIV-infected chimpanzees, which rarely develop immunodeficiency (13, 14). However, in these studies both CD4⁺ and CD8⁺ T cells are affected (8, 13), raising the question of whether the increased

apoptosis is directly due to HIV-1 infection or due to indirect consequences of the disease.

The Fas/Fas ligand (FasL)¹ system is a key cellular apoptotic pathway that has been proposed to play a role in HIV-induced cell death (15). This pathway is important in regulation of lymphocyte survival and in antigen-induced T cell death (16). Since T cell activation augments HIV-induced apoptosis, the Fas pathway has been examined in patients with HIV-1. Infected patients have a higher percentage of Fas-expressing T cells as compared with uninfected people (17), and T cells from these patients are more sensitive to killing by anti-Fas antibody (15). In addition, FasL mRNA levels have been found to be elevated in PBMCs from patients with HIV-1 infection (18). Soluble exogenous Tat combined with CD4 cross-linking by antibody has been shown to increase FasL mRNA expression in uninfected PBMCs (19). Whether HIV-1 directly or indirectly affects the Fas pathway is not settled because previous studies have not measured Fas or FasL levels specifically in those cells that are infected.

To clarify the mechanisms of HIV-induced cell death, we used a reporter virus system in which a cell surface protein, placental alkaline phosphatase (PLAP), is expressed by HIV-1, thereby marking infected cells in a culture. This system allows us to distinguish direct from indirect effects of the virus on its host cell. We show by TUNEL (TdT-mediated dUTP nick-end labeling) assay that HIV-1 infection induces apoptosis in T cell lines and primary T cells in vitro. The reporter virus system is then used to show that HIV-1 induces apoptosis predominantly in infected cells. Finally, we use the reporter virus system to study the specific effects of viral infection on the Fas pathway. We find that HIV-1 infection does not specifically upregulate Fas expression on the surface of infected cells and that HIV-1 is able to kill T cells in which the Fas pathway is defective or cells in which the downstream effectors of the pathway are blocked.

Materials and Methods

Cells. CEM is a human CD4⁺ T lymphoblastoid cell line originally isolated from a child with acute leukemia (20, 21). SupT1 is a CD4⁺ T cell line isolated from a pleural effusion of a non-Hodgkin's lymphoma patient (22). Jurkat clone E6 is a CD4⁺CD3⁺ acute T cell leukemia line (23). CEM (from Peter L. Nara), SupT1 (from James Hoxie), and Jurkat clone E6 (from Arthur Weiss) were obtained from the NIH AIDS Research and Reference Reagent Program. The hFasL/L5178 cell line, which stably expresses human FasL (hFasL; reference 24), was provided by Hideo Yagita of the Juntendo University School of Medicine, Tokyo, Japan. The CEM, SupT1, Jurkat, and hFasL/L5178 cell lines were grown in RPMI 1640 medium supplemented with

10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. PBMCs from HIV-seronegative blood donors were purified by centrifugation of buffy coat samples over a Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient. CD4⁺ T cells were purified by negative selection of PBMCs using a CD4⁺ T cell enrichment column (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. Patients with autoimmune lymphoproliferative syndrome (ALPS) arising as a result of mutations in Fas and their families were evaluated and followed at the Warren Magnuson Clinical Center at the National Institutes of Health as part of an Institutional Review Board-approved clinical research protocol. PBMCs were isolated as above and T cells and EBV-transformed B cells were tested for sensitivity to anti-Fas antibody (CH11; Kamiya Biomedical, Seattle, WA) and plate-immobilized anti-CD3ε monoclonal antibody (64.1) as previously described (25, 26). PBMCs from these families were cryopreserved after donation. Before infection with HIV-1, PBMCs and purified CD4⁺ T cells were activated for 2 d in culture medium containing 5 µg/ml PHA (Sigma Chemical Co., St. Louis, MO), and then maintained in culture medium with 100 U/ml recombinant IL-2 (Genzyme Corp., Cambridge, MA).

Viruses. The HIV molecular clone NL43 (27) was obtained from Dr. Malcolm Martin through the AIDS Research and Reference Reagent Program. The HIV-1 molecular clone HXB2 was provided by Dr. Mark Feinberg (Emory University, Atlanta, GA; R7 clone) and contains a repaired nef open reading frame (28). The construction of PLAP-expressing NL43 (NL-PI) and PLAP-expressing HXB2 (HXBnPLAP) have been previously described (29, 30). Virus was produced by calcium phosphate transfection of HIV DNA constructs into 293 cells and viral supernatants were harvested at 48 h after transfection as previously described (30). Virus was quantitated by p24 ELISA to normalize all infections to equivalent antigenic input (31).

Infections. Target cells at 1–2 × 10⁶ cells/ml were incubated with HIV-1 (NL43 or HXB2) or HIV-PLAP at different multiplicities of infection in the presence of 4 µg/ml polybrene for 8–12 h at 37°C in a humidified incubator. Infection of PBMCs with HIV-PLAP was carried out by spin infection as follows: 1–2 × 10⁶ cells in 1 ml of culture medium containing HIV-PLAP at different multiplicities of infection and 4 µg/ml polybrene were centrifuged in a sealed biosafety container for 90 min at 2,000 rpm in a Jouan CR4.12 centrifuge (Jouan Inc., Winchester, VA), and then returned to a humidified incubator for 8–12 h. After infection, cells were washed with 5 volumes of PBS and then resuspended in culture medium. Cultures were split every 2–3 d to maintain a concentration of 5–20 × 10⁵ cells/ml. For experiments involving the caspase inhibitor z-VAD-fmk (Enzyme Systems Products, Livermore, CA), after infection CEM and Jurkat T cells were resuspended in culture medium in the presence or absence of 50 µM z-VAD-fmk. Cells were maintained at 5–20 × 10⁵ cells/ml and fresh z-VAD-fmk was added every 2–3 d.

Flow Cytometry. Flow cytometric analyses for PLAP and CD4 were performed as previously described (30). In brief, cells were stained with a 1:100 dilution of rabbit anti-human PLAP (Zymed Laboratories, So. San Francisco, CA) and a 1:100 dilution of anti-human CD4-biotin (Caltag Labs., Burlingame, CA). Goat anti-rabbit FITC that was human and mouse serum adsorbed (BioSource International, Camarillo, CA) at 1:250 and Ultra-avidin PE (Leinco Technologies, Ballwin, MO) at 1:100 were used as secondary stains. The control for the anti-PLAP staining was incubation of cells with secondary antibody only. Triple staining for CD3, CD4, and CD8 was done using anti-

¹Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; FasL, Fas ligand; hFasL, human FasL; HXBnPLAP, PLAP-expressing HXB2; NL-PI, PLAP-expressing NL43; PLAP, placental alkaline phosphatase; TUNEL, TdT-mediated dUTP nick end labeling.

CD3-FITC (PharMingen, San Diego, CA) at a 1:25 dilution, anti-CD4-PE (Caltag Labs.) at a 1:100 dilution, and anti-CD8-CyChrome (PharMingen) at a 1:25 dilution. Two-color staining for surface PLAP and Fas was performed using rabbit anti-human PLAP as above and mouse anti-human Fas IgM (Upstate Biotechnology, Inc., Lake Placid, NY) at a 1:200 dilution; secondary staining was done with goat anti-rabbit PE (Southern Biotechnology Associates, Birmingham, AL) at 1:200 and goat anti-mouse IgM FITC (Caltag) at 1:250. FasL staining was performed using the NOK-1 antibody (PharMingen) at 20 μ l per reaction followed by biotin-conjugated goat anti-mouse immunoglobulin (PharMingen) at 1:100 and then Ultra-avidin PE at 1:100. Cells that were stained for FasL were grown and stained in the presence of 10 μ M of the metalloproteinase inhibitor KB8301 (24), which was provided by Hideo Yagita. After staining, cells were fixed in 1% paraformaldehyde overnight. Flow cytometry was performed on a FACScan® (Becton Dickinson, San Jose, CA) as previously described.

Detection of Cytopathicity and Apoptosis. The number of viable cells in each culture was determined by using trypan blue exclusion. To perform two-color staining for PLAP and TUNEL, cells were first stained for PLAP, and goat anti-rabbit PE was used as a secondary stain as above. The cells were then fixed in 1% paraformaldehyde for 1 h, washed in PBS, resuspended in 70% EtOH, and stored overnight at 4°C. The cells were then washed twice in PBS and a TUNEL assay was carried out using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim, Indianapolis, IN) as per the manufacturer's recommendations, except that the cells were permeabilized with 70% EtOH (see above) rather than with Triton X-100. After the TUNEL reaction, cells were washed in 20 volumes of PBS twice. Cells were then analyzed by flow cytometry for both PLAP and TUNEL. Apoptosis was also assessed by staining cells with propidium iodide for DNA content and by determining the percentage of cells

that had hypodiploid DNA content. Cells were permeabilized in 70% EtOH for 1 h, washed in PBS, and then resuspended in PBS containing 0.1 mg/ml propidium iodide and 1 mg/ml RNase. After 90 min in the dark at room temperature the cells were analyzed by flow cytometry.

Results

Infection of T Cell Lines and Primary Cells with PLAP-expressing HIV-1 Leads to CD4 Cell Depletion. To study how HIV-1 kills CD4⁺ T cells, we infected two human CD4⁺ T lymphoblastoid cell lines, CEM and SupT1, with either the HIV-1 molecular clone NL43 or NL-PI (Fig. 1 A), and then determined the number of viable cells every 2–4 d. We observed consistent depletion of both cell types in the HIV-infected cultures by 7–10 d after infection (Fig. 2 A). Although the reporter virus NL-PI grew more slowly than did the wild-type virus (data not shown), it also induced significant T cell depletion in these cells, thereby allowing us to study the mechanism of HIV-induced T cell killing.

We also examined the effect of HIV-1 infection of PHA-activated PBMCs from uninfected donors on the percentage and absolute number of CD4⁺ T cells. After infection with NL43 or NL-PI, a portion of each culture was stained daily for surface CD3, CD4, and CD8 expression and analyzed by flow cytometry. This method allowed us to distinguish loss of CD4 cells from the CD4 downmodulation induced by multiple HIV-1 gene products (30). CD4 downmodulation was detected by the appearance of CD3⁺CD4⁻CD8⁻ T cells in the culture, and these cells were included in the determination of CD4⁺ cells present in the sample. We found that infection with NL43 led to a rapid depletion of CD4⁺ T cells from the culture (Fig. 2 B). Similar results were obtained with NL-PI (data not shown). At early time points (2–3 d after infection), the extent of depletion was proportional to the amount of virus added to the culture (as measured by nanogram per milliliter of HIV p24). When PBMCs were infected with 50 ng/ml p24 of NL43 or 200 ng/ml p24 of NL-PI, the majority of CD4⁺ cells were lost within 2 to 4 d after infection.

We then asked whether depletion of primary CD4⁺ T cells by HIV-1 depends upon the presence of other cells because it has been suggested that macrophages may upregulate FasL after HIV-1 infection and then kill CD4⁺ T cells expressing Fas (32). We purified CD4⁺ T cells to >95% purity using a column that removes CD8⁺ T cells, macrophages and B cells. Infection of PHA-activated purified CD4⁺ T cells with HXB2 led to rapid depletion of all the cells in culture (Fig. 2 C), suggesting that HIV-1 can cause the death of CD4⁺ T cells in the absence of other cell types.

HIV-1 Infection Leads to Apoptosis by a Direct Mechanism. To determine whether HIV-1 infection was killing T cells by apoptosis, we performed a TUNEL assay on infected and uninfected cell cultures. In both T cell lines and primary CD4⁺ T cells, the cultures infected with HIV-1 had a significantly higher percentage of TUNEL-positive cells as compared with an uninfected culture (Fig. 3, A and

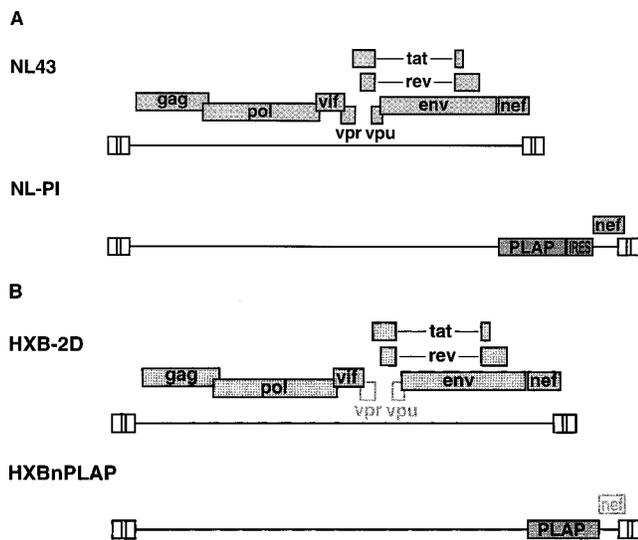


Figure 1. Genomic organization of HIV constructs. NL-PI is derived from NL43 and expresses all of the accessory genes of HIV-1. *IRES* indicates insertion of an internal ribosomal entry site (from encephalomyocarditis virus) which restores expression of nef in NL-PI. HXBnPLAP is derived from HXB-2D and does not express the accessory genes vpr, vpu and nef. Gray boxes indicate genes expressed by the virus; open boxes indicate genes that are not expressed.

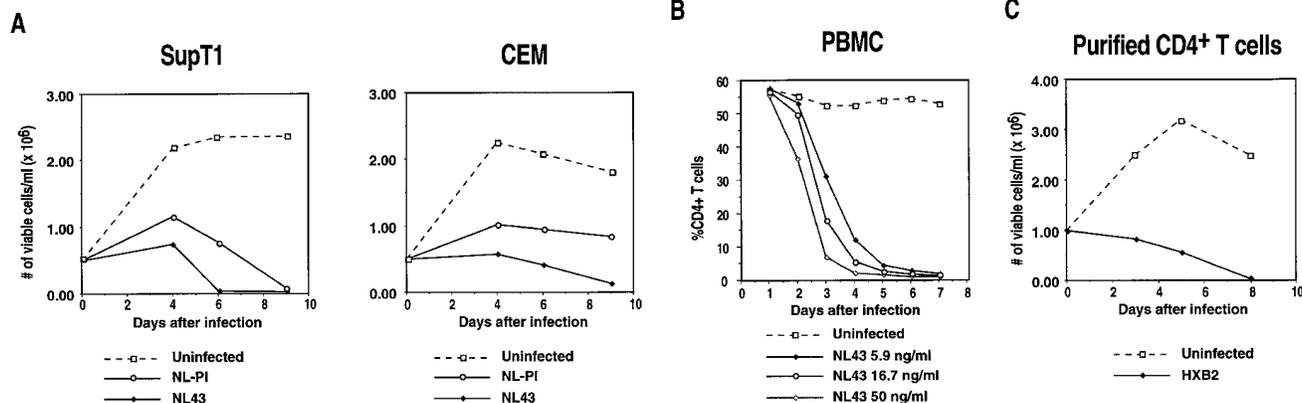


Figure 2. CD4⁺ T cell depletion after infection with HIV-1. (A) SupT1 and CEM cells were infected with either NL43 or NL-PI or mock-infected and the number of viable cells was determined by trypan blue exclusion on the indicated days after infection. (B) PBMCs from a normal donor were infected with different concentrations of NL43 (measured in nanograms per milliliter of p24) or mock-infected. Samples were stained daily for CD3, CD4, and CD8 and analyzed by flow cytometry. The percentage of CD4⁺ cells remaining on each day is plotted. Cells that were CD3⁺CD4⁻CD8⁻ were considered to have downregulated CD4 and were included in the percentage of CD4⁺ T cells remaining. (C) CD4⁺ T cells were purified from a normal donor and infected with HXB2 or mock-infected. The number of viable cells was determined by trypan blue exclusion on the indicated days after infection. All experiments were performed at least three times and representative results are shown.

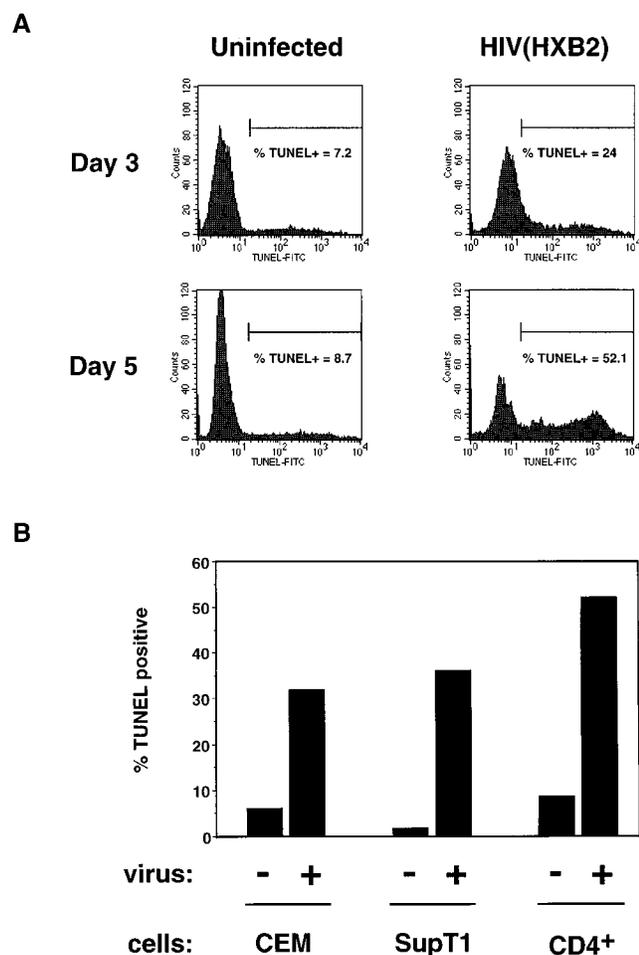


Figure 3. TUNEL assay on infected and uninfected CD4⁺ T cells. (A) CD4⁺ T cells from a normal donor were purified and infected with HXB2 or mock-infected. TUNEL assays were performed 3 and 5 d after infection. The TUNEL-positive gate was determined by analyzing each

(B). In purified CD4⁺ T cells, there was a sixfold increase in apoptosis in the infected culture 5 d after infection.

We then determined whether infected or uninfected cells within a culture are preferentially undergoing apoptosis by infecting cultures with HIV-PLAP and performing two-color flow cytometric analysis for detection of PLAP and TUNEL. After infection of both CEM and primary CD4⁺ T cells with HIV-PLAP, a higher fraction of PLAP-positive cells was TUNEL-positive as compared with PLAP-negative cells (Fig. 4). In CEM cells, we infected 75.6% of cells by day 4 after infection (Fig. 4 B, upper right plus lower right quadrants). The percentage of TUNEL-positive and PLAP-positive cells was 16.1% (Fig. 4 B, upper right quadrant). Therefore, 21.3% (16.1 out of 75.6%) of infected (PLAP-positive) cells were undergoing apoptosis (TUNEL-positive). The percentage of TUNEL-positive and PLAP-negative cells undergoing apoptosis was 1.7% (Fig. 4 B, upper left quadrant), which is comparable to the background apoptosis in CEM cells. In purified CD4⁺ cells, we infected 7.75% of cells by day 5 after infection and 1.94% of cells were TUNEL- and PLAP-positive (Fig. 4 E). Thus, 25% (1.94 out of 7.75%) of infected (PLAP-positive) CD4⁺ T cells were undergoing apoptosis (TUNEL-positive). The fraction of PLAP-negative cells that were TUNEL-positive in the infected culture was nearly the same as that in the uninfected culture (8%), which is the background apoptosis after activation of primary cells. A similar result was obtained when primary cells were examined 3 d after infection (data

sample in parallel without TdT in the TUNEL reaction mix. (B) CEM and SupT1 cells were infected with NL43 or mock-infected and purified CD4⁺ T cells were infected with HXB2 or mock-infected. TUNEL assays were performed on days when the cell counts were declining. The percentage of TUNEL-positive cells is plotted for CEM, SupT1 (both day 6 after infection) and purified CD4⁺ T cells (day 5 after infection). These results are representative of three separate experiments.

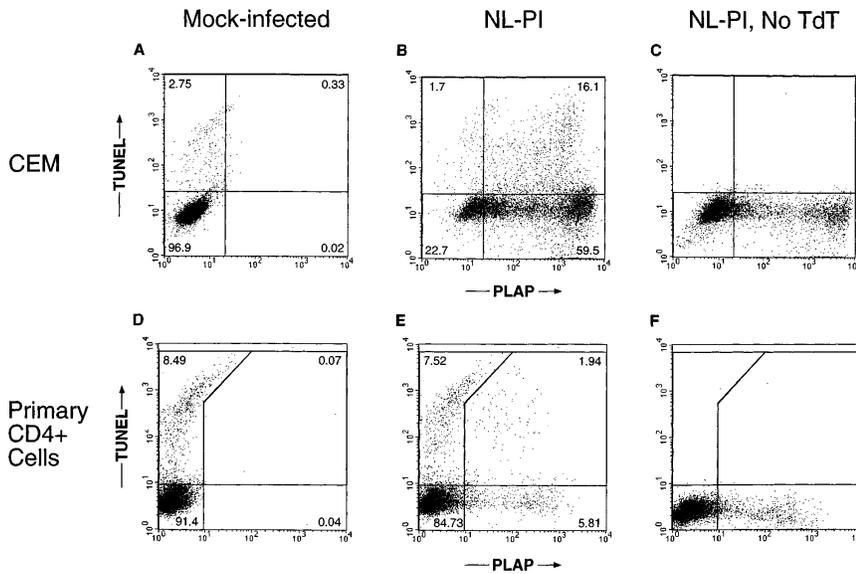


Figure 4. Two-color flow cytometric analysis of cells for PLAP and TUNEL. CEM cells (A–C) and purified CD4⁺ T cells (D–F) were mock-infected or infected with NL-PI. CEM cells and purified CD4⁺ T cells were stained 4 and 5 d after infection, respectively, for PLAP and TUNEL. (A and D) uninfected cultures. (B and E) Infected cultures. (C and F) Infected cultures in which TdT was left out of the TUNEL reaction mix. The percentage of cells in each region is indicated in the corners of each plot. These results are representative of three separate experiments.

not shown). Therefore, in both CEM and primary CD4⁺ T cells, virtually all of the HIV-induced apoptosis occurs in the infected population. We find *in vitro* that HIV-1 infection has little influence on levels of apoptosis occurring in uninfected bystander cells.

HIV-1 Infection Does Not Upregulate Fas Surface Expression. The Fas pathway plays a key role in regulating the survival of T lymphocytes, and induction of this pathway has been proposed as a mechanism by which HIV-1 causes T cell apoptosis. In PBMCs from HIV-infected patients, a higher percentage of T cells express Fas, and the T cells are more sensitive to killing by anti-Fas antibody (15, 17). However, it is not known whether these effects are specific to HIV-infected cells. Therefore, we examined whether Fas expression is upregulated in HIV-infected cells by analyzing cultures infected with HXBnPLAP (Fig. 1 B) for expression of Fas and PLAP. PLAP-positive PBMCs did not have an increase in surface Fas expression as compared with PLAP-negative cells (Fig. 5). Infection with NL-PI also did not affect surface expression of Fas on PBMCs when assessed 4–9 d after infection (data not shown). Since NL-PI expresses all of the regulatory genes of HIV-1, the lack of Fas modulation was not due to the absence of a specific HIV-1 gene product. Similar results were obtained when CEM cells were infected with NL-PI (data not shown). This finding suggests that HIV-1 does not specifically upregulate Fas expression on the surface of infected cells.

We also stained uninfected and infected CEM cells and activated PBMCs for FasL. In uninfected cells, levels of surface FasL were extremely low even in the presence of a metalloproteinase inhibitor, which previously has been shown to stabilize FasL on the cell surface (24). We observed no difference in FasL levels in infected cultures as compared with uninfected cultures (data not shown). As a control, surface FasL was easily detected on hFasL/L5178Y cells stably expressing hFasL (data not shown). The low level of FasL detected on T cells makes it difficult to rule out that HIV-1 infection subtly affects its surface expression.

HIV-induced Killing of T Cells Does Not Require the Fas Pathway. We found that the ability of HIV-1 to induce cell death in a T cell line was not correlated with the Fas sensitivity of the cell line. As shown in Fig. 2 A, HIV-1 was able to deplete both SupT1 and CEM cells although these cells differ significantly in their susceptibility to Fas-induced killing. This was shown by incubating SupT1 and CEM cells with increasing amounts of anti-Fas antibody (CH11) for 24 h and then staining with propidium iodide to determine their DNA content; the percentage of hypodiploid cells was used as a measure of apoptosis. CEM cells underwent apoptosis with small amounts of anti-Fas antibody, whereas SupT1 cells were resistant to anti-Fas antibody over a wide range of antibody concentration (Fig. 6). This difference was not due to a lack of expression of Fas since both CEM and SupT1 cells expressed Fas on their surface (data not shown).

We also determined whether inhibiting caspases, which are critical downstream effectors of the Fas pathway, would block HIV-induced cell death. Z-VAD-fmk is a cell-permeable irreversible peptide inhibitor of multiple caspase family members (33). When present at a concentration of

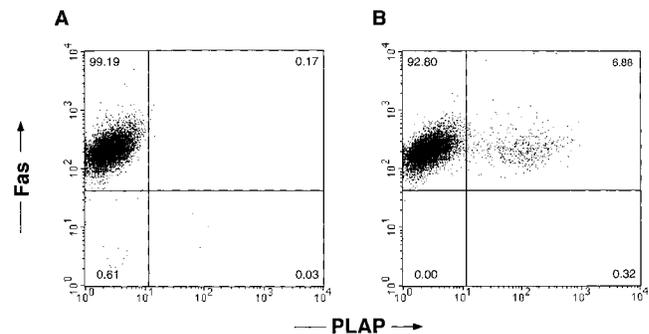


Figure 5. Two-color flow cytometric analysis of PBMC for Fas and PLAP. (A) Uninfected cells. (B) PBMCs on day 6 after infection with HXBnPLAP.

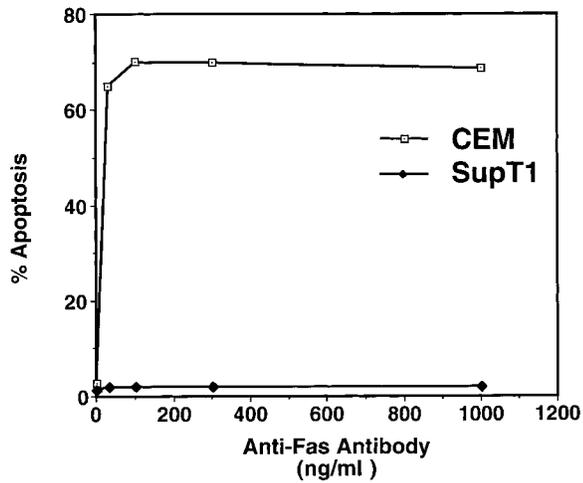


Figure 6. Sensitivity of CEM and SupT1 cells to anti-Fas antibody. Cells were incubated for 24 h in the presence of the indicated concentrations of mouse anti-human Fas IgM antibody (CH11). The percentage of apoptosis was assessed by staining the cells with propidium iodide and determining the fraction of cells that had hypodiploid DNA content.

50 μ M, z-VAD-fmk completely blocked apoptosis of CEM T cells induced by 1 μ g/ml of anti-Fas antibody (Fig. 7 A). CEM cells were infected with HXBnPLAP in the presence or absence of 50 μ M of z-VAD-fmk. HIV-1 infection depleted CEM T cells as rapidly in the presence of z-VAD-fmk as in its absence (Fig. 7 B). HIV-PLAP also depleted Jurkat T cells in the presence of z-VAD-fmk (data not shown). In fact, we observed a more rapid depletion of Jurkat cells by HXBnPLAP when z-VAD-fmk was present. Staining for PLAP demonstrated that Jurkat cells were

more rapidly infected by HIV-1 in the presence of z-VAD-fmk (data not shown), as has been previously reported (34). In CEM cells, the rate of infection was not significantly affected by the caspase inhibitor. In these cells, z-VAD-fmk decreased the percentage of TUNEL-positive cells from 37.7 to 15.5% on day 8 after infection (background apoptosis in uninfected cells was <2%). However, by day 10 virtually all cells were killed by HIV-1 in the z-VAD-fmk-treated and untreated cultures. Therefore, despite blocking Fas-induced apoptosis and decreasing HIV-triggered DNA cleavage, the caspase inhibitor did not prevent virus-associated depletion of CEM cells. There was no effect of z-VAD-fmk alone on growth of uninfected CEM or Jurkat cells.

Finally, we examined whether HIV-1 could kill CD4⁺ T cells from patients who have a genetically defective Fas signaling pathway. A number of patients with ALPS are known to harbor heterozygous dominant-negative mutations in the Fas molecule which renders them relatively insensitive to killing by anti-CD3 stimulation or anti-Fas antibody (25, 35, 36). Many of these patients have missense mutations in the Fas death domain. We studied cells from two ALPS patients and their healthy (Fas-normal) mothers for susceptibility to HIV-induced cell death. These two patients had mutations in exon 9 of the Fas gene that rendered their T cells significantly less susceptible to anti-CD3- and anti-Fas-induced killing (Table 1). We infected PBMCs from these two ALPS patients and their healthy mothers with serial dilutions of NL-PI. Using the PLAP marker, we found that the percentage of infection is proportional to the amount of input HIV over a range of 20–200 ng/ml p24, and that after spin infection with the highest concentration of NL-PI, >75% of CD4⁺ T cells are infected after 1 d. Importantly, both normal and Fas pathway-defective CD4⁺ T cells were almost completely de-

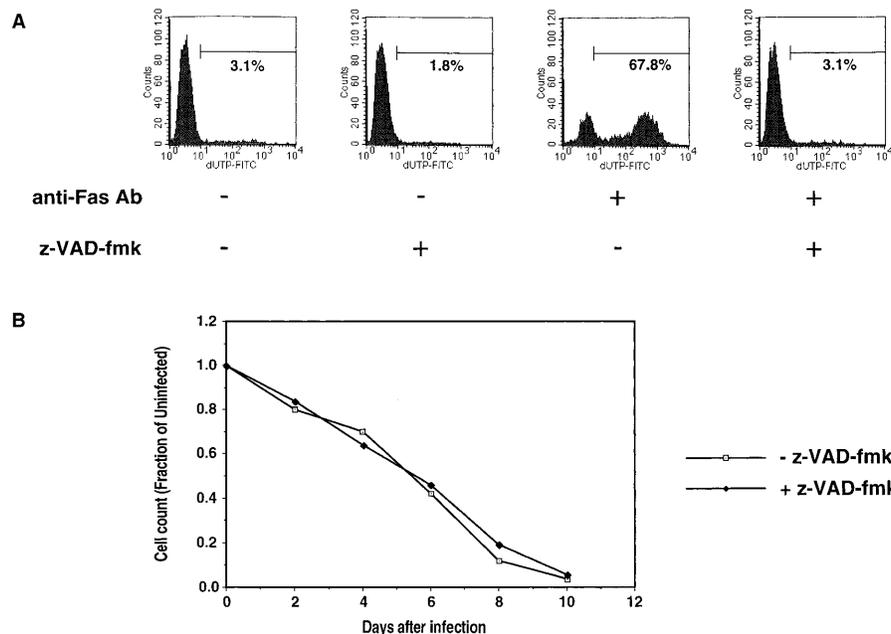


Figure 7. The effect of the caspase inhibitor z-VAD-fmk on apoptosis induced by Fas and on killing of T cells by HIV-1. (A) Anti-Fas antibody (CH11) at 1 μ g/ml was added to the indicated samples of CEM T cells that had been grown in the presence or absence of 50 μ M of z-VAD-fmk. At 18 h after addition of anti-Fas antibody, the samples were analyzed for apoptosis by the TUNEL assay. (B) CEM T cells were infected with HXBnPLAP in the presence or absence of 50 μ M z-VAD-fmk. Fresh z-VAD-fmk was added to the culture every 2 d. On the indicated days, equal sample volumes were counted for 1 min on a FACS-can® and the number of cells with forward and side scatter characteristics consistent with viable cells was determined. This number in infected samples is plotted as a fraction of the number in the mock-infected culture.

Table 1. Characteristics of Cells from Patients

Patient	Fas genotype	Cell type	Treatment	Percentage of killing
ALPS patient 3 13-yo M with ALPS	A915C (Thr225 to Pro)	EBV-transformed B cells	anti-Fas Ab	11
		T cells	anti-Fas Ab	35
		T cells	anti-CD3	14
Mother of patient 3	WT	EBV-transformed B cells	anti-Fas Ab	41
		T cells	anti-Fas Ab	66
ALPS patient 27 8-yo M with ALPS	C916A (Thr225 to Lys)	T cells	anti-Fas Ab	37
		T cells	anti-CD3	21
Mother of patient 27	WT	T cells	anti-Fas Ab	79
		T cells	anti-CD3	41

M, male; WT, wild-type; yo, year-old.

pleted by day 7 after infection with NL-PI (Fig. 8). Similar results were obtained when these cells were infected with NL43 (data not shown). These results indicate that HIV-1 can kill primary cells that have a defect in their Fas pathway.

Discussion

The process by which HIV-1 induces CD4⁺ T lymphocyte death was studied using a reporter virus system that distinguishes direct from indirect effects of the virus on its host cell. We find that, in vitro, T-tropic HIV-1 depletes and induces apoptosis in CD4⁺ primary cells and cell lines. After infection of PBMCs with high titers of HIV-1, >50% of CD4⁺ T cells are depleted in 2–3 d. This finding is in agreement with in vivo studies of viral dynamics that indicate that the half-life of the infected cell is ~2 d (3). Earlier studies found a slower in vitro depletion of primary CD4⁺ T cells by HIV-1 (37); in those experiments HIV-1 was amplified by prolonged cultivation with producer cells, possibly resulting in accumulation of less infectious viral variants. We avoid this problem by producing HIV-1 by transient transfection of 293 cells with viral DNA constructs, generating high titer, homogeneous HIV-1 able to infect at high multiplicity.

We have used the PLAP reporter virus system to demonstrate in vitro that HIV-1 directly induces apoptosis in the cells that it infects. The majority of HIV-induced cell death in vitro occurs in cells that have expressed the PLAP marker. This result is important in light of the continuing debate over whether HIV-1 directly kills infected cells or whether it predominantly induces death of uninfected bystander cells (6). Our finding that in the context of viral infection HIV-1 predominantly causes direct cell death argues against in vitro models in which isolated HIV-1 gene products, such as Tat or Env, induce killing of uninfected bystander cells (38–40). In addition, since HIV-1 is able to directly kill purified primary CD4⁺ T cells in the absence of other cell types, the proposed upregulation of FasL on infected macrophages and resulting parricide of uninfected

T cells (32) does not appear to be necessary to explain how HIV-1 induces T cell death. Our demonstration in vitro that HIV-1 directly kills infected cells is consistent with recent indications that HIV-infected patients with different degrees of immune dysfunction have similar rates of infected cell clearance, suggesting that direct cytopathicity of the virus determines the lifespan of the infected cell (1, 3, 41).

The finding that the majority of HIV-specific cell death occurs in cells that express viral gene products appears to contradict observations that in HIV-infected patients apoptosis is increased in CD8⁺ T cells (8, 13) and B lymphocytes (42) as well as in CD4⁺ T cells. We suggest that this increased apoptosis may be due to the generalized immune

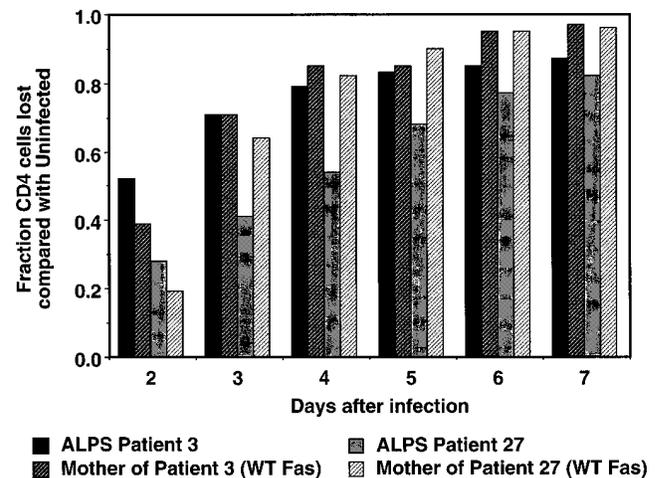


Figure 8. Fraction of CD4⁺ T cells lost after HIV-1 infection of PBMCs from patients with ALPS and normal controls. PBMCs from the patients characterized in Table 1 were infected with 200 ng/ml p24 of NL-PI and samples were stained daily for CD4 and PLAP. The fraction of CD4⁺ cells lost compared with uninfected is 1 – (percentage of CD4⁺ cells in the infected sample/percentage of CD4⁺ cells in the uninfected sample). Cells that were PLAP⁺ and CD4^{lo} were considered to be infected cells that had downmodulated CD4, and, thus, were included in the determination of CD4⁺ cells present in the sample.

activation which occurs in response to HIV-1 infection and, thus, may not be responsible for selective CD4⁺ T cell depletion. Other viral infections, such as with EBV, have also been associated with increased apoptosis of uninfected lymphocytes (43). In addition, a study that examined lymph nodes of patients with and without HIV infection concluded that the intensity of apoptosis correlated with the generalized state of activation of the lymph node (i.e., the degree of follicular hyperplasia and germinal center formation) and not with the stage of disease and viral burden (12). The relative role of increased apoptosis secondary to global immune activation in the pathogenesis of the selective CD4⁺ T cell depletion in AIDS is not clear.

Because of its critical role in regulating lymphocyte survival, we also examined the importance of the Fas pathway in HIV-induced cell death. This pathway has been proposed to mediate HIV-induced cell death based in part on observations that patients with HIV-1 infection have a higher percentage of Fas-expressing lymphocytes than uninfected people (17). However, our analysis demonstrates that HIV-1 infection does not directly lead to upregulation of Fas on the surface of the infected cell. Using the HIV-PLAP reporter virus system to distinguish infected from uninfected cells, we find that PLAP-positive primary T cells and CEM cells have the same level of surface Fas as PLAP-negative cells. The elevated percentage of PBMCs expressing Fas in patients with HIV-1 infection may again be a nonspecific manifestation of immune activation. Activation through the antigen receptor is known to induce expression of Fas in T lymphocytes (44–46), and this could explain why both CD8⁺ and CD4⁺ T cells from patients with HIV-1 have been found to have elevated levels of surface Fas (17).

Our findings also raise doubts as to whether the Fas pathway is necessary for HIV-1 to kill CD4⁺ T cells. Although both CEM and SupT1 cells are efficiently killed by HIV-1, only the CEM cells are sensitive to Fas-induced killing. SupT1 cells are resistant to apoptosis induced by anti-Fas antibody even though they express surface Fas. HIV-1 infection also leads to rapid depletion of CD4⁺ T cells from the PBMCs of patients who have a dominant-negative mutation in Fas that impairs Fas-mediated killing. Since the cells from these patients have some residual Fas function (Table 1), this finding does not rule out the possibility that this pathway is involved in HIV-induced cell death. However, we also find that HIV-1 kills CEM and Jurkat T cells in vitro despite the presence of a caspase inhibitor (z-VAD-fmk) that completely abrogates Fas-mediated apoptosis. In fact, we find, in agreement with a previous report (34), that the presence of z-VAD-fmk augments the spread of HIV-1 through Jurkat T cell cultures, which in turn leads to their more rapid death. The caspase inhibitor does decrease the percentage of TUNEL-positive CEM cells after HIV-1 infection, but it does not prevent virus-induced depletion of these cells. This finding is analogous to Bax-induced apoptosis, in which z-VAD-fmk inhibits DNA cleavage but does not block cell death (47). Similarly, HIV-1 may induce cell death both by triggering caspases that cause DNA cleavage as well as by activating other pathways that lead to the death of the cell. These unknown effectors allow HIV-1 to kill CD4⁺ T cells even when the Fas-induced caspases are inhibited. In conclusion, these results suggest that HIV-1 directly kills CD4⁺ T lymphocytes in vitro by a Fas-independent mechanism. The details of this mechanism will be an important topic for future study.

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References

1. Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and lymphocytes in HIV-1 infection. *Nature*. 373: 123–126.
2. Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emmini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature*. 373:117–122.
3. Perelson, A.S., A.U. Neumann, M. Markowitz, J.M. Leonard, and D.D. Ho. 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. *Science*. 271:1582–1586.
4. Mellors, J.W., C. Rinaldo, P. Gupta, R.M. White, J.A. Todd, and L.A. Kingsley. 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science*. 272: 1167–1170.
5. Levy, J.A. 1993. Pathogenesis of human immunodeficiency virus infection. *Microbiol. Rev.* 57:183–289.

6. Finkel, T.H. and N.K. Banda. 1994. Indirect mechanisms of HIV pathogenesis: how does HIV kill T cells? *Curr. Opin. Immunol.* 6:605–615.
7. Gougeon, M.L., A.G. Laurent-Crawford, A.G. Hovanessian, and L. Montagnier. 1993. Direct and indirect mechanisms mediating apoptosis during HIV infection: contribution to in vivo CD4 T cell depletion. *Semin. Immunol.* 5:187–194.
8. Meyaard, L., S.A. Otto, R.R. Jonker, M.J. Mijster, R.P. Keet, and F. Miedema. 1992. Programmed death of T cells in HIV-1 infection. *Science.* 257:217–219.
9. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J.C. Ameisen. 1992. Activation-induced death by apoptosis in CD4⁺ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J. Exp. Med.* 175:331–340.
10. Oyaizu, N., T.W. McCloskey, M. Coronese, N. Chirmule, V.S. Kalyanaraman, and S. Pahwa. 1993. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. *Blood.* 82:3392–3400.
11. Finkel, T.H., G. Tudor-Williams, N.K. Banda, M.F. Cotton, T. Curiel, C. Monks, T.W. Baba, R.M. Ruprecht, and A. Kupfer. 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat. Med.* 1:129–134.
12. Muro-Cacho, C.A., G. Pantaleo, and A.S. Fauci. 1995. Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. *J. Immunol.* 154:5555–5566.
13. Gougeon, M.L., S. Garcia, J. Heeney, R. Tschopp, H. Lecoecur, D. Guetard, V. Rame, C. Dauguet, and L. Montagnier. 1993. Programmed cell death in AIDS-related HIV and SIV infections. *AIDS Res. Hum. Retroviruses.* 9:553–963.
14. Gougeon, M.L., H. Lecoecur, F. Boudet, E. Ledru, S. Marzabal, S. Boullier, R. Roue, S. Nagata, and J. Heeney. 1997. Lack of chronic immune activation in HIV-infected chimpanzees correlates with the resistance of T cells to Fas/Apo-1 (CD95)-induced apoptosis and preservation of a T helper 1 phenotype. *J. Immunol.* 158:2964–2976.
15. Katsikis, P.D., E.S. Wunderlich, C.A. Smith, L.A. Herzenberg, and L.A. Herzenberg. 1995. Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals. *J. Exp. Med.* 181:2029–2036.
16. Nagata, S. 1997. Apoptosis by death factor. *Cell.* 88:355–365.
17. Debatin, K.M., A. Fahrig-Faissner, S. Enenkel-Stoodt, W. Kreuz, A. Benner, and P.H. Krammer. 1994. High expression of APO-1 (CD95) on T lymphocytes from human immunodeficiency virus-1-infected children. *Blood.* 83:3101–3103.
18. Mitra, D., M. Steiner, D.H. Lynch, L. Staiano-Coico, and J. Laurence. 1996. HIV-1 upregulates Fas ligand expression in CD4⁺ T cells in vitro and in vivo: association with Fas-mediated apoptosis and modulation by aurointricarboxylic acid. *Immunology.* 87:581–585.
19. Westendorp, M.O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K.M. Debatin, and P.H. Krammer. 1995. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature.* 375:497–500.
20. Foley, G.E., H. Lazarus, S. Farber, B.G. Uzman, B.A. Boone, and R.E. McCarthy. 1965. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer.* 18:522–529.
21. Nara, P.L. and P.J. Fischinger. 1988. Quantitative infectivity assay for HIV-1 and-2. *Nature.* 332:469–470.
22. Smith, S.D., M. Shatsky, P.S. Cohen, R. Warnke, M.P. Link, and B.E. Glader. 1984. Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. *Cancer Res.* 44:5657–5660.
23. Weiss, A., R.L. Wiskocil, and J.D. Stobo. 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. *J. Immunol.* 133:123–128.
24. Kayagaki, N., A. Kawasaki, T. Ebata, H. Ohmoto, S. Ikeda, S. Inoue, K. Yoshino, K. Okumura, and H. Yagita. 1995. Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med.* 182:1777–1783.
25. Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell.* 81:935–946.
26. Sneller, M.C., J. Wang, J.K. Dale, W. Strober, L.A. Middleton, Y. Choi, T.A. Fleisher, M.S. Lim, E.S. Jaffe, J.M. Puck, M.J. Lenardo, and S.E. Straus. 1997. Clinical, immunologic, and genetic features of an autoimmune lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. *Blood.* 89:1341–1348.
27. Adachi, A., H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M.A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* 59:284–291.
28. Feinberg, M.B., D. Baltimore, and A.D. Frankel. 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. USA.* 88:4045–4049.
29. Collins, K.C., B.K. Chen, S.A. Kalams, B.D. Walker, and D. Baltimore. 1998. HIV-1 Nef protein protects infected primary human cells from killing by cytotoxic T lymphocytes. *Nature.* 391:397–401.
30. Chen, B.K., R.T. Gandhi, and D. Baltimore. 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. *J. Virol.* 70:6044–6053.
31. Moore, J.P., J.A. McKeating, R.A. Weiss, and Q.J. Sattentau. 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science.* 250:1139–1142.
32. Badley, A.D., J.A. McElhinny, P.J. Leibson, D.H. Lynch, M.R. Alderson, and C.V. Paya. 1996. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J. Virol.* 70:199–206.
33. Margolin, N., S.A. Raybuck, K.P. Wilson, W. Chen, T. Fox, Y. Gu, and D.J. Livingston. 1997. Substrate and inhibitor specificity of interleukin-1 beta-converting enzyme and related caspases. *J. Biol. Chem.* 272:7223–7228.
34. Chinnaiyan, A.M., C. Woffendin, V.M. Dixit, and G.J. Nabel. 1997. The inhibition of pro-apoptotic ICE-like proteases enhances HIV replication. *Nat. Med.* 3:333–337.
35. Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science.* 268:1347–1349.
36. Puck, J.M., and M.C. Sneller. 1997. ALPS: an autoimmune

- human lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. *Semin. Immunol.* 9:77–84.
37. Yu, X., M.F. McLane, L. Ratner, W. O'Brien, R. Collman, M. Essex, and T.H. Lee. 1994. Killing of primary CD4⁺ T cells by non-syncytium-inducing macrophage-tropic human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA.* 91:10237–10241.
 38. Li, C.J., D.J. Friedman, C. Wang, V. Metelev, and A.B. Pardee. 1995. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science.* 268:429–431.
 39. Westendorp, M.O., M. Li-Weber, R.W. Frank, and P.H. Krammer. 1994. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *J. Virol.* 68:4177–4185.
 40. Banda, N.K., J. Bernier, D.K. Kurahara, R. Kurrle, N. Haigwood, R.P. Sekaly, and T.H. Finkel. 1992. Cross-linking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J. Exp. Med.* 176: 1099–1106.
 41. Feinberg, M.B., and A.R. McLean. 1997. AIDS: decline and fall of immune surveillance? *Curr. Biol.* 7:R136–140.
 42. Samuelsson, A., A. Sonnerborg, N. Heuts, J. Coster, and F. Chiodi. 1997. Progressive B cell apoptosis and expression of Fas ligand during human immunodeficiency virus type 1 infection. *AIDS Res. Hum. Retroviruses.* 13:1031–1038.
 43. Akbar, A.N., N. Borthwick, M. Salmon, W. Gombert, M. Bofill, N. Shamsadeen, D. Pilling, S. Pett, J.E. Grundy, and G. Janossy. 1993. The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infections. The role of apoptosis in T cell memory. *J. Exp. Med.* 178:427–438.
 44. Brunner, T., R.J. Mogil, D. LaFace, N.J. Yoo, A. Mahboubi, F. Echeverri, S.J. Martin, W.R. Force, D.H. Lynch, C.F. Ware, et al. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature.* 373:441–444.
 45. Dhein, J., H. Walczak, C. Baumler, K.M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature.* 373:438–441.
 46. Ju, S.T., D.J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D.H. Sherr, B.Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature.* 373:444–448.
 47. Xiang, J., D.T. Chao, and S.J. Korsmeyer. 1996. BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc. Natl. Acad. Sci. USA.* 93: 14559–14563.