

Activation-induced peripheral blood T cell apoptosis is Fas independent in HIV-infected individuals

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Abstract

T cell apoptosis has been proposed as an important contributor to the functional defects and depletion of T cells in HIV-infected individuals. However, the mechanisms involved in this apoptosis have not been elucidated. We recently showed that peripheral blood T cells from HIV-infected individuals are especially susceptible to Fas antigen-induced apoptosis. In this study we examine the role of Fas, CTLA-4, tumor necrosis factor (TNF) receptors (TNFR) and CD30, receptors known to be involved in T cell activation-induced cell death (AICD), in the spontaneous and activation (anti-CD3)-induced apoptosis of peripheral blood T cells from asymptomatic HIV-infected individuals. We report here that spontaneous and activation-induced T cell apoptosis cannot be inhibited by reagents that block interactions of Fas, CTLA-4, p55 and p75 TNFR and CD30 with their respective ligands. We also show that IL-12, IFN- γ , IL-4 and IL-10 cannot modify spontaneous, activation- and anti-Fas-induced apoptosis. Anti-Fas preferentially induced CD4⁺ T cell apoptosis whereas AICD induced apoptosis equally in CD4⁺ and CD8⁺ T cells. We conclude that T cell AICD in HIV infection is not mediated by Fas, thus indicating that Fas-induced and activation-induced T cell apoptosis are independent mechanisms of apoptosis which may play different roles in the pathogenesis of HIV infection.

Introduction

Apoptosis has been proposed as an important mechanism in the pathogenesis of HIV disease, involved in both the functional defects and depletion of CD4⁺ T cells. A number of studies have shown that spontaneous and activation-induced T cell apoptosis occur in peripheral blood mononuclear cells (PBMC) and purified T cells from HIV-infected individuals (1–3). Animal models of lentiviral infection have shown that apoptosis of CD4⁺ T cells is observed only in pathogenic models, thus strongly suggesting an association of CD4⁺ T cell apoptosis with disease pathogenesis (4,5). The mechanisms involved in the spontaneous and activation-induced T cell apoptosis of HIV disease, however, have not yet been elucidated.

We recently showed that peripheral blood T cells from HIV-infected individuals are especially susceptible to Fas-induced

apoptosis. This apoptosis involves both CD4⁺ and CD8⁺ T cells and correlates with disease progression and severity (6). Fas, a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily (7), and its ligand (FasL) have recently been cloned (8,9). Fas signaling has been shown to induce apoptosis of T cell clones and lines (10–12), to co-stimulate proliferation and cytokine production of T cells from healthy individuals (12), and to be involved in cytotoxic T lymphocyte-mediated killing (13,14). Previously a number of investigators have shown that activation-induced cell death in human T lymphocytes is mediated by Fas–FasL interactions (15–18). Our initial study on the role of Fas in T cell apoptosis of HIV disease raises the question whether Fas–FasL interactions are involved in the spontaneous and activation-induced T cell apoptosis observed in HIV.

Fas–FasL interactions, however, may not be the only mediators of T cell spontaneous apoptosis and activation-induced cell death (AICD) in HIV; two other receptors that may be involved are CTLA-4 and CD30. A recent study indicated that signaling through CTLA-4 under certain conditions can also induce apoptosis in previously activated T cells (19). Such apoptosis occurs when a putative third ligand of CTLA-4 signals in the absence of CD28 co-stimulation. Lack of CD28 co-stimulation during antigen stimulation of peripheral blood T cells from HIV-infected individuals has been previously reported (3), such a lack of B7-1 and B7-2 signaling in the presence of the putative third ligand could result in CTLA-4-induced T cell apoptosis. CD30, another member of the TNF/NGF receptor family, also can induce apoptosis in cell lines and has been shown to co-stimulate T cell proliferation and cytokine production (20,21).

Cytokines have also been proposed as modifiers of T cell apoptosis in HIV. IL-12 has recently been shown to enhance antigen-specific T cell proliferation and inhibit spontaneous and activation-induced T cell apoptosis in HIV-infected individuals (22,23). T_H2 -type cytokines such as IL-4 and IL-10 were shown to enhance T cell activation-induced apoptosis (23). These observations suggest a possible role for cytokines in the regulation of apoptosis in HIV.

These findings prompted us to investigate whether Fas, CTLA-4, p55 and p75 TNFR or CD30 are involved in the spontaneous T cell apoptosis or T cell AICD of HIV disease. We report here that spontaneous and activation-induced T cell apoptosis in PBMC from asymptomatic HIV-infected individuals cannot be inhibited by reagents that block Fas, TNF- α , lymphotoxin (LT)- α , CTLA-4 or CD30 interaction with their respective ligands or receptors. AICD and anti-Fas-induced apoptosis of PBMC from HIV-infected individuals resulted in different CD4⁺ and CD8⁺ T cell subset apoptosis profiles, with anti-Fas inducing apoptosis preferentially in CD4⁺ T cells and AICD inducing apoptosis equally in CD4⁺ and CD8⁺ T cells. In addition, cytokines such as IL-12, IFN- γ , IL-4 and IL-10 could not modify spontaneous, activation-induced or Fas-induced apoptosis. The above findings indicate that AICD of T cells in HIV infection is Fas independent. These two independent mechanisms of T cell apoptosis may play different roles in the pathogenesis of HIV disease.

Methods

Samples and materials

Heparinized blood samples were obtained following informed consent of asymptomatic HIV⁺ and HIV⁻ individuals. Blocking mouse mAb to Fas antigen (M3, IgG1 isotype) and CD30L (M81, IgG2b), and recombinant Fas–Fcy, CTLA-4–Fcy and p75TNF–Fcy fusion proteins were provided by Immunex (Seattle, WA). Blocking mouse mAb to FasL (NOK1, IgG1 isotype) was provided by Dr H. Yagita (Juntendo University, Tokyo, Japan). The blocking anti-hTNF- α mouse mAb (A2, IgG1 isotype) was a kind gift from Dr James Woody (Centocor, PA). Anti-CD3 mAb OKT3 and the isotype control antibody DS-1 (anti-mouse IgM of the a allotype) were kindly provided by Drs M. Feldmann (London, UK) and A. B. Kantor (Stanford, CA) respectively. Recombinant IL-12 (kind gift from Dr M.

Gately, Roche, NJ), IFN- γ (Boehringer Mannheim, Germany), IL-10 (Genzyme, Cambridge, MA) and IL-4 (kindly provided by Dr F. Di Padova, Sandoz, Switzerland) were all used at 10 ng/ml in culture. RPMI 1640 (Gibco/BRL, Gaithersburg, MD) and FCS (Gibco/BRL) contained <0.03 and <0.3 EU/ml of endotoxin respectively.

Anti-Fas- and anti-CD3-induced apoptosis and inhibition experiments.

Freshly isolated PBMC by density centrifugation on Ficoll-Hypaque from asymptomatic HIV⁺ and HIV⁻ individuals were cultured for 24 h at 1×10^6 cells/ml in RPMI 1640 + 10% FCS in 24 well plates coated with or without monoclonal anti-Fas antibody M3, anti-CD3 antibody OKT3 or isotype control mAb DS-1. Plates were coated with 500 μ l/well of 10 μ g/ml of antibodies in RPMI 1640 for 2 h at 37°C. Plates were then washed twice briefly with 500 μ l/well RPMI 1640/10% FCS and then washed one more time as above for 30 min at 37°C. In the blocking experiments soluble mAb to Fas (M3) ($n = 15$), anti-FasL mAb (NOK-1) ($n = 5$), Fas–Fcy fusion protein ($n = 6$), CTLA-4–Fcy fusion protein ($n = 8$), p75TNFR–Fcy fusion protein ($n = 5$), neutralizing anti-human TNF- α mAb A2 ($n = 5$) and mAb to CD30L ($n = 4$) were all used at 10 μ g/ml in solution and added simultaneously with PBMC to coated plates. In some experiments ($n = 5$) anti-Fas blocking mAb M3 was added together with either the anti-TNF- α mAb A2 or the p75TNFR–Fcy fusion protein each at 10 μ g/ml. In the cytokine experiments PBMC from asymptomatic HIV⁺ individuals ($n = 7$) were treated with 10 ng/ml of cytokine immediately following the addition of the PBMC to uncoated or OKT3- or M3-coated plates. Biological activity of the above cytokines was tested by measuring TNF- α production by PBMC from HIV⁻ individuals stimulated with 1 μ g/ml lipopolysaccharide (LPS). TNF- α was measured by specific ELISA (PharMingen, San Diego, CA). In all the above experiments cells were cultured for 24 h at 1×10^6 cells/ml in RPMI 1640 + 10% FCS in 24-well plates.

Apoptosis measurement and DNA fragmentation

Apoptosis was determined as previously described (6), by staining cells with 1 μ g/ml Hoechst 33342 (HO342; Molecular Probes, Eugene, OR) for 7 min on ice. Cells were co-stained with anti-CD8–phycoerythrin (PE) (Becton Dickinson, Mountain View, CA), anti-CD4–FITC (PharMingen) and anti-CD5–CyChrome 5 (Cy5) (PharMingen) for 15 min on ice. Following washes and fixing in paraformaldehyde, 30,000 cells were analyzed by flow cytometry using a FACStar (Becton Dickinson) and Desk software (24). Apoptosis was determined by measuring HO342 staining cells gated on forward and side scatter for lymphocytes. Live CD4⁺ and CD8⁺ T cell absolute numbers in culture were determined by measuring the percent of live non-apoptotic cells by flow cytometry as above and counting the total number of PBMC using a Coulter Counter model ZM (Coulter, Hialeah, FL).

DNA fragmentation analysis was performed on PBMC (2×10^6 cells) treated as described above for 5 h. Cells were centrifuged and DNA was extracted by resuspending cells in 20 μ l of 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosylate, 0.5 mg/ml proteinase K and incubating for 1 h at 50°C. RNase was then added at 100 μ g/ml and the

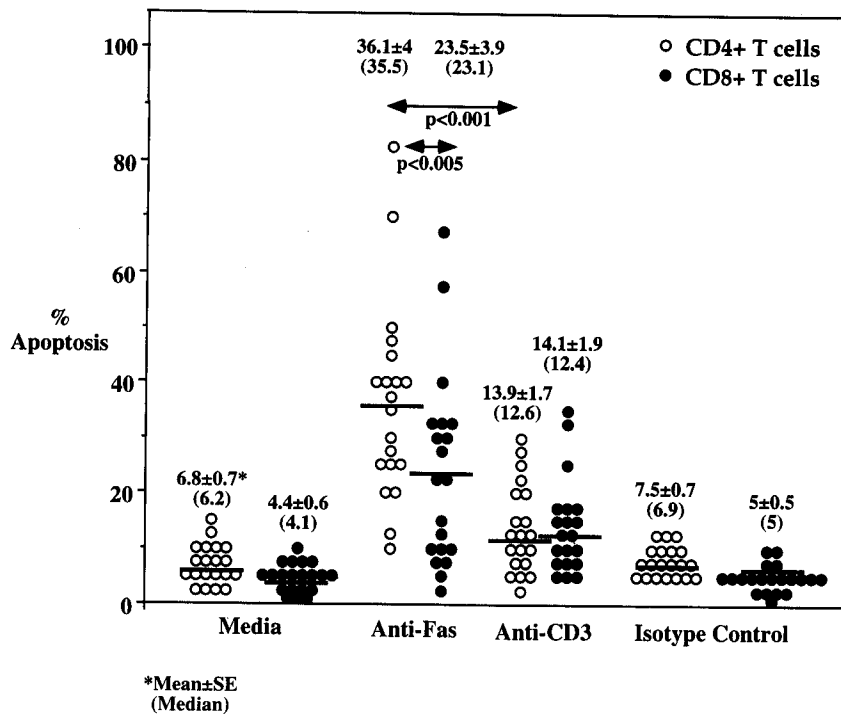


Fig. 1. Activation and Fas-induced apoptosis of peripheral blood T cells from asymptomatic HIV⁺ individuals. Immobilized anti-Fas mAb induced significantly higher levels of CD4⁺ T cell apoptosis compared to CD8⁺ T cells ($n = 20$, $P < 0.005$, Wilcoxon's signed-rank test for paired data). No difference between CD4⁺ and CD8⁺ T cell apoptosis was found when cultures were treated with immobilized anti-CD3 mAb. Anti-Fas antibody induced significantly higher levels of CD4⁺ T cell apoptosis compared to anti-CD3 treatment ($n = 20$, $P < 0.001$, Mann-Whitney U -test). Mean \pm SE with median value in parenthesis is shown above each group. Horizontal bars depict median values.

extract was further digested for 1 h at 50°C. DNA was run on a 2% agarose gel in 45 mM Tris-borate, 1 mM EDTA, pH 8.0, stained with 0.5 μ g/ml ethidium bromide and visualized under UV light.

Statistical analysis

Data were analyzed by the Mann-Whitney U -test and Wilcoxon's signed-rank test for paired data, using JMP Statistics Guide (SAS Institute, Cary, NC).

Results

Anti-Fas- and anti-CD3- induced CD4⁺ and CD8⁺ T cell apoptosis

Immobilized anti-Fas and anti-CD3 treatment of PBMC from asymptomatic HIV-infected individuals ($n = 20$) resulted in both CD4⁺ and CD8⁺ T cell apoptosis. Apoptosis induced by 24 h anti-Fas treatment resulted in significantly higher CD4⁺ T cell apoptosis compared with that of CD8⁺ T cells ($P < 0.005$), whereas anti-CD3 treatment resulted in similar levels of apoptosis in these two T cell subpopulations (Fig. 1). Anti-Fas treatment induced significantly higher CD4⁺ T cell apoptosis compared to anti-CD3 ($P < 0.001$). CD8⁺ T cell apoptosis was also higher in anti-Fas-treated cultures; however, this was not found to be statistically significant when compared with anti-CD3-induced CD8⁺ apoptosis ($P > 0.05$) (Fig. 1). Cell numbers of live CD4⁺ and CD8⁺ T cells

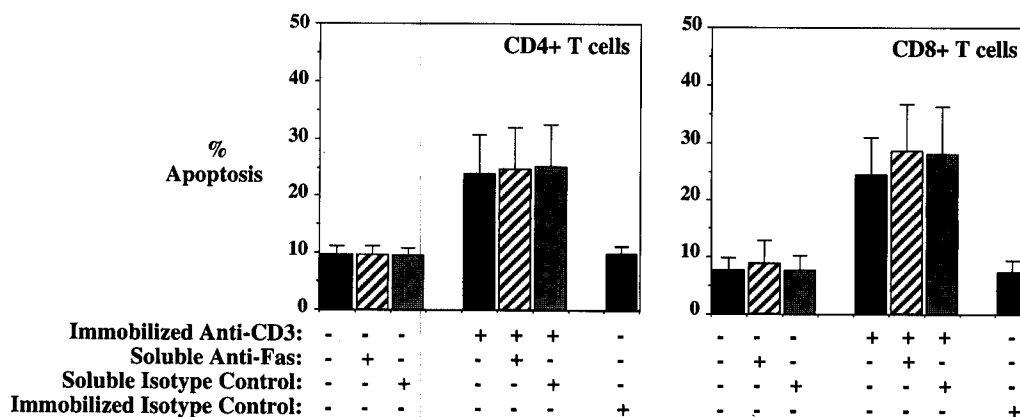
counted in these cultures gave similar results to the apoptosis measurement (data not shown).

AICD inhibition experiments

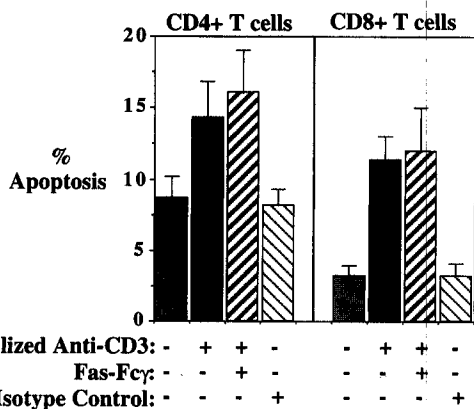
Spontaneous and anti-CD3-induced T cell apoptosis in PBMC from asymptomatic HIV⁺ individuals could not be inhibited in 24 h cultures with reagents that block Fas-FasL interactions. These reagents, i.e. blocking mAb to Fas antigen (number of patients: $n = 15$) and FasL ($n = 5$) and a Fas-Fc γ fusion protein ($n = 6$), all failed to block spontaneous and anti-CD3-induced apoptosis measured by flow cytometry (Fig. 2A-C). They also failed to inhibit DNA fragmentation and loss of cell numbers in culture (data not shown). These reagents at 10 μ g/ml, however, could inhibit AICD induced by anti-CD3 stimulation of the Jurkat T cell line (Fig. 3 and data not shown). These reagents also failed to inhibit T cell AICD induced by staphylococcal enterotoxin A and pokeweed mitogen in PBMC from HIV⁺ individuals (data not shown).

A CTLA-4-Fc γ fusion protein ($n = 8$) and a blocking mAb to CD30L ($n = 4$) also failed to block spontaneous and anti-CD3-induced apoptosis in HIV⁺ PBMC (Fig. 4). The CTLA-4-Fc γ fusion protein also failed to inhibit anti-CD3-induced DNA fragmentation in these cultures (data not shown). TNF- α and LT- α blocking by a p75TNFR-Fc γ fusion protein ($n = 5$) and the neutralizing anti-human TNF- α mAb A2 ($n = 5$) also failed to block spontaneous and anti-CD3-induced T cell apoptosis (data not shown). Spontaneous and activation-induced T cell

A)



B)



C)

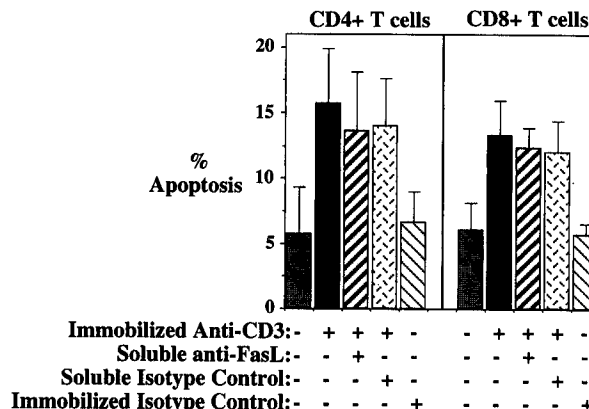


Fig 2. Anti-CD3-induced apoptosis of peripheral blood CD4⁺ and CD8⁺ T cells from asymptomatic HIV⁺ individuals is Fas-FasL independent. (A) Soluble blocking monoclonal anti-Fas antibody does not inhibit anti-CD3-induced T cell apoptosis (*n* = 15). (B) Fas-Fc γ fusion protein cannot block anti-CD3-induced T cell apoptosis (*n* = 6). (C) Anti-FasL blocking mAb does not inhibit anti-CD3-induced apoptosis (*n* = 5).

death was also not inhibited by the simultaneous addition to these cultures of anti-Fas blocking mAb together with either the anti-TNF- α mAb A2 or the p75TNFR-Fc γ fusion protein (*n* = 5) (data not shown). All the above treatments also failed to inhibit the loss of absolute numbers of CD4⁺ and CD8⁺ T cells in these cultures (data not shown).

Cytokines and apoptosis.

To investigate whether cytokines can modulate spontaneous, AICD and Fas-induced T cell apoptosis in PBMC cultures from asymptomatic HIV-infected individuals (*n* = 7), we incubated untreated, anti-CD3- and anti-Fas-treated cultures with 10 ng/ml of IL-12, IFN- γ , IL-4 and IL-10 for 24 h. Cells were then counted and apoptosis was measured by flow cytometry. None of the above cytokines enhanced or inhibited T cell apoptosis (Fig. 5), cell loss and DNA fragmentation (data not shown) in any of these cultures. The biological activity of the above recombinant cytokines was confirmed by measuring TNF- α produced by PBMC stimulated with LPS in the presence of the above cytokines. IL-12 and IFN- γ were potent enhancers whilst IL-4 and IL-10 were potent inhibitors of TNF- α production (data not shown).

Discussion

A number of studies have recently shown that Fas is involved in T cell activation-induced cell death of T cell clones, lines and hybridomas (15-18). These observations raise the question whether Fas also is involved in both the spontaneous and activation-induced T cell apoptosis of HIV infection. We previously showed that peripheral blood T cells from HIV-infected individuals are especially susceptible to undergo apoptosis in response to Fas cross-linking (6). We extend these observations here by asking whether Fas also mediates spontaneous or activation-induced apoptosis in PBMC from asymptomatic HIV-infected individuals. Using a number of different reagents that block Fas-FasL interactions we find that spontaneous and activation-induced T cell apoptosis in PBMC from HIV⁺ individuals is independent of Fas-FasL interactions. A recent study has indicated that FasL and TNF- α may both participate in AICD of mature mouse T cells (25), raising the possibility that these factors may be synergizing in peripheral blood T cell apoptosis of HIV⁺ individuals. In this study we find that simultaneous blocking of Fas and TNF- α also fails to inhibit T cell spontaneous apoptosis and AICD

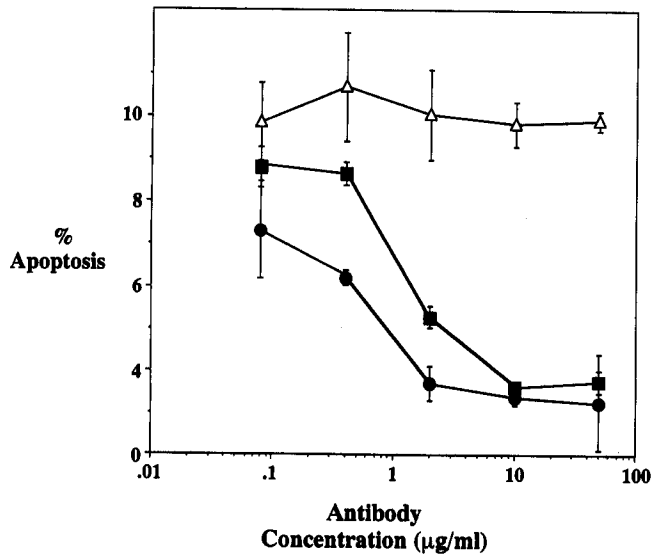


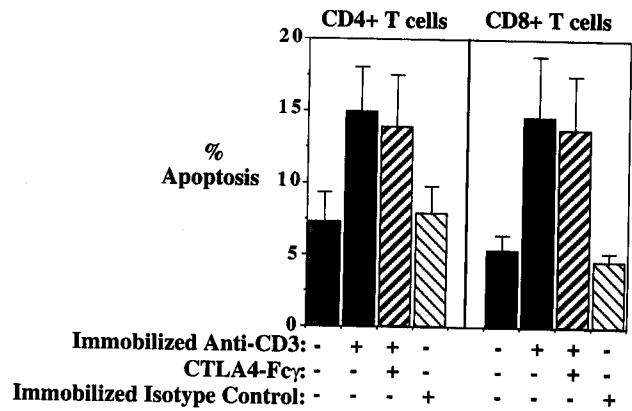
Fig. 3. Dose response of inhibition of Jurkat T cell line AICD by soluble antibodies to Fas and FasL. Soluble anti-Fas antibody (closed circles), anti-FasL antibody (closed squares) and isotype control (open triangles).

in PBMC from HIV⁺ individuals. The mechanism responsible for this Fas-independent AICD in HIV infection is not known; however, such Fas-independent AICD has been previously described for mouse CD4⁺CD8⁻, CD4⁺CD8⁺ and CD4⁺CD8⁺ thymocytes (26), and more relevantly in mature peripheral lymphocytes (27,28), raising the question as to whether AICD in these systems is similar to that of HIV infection. Further studies should address this.

CTLA-4 has recently been suggested to be involved in AICD as it has been shown to mediate T cell apoptosis in some mouse T cell clones. Such apoptosis occurs when a putative third ligand of CTLA-4 signals in the absence of CD28 co-stimulation (19). Lack of CD28 co-stimulation has been reported during antigen stimulation of peripheral blood T cells from HIV-infected individuals (3), such a lack of B7-1 and B7-2 signaling in the presence of the putative third ligand could result in CTLA-4-induced T cell apoptosis. In the present study we find that spontaneous and activation-induced peripheral blood T cell apoptosis in HIV⁺ individuals is CTLA-4 independent since it cannot be blocked by a CTLA-4-Fcγ fusion protein. CD30 has been reported to induce apoptosis in some B cell lines and can co-stimulate T cells (20,21), we therefore investigated whether CD30L participates in the T cell apoptosis of HIV. We found that blocking CD30L had no effect on spontaneous or activation-induced peripheral blood T cell death of HIV-infected individuals.

Cytokines have also been proposed as modifiers of T cell apoptosis in HIV. IL-12 has recently been shown to enhance antigen-specific T cell proliferation and inhibit spontaneous and activation-induced and anti-Fas-induced T cell apoptosis in HIV-infected individuals (22,23,29). T_H2-type cytokines such as IL-4 and IL-10 were shown to enhance T cell activation-induced apoptosis (23). However, we found that recombinant IL-12, IFN-γ, IL-4 and IL-10 at 10 ng/ml concentration failed

A)



B)

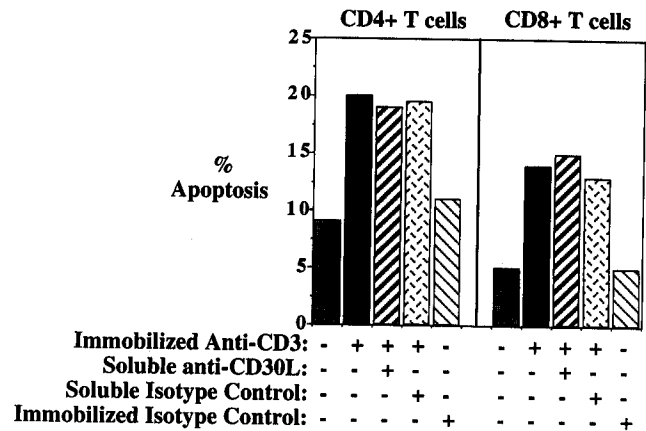


Fig. 4. CTLA-4 and CD30 do not mediate anti-CD3-induced T cell apoptosis of PBMC from asymptomatic HIV⁺ individuals. (A) CTLA-4-Fcγ fusion protein fails to inhibit AICD (*n* = 8). (B) Anti-CD30L blocking mAb does not inhibit anti-CD3-induced T cell apoptosis (representative experiment of four performed shown).

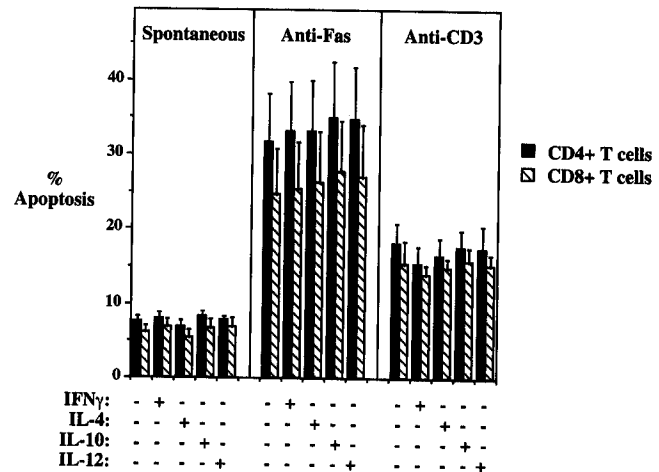


Fig. 5. T_H1 and T_H2 type cytokines have no effect on spontaneous, anti-CD3-induced and anti-Fas-induced apoptosis of peripheral blood T cells from asymptomatic HIV⁺ individuals (*n* = 7).

to have any effect on spontaneous, activation-induced or anti-Fas-induced T cell apoptosis in PBMC from asymptomatic HIV⁺ individuals. These same cytokines were very potent in modulating TNF- α production by LPS-stimulated PBMC from HIV⁻ individuals. The reason for the discrepancies between our data and the previous reports are not clear, but may have to do with the use of PBMC in our experiments instead of purified T cells. Also, our experiments were all carried out under LPS-free conditions thus possibly contributing to these discrepancies. Our data are in agreement with a previous report showing that cytokines cannot modulate AICD of mouse T cell clones (30). Our findings exclude a regulatory role of T_H1- or T_H2-type cytokines in the T cell apoptosis of HIV infection.

Recent reports have shown that T cells undergoing apoptosis in lymph nodes of HIV-infected individuals are not directly infected with HIV virus (31). Similarly, apoptotic T cells in the thymus of SCID mice reconstituted with human fetal thymus and liver and infected with HIV are also not infected (32). T cell apoptosis in HIV infection is due to indirect priming for apoptosis in response to activation and Fas stimulation. Some studies have suggested that cross-linking of CD4 on T cells by gp120 protein results in cells undergoing AICD when this is followed with stimulation through the TCR (2,33). CD4 cross-linking in mice results in T cell apoptosis and this apoptosis is mediated by Fas (34). HIV Tat protein has also been shown to prime normal peripheral blood T cells to undergo AICD in response to TCR or anti-CD3 stimulation (35,36). We have found that although Tat enhances AICD it does not prime normal T cells to undergo Fas-induced apoptosis (P. Katsikis, in preparation). The possibility that spontaneous, activation-induced as well as Fas-induced T cell apoptosis may result from non-specific immune activation and not be the result of an HIV-specific mechanism cannot be excluded at the moment. However, our preliminary data examining apoptosis in chronic hepatitis C patients show that no such peripheral blood T cell apoptosis occurs in these patients (P. Katsikis, in preparation). The above studies indicate that there may be more than one mechanism of apoptosis priming in HIV infection.

The possible importance of apoptosis in the pathogenesis of HIV infection has been shown in animal models of chronic lentiviral infections in which apoptosis is observed only in pathogenic models (4,37). Animal models have also suggested that two different mechanisms of T cell apoptosis may be operative in chronic lentiviral infections, one involving primarily CD4⁺ T cell apoptosis and observed only in pathogenic models, CD8⁺ T cell apoptosis being observed in both pathogenic and non-pathogenic models (5). Our findings comparing Fas- and activation-induced apoptosis shows a preference for CD4⁺ T cell killing by Fas indicating that Fas-induced apoptosis may be an important player in HIV pathogenesis. Cells primed to undergo Fas-induced apoptosis circulating through sites where activated CD8⁺ T cells may be residing and expressing FasL would be killed in a bystander manner and this could be an antigen-independent mechanism of apoptosis which leads to T cell depletion. AICD, on the other hand, is an antigen-dependent mechanism of apoptosis which could lead to depletion and hyporesponsiveness of T cells to recall antigen. The contribution of these

two mechanisms of apoptosis in the pathogenesis of HIV disease remains to be determined.

Our findings support the hypothesis that activation and Fas-induced apoptosis of peripheral blood T lymphocytes from HIV-infected individuals are independent. Fas- and activation-induced apoptosis resulted in different profiles of cell death with Fas being a more efficient killing mechanism with a preference for CD4⁺ T cell killing over CD8⁺ T cells. In addition activation-induced T cell apoptosis is Fas, TNF- α , LT- α , CTLA-4 and CD30 independent. We also show that spontaneous, activation and anti-Fas-induced apoptosis are not affected by T_H1- or T_H2-type cytokines. Our findings suggest that Fas- and activation-induced apoptosis of peripheral blood T cells from HIV⁺ individuals are two independent mechanisms of apoptosis that may play different roles in HIV pathogenesis. Understanding the mechanisms responsible in HIV infection for priming of T cells to undergo apoptosis in response to Fas antigen and TCR stimulation may prove important in elucidating the immunopathogenesis of HIV infection and designing novel therapeutic approaches.

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Abbreviations

AICD	activation-induced cell death
FasL	Fas ligand
LPS	lipopolysaccharide
LT	lymphotoxin
NGF	nerve growth factor
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor

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