Cell Death and Differentiation (1999) 6, 420–432

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# Dynamic correlation of apoptosis and immune activation during treatment of HIV infection

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Received 28.9.98; revised 8.12.98; accepted 4.2.99
Edited by M.L. Gougeon

## Abstract

**T cells from HIV infected patients undergo spontaneous apoptosis at a faster rate than those from uninfected patients, are abnormally susceptible to activation induced cell death (AICD), and undergo increased apoptosis in response to Fas receptor ligation. These observations have led to the hypothesis CD4 T cell apoptosis may be a mechanism of CD4 T cell depletion and the pathogenesis of AIDS. Successful treatment of HIV infected patients is accompanied by quantitative and qualitative improvements in immune function reflecting at least partial reversibility of the underlying pathogenesis of HIV. In this report we correlate improvements in markers of immune function with a decrease in apoptosis, and changes in its regulation. Therapy with nelfinavir plus saquinavir in combination with two nucleoside analogue inhibitors of reverse transcriptase dramatically reduces plasma viremia and increases CD4 T cell counts. Coincident with these improvements, CD38 and HLA-DR coexpression on both CD4 and CD8 T cells decrease, and CD45RA and CD62L coexpression increase. Furthermore, spontaneous apoptosis decreases in both CD4 and CD8 T cells (CD4 apoptosis 17.4 *vs* 2.6%, $P=0.005$; CD8 apoptosis 15.0 *vs* 1.0%, $P<0.001$), as does both Fas mediated apoptosis (CD4 apoptosis 19.0 *vs* 3.5%, $P=0.03$; CD8 apoptosis 13.7 *vs* 1.5%, $P=0.002$) and CD3 induced AICD (CD4 apoptosis 13.7 *vs* 3.2%, $P=0.001$; CD8 apoptosis 29 *vs* 2.2%, $P=0.08$). Changes in apoptosis are not associated with changes in Fas receptor expression, but are significantly correlated with changes in activation marker profiles. Although this suggests a possible regulatory role for the apoptosis inhibitory protein FLIP, direct assessment did not reveal quantitative differences in FLIP expression between apoptosis resistant PBL's from HIV negative patients, and apoptosis sensitive PBL's from HIV positive patients. These findings support the hypothesis that apoptosis mediates HIV induced CD4 T cell depletion, but indicate the need for further studies into the molecular regulation of HIV induced apoptosis.**

**Keywords:** HIV; apoptosis; AICD; Fas; FLIP; activation

**Abbreviations:** AICD, activation induced cell death; AIDS, acquired immunodeficiency syndrome; AZT, azithothymidine; BSA, bovine serum albumin; CTL, cytotoxic T lymphocytes, DDC, 2′, 3′-dideoxycytidine; ECL, electrochemoluminescence; FITC, fluorescein isothyocyanate; FLICE, FADD like ICE (Caspase 8); FLIP, FLICE inhibitory protein; HIV, human immunodeficiency virus; MHC, major histocompatibility complex; NS, non significant; PBL, peripheral blood lymphocytes; PBMC's, peripheral blood mononuclear cells; PBS, phosphate buffered saline; TCR, T cell receptor; TNF, tumor necrosis factor; TRAIL, TNF related apoptosis inducing ligand

## Introduction

Recent advances in the understanding of the pathogenesis of human immunodeficiency virus (HIV) infection have led to treatments that restore CD4 T cell counts and improve immune function of HIV infected individuals.[1–9] One approach to therapy has been combinations of HIV protease inhibitors[10] and nucleoside inhibitors of HIV reverse transcriptase, which together result in partial immune reconstitution of some patients.[1] The effectiveness of these treatments suggests that the pathogenic mechanism(s) responsible for the induction of CD4 T cell loss in untreated patients are reversed by such therapies.

Although considerable controversy exists, apoptosis is emerging as the most plausible mechanism responsible for CD4 T cell depletion in patients infected with HIV.[11–13] Apoptosis is a tightly regulated process of cellular death that may be induced by both death receptor initiated signalling,[14] or by mechanisms which bypass the requirement for receptor ligation.[15] T cells from HIV infected persons when cultured *ex vivo* demonstrate enhanced apoptosis in the absence of stimuli (spontaneous apoptosis) in comparison with cells from HIV negative patients.[16,17] Increased spontaneous apoptosis has consistently been observed to correlate directly with the rate of disease progression: rapid progressors have the highest rates of spontaneous apoptosis,[18–20] whereas long term non progressors have rates of apoptosis similar to HIV negative subjects.[18,21,22] In addition to enhanced spontaneous apoptosis, peripheral blood mononuclear cells from HIV

infected patients and HIV infected monocyte derived macrophages develop the ability to induce apoptosis of autologous CD4 and CD8 T cells.[23–30] This phenomenon, in addition to enhanced susceptibility of T cells from HIV infected patients to undergo Fas mediated apoptosis,[31–36] suggest that the Fas/Fas Ligand (FasL) system is involved in HIV induced apoptosis. Indeed, interruption of Fas/FasL interaction abrogates the ability of macrophages to induce autologous cell killing.[23,24,26,28] Recent demonstration of increased T lymphocyte apoptosis in lymphoid tissues of HIV infected patients,[37–41] elevated macrophage associated FasL within the lymphatic tissues of infected patients[40,41] and the correlation of FasL with the presence of increased T lymphocyte apoptosis[41] lends support to the importance of Fas mediated T cell death *in vivo*. A third mechanism of T cell apoptosis in HIV infected patients is related to the profound immune activation which occurs in HIV infected patients.[42,43] T cell activation leads to a state of enhanced susceptibility to apoptotic cell death. When activated cells then encounter another activating stimulus, *de novo* production of FasL results in apoptosis.[44,45] Experimental systems which study this activation induced cell death (AICD), use mitogens or T cell receptor (TCR) ligation as an activation stimulus to induce AICD. In this regard, T cells from HIV infected patients are more prone to AICD than those from HIV negative patients,[46–48] possibly due to activation induced by viral gp120.[26,49]

Since apoptosis is one potential pathogenic mechanism responsible for HIV mediated T cell death and CD4 T cell depletion in AIDS, we have evaluated the amount of apoptosis that occurs before and after the initiation of

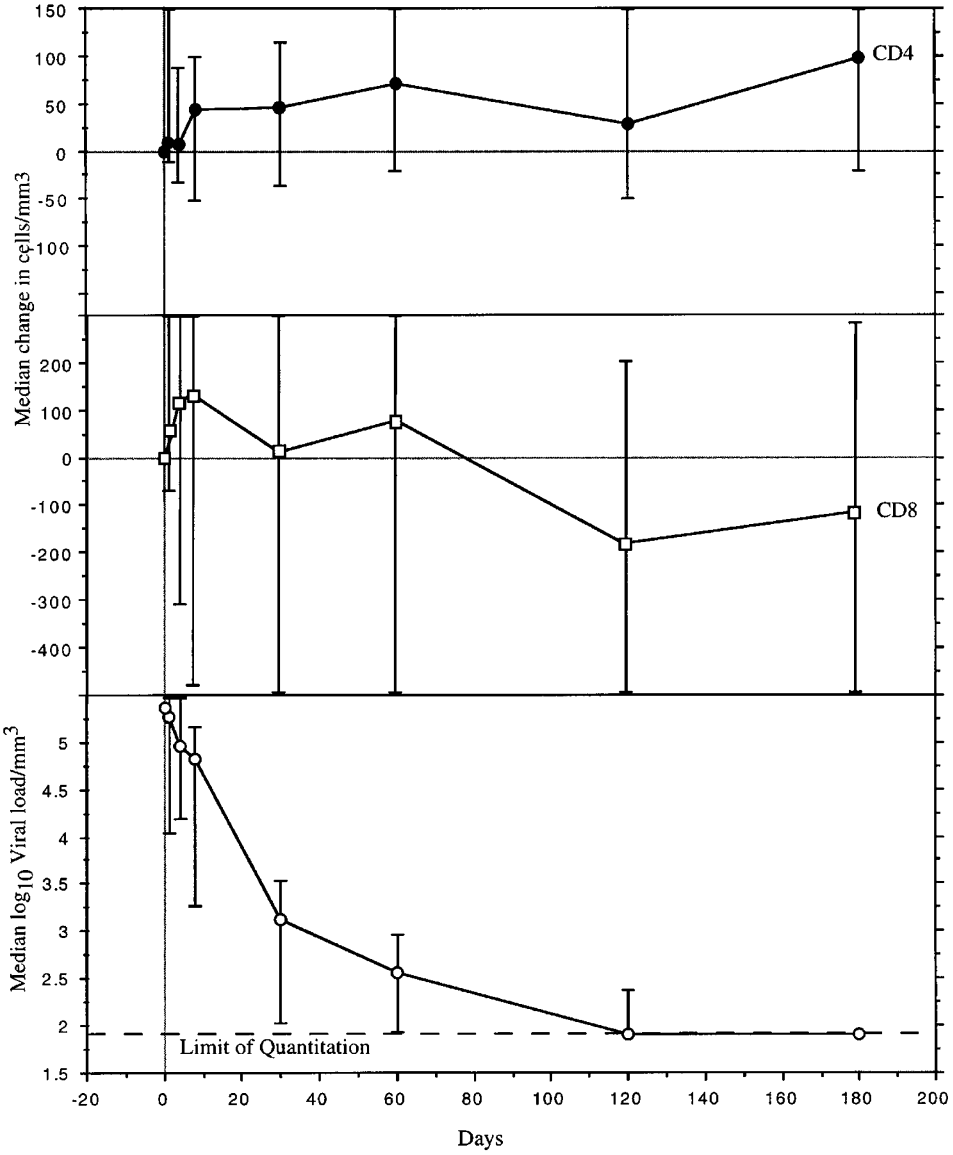


**Figure 1** Virologic and T cell response to therapy. CD4 and CD8 T cell counts and plasma HIV viral RNA levels were determined prior to and during therapy with nelfinavir, saquinavir and two nucleoside analogue inhibitors of HIV reverse transcriptase. (Bars represent 25th and 75th percentiles)

potent antiretroviral therapy. Infected patients that were not previously treated with protease inhibitors were enrolled in a pharmacokinetic and anti-HIV activity trial of nelfinavir and saquinavir in combination with two nucleoside analogue inhibitors of HIV reverse transcriptase. Blood samples were analyzed before and during therapy for enumeration of T cell subsets, quantitation of plasma HIV viral load, measurement of markers of immune function, and for apoptosis markers.

## Results

### Virologic and T cell response to therapy

Ten patients were enrolled and followed for 6 months. One patient discontinued treatment after 4 weeks for reasons unrelated to study medications. In the remaining patients, pre-treatment median plasma viremia was 5.3 $\log_{10}$ copies/ml (range 3.61 – 6.38 $\log_{10}$ HIV RNA genomes/ml), median CD4 count was 301 cells/$\mu$l (range 21 – 114 cells/$\mu$l) and median CD8 T cell count was 1162 cells/$\mu$l (range 575 – 1567 cells/$\mu$l). A rapid decline in plasma viremia was observed following the initiation of treatment with a median 2.23 $\log_{10}$ reduction from baseline observed by day 30 (Figure 1). Following 120 days of treatment, median viral load was below the lower limit of quantitation of 80 copies (seven to nine patients below detection). By day 180, eight to nine evaluable patients had plasma viremia below 80 copies/ml (overall $P$=0.05). Sustained increases from baseline in CD4 T cell counts were observed, with a 47 cell $\mu$l/median increase (range −185 to +342 cells; P=NS) occurring after 8 days of treatment; following 180 days of treatment, the median CD4 T cell count had risen by 97 cell/$\mu$l (range −77 to +183 cells; day 0 *vs* day 180 $P$=0.05). Similar to previous observations in patients treated with protease inhibitor based antiretroviral therapy,[7] the CD8 T cell response to therapy was biphasic, within an initial increase in CD8 T cell counts being followed by a decline. By day 8 after therapy, the median CD8 T cell count had increased by 134 cells/$\mu$l (range −518 to +744 cells P=NS). After 180 days the median CD8 T cell count had fallen by 126 cells/$\mu$l (range −724 to +579 cells P=NS).

### Immune reconstitution following therapy

Considerable recent attention has been focused on immune reconstitution following effective anti-retroviral therapy. These studies have demonstrated that suppressed viral replication is followed by decreased immune activation, increased expression of co-stimulatory molecules, and increased numbers of naive circulating T cells.[1–9] Other studies indicate that abnormal T cell activation occurs in HIV infection,[42,43,50,51] and that this abnormality increases susceptibility of T cells to become infected,[52–54] or to die by apoptosis.[35,55,56] The abnormal state of activation may therefore be central to the pathogenesis of HIV disease. Consequently, we analyzed the activation profile of CD4 and CD8 T cells before and during therapy. Co-expression of CD38 and HLA-DR were analyzed in CD4 and CD8 T lymphocytes at day zero, 60, and 180 (Figure 2). Despite rising CD4 T cell counts, the median number of CD4 T cells expressing CD38 and HLA-DR

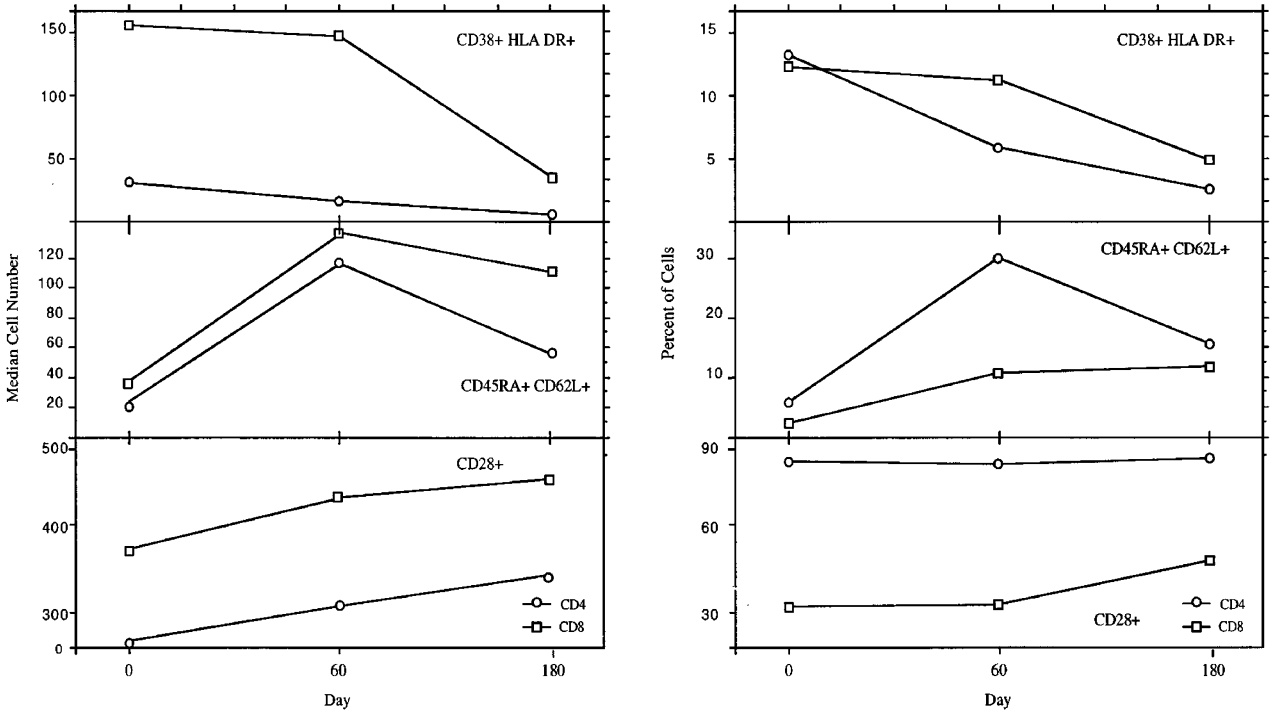


**Figure 2** Immunophenotype subset analysis. Prior to and during antiretroviral therapy, the absolute number of CD4 (○) and CD8 (□) T cells which co-expressed CD38 and HLADR (top panels) CD45RA and CD62L (middle panels) or CD28 (lower panels) were analyzed by flow cytometry. Data is expressed either as median cell number (left panels) or as percentage of CD4 or CD8 T cells (right panels)

decreased from a pre treatment level of 30.5/$\mu$l cells (range 5–163, standard error=14.7) to 24 cells/$\mu$l (range 3–40, standard error 4.8) by day 60, and to 5.5 cells/$\mu$l (range 1–30, standard error 3.5) by day 180 (overall $P$=0.04). A reduction in activation profile was also observed in CD8 T cells, with the mean baseline number of CD8 T cells expressing CD38 and HLA-DR of 161 cells/$\mu$l (range 20–566, standard error 59.6) decreasing to 155.5 cells/$\mu$l (range 53–244, standard error=26.1) by day 60 and to 40.0 cells/$\mu$l (range 30–85, standard error=9.7) by day 180 (overall $P$=0.005).

We next analyzed the number of naive CD4 and CD8 T cells before and during therapy. Co-expression of CD45RA and CD62L was used to identify the naive T cell subsets.[6,57] At baseline, the median number of CD45RA and CD62L positive (naive) CD4 T cells was 19.5 cells/$\mu$l (range 2–392, standard error 37.4), which increased to 114 (range 5–346, standard error 35.3) at day 60 (overall $P$=0.02). At day 180, median 58.5 (range 13–113, standard error=11.9) naive CD4 T cells/$\mu$l were present. This was less on day 60, but still greater than baseline (overall $P$=0.03, Figure 2). The median number of naive CD45RA and CD62L positive CD8 T cells also exhibited an initial increase from 37.5 cells/$\mu$l (range 10–319, standard error 37.7) to 134 cells/$\mu$l (range 22–452, standard error=49.9) 60 days after starting treatment (overall $P$=0.03). At 180 days post treatment, the number of naive cells decreased to 114.5/$\mu$l (range 34–287, standard error=33.7), but this was still significantly greater than at baseline (day 0 *vs* day 180 $P$=0.04).

Having demonstrated a decrease in activation markers and an increase in naive CD4 and CD8 T cells, we next assessed CD28 as a surrogate marker of immune function. Optimal T cell function requires the interaction of T cells with antigen presenting cells. This interaction occurs primarily through the T cell receptor (TCR) and MHC class II molecules, and is further enhanced by the co-stimulatory molecule B7 (expressed by antigen presenting cells) and CD28[58,59] (expressed by T cells). Previous reports in patients with HIV infection have demonstrated a decrease in CD28 expression on CD8 T cells and to a lesser extent, on CD4 T cells.[60,61] Indeed, defects in proliferative function of CD4 T cells from HIV positive patients may be due to a decrease in CD28 expression.[62] We therefore analyzed the number of cells expressing

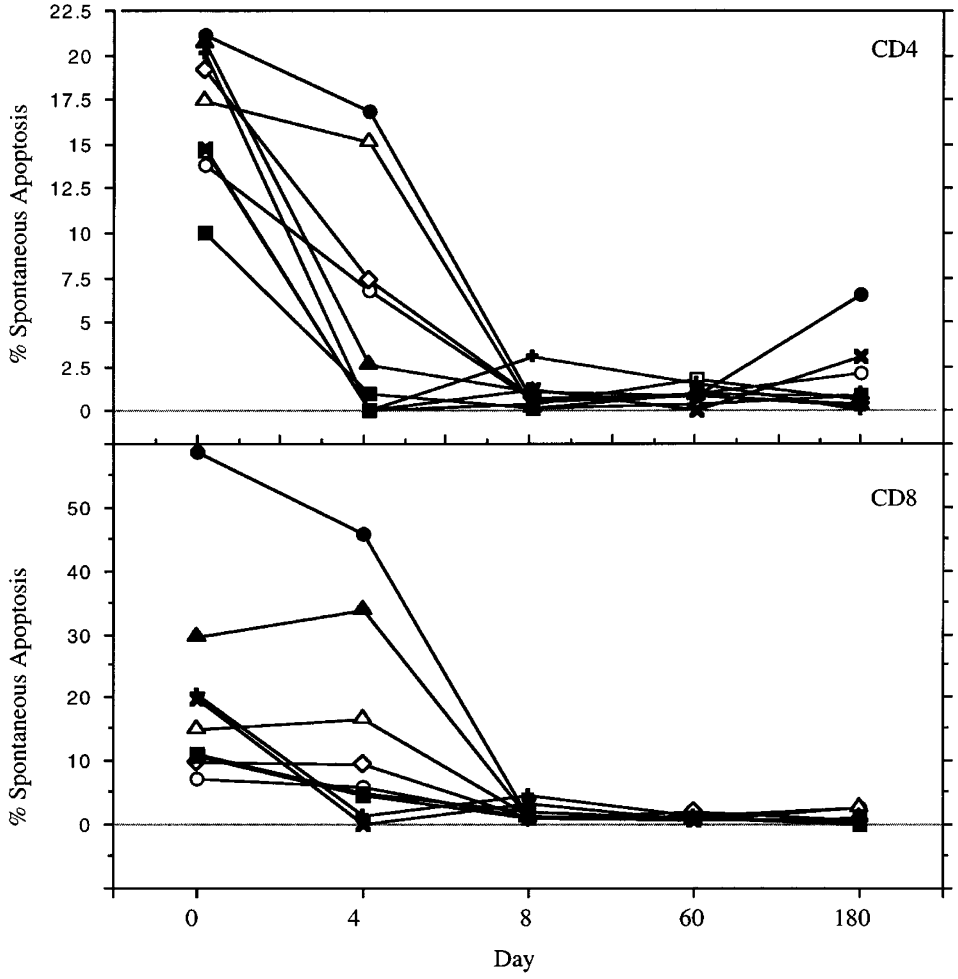


**Figure 3** Analysis of spontaneous apoptosis. Prior to and during antiretroviral therapy, the number of CD4 (top panel) and CD8 T cells (bottom panel) undergoing spontaneous apoptosis was determined using annexin V and analyzed by multiparameter flow cytometry

CD28 before and following treatment. The median number of CD4 T lymphocytes expressing CD28 rose from 253 cells/$\mu$l (range 3–993, standard error 89.7) to 306 (range 27–625, standard error 69.6) by day 60 and was 323 cells/$\mu$l (range 70–1134, standard error 118.4) at 180 days (overall P=NS, Figure 2). CD8 T cell expression of CD28 also increased from 381.5 cells/$\mu$l (range 10–972, standard error 90.7) to 404 (310–957, standard error 72.9) by day 60, and to 445 (range 257–579, standard error 43.6) after 180 days of treatment, although differences did not reach significance.

## Analysis of apoptosis following treatment

Of the candidate mechanisms responsible for the induction of CD4 T cell death, aberrant apoptosis has consistently been observed *in vitro*, *ex vivo*, and *in vivo*. In order to implicate apoptosis in the CD4 T cell loss seen in untreated HIV infection, then therapies which reverse the CD4 T cell decline must necessarily reduce CD4 T cell apoptosis. Since three distinct forms of apoptosis have been described in HIV infection (enhanced spontaneous apoptosis, enhanced AICD, and enhanced anti-Fas stimulated apoptosis), we therefore analyzed each of these forms of apoptosis before and during therapy.

Dramatic reductions in all three forms of apoptosis were observed in both CD4 and CD8 T cell subsets within a few days of starting therapy. Prior to antiretroviral therapy, 17.4% (range 10–21.1) of CD4 T cells were undergoing spontaneous apoptosis. Within 4 days of therapy this rapidly declined to 2.6% (range 0–16.8%; $P$=0.005) (Figure 3), and further decreased by day 8 to 0.6% (range 0.2–3.1), continuing at a low level throughout the period of observation (overall $P=<0.001$). Similarly, spontaneous CD8 T cell apoptosis which was elevated before therapy with a median value of 15.0% (range 7.3–58.6) fell within 4 days to 5.9% (range 0–45.7; $P$=0.05) and by day 8 spontaneous apoptosis was reduced to 1% (range 0.9–4.5%), a level that persisted throughout the period of observation (overall $P=<0.001$).

A similar analysis was performed in the CD4 and CD8 T cell subsets following stimulation with either anti Fas antibody or anti CD3 antibody (Figure 4). In order to differentiate changes in anti Fas or anti CD3 specific apoptosis from changes in spontaneous apoptosis, cells were coincubated in the presence of an isotype control antibody, and the amount of specific apoptosis was determined by subtracting isotype control apoptosis from the observed apoptosis following either anti Fas or anti CD3 stimulation. Prior to treatment, anti Fas stimulation resulted in 19.0% (range 1.3–73.9%) specific apoptosis of CD4 T cells, and in 13.7% (range 1.7–86.1%) specific CD8 T cell apoptosis. Similar to the findings with spontaneous apoptosis, CD4 T cell Fas specific apoptosis decreased during treatment, finally resulting in 3.5% (range 0.4–20.5) specific apoptosis by day 180, and 1.5% (range 0.1–3.8) CD8 T cell specific apoptosis by day 180 (overall CD4 T cell anti Fas apoptosis, overall $P$=0.03; overall CD8 T cell anti Fas apoptosis $P$=0.002). Analysis of CD3 induced apoptosis before and after therapy revealed a similar pattern: ranging from 13.7% in CD4 T cells (range 1–30.2) before therapy to 3.2% (range 0.4–11.5) after 180 days of treatment (overall $P$=0.001). CD3 induced AICD of CD8 T cell also decreased from 29% (range 1.9–84.7) prior to treatment to 2.2% (range 0.4–4.8) following 180 days of treatment (overall $P$=0.03).

## Analysis of Fas expression

In comparison to resting CD4 T cells, activated CD4 T cells are preferentially infected by HIV[52–54] and preferentially susceptible to die by Fas induced apoptosis.[63–65] These observations suggest that in the context of HIV infection both increased Fas sensitivity, and increased FasL production favour CD4 T cell apoptosis.

We therefore evaluated whether changes in Fas expression could account for the decreases in apoptosis and sensitivity to apoptosis that were observed. Before initiation of therapy the median percentage of Fas positive CD8 T cells was 55% (range 25.5–81). The percentage of Fas positive cells did not change after initiation of therapy: median percentage of Fas positive cells on day 4 was 43.4% (range 14–74.7), day 8 was 47.4% (range 14.7–68.8), on day 60 was 60.1% (range 35.9–75) and on day 180 was 43.2% (range 18.6–52.1), (overall $P$=NS). Similarly, the median percentage of CD4 T cells expressing Fas was 56.7% (range 36.5–73.3) prior to therapy, 57.5% (range 43.7–78.2) on day 4, 49.6% (range 25.8–78.8) on day 8, 61.5% (range 34.4–85.9) on day 60 and was 52.3% (range 34.6–82.2) on day 180 (overall $P$=NS).

In order to assess whether small changes in Fas expression were responsible for the observed changes in apoptosis, we correlated Fas receptor expression with measures of apoptosis, evaluating the number of Fas positive cells and amount of CD4 and CD8 T cell apoptosis that occurred spontaneously, in response to Fas ligation, or in response to CD3 stimulation. The percentage of CD4 T cells expressing Fas was not significantly associated with spontaneous CD4 T cell apoptosis ($P$=NS, $R^2$=0.029), anti CD3 induced CD4 T cell apoptosis ($P$=NS, $R^2$=0.04) or anti Fas induced CD4 T cell apoptosis ($P$=NS, $R^2$=0.06). Similarly, the percentage of CD8 T cells expressing Fas was not associated with spontaneous CD8 T cell apoptosis ($P$=NS, $R^2$=0.10), and CD3 induced CD8 T cell apoptosis ($P$=NS, $R^2$=0.04) or with anti Fas induced CD8 T cell apoptosis ($P$=NS, $R^2$=0.02). These data suggest that in contrast to prior cross sectional data,[31–33,36] in HIV infected patients followed longitudinally, changes in susceptibility to apoptosis is not a consequence of changes in Fas receptor expression.

## Correlation between activation and apoptosis

Previous work by several groups has demonstrated that sensitivity towards Fas receptor mediated apoptosis is correlated with activation.[63–65] In addition, HIV gp120 both directly activates CD4 T cells and has been implicated as a mechanism for priming cells towards both AICD and spontaneous apoptosis.[49,55,66,67] We therefore postulated that in the context of HIV infection, decreasing sensitivity to

apoptosis following effective therapy should correlate with changes in activation profiles. We therefore performed linear regression analyses to assess for a potential relationship between the percentage of cells coexpressing the activation markers CD38 and HLA-DR, and the percentage of apoptotic cells present either spontaneously or in response to anti Fas or anti CD3 ligation (Figure 5). Activation markers of CD4 T cells were significantly correlated with spontaneous apoptosis ($P$=0.003, $R^2$=0.542), anti Fas induced apoptosis ($P$=0.01, $R^2$=0.246) and with CD3 induced apoptosis ($P$=0.001, $R^2$=0.389). CD8 T cell activation profiles were also associated with spontaneous apoptosis ($P$=0.02, $R^2$=0.303) with Fas induced apoptosis ($P$=0.01, $R^2$=0.248), but not CD3 induced apoptosis ($P$=0.06, $R^2$=0.151). Since the nature of our data set was serial longitudinal observations, in nine different patients, linear regression analyses may be affected by bias introduced by multiple comparisons of individual subjects. Therefore, since linear regression analyses suggested potential correlation between activation and apoptosis, we next performed a Liang-Zeger regression.[68,69]

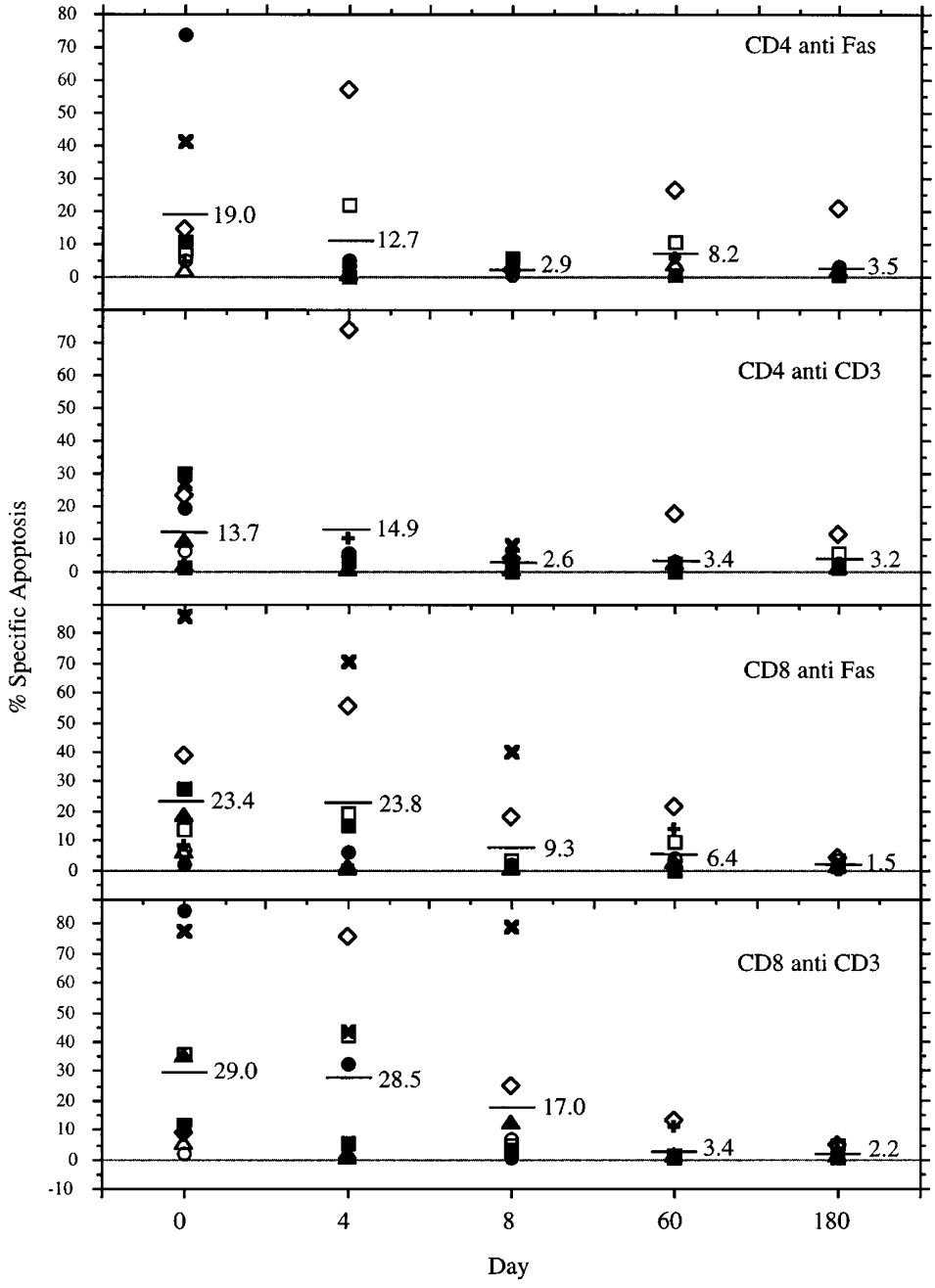


**Figure 4** Analysis of anti Fas induced apoptosis and AICD. Prior to and during antiretroviral therapy, the number of CD4 (top two panels) of CD8 T cells (bottom two panels) undergoing anti Fas specific (first and third panels) or anti CD3 specific (second and fourth panels) specific apoptosis was determined using annexin V. Horizontal bars represent group means, and each symbol represents a different patient

These analyses confirmed the association of activation markers of CD4 T cells with spontaneous apoptosis ($P$=0.008), antiFas induced apoptosis ($P$=0.001) and anti CD3 induced apoptosis ($P$=0.005). Similarly, the associations of CD8 T cell activation with spontaneous apoptosis ($P$=0.001) anti Fas induced apoptosis ($P$=0.001) and anti CD3 induced apoptosis ($P$=0.001) were confirmed.

## Analysis of FLIP expression

Our observation of decreasing Fas sensitivity without changes in Fas receptor expression, is consistent with prior observations indicating a discordance between Fas expression and Fas sensitivity.[70,71] One molecular explanation for this revolves around a recently characterized protein, FLIP, (FLICE inhibitory peptide) which inhibits caspase activation.[72] In resting naive T cells (which express Fas but are Fas resistant) FLIP is expressed at high levels, whereas activation of T cells results in decreased levels of FLIP and the development of Fas susceptibility. Importantly, within PBLs, protein expression is concordent with mRNA levels.[72] Since patients enrolled in our study initially have Fas expressing and Fas sensitive CD4 and CD8 T cells, we postulated that these cells would contain less FLIP content, (making them Fas sensitive) when compared to cells from uninfected donors.

FLIP expression was therefore analyzed in PBL's from six untreated HIV positive patients with high levels of spontaneous apoptosis (range=22–61%) and from one HIV negative volunteer with 6% spontaneous apoptosis (Figure 6). In HIV negative patients 55 Kd FLIP was detectable in PBL's. Surprisingly, analysis of PBL's from untreated HIV infected patients revealed similar levels of FLIP expression.

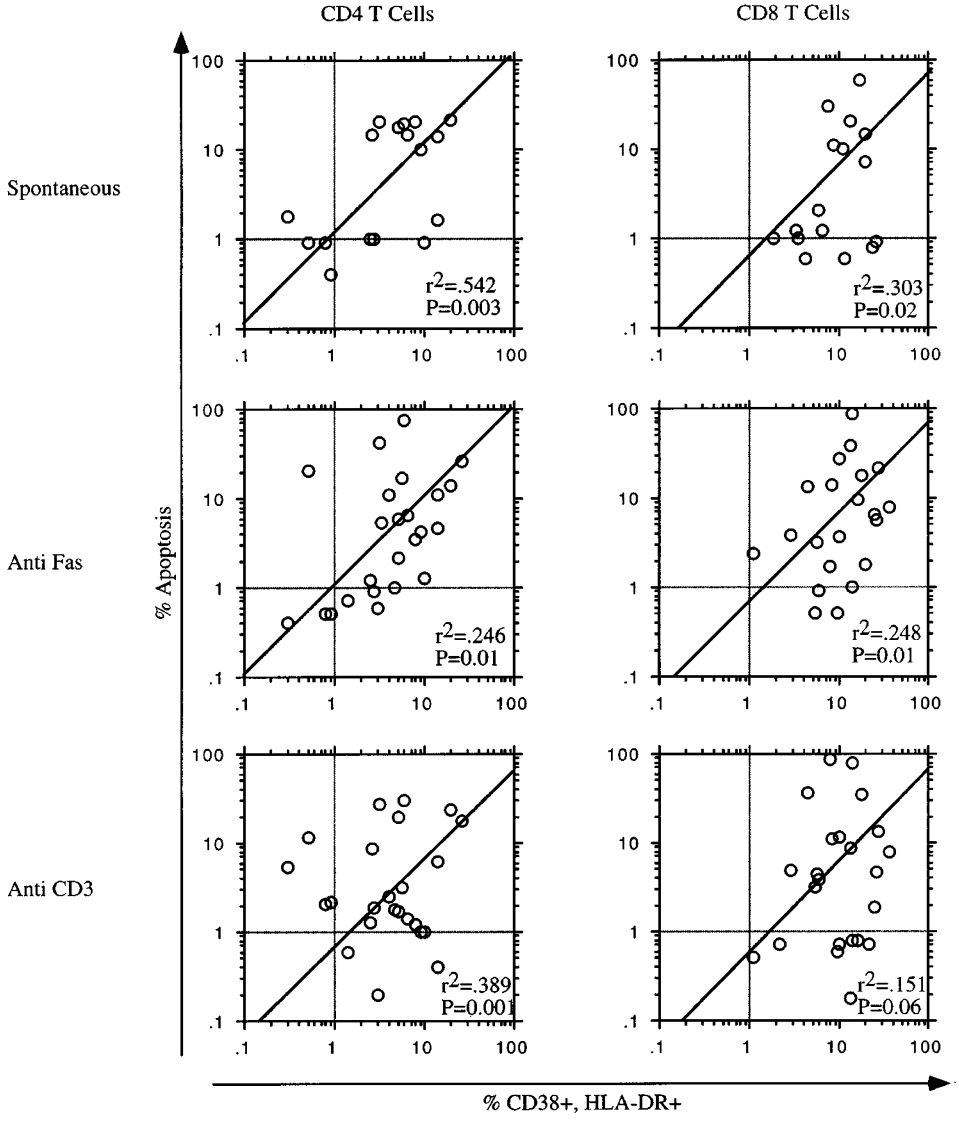


**Figure 5** Correlation between apoptosis and activation profile. Linear regression analyses were performed using paired data of percentage of apoptotic cells as determined by annexin V (Y axis) *versus* percentage cells co-expressing CD38 and HLADR (X axis). Separate analyses were performed for CD4 T cells (left panels) and CD8 T cells (right panels). Confirmatory statistical analyses using Liang-Zeger regression was also performed (see text)

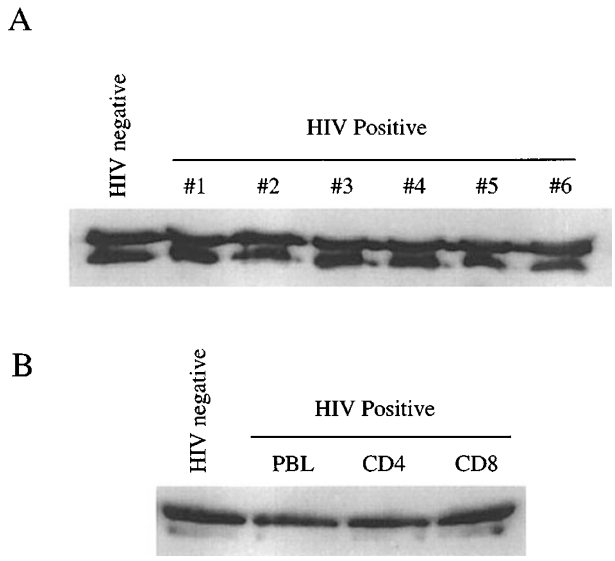


**Figure 6** Analysis of FLIP expression. Whole cell lysates from either bulk PBLs (**A**) or purified CD4 or CD8 T lymphocytes (**B**) form HIV infected or uninfected patients were analyzed by Western blot analysis for intracellular FLIP content. All HIV infected patients included in these experiments had high levels of apoptosis (see text)

To determine whether differences in FLIP expression were present between T cell subsets, we purified CD4+lymphocytes and CD8+lymphocytes from an HIV positive patient with high levels (46%) of spontaneous apoptosis. No differences in FLIP expression were observed between bulk PBL's from an HIV negative control, bulk PBL's from an HIV infected but untreated patient, or either purified CD4 or CD8 T cells from the same HIV infected patient.

## Discussion

It is clear that in HIV infected patients, CD4 T cell death occurs by apoptosis that may result from one of several mechanisms (see below). In each, the presence of T cells which are primed to undergo apoptosis favors the initiation of apoptosis, and therefore may be critical to AIDS pathogenesis. This paper defines the relationship between apoptosis, apoptosis susceptibility, viral load, and immunologic reconstitution.

Patients enrolled in our trial experienced a rapid decline in viral load, with a concomitant increase in CD4 T cell counts. At the same time, the proportion of activated T lymphocytes decreased, the number of naive T lymphocytes increased, and apoptosis (induced by a variety of stimuli) was reduced in a manner that correlated with activation profiles. Our results extend prior observations that apoptosis and sensitivity towards apoptosis are closely related to activation state.[73] This association has been observed in cross-sectional[57,74] and longitudinal studies.[46] Indeed, the correlation of apoptosis with activation has been suggested as a mechanism to explain the enhanced CD4 T cell loss seen in HIV positive patients coinfected with other pathogens,[75,76] and the lack of apoptosis in HIV infected chimpanzees (in whom activation does not occur).[77] It remains unknown whether effective antiretroviral therapy, which leads to immune deactivation, is associated with decreases in apoptosis and apoptosis sensitivity. The current report addresses this question and demonstrates a significant correlation between changes in apoptosis and changes in activation. These data suggest that immune activation, perhaps due to virally encoded antigen(s), may be responsible for the enhanced susceptibility to apoptosis that is seen in HIV infected patients. Importantly, we demonstrate the reversibility of activation, which is associated with reversal of enhanced apoptosis. Together these results support an etiologic role for apoptosis as a mechanism of CD4 T cell depletion in AIDS that is efficiently reversed by therapies that lead to immunologic reconstitution. Whether the inhibition of apoptosis is due to direct antiapoptotic effects of therapy, or is due solely to immune deactivation, remains to be elucidated.

Apoptosis in patients infected with HIV has been widely studied *in vitro* and to a lesser extent *in vivo*. Most of the published literature indicates that apoptosis is highly and temporally associated to the CD4 T cell decline that is seen in HIV infected patients. Furthermore at least three distinct apoptotic mechanisms have been suggested. The first mechanism is modelled *in vitro* by direct HIV infection of CD4 T cells, in which apoptosis occurs following direct HIV infection of T cells and is closely related to the kinetics of HIV replication.[78] Detailed analyses have indicated that, although caspase activation occurs following direct HIV infection,[79] this form of cell death is independent of Fas receptor signalling,[80,81] thereby suggesting a direct role for an HIV encoded protein to activate apoptosis independent of Fas. In this regard, HIV protease,[82–84] HIV vpr,[85–87] HIV Nef[88–90] and HIV Tat,[91,92] have all been suggested as potential mediators of direct CD4 T cell induced apoptosis. In patients infected with HIV, this form of CD4 T cell death is represented by spontaneous T cell apoptosis. Previous studies have evaluated spontaneous apoptosis in HIV infected patients and showed that apoptosis of CD4 T cells was significantly increased in comparison to HIV uninfected patients.[16,17] Furthermore the magnitude of apoptosis directly correlated with stage of HIV disease when examined either longitudinally[20] or cross-sectionally.[19] Furthermore, while spontaneous apoptosis is increased in HIV infected patients with progressive disease compared to HIV uninfected patients[18,20] levels of spontaneous apoptosis in patients with long term non progressive HIV infection are similar to that of HIV negative patients.[21,22] Since spontaneous apoptosis is inhibited *in vitro* by blocking viral replication, it is reasonable to suspect that spontaneous apoptosis *in vivo* would similarly be decreased. This study supports that hypothesis by showing that levels of spontaneous apoptosis revert to values seen in HIV uninfected patients within 8 days of treatment, and that within this short time frame, CD4 and CD8 T cell counts increase.

AICD has been implicated as a second form of apoptosis in the pathogenesis of HIV. This form of apoptosis occurs in both HIV uninfected patients[44,45,93–95] and HIV infected

patients[46–48,96] in response to T cell activation taking place in the absence of appropriate MHC class II antigen presentation. In this report, we confirm increased levels of AICD in HIV infected patients prior to the initiation of antiretroviral therapy. Although the precise signals responsible for AICD in patients with HIV infection are unclear,[36,47,97,98] gp120 mediated activation of the CD4 receptor has been demonstrated to enhance AICD, to increase Fas susceptibility, and to increase Fas ligand expression (see above). Therefore, decreased production of viral gp120, such as would occur in the context of effective antiretroviral therapy, would be expected to result in decreased cellular activation, as a concomitant decrease in AICD. This study demonstrates striking decreases in activation and a significant correlation between the activation profile and the magnitude of AICD.

A third postulated mechanism for HIV governed apoptosis of CD4 T cells suggests that HIV infected macrophages, a source of apoptosis inducing ligands, are capable of killing apoptosis prone CD4 T cells. This mechanism has been extensively studied *in vitro*,[23–30] and recently has been found to occur *in vivo*.[40] FasL has been implicated in HIV pathogenesis as a molecule that is selectively upregulated in monocytes/macrophages after CD4 crosslinking[26] or HIV infection.[23,24] Furthermore, FasL transcripts are seen in peripheral blood mononuclear cells (that contain monocytes) from HIV infected but not HIV uninfected patients.[99] This has led to a model wherein HIV infected monocytes/macrophages are a source of ligands that induce apoptosis of uninfected CD4 T cells. Data using HIV patient PBMC's,[29] or macrophages infected with HIV [20,23,24,28] demonstrate an ability to selectively kill CD4 T cells, that is blocked by masking Fas receptor. Recent demonstration of a selective upregulation of FasL in lymph node macrophages from HIV infected patients supports this model.[41] In untreated HIV patients, tissue levels of FasL are correlated with levels of T cell apoptosis in the same tissue. After effective therapy apoptosis decreases but FasL expression does not,[40] suggesting an important regulatory role for T cell susceptibility towards Fas mediated apoptosis. Consistent with these previous observations, our data demonstrate a dramatic change in Fas sensitivity in response to suppressed viral replication.

This report provides additional insights into the pathogenesis of HIV disease. FLIP is a recently described inhibitor of the TNF receptor superfamily induced apoptosis.[72] Transfection of FLIP into cell lines blocks FasL and TRAIL (TNF related apoptosis inducing ligand) induced apoptosis (which utilize the proximal caspase FLICE) but not staurosporin induced apoptosis (which bypasses FLICE). The important correlation of apoptosis with activation suggests a potential role for FLIP in the regulation of apoptosis susceptibility that is increased in HIV infected patients. However, our observation that FLIP expression in PBL's from HIV infected individuals is the same as in PBL's from HIV uninfected individuals suggests that if FLIP is an inhibitor of FLICE then HIV mediated direct T cell killing occurs through a receptor independent mechanism that activates apoptosis at or distal to FLICE. Alternately, FLIP may be a prototype of a new family of apoptosis regulating protein, whose cumulative cellular effects depend upon the relative expression of each family member, as opposed to the isolated expression of one (analogous to the $Bcl_2$ family of apoptosis regulating proteins.[100] The existence of multiple FLIP RNA species in Northern blot analyses from human tissues supports this theory. Furthermore, the presence of FLIP (short) RNA but no FLIP (short) protein in resting T cells suggests an element of post transcriptional FLIP regulation.[72] Lastly, recent characterization of I-FLICE[101] and CLARP[102] both of which have multiple transcripts in human tissues further demonstrates the complexity and multiplicity of the molecular interactions involved in the regulation of apoptosis.

Our data indicate that protease inhibitor based antiretroviral therapy results in decreased activation and decreased apoptosis, that are statistically correlated. It remains possible that HIV protease inhibitors may directly influence apoptosis, potentially in a manner which inhibits apoptosis. In support of this concept, the direct inhibitory effects of the protease inhibitor ritonavir on CTL function and on antigen presentation, demonstrate that this class of agents directly modulates immune function.[103] Furthermore, several lines of evidence suggest that protease inhibitors may affect T cell turnover in a manner that is independent of changes in viral load. If rebound in CD4 T cell counts are solely related to decreases in viral load, one would predict similar increases in CD4 T cell levels with similar degrees of viral suppression whether this was achieved by protease inhibitor or non-protease inhibitor based therapy. In one such trial, despite similar levels of viral suppression, therapy with AZT and the protease inhibitor saquinavir results in superior rises in CD4 T cell counts, after 8 and 16 weeks therapy when compared to non protease inhibitor therapy with AZT and DDC.[104] In addition, recent reports describe improvements in CD4 counts despite unsuppressed viral replication in patients treated with protease inhibitor based combination antiretroviral salvage regimens.[105–107] A second line of evidence which suggests that protease inhibitors directly affect T cell homeostasis is the observation that in patients treated with protease inhibitor based therapy, CD8 T cell numbers rise in a manner which is greater than that seen with non protease inhibitor based regimens.[108] Together these observations support the possibility that HIV protease inhibitors may directly influence T cell apoptosis.

In conclusion, this series of experiments demonstrates changes in apoptosis and its regulation which occur in response to initiation of antiretroviral therapy. Furthermore, since these changes occur both rapidly and parallel to improvements in CD4 T cell counts and activation profiles, the data provide further evidence in support of a causal role of apoptosis in CD4 T cell depletion following HIV infection. The observation that decreased spontaneous apoptosis and decreased apoptosis sensitivity is closely correlated with immune deactivation suggests that the process by which HIV activates cells also is responsible for enhancing cell susceptibility towards apoptosis. Finally, our observation that the expression of FLIP was not different between HIV positive and negative patients indicates the need for

further studies designed to identify the mechanism(s) by which apoptosis and susceptibility to apoptosis is regulated in HIV infection.

## Materials and methods

### Patients

All patients attending the Ottawa Hospital (General Site) Immunodeficiency Clinic, were eligible for enrollment if they were between 18–60 years of age, with a positive serologic test for HIV and a baseline HIV RNA plasma virus load of greater than or equal to 400 copies/ml. Patients were excluded if they had been treated with reverse transcriptase inhibitors for more than 6 months, or had been previously treated with HIV protease inhibitors. After obtaining informed consent, patients were enrolled into one of two treatment arms of a trial designed to compare the pharmacokinetics of different doses of nelfinavir and saquinavir in combination with two reverse transcriptase inhibitors. All patients evaluated in the current study received saquinavir 600 mg tid; after 4 days, 750 mg tid of nelfinavir was added. Eight days after starting saquinavir, two reverse transcriptase inhibitors were added. Blood was collected in heparinized tubes on days 0, 1, 4, 8, 30, 60, 120, 180 of the trial.

### Cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, using Ficoll hypaque (Pharmacia, Uppsala, Sweden). Cells were centrifuged, washed, and resuspended in RPMI 1640 (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% AB serum (Gibco Laboratories, Burlington, Ontario, Canada), penicillin, streptomycin and glutamine (Sigma-Aldrich, Oakville, Ontario, Canada) and incubated at 37°C in a 5% $CO_2$ humidified environment. Where indicated, monocytes were removed by two cycles of plastic adherence, and the resultant peripheral blood lymphocytes (PBL) populations incubated as above.

### Measurement of lymphocyte subsets and viral load

CD4 and CD8 T cell counts were measured by a Federally certified independent flow cytometry facility using standardized techniques. The levels of HIV-1 RNA in plasma were quantified using a commercially available NASBA (Nucleid Acid Sequence Based Amplification) and electro-chemoluminescence (ECL) detection system (nuclisens™ HIV QT; Organon Teknika, Durham, North Carolina, USA). This assay has a quantification limit 80 copies/ml of liquid.

### Immunophenotyping

Whole blood was used for immunophenotype analysis, following Q prep treatment (Coulter Labs, Burlington, Ontario, Canada) according to the manufacturers' instructions. Cells were washed, and then resuspended in PBS containing 1 $\mu$g/ml of the indicated antibody or isotype control antibody. Thereafter cells were analyzed using a Coulter Epics XL flow cytometer, and 30 000 total events were recorded. Specific fluorescence was determined after gating against an isotype control antibody and an autofluorescence tube, and colour compensation was performed using CD3FITC, CD4PE and CD45PE-Cy5. Antibodies used for these experiments were CD4-PE, CD8-PE, CD45RA-PE-Cy5, CD62L-FITC, CD38-FITC, anti HLADR-PE-Cy5 and anti CD28-FITC; all were obtained from Coulter Labs (Burlington, Ontario, Canada).

### Induction and measurement of apoptosis

To measure anti Fas induced apoptosis, cells were stimulated with either 1 $\mu$g/ml of agonistic anti Fas IgM (CH11, Coulter Labs, Burlington, Ontario, Canada) or with an isotype matched control antibody (gc323, Coulter Labs, Burlington, Ontario, Canada). To measure AICD induced by anti CD3 treatment, cells were incubated with 1 $\mu$g/ml of anti CD3 (HIT3.a; Pharmingen, Mississauga, Ontario, Canada), or with an isotype control (IgG2a; Becton Dickinson, Mississauga, Ontario, Canada). To determine spontaneous apoptosis, cells were immediately analyzed for apoptosis. Following 4 h of incubation with antibody (or immediately in the case of spontaneous apoptosis), cells were gently harvested, washed with PBS, and incubated sequentially with 10 $\mu$g/million cells of immune globulin (Sigma Aldrich, Oakville, Ontario, Canada), then either 1 $\mu$g/million cells of CD4 PE (13B8.2; Coulter, Burlington, Ontario, Canada) or 1 $\mu$g/million cells of CD8 PE (B9.11; Coulter, Burlington, Ontario, Canada) (or isotype control; 1gG1 Becton Dickinson, Mississauga, Ontario, Canada). Thereafter cells were stained with annexin V-FITC (BioWhittaker Inc., Walkersville, MD, USA) according to the manufacturers instructions. To exclude dead cells (which are annexin positive) forward *versus* side scatter plots were gated to include only the viable population, and the expression of CD4, CD8 and annexin determined. This method has previously been validated,[20,109] and prior to initiation of this study its sensitivity confirmed in comparison to the combination of annexin - V FITC and Propidium iodide (data not shown). To determine Fas specific and CD3 specific apoptosis, the amount of apoptosis observed after isotype antibody treatment was subtracted from the amount of apoptosis present after either anti Fas or anti CD3 treatment. For each analysis, 30 000 total events were collected using a Coulter Epics XL flow cytometer.

### Analysis of FLIP expression

FLIP expression was analyzed by Western blot as previously described.[72] Briefly, cells were harvested by Ficoll hypaque as above, and either bulk PBL's or purified CD4 or CD8 T cell populations were analyzed for FLIP content. For purification of CD4 or CD8 T cell populations, monocyte depleted PBL's were first incubated with either CD4 monoclonal antibody (Pharmingen) or CD8 monoclonal antibody (Pharmingen) for 30 min on ice. Cells were then washed in PBS - 1% BSA and incubated with a secondary anti mouse biotinylated antibody for 30 min on ice. Following washing in PBS 1% BSA, purified CD4 or CD8 T cell populations were recovered using an avidin column, according to the manufacturers' instructions (Cell Pro, Ceprate LC). The resultant CD4 or CD8 T cell populations were at least 95% pure as measured by flow cytometry. The indicated cell populations were then lysed in PBS, 1% NP40 with 0.35 mg/ml PMSF, 9.4 $\mu$g/ml leupeptin, 13.7 $\mu$g/ml pepstatin A and 10 $\mu$g/ml aprotinin. Fifty microliters of lysis buffer were used for every million cells, and lysis was performed on ice for 5 min. Cell lysates were then assayed for protein content using a Bradford technique (Biorad) and 10 $\mu$g of lysates were separated on a 12% polyacrylamide gel. Following semi dry transfer, membranes were blocked in PBS 0.1% tween containing 5% skim milk. FLIP was detected using a 1 : 1000 dilution of anti-FLIP antibody AL129 at 4°C overnight. A secondary anti rabbit HRP conjugated antibody (Amersham Laboratories) was then used, and bands were visualized using ECL (Amersham Laboratories).

### Statistics

Comparisons of parameters measured at different time points were analyzed by ANOVA to assess for overall effects of drug treatment.

Thereafter paired Student's *t*-tests were performed to identify differences between individual time points. Linear regression analyses comparing paired data points (e.g. Fas expression and % apoptosis) were analyzed by Spearman correlation. If the linear regression analyses suggested potential correlation, then a Liang-Zeger regression[68,69] was performed. This form of analysis was developed specifically for analyzing longitudinal data using regression models. For each subject, a regression coefficient is estimated for each variable. If the data sets are truly independent (i.e. not correlated) then the mean regression coefficient will be zero (i.e. the null hypothesis). Regression coefficients are directly compared using a Mann Whitney statistic for non parametric data, and the significance level determined. All analyses were done using Statview 4.0 (Abacus Concepts, Berkley CA, USA).

## Acknowledgements

This work was supported by grants from Physicians Services Incorporated (to A.D. Badley, #97–30) the Doris Duke foundation (to A.D. Badley, #T98026), the Medical Research Council of Canada (to J.B. Angel, #PA-14913) and the Canadian Foundation for AIDS Research (to J.B. Angel), and a portion of the research was supported by Roche Laboratories. D.W. Cameron is an Ontario Ministry of Health Career Scientist (#02484). Informed consent was obtained from patients and the protocol was reviewed by the Ottawa General Hospital/University of Ottawa Ethics Review Committee. The authors gratefully acknowledge the efforts of I. Seguin, N. Hawley-Foss and Drs G. Garber, S. Cassol, Y. Khaliq, K. Gallicano and J. Sahai for their roles in this study. The efforts of S. Giguère during manuscript preparation are gratefully acknowledged.

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