

Mechanisms of HIV-associated lymphocyte apoptosis

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Infection with the human immunodeficiency virus (HIV) is associated with a progressive decrease in CD4 T-cell number and a consequent impairment in host immune defenses. Analysis of T cells from patients infected with HIV, or of T cells infected in vitro with HIV, demonstrates a significant fraction of both infected and uninfected cells dying by apoptosis. The many mechanisms that contribute to HIV-associated lymphocyte apoptosis include chronic immunologic

activation; gp120/160 ligation of the CD4 receptor; enhanced production of cytotoxic ligands or viral proteins by monocytes, macrophages, B cells, and CD8 T cells from HIV-infected patients that kill uninfected CD4 T cells; and direct infection of target cells by HIV, resulting in apoptosis. Although HIV infection results in T-cell apoptosis, under some circumstances HIV infection of resting T cells or macrophages does not result in apoptosis; this may be a critical step in the development of viral reservoirs. Recent

therapies for HIV effectively reduce lymphoid and peripheral T-cell apoptosis, reduce viral replication, and enhance cellular immune competence; however, they do not alter viral reservoirs. Further understanding the regulation of apoptosis in HIV disease is required to develop novel immune-based therapies aimed at modifying HIV-induced apoptosis to the benefit of patients infected with HIV. (*Blood*. 2000;96:2951-2964)

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Introduction

Patients infected with the human immunodeficiency virus (HIV) experience a progressive decline in CD4 T-cell number, resulting in immunodeficiency and increased susceptibility to opportunistic infections and malignancies. Although CD4 T-cell production is impaired in patients infected with HIV,¹ there is now overwhelming evidence that the primary basis of T-cell depletion in patients infected with HIV is increased apoptosis of CD4 and CD8 T cells. Since it was first proposed as a potential mechanism of CD4 T-cell depletion in patients infected with HIV,² apoptosis and its dysregulation after HIV infection has become a major focus of research. Although apoptosis may result from the effects of continuous immune activation that occurs in HIV-infected patients, considerable data indicate that there are additional distinct mechanisms by which HIV (and HIV-specific proteins) enhances apoptosis. Importantly, only a minor fraction of apoptotic lymphocytes are physically infected by HIV, indicating that the enhanced apoptosis of lymphocytes seen in infected persons results from mechanism(s) other than direct infection. Thus, understanding of the mechanisms of HIV-associated lymphocyte apoptosis may lead to new and more effective therapies for HIV disease and acquired immunodeficiency syndrome.

such as cytomegalovirus, cause enhanced apoptosis and lymphopenia. Chronic HIV infection provides a chronic immunologic stimulus; however, it may be unique in its ability to induce lymphocyte apoptosis through direct or indirect mechanism(s) that are distinct from immune activation alone. Although numerous pathogenic viruses have developed mechanisms to prevent apoptosis of host cells, no such antiapoptotic machinery is present in HIV. Indeed, HIV-encoded proteins may induce apoptosis of infected cells and uninfected cells (ie, paracrine death) through various mechanisms, some of which are defined; others are as yet unidentified (Table 1).

Overview of HIV-associated lymphocyte apoptosis

Chronic uncontrolled infections provide continuous antigenic stimulation that causes persistent immune activation and consequent apoptosis. This is the mechanism by which infectious diseases,

Overview of the regulation of apoptosis

Apoptosis is a highly regulated and coordinated cellular death process that is essential for cellular homeostasis. Alterations in the regulation of apoptosis may lead to malignancies,³ immunodeficiencies,⁴ and autoimmune phenomena.⁵

Apoptosis regulatory proteins

Many elements influence whether a cell will undergo apoptosis⁶ (Figure 1). Four cellular receptors induce apoptosis after ligation; they are the Fas receptor,⁷ p55 tumor necrosis factor (TNF) receptor,⁸ and TRAIL/APO 2-L (TNF-related apoptosis-inducing ligand) receptors 1 and 2.⁹ Fas Ligand (FasL), TNF, and TRAIL/APO 2-L, respectively, bind these receptors to initiate apoptosis. In the case of FasL and TNF, membrane-associated proteins may be cleaved by the action of matrix metalloproteases to release soluble

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Table 1. Proposed mechanisms of HIV-associated lymphocyte apoptosis

Effector	Proposed mechanism	Target cell
HIV Tat	Enhanced Fas sensitivity Enhanced Fas ligand production	Infected + uninfected cells
HIV Nef	Activation Enhanced FasL production ? Binding to unidentified receptor	Infected + uninfected cells
HIV vpr	Cell cycle arrest Direct effect on mitochondrial permeability	Infected + uninfected cells
HIV protease	Cleavage of host structural proteins	Infected cells
Activation-induced cell death	HIV-associated activation Increased TRAIL/APO-2L, FasL, or both	Uninfected cells
gp 120/160	Inappropriate activation after CD4 ligation Enhanced Fas susceptibility/FasL production ? Nonapoptotic death by CXCR4	Uninfected cells
Autologous cell-mediated killing	Enhanced production of cytotoxic ligands by HIV-infected cells	Uninfected cells

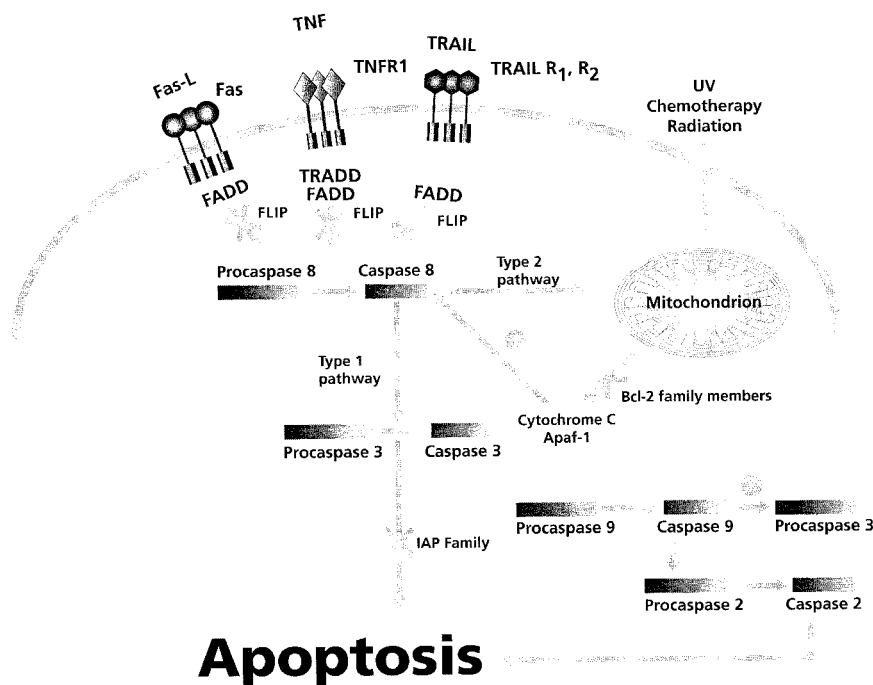
ligands that maintain their biologic activity.¹⁰⁻¹² It is unknown whether TRAIL/APO 2-L exists as a soluble molecule. Ligation of these death receptors recruits the adaptor proteins FADD (Fas-associated death domain)¹³⁻¹⁵ TRADD (TNF receptor-associated death domain), or both,^{16,17} which sequentially activate a family of cysteine proteases that cleave at aspartate residues (cysteine-dependent, aspartate-specific protease), or caspases. Caspases are synthesized as inactive zymogens and become activated after proteolytic removal of a terminal prodomain.¹⁸⁻²⁰ Fourteen mammalian caspase family members have been identified, each with varying involvements in the regulation of apoptosis. For example, caspase 8 (FLICE)²¹⁻²³ and caspase 3 (CPP32)²⁴⁻²⁶ are involved in apoptosis mediated by Fas, p55 TNF receptor, and TRAIL/APO 2-L receptor ligation. Activated caspases catalyze the cleavage of other caspases, which, in turn, activate various cellular proteases and endonucleases that cleave host cell structural and regulatory proteins and host nuclear DNA,²⁷ ultimately causing the cell to undergo the morphologic and biochemical changes that are characteristic of apoptosis.²⁸

In addition to receptor-mediated apoptosis, other stimuli (eg,

chemotherapy, ultraviolet radiation, and ionizing radiation) induce changes in mitochondria that include opening of the permeability transition pore and loss of mitochondrial inner transmembrane potential, which allows the release of apoptosis regulatory proteins (including cytochrome c, Apaf-1, and caspase 9)²⁹⁻³² that initiate further caspase activation, ultimately leading to apoptosis. Although classical Fas-induced apoptosis (see above) involves direct caspase activation without mitochondrial involvement (type 1), in certain cell types Fas-induced apoptosis may also require mitochondrial activation (type 2).⁷

Antiapoptosis regulatory molecules

In addition to the proteins involved in mediating apoptosis described above, other proteins act to inhibit apoptosis. One such family of regulatory proteins is cellular FLICE-like inhibitory protein (c-FLIP), which inhibits apoptosis by binding to FADD and thus prevents the activation of caspase 8.^{33,34} The inhibitor of apoptosis proteins (IAP) family, including HIAP, XIAP, and others, acts by inhibiting the activation of caspase 3 and possibly other caspases.³⁵⁻³⁷

**Figure 1. Schematic overview of the regulation of apoptosis.**

Bcl2 and related family members,^{38,39} including BclXS, BclXL, Bad, and Bax, influence apoptosis by regulating the intracellular signals that induce apoptosis. Some family members (Bcl2) are antiapoptotic, whereas others (Bax) are proapoptotic. Cells that contain a predominance of proapoptotic Bcl2 family molecules promote apoptosis, and cells with a predominance of antiapoptotic Bcl2 family proteins are relatively apoptosis resistant. Bcl2 consistently blocks apoptosis induced by anticancer and nitric oxide,⁴¹ and these effects may result from the inhibition of calcineurin activation,^{40,42,43} NFAT activation,⁴⁰ or transcription of Fas ligand.⁴⁰ Conversely, reports on the effects of Bcl2 on Fas-induced apoptosis are conflicting: Bcl2 may variably inhibit⁴⁴ or not inhibit⁴⁵ Fas-induced death. Because members of the Bcl2 family are principally localized within mitochondria, their influence may be greatest in forms of apoptosis that are associated with mitochondrial activation. Thus, Bcl2 overexpression may not inhibit death receptor-initiated apoptosis in cells with a type 1 (mitochondria-independent) Fas pathway, but it may block Fas-initiated death in type 2 (mitochondria-dependent) cells.⁴⁶

Physiologic T-cell apoptosis

Healthy subjects orchestrate a physiologic immune response to a foreign antigen by T-cell activation and proliferation. If this T-cell proliferative response were not regulated, each encounter with a foreign antigen would lead to unending T-cell expansion. Down-regulation of T-cell proliferation occurs by an apoptotic program that is initiated after activation⁴⁷ (Figure 2, top). After T-cell activation, c-FLIP expression is reduced, and the cells become susceptible to Fas ligation and to caspase 8-mediated apoptosis.³³ Exposure to a second activation stimulus (eg, CD3 stimulation in the absence of CD28 costimulation) promotes de novo production of FasL, leading to both autocrine and paracrine Fas/FasL-mediated T-cell apoptosis.⁴⁸⁻⁵² It is important to note that not all physiologic T-cell apoptosis is regulated solely by Fas/FasL interactions; Fas-deficient cells maintain T-cell receptor (CD3)-induced apoptosis that is inhibited by TNF antagonists.^{53,54}

Measurement of apoptosis

As noted, apoptosis is characterized by distinct morphologic and biochemical changes, including chromatin condensation, shrinkage of the cytoplasm, membrane blebbing, and formation of apoptotic bodies. Apoptosis is a complex and sequential process, and, as such, some assays detect changes that occur early, whereas other assays detect later events. The most common assays used in the detection of apoptosis are listed in Table 2⁵⁵⁻⁹⁹; many have been used to evaluate apoptosis in patients infected with HIV. In a direct comparison of the relative benefits of these assays for use in the evaluation of apoptosis of HIV-infected patients, TUNEL staining was the most specific and therefore may be the most accurate assay to use in this patient population.¹⁰⁰

HIV-mediated alterations in molecules that regulate the apoptotic process

Cells obtained from HIV-infected patients and cells infected with HIV in vitro show changes in the regulation of Fas and Fas ligand (reviewed in¹⁰¹). Acute HIV infection of the promonocytic cell line U937 is associated with viral replication-dependent apoptosis¹⁰² that is characterized by the increased membrane expression of Fas¹⁰² and FasL,¹⁰² by the down-regulation of antiapoptotic proteins Bcl2 and BclXL,^{103,104} and by a concomitant increase in proapoptotic BclXS and Bax.^{103,104} The hypothesis that Fas/FasL interactions may be responsible for HIV-induced apoptosis is supported by the observation that soluble Fas receptor decoys block HIV-associated death in U937 cells.¹⁰² This is in marked contrast to the effects of acute HIV infection of T-cell lines, which is Fas independent despite increased Fas expression.¹⁰⁵⁻¹⁰⁸ Interestingly, though T cells from HIV-infected patients have altered expression of Bcl2, the expression of Bax, BclXL, and BclXS does not differ from that of uninfected controls.¹⁰⁹

T cells from HIV-infected patients exhibit both increased Fas receptor expression and enhanced susceptibility to Fas-mediated death.¹¹⁰⁻¹¹⁷ FasL is elevated in peripheral blood mononuclear cells

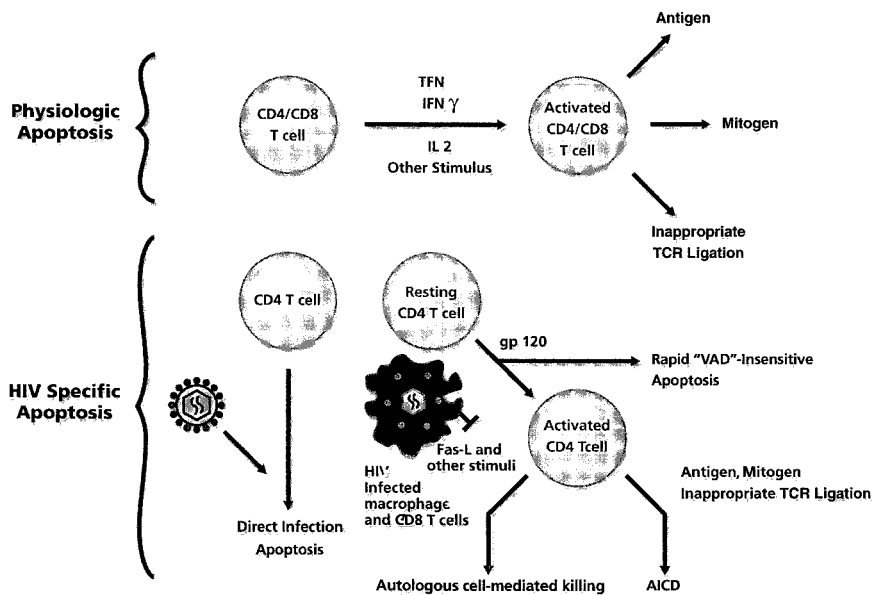


Figure 2. T-cell apoptosis. Mechanisms of physiologic T-cell apoptosis (top) and mechanisms of increased T-cell apoptosis associated with HIV infection (bottom). VAD refers to the pan-caspase inhibitor 2-VAD-Fmk.

Table 2. Assays of apoptosis and their relationship to events of apoptosis

Event	Assays	Detection
Changes in nuclear morphology: Chromatin condensation, segmentation, and formation of apoptotic bodies	DNA stains (DAPI)	Microscopy
Changes in membrane permeability	Vital dyes (PI) Permeable DNA stains: (DAPI, Hoechst 33258)	Microscopy Flow cytometry with simultaneous size determination
Changes in membrane composition: Externalization of phosphatidylserine	Annexin V binding	Flow cytometry Confocal and epifluorescence microscopy
Cleavage of nuclear proteins	Poly ADP ribose polymerase	Western blot
Mitochondrial function and integrity		
Changes in permeability transition ($\Delta\Psi_m$)	Vital dyes (DiOC ₆ , JC-1)	Flow cytometry
Accessibility to mitochondrial antigens	Apo 2,7 antibody	Flow cytometry
Release of cytochrome-c	Anti-cytochrome-c antibody	Flow cytometry, Western blot
Production of free radicals	DPPP/dihydroethidium	Flow cytometry
Caspase activation		
Detection of caspase cleavage product	Known caspase substrates; PARP, caspase 3, caspase 8, DNA-PK, PK-C	Western blot
Detection of active caspase	Anti-activated caspase 3 antibody	Western blot
Detection of caspase activity	Cleavage of fluorescent or colorimetric substrate(s)	Fluorometer, plate reader
DNA degradation		
Large fragments	DNA stains (EtBr, SYBR green) DNA stains (EtBr) Radioactivity (C ¹⁴)	Pulse-field gel electrophoresis Comet Detection of radio-labeled DNA by filter binding
Small fragments	DNA stains (EtBr) Radioactivity (C ¹⁴)	Agarose gel electrophoresis (DNA ladder) Detection of radio-labeled DNA by filter binding
Sub-G1 peak detection	DNA stains (PI, Hoechst)	Flow cytometry
Detection of DNA strand breaks	Terminal dUTP nick end labeling (TUNEL)	In situ hybridization Flow cytometry
	Ligation-mediated polymerase chain reaction	Agarose or polyacrylamide gel electrophoresis

(which contain monocytes)^{114,118,119} from HIV-infected patients, and the plasma level of soluble FasL is increased in HIV-positive patients and correlates with HIV RNA burden.¹²⁰ The demonstrated increases in Fas expression, Fas susceptibility, and Fas ligand expression suggest that these molecules may be important in some forms (see below) of HIV-induced cell death, though direct T-cell killing is independent of Fas.¹⁰⁵⁻¹⁰⁸

Intracellular levels of c-FLIP in resting cells from HIV-negative patients decrease after activation, resulting in enhanced sensitivity to Fas-mediated apoptosis.³³ This observation, coupled with observations that apoptosis in patients infected with HIV occurs in activated CD45 RO⁺, HLA-DR⁺, CD28⁻ cells,¹²¹⁻¹²⁴ suggests that decreased c-FLIP expression may be responsible for the enhanced susceptibility of cells from these patients to apoptosis. However, c-FLIP expression in bulk peripheral blood lymphocytes (PBL) or in purified CD4 or CD8 T cells from HIV-infected patients does not differ from that of HIV-negative patients.¹²⁵ It remains possible that defined cellular subsets may have reduced levels of c-FLIP that are missed in bulk analysis.

The regulation of TNF, TNF receptors, or both is fundamentally altered in HIV-infected patients.¹²⁶⁻¹²⁹ Both cognate receptors for TNF, p75 TNFR and p55 TNFR,¹³⁰ are expressed in a variety of cell types. However, only ligation of the p55 TNF receptor leads to apoptosis.^{16,17,53,131,132} Elevated serum TNF levels are seen in symptomatic HIV-infected patients¹²⁶⁻¹²⁹ but not in asymptomatic patients.^{133,134} Furthermore, (1) HIV infection of lymphocytes or monocytes results in TNF production,^{135,136} and (2) TNF activates the transcription factor NF κ B, which, in turn, activates HIV transcription,^{137,138} initiating an autocrine loop that results in high levels of TNF production and increased levels of HIV transcription. In addition, elevated serum levels of soluble p75 TNFR are predictive of HIV disease progression, independent of other

immunologic or virologic prognostic markers.¹³⁹ Although little is known of the ability of TNF to induce apoptosis in HIV-infected cells, HIV-infected macrophage-mediated killing of uninfected CD4 T-cell blasts (see below) can be partially reduced by the administration of soluble TNFR decoys,⁴⁰ and TNF may contribute to apoptosis induced by gp120-mediated cross-linking of CD4¹³⁸ (see below). The potential role of TNF as a mediator of HIV disease has prompted trials of anti-TNF therapy to retard HIV disease progression. Thalidomide reduces TNF secretion,¹⁴¹ and pentoxifylline reduces TNF mRNA half-life.¹³⁸ However, clinical trials with each of these agents have consistently failed to show improvement in either immunologic or virologic outcomes.¹⁴²⁻¹⁴⁴ Other studies using soluble TNF antagonists have had similarly disappointing results.¹⁴⁵

There is also relatively little information concerning the potential role of TRAIL/APO 2-L in apoptosis in HIV-infected patients. Current data suggest that TRAIL/APO 2-L can bind to 1 of 5 receptors, TRAIL/APO 2-L-R1, TRAIL/APO 2-L-R2, TRAIL/APO 2-L-R3, TRAIL/APO 2-L-R4,¹⁴⁶ and osteoprotegerin.¹⁴⁷ Binding of TRAIL/APO 2-L to TRAIL/APO 2-L-R1 or R2 transduces apoptotic signals, whereas binding to TRAIL/APO 2-L-R3 or TRAIL/APO 2-L-R4 does not. The effects of TRAIL/APO 2-L binding to osteoprotegerin are unknown. Although it has been suggested that the relative expression of TRAIL/APO 2-L-R3 and TRAIL/APO 2-L-R4 to TRAIL/APO 2-L-R1 and TRAIL/APO 2-L-R2 influences susceptibility to TRAIL/APO 2-L-mediated killing,¹⁴⁸⁻¹⁵⁰ recent studies do not support this hypothesis. Rather, intracellular levels of c-FLIP may correlate with the sensitivity or resistance to TRAIL/APO 2-L-induced apoptosis in target cells.^{151,152}

Although no studies to date have evaluated the relative expression of TRAIL/APO 2-L receptor(s) or TRAIL/APO 2-L expression in patients infected with HIV, it has been observed that (in

contrast to cells from HIV-uninfected patients) cells from HIV-infected patients are susceptible to TRAIL/APO 2-L-mediated killing.¹⁵³ This finding, together with the fact that activation-induced cell death in patients with HIV infection may be partially inhibited using antagonistic TRAIL/APO 2-L-specific antibodies,¹⁵⁴ suggests that TRAIL/APO 2-L and TRAIL/APO 2-L receptor dysfunction may contribute to HIV pathogenesis.

Apoptosis of uninfected and infected T cells induced by HIV proteins

HIV infection is associated with enhanced apoptosis in CD4 T cells infected by HIV and in uninfected T cells. In this section we review proposed mechanisms of CD4 T-cell apoptosis, focusing on whether the proposed mechanisms affect infected cells, uninfected cells, or both.

Gp120-induced apoptosis

Gp120 is an HIV viral envelope glycoprotein that can bind to and cross-link the CD4 receptor and the chemokine coreceptors. Cross-linking of CD4 T cells by gp120 causes the induction of enhanced susceptibility to Fas-mediated killing.¹⁵⁵ In previously activated cells, gp120 cross-linking results in apoptosis¹⁵⁶ (possibly mediated by IFN- γ , TNF, or both¹⁵⁷), down-regulation of Bcl-2 expression,¹⁵⁸ and activation of caspase 3.¹⁵⁹ The apoptotic response to gp 120 is almost completely inhibited by soluble CD4 and by anti-gp120 antibodies.¹⁶⁰ Further evidence for the specificity of this interaction is provided by the observation that a point mutation in the V3 loop of gp120 inhibits the induction of apoptosis in CD4 T cells.¹⁶¹ Finally, this interaction must also involve CD4 signaling because deletion or mutation of the intracytoplasmic portion of CD4 also abrogates the apoptotic response.^{162,163}

Most of the experiments involving gp120-induced apoptosis evaluate apoptosis that occurs after several days. However, recent reports¹⁶⁴⁻¹⁶⁶ show that gp120 cross-linking of CD4 and CXCR4 chemokine receptor results in nonapoptotic death within several hours of stimulation by a mechanism that appears to be independent of p56LCK,¹⁶⁴ g-protein-coupled signaling,¹⁶⁶ Fas, or TNF receptors.¹⁶⁵ The administration of CXCR4 antagonists blocks this apoptosis response to the HIV envelope.¹⁶⁷

It is appealing to invoke gp120 as a responsible mechanism for CD4 T-cell death in patients infected with HIV because it does *not* depend on the infection of all cells that become apoptotic and it does not require the presence of viable virions. Circulating immune complexes and replication-incompetent viruses that contain gp120 can induce death in a similar manner.¹⁶⁸⁻¹⁷⁰

Apoptosis induced by other HIV proteins

Transfection experiments demonstrate that the ectopic expression of HIV Tat induces apoptosis. Further, gp120/160-deleted HIV maintains its ability to induce infected cell apoptosis, potentially because of the Tat-directed up-regulation of caspase 8¹⁷¹ or because of Fas ligand.¹⁷² Importantly, Tat has also been implicated as an inducer of apoptosis in uninfected T cells, potentially by Fas-dependent mechanisms, superoxide dismutase inhibition, or activation of cyclin-dependent kinases.¹⁷³⁻¹⁷⁵ The ability of Tat to induce uninfected cell death has also been demonstrated *in vitro* for neurons, lymphocytes, and CD4 T-cell lines. Its clinical relevance

is suggested by observations that Tat is readily secreted by infected cells¹⁷⁶ and cellular or humoral immunity to Tat may have protective effects against HIV disease progression.¹⁷⁷

Because Nef is essential for viral pathogenicity, HIV-encoded Nef has been suggested as a potential mediator of apoptosis.¹⁷⁸ This proposal is supported by the following findings: (1) human infection with naturally occurring Nef deletion mutants leads to less rapid CD4 T-cell depletion (compared to strains with Nef),^{179,180} though the differences may be related to the decreased efficiency of viral replication¹⁸¹⁻¹⁸⁴; (2) Nef synergistically enhances the activating effects of T-cell receptor ligation,¹⁸⁵⁻¹⁸⁷ though this enhancement may be stimulus dependent¹⁸⁷⁻¹⁹¹; (3) Nef-expressing T cells coexpress FasL,¹⁹² as do infected T cells from SIV-infected macaques but not T cells from macaques infected with similar strains of SIV that contain mutations within the Nef gene¹⁹³ (the mechanism(s) by which Nef results in activation and FasL production remain unclear, yet mutational analysis indicates that the carboxy terminus of the CD4 receptor associates with both Nef and p56LCK¹⁹⁴); lastly, (4) Nef may exert an apoptotic effect on uninfected CD4 T cells by binding to unidentified receptor(s),¹⁹⁵ resulting in Fas-independent death.¹⁹⁶ In this regard, Nef may induce apoptosis of infected and uninfected cells.

HIV-encoded vpr also has the ability to induce apoptosis through transfection and exogenous treatment. Proposed mechanisms include the induction of G2/M cell cycle arrest^{197,198} and a direct effect on mitochondrial permeability.¹⁹⁹ Vpr also influences viral LTR transcription,^{200,201} cellular activation, and differentiation,^{202,203} suggesting a role in the development of HIV reservoirs. The seeming paradox of inducing apoptosis while promoting viral reservoirs is elucidated by data that vpr may, in certain situations, inhibit apoptosis.²⁰⁴⁻²⁰⁶ The observation that virion-associated vpr acts as an immediate early viral protein to induce apoptosis²⁰⁷ is inconsistent with the apparent requirement that viral replication must occur before the onset of infected T-cell apoptosis. In addition, the finding that direct HIV-induced T-cell apoptosis occurs in all phases of the cell cycle¹⁰⁷ brings into question the role of vpr in direct infection apoptosis. Vpr is more likely to be involved in regulation of latency, control of replication, and resistance to antiretroviral agents.²⁰⁸

Knowledge that HIV-encoded protease is a cytotoxic protein that leads to apoptosis in human and bacterial cells after transfection²⁰⁹⁻²¹² has been exploited as a method of screening compounds for potential HIV protease inhibitory activity.²¹³ However, the relevance of HIV protease to HIV-infected T-cell death *in vitro* and *in vivo* is unknown. HIV protease expression (by Western blotting) correlates with the presence of apoptosis *in vitro* and *in vivo*.²¹⁴ Further studies demonstrate that HIV protease directly cleaves caspase 8²¹⁴ and modifies cellular susceptibility to apoptosis by virtue of proteolytic degradation of the antiapoptotic protein Bcl2.²¹⁵ Together these findings indicate that HIV protease may also play a role in the death of HIV-infected T cells. There are no data to suggest that HIV protease may influence the death of uninfected cells.

Indirect mechanisms of HIV-associated apoptosis

In addition to apoptosis induced directly by HIV proteins, HIV infection may induce T-cell apoptosis through indirect mechanisms, including activation-induced cell death and autologous infected cell-mediated killing. The indirect mechanisms of T-cell death mediate the deaths principally of uninfected T cells (Figure 2).

Activation-induced cell death

T cells obtained from HIV-infected patients undergo spontaneous apoptosis at a greater rate than cells from HIV-seronegative subjects.^{111,216-219} Furthermore, the *ex vivo* activation of CD4 T cells from HIV-infected patients (using a variety of stimuli) consistently enhances apoptosis compared with cells from uninfected subjects.^{111,122,124,217-219} This phenomenon, termed activation-induced cell death (AICD), occurs only in cells that have been previously activated,^{49,52} and it may represent the *in vitro* model of the effects of repeated antigenic stimulation.^{49,52,220,221} Naive peripheral blood T cells from HIV-negative patients, when stimulated through the T-cell receptor, undergo proliferation, cytokine secretion,²²²⁻²²⁶ and the development of susceptibility to apoptosis induced by Fas ligation.^{48,52,220,221} Subsequent stimulation results in AICD by the *de novo* production of FasL, which mediates autocrine and paracrine apoptosis.^{48,52,220,221} Both *in vivo* and *in vitro*, HIV infection is associated with an activated T-cell phenotype,²²⁷⁻²³¹ increased expression of Fas, enhanced susceptibility to Fas-mediated killing,^{111,115,116,174,232,233} and increased T-cell-expressed FasL after T-cell receptor stimulation,^{114,234} suggesting a role for Fas/FasL in HIV-associated AICD. Findings that retinoic acid inhibits FasL expression and resultant apoptosis *in vitro*²²¹ and that retinoic acid therapy in HIV-infected patients reduces CD4 T-cell depletion²³⁵ support a causal role for Fas/FasL interactions in T-cell death induced by HIV.

Elevated levels of apoptosis are seen after mitogenic stimulation or TCR cross-linking of PBL from HIV-seropositive patients.^{124,154,216-218,227,236,237} The molecular signals responsible for apoptosis in these patients are unclear, but the administration of Fas, TRAIL/APO 2-L, or TNF antagonists reduces AICD in cells from patients infected with HIV,^{154,237} suggesting that all 3 signals—Fas, TNF, and TRAIL/APO 2-L—may be involved.

Autologous infected cell-mediated killing

Macrophages,^{102,140} monocytes,^{213,238,239} peripheral blood mononuclear cells,²⁴⁰ CD4 T cells,²⁴¹ and CD8 T cells²⁴² derived from HIV-infected patients may induce the death of uninfected CD4 T lymphocytes. Autologous infected cell-mediated killing may involve gp120 interactions (see below), the Fas/FasL system, or both. Macrophages express basal levels of FasL that are significantly up-regulated after infection with HIV,¹⁰² and monocytes from HIV-infected patients have significantly increased FasL expression compared with monocytes from HIV-negative controls.²⁴³ HIV-infected macrophages (and, to a lesser extent, uninfected macrophages) have been shown to kill Fas-sensitive T-cell targets¹⁰² in a major histocompatibility complex-unrestricted and Fas/TNF-dependent manner.¹⁴⁰ Macrophage-mediated killing appears to be selective for uninfected T cells,²⁴⁴ as opposed to the mechanisms involved in infected T-cell death described above. Macrophage-mediated CD4 T-cell apoptosis has implications *in vivo* because levels of tissue apoptosis directly correlate with levels of macrophage-associated FasL.²⁴⁵ Thus, FasL may be the mediator of uninfected CD4 T-cell death by monocytes, macrophages,^{213,239} and CD8 T cells.^{242,246}

CD8 T-cell apoptosis

Although levels of CD8 T-cell apoptosis are consistently elevated in patients infected with HIV (whether this occurs spontaneously, in response to activation stimuli [AICD] or after coinubation with

autologous infected cells^{102,122-124,154,216,227,240,244,247-249}), the CD8 T-cell count is not significantly reduced in these patients. This apparent paradox may be resolved by observations in SIV-infected primates receiving total body irradiation, in which it was observed that CD8 T-cell recovery significantly precedes the recovery of CD4 T cells.²⁵⁰ A similar delay in CD4 repopulation is also seen in humans receiving high-dose chemotherapy.^{251,252} These data have several potential interpretations, yet they demonstrate that CD8 T-cell rebound occurs earlier than CD4 T-cell rebound after PBL depletion. In HIV-infected patients, it may therefore be expected that if rates of CD4 and CD8 T-cell loss were equal, the steady state CD8 number may be greater than the CD4 number because of the quicker recovery times. Further, HIV-associated apoptosis may lead to greater absolute numbers of CD4 T-cell apoptosis than CD8 T-cell apoptosis, because direct infection and gp120-mediated apoptosis selectively target cells that express CD4, whereas gp120 does not bind to (and thus cross-link) CD8. Nonetheless, it has recently been proposed that macrophage-associated gp120 may mediate CD8 T-cell apoptosis through interaction with CXCR4.²⁵³ Alternative potential mechanisms may also be involved (see below).

The fact that CD8 T cells from patients with HIV infection are more activated than are similar cells from HIV-uninfected persons^{227,254-257} suggests that the enhanced state of susceptibility to apoptosis is present in CD8 and in CD4 T cells and that CD8 T cells would be expected to die by apoptosis after exposure to another activation stimulus or with a preformed apoptosis-inducing ligand (eg, macrophage-associated FasL¹⁴⁰). Furthermore, CD8 T cells express the CD4 receptor after activation, thereby rendering them susceptible to direct infection by the virus.^{258,259} In addition, the enhanced expression of CD4 antigen on CD8 T cells would be expected to render these double-positive cells more susceptible to the effects of gp120 cross-linking and subsequent apoptosis. Despite the several possible pathways that may be responsible for CD8 T-cell apoptosis in HIV-infected patients, chronic antigenic stimulation most likely contributes to CD8 T-cell apoptosis. The relative role of direct infection leading to CD8 T cell death remains untested.

Associations of apoptosis with HIV disease progression and response to therapy

Clinical studies in patients infected with HIV measure spontaneous apoptosis, Fas ligation-induced apoptosis, and apoptosis occurring in response to mitogenic activation or TCR cross-linking. In relation to the various mechanisms of apoptosis outlined above, spontaneous apoptosis may reflect infected cell apoptosis or gp120-induced apoptosis; Fas-induced apoptosis may reflect autologous cell-mediated killing of uninfected bystander cells or AICD; apoptosis in response to mitogen or CD3 ligation reflects AICD. In studies in which tissue apoptosis has been measured,²⁶⁰⁻²⁶² few apoptotic cells are found to be physically infected by virus,²⁶⁰ suggesting that tissue apoptosis reflects the killing of uninfected cells by gp120-induced or autologous cell-mediated killing of uninfected cells.

The magnitude of apoptosis observed in HIV-infected patients correlates well with the stage of HIV disease in longitudinal and cross-sectional analyses.²⁶³⁻²⁶⁵ Spontaneous apoptosis is greater in HIV-infected patients with progressive disease than in uninfected patients.^{266,267} In addition, spontaneous apoptosis in patients with long-term nonprogressive HIV infection are similar to those of

HIV-negative patients.²⁶⁸ Thus, the rate of apoptosis correlates inversely with CD4 T-cell depletion. Because recent advances in HIV therapy have resulted in sustained increases in CD4 T cell number, if enhanced apoptosis causes CD4 T-cell depletion then apoptosis must decrease during therapy.

Numerous studies have shown that apoptosis in lymph nodes, rectal mucosa, and PBL subsets from patients infected with HIV decreases dramatically in response to protease inhibitor-based HIV treatment.^{125,269-274} This effect is seen for spontaneous apoptosis, apoptosis in response to T-cell receptor ligation, apoptosis in response to mitogenic stimulation, and apoptosis in response to Fas receptor ligation.^{125,271,272,274} The decrease in apoptosis is rapid and is seen as early as 4 days after protease inhibitor therapy is initiated¹²⁵; it occurs in all patients within 14 days.²⁷¹⁻²⁷⁴ Because the decrease precedes significant changes in viral replication, it has been suggested that protease inhibitors may be antiapoptotic,^{275,276} possibly by virtue of inhibiting the activity of effector proteases involved in apoptosis.

Effects of cytokines on HIV-associated apoptosis

One hallmark of infection with HIV is progressive T-helper-cell dysfunction. As HIV disease progresses, the balance of Th1 cytokines (IL-2 and IFN- γ) that enhance cellular immunity eventually shift to a Th2 cytokine profile (IL-4, IL-5, IL-6, and IL-10) that promotes humoral responses. The suggestion that helper cell dysfunction is central to the pathogenesis of HIV infection²⁷⁷ is supported by observations²⁷⁸⁻²⁸⁰ that the Th1-promoting cytokine IL-12, or the use of antagonistic antibodies specific for the Th2 cytokines IL-4 and IL-10, restores T-cell proliferative responses to recall antigens in HIV-infected patients. Because of the pervasive effects of cytokines in modulating apoptosis and apoptosis susceptibility, cytokine-based therapy may result in changes in apoptosis. Indeed, it has been reported that resistance to apoptosis in HIV and SIV infection is associated with a predominance of a Th1 phenotype,²⁸¹ arguing that chronic immune activation and a Th2 shift may promote apoptosis. Consistent with this hypothesis, spontaneous apoptosis in cells from HIV-infected patients is blocked by the administration of IL-12, IFN- γ , anti-IL-4, anti-IL-10, and antilymphotoxin, but not by anti-IL-12 therapy.²⁸² Furthermore, IL-12 protects against the enhanced sensitivity to Fas-mediated apoptosis and enhanced sensitivity to AICD seen in HIV-infected patients.²¹⁹

Apoptosis in patients with HIV infection is modulated by exogenous cytokines or cytokine antagonists that promote a Th1 helper cell phenotype and by cytokines that promote T-cell proliferation. IL-2 therapy in patients infected with HIV results in increased CD4 T-cell numbers unrelated to decreases in viral replication. Thus, IL-2 may modulate CD4 T-cell survival directly, possibly through an antiapoptotic mechanism, a hypothesis supported by *in vitro* studies in which clinically relevant concentrations of IL-2 significantly reduce spontaneous apoptosis in CD4+ T cells from HIV-infected patients but not from HIV-uninfected patients.²⁸³

IL-15 is a T-cell growth factor whose effects include T-cell proliferation, enhanced cytotoxicity of T cells and natural killer cells, B-cell proliferation, and immunoglobulin secretion.²⁸⁴ The effects of IL-15 on T cells are related to its ability to bind to a trimeric receptor consisting of the IL-15R α subunit and the shared IL-2R β and IL-2R γ subunits. Thus, many physiologic effects of IL-15 parallel those of IL-2. In addition, the incubation of peripheral

blood mononuclear cells from HIV-infected patients with IL-15 results in enhanced production of the Th1 cytokine IFN- γ ,²⁸⁵ CD8 T-cell activation, increased numbers of CD8 T cells,²⁸⁶ enhanced lymphoproliferative responses,²⁸⁷ and decreased spontaneous T-cell apoptosis,²⁸⁸ possibly mediated by increases in Bcl-2 expression.²⁸⁸ It is significant that although IL-2 increases HIV replication, IL-15 does not share this effect.^{287,288} Finally, IL-16 may have therapeutic implications for HIV-associated apoptosis.

IL-16 is a chemoattractant²⁸⁹ that inhibits lymphocyte activation²⁹⁰ and may also inhibit HIV replication.²⁹¹ Possibly because of its antiproliferative effects, IL-16 treatment *in vitro* decreases levels of anti-CD3- or anti-Fas-induced apoptosis in lymphocytes from HIV-infected patients.²⁹² However, the inhibitory effects of IL-16 on apoptosis are not seen in the context of spontaneous apoptosis.²⁹²

T-cell regeneration in response to therapy

The institution of highly active antiretroviral therapy (HAART) has witnessed a major impact on immune reconstitution: sustained increases in numbers of circulating CD4 T cells associated with a rapid drop in plasma viral RNA levels. The mechanisms proposed to explain the increase in numbers of CD4 T cells include cellular redistribution from lymphoid tissue,²⁹³ cellular proliferation of the peripheral T-cell pool,²⁹⁴ new T-cell synthesis from a thymic source,^{295,296} and reduced levels of apoptosis (see above). We have previously demonstrated that HAART therapy rapidly reduces apoptosis in lymphoid tissue²⁷³ and significantly decreases apoptosis in PBL.^{125,273} The decrease in apoptosis occurs before significant changes on plasma viral RNA levels and when patients are receiving only the protease inhibitor component of the HAART regimen.¹²⁵ This finding has led to the proposal that protease inhibitors have an effect on immune reconstitution that is independent of their ability to suppress HIV replication.^{275,276,297} *In vitro* therapy with protease inhibitors has been shown to reduce the expression of selected caspases in treated cells and to reduce the rate of caspase 3 activation.^{275,297} Additional evidence for an indirect protease inhibitor effect comes from studies that demonstrate sustained CD4 rises in patients who experience virologic failure²⁹⁸⁻³⁰¹ and who are receiving protease inhibitor-containing HAART regimens.

The early rise (2 weeks) in CD4 cells attributable to a reduction in apoptosis appears to be followed by a phase of CD4 cell increase due to cellular redistribution and proliferation of predominately memory CD4 T cells³⁰²⁻³⁰⁶ (Figure 3). A possible third phase

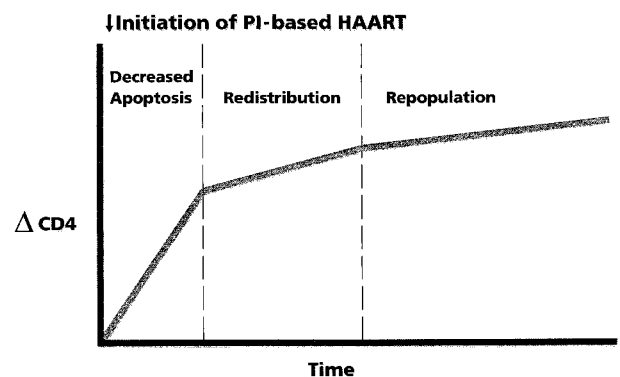


Figure 3. Kinetics of change in CD4 T-cell number after the initiation of protease inhibitor (PI)-based HAART.

consists mainly of new T-cell synthesis characterized by cells with a naive phenotype.³⁰⁷ This third phase of T-cell regeneration is characterized by the presence of circular DNA elements formed after the rearrangement of possible T-cell receptor alleles, thereby indicating that these are newly produced T cells that have matured in the thymus.^{295,296}

Alteration of apoptosis as a therapeutic approach in HIV infection

If CD4 T-cell depletion in HIV infection results from enhanced apoptosis, then the prevention of apoptosis might be expected to modify the course of HIV disease. In vitro studies using apoptosis inhibitors (with no intrinsic antiviral properties) on PBL from HIV-infected patients cause increased viral production and increased cell survival.³⁰⁸ These findings suggest that non-apoptotic-infected cells serve as viral reservoirs and that it is unlikely that phenotypic and functional abnormalities of infected cells will be reversed by merely inhibiting apoptosis. Thus, blocking apoptosis alone fails to meet 2 objectives of effective HIV therapy: it does not decrease viral replication or decrease viral reservoirs, and it does not increase cellular immune competence.

The main obstacle to viral eradication in HIV-infected patients (reviewed in Chun and Fauci³⁰⁹) is the presence of chronically infected latent reservoir cells, such as macrophages, and latently infected CD4 T lymphocytes.³¹⁰⁻³¹³ In these cellular populations, HIV infection is not associated with apoptosis but with a chronic

productively infected phenotype. Indeed, latently infected CD4 T cells have a markedly prolonged half-life (estimated at 6 months), which limits the probability that viral reservoirs can be eliminated by interference in viral replication alone.³¹⁴ In fact, recent estimates based on the half-life of latently infected cells suggest that 60 years of viral suppression would be required to eliminate viral reservoirs.³¹¹ A possible way to achieve viral eradication is to target infected macrophages and latently infected CD4 T cells to undergo apoptosis after infection. Along these lines, it has recently been proposed that treatment with a pro-caspase 3 analogue, which contains an HIV protease-specific sequence in its prodomain, may cause apoptosis of all infected cells.³¹⁵ Additional research is required to evaluate the clinical usefulness of this and other approaches designed to enhance the apoptosis of cells that normally function as reservoirs for HIV. The concept of enhancing HIV-associated apoptosis is, however, a potentially significant step forward in attempts to modify apoptosis for the benefit of patients infected with HIV. It further underscores the need for continued efforts to understand the regulation of apoptosis induced by HIV infection.

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