T Helper Type 1/T Helper Type 2 Cytokines and T Cell Death: Preventive Effect of Interleukin 12 on Activation-induced and CD95 (FAS/APO-1)–mediated Apoptosis of CD4$^+$ T Cells from Human Immunodeficiency Virus–infected Persons

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Summary

Human immunodeficiency virus (HIV) infection leads to a progressive loss of CD4$^+$ T helper (Th) type 1 cell–mediated immunity that is associated with defective in vitro CD4$^+$ T cell proliferation and abnormal T cell death by apoptosis in response to T cell receptor (TCR) stimulation. Quantification of interleukin (IL)-2, interferon γ, IL-4, IL-5, and IL-10 secretion by immunoassays, and of interferon γ, IL-4 and IL-10 messenger RNA expression by competitive reverse transcriptase polymerase chain reaction after in vitro stimulation of the TCR revealed a similar Th1 cytokine profile in T cells from HIV-infected persons and from controls. These data indicated that the loss of CD4$^+$ Th1 cell function in HIV-infected persons is not related to a Th1 to Th2 cytokine switch as previously proposed, but to a process of activation-induced death of CD4$^+$ Th1 cells. Despite the absence of elevated levels of Th2 cytokines, apoptosis of CD4$^+$ T cells, but not of CD8$^+$ T cells, was prevented in vitro by antibodies to IL-10 or IL-4, two Th2 cytokines that downregulate Th1 cell responses, or by the addition of recombinant IL-12, a cytokine that upregulates Th1 functions. TCR-induced apoptosis of T cell hybridomas and preactivated T cells has been shown to involve the CD95 (Fas/Apo-1) molecule. CD4$^+$ and CD8$^+$ T cells from HIV-infected persons expressed high levels of the CD95 molecule, and, in contrast to T cells from controls, were highly sensitive to antibody-mediated CD95 ligation, which induced apoptosis in a percentage of T cells similar to that induced by TCR stimulation. As TCR-induced apoptosis, CD95-mediated apoptosis of CD4$^+$ T cells, but not of CD8$^+$ T cells, was prevented by the addition of recombinant IL-12. Together, these findings suggest that apoptosis of CD4$^+$ T cells from HIV-infected persons involves an abnormal sensitivity to CD95 ligation, and to TCR stimulation in the presence of normal levels of Th2 cytokines. The preventive effect of IL-12 on both mechanisms has potential implications for the design of immunotherapy strategies aimed at the upregulation of CD4$^+$ Th1 cell functions in AIDS.

In HIV-1–infected persons, CD4$^+$ T cell depletion is preceded by early functional defects of cell-mediated immunity (1–3), characterized in vitro by a failure of T cells to proliferate in response to TCR stimulation by recall antigens and by various mitogens, and in vivo by a loss of cell-mediated, delayed-type hypersensitivity reaction, a response that involves the CD4$^+$ Th1 lymphocyte population (3). We have proposed that T cell defects in HIV-1–infected persons may be related to abnormal induction of apoptosis, or programmed cell death (PCD)[1] caused by interference of HIV mediated with intercellular signalling (4). Experimental support for this model has been provided by a series of observations from several laboratories showing abnormal

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[1] *Abbreviations used in this paper:* CSA, cyclosporin A; CHX, cycloheximide; PCD, programmed cell death; RT-PCR, reverse transcriptase PCR; SEB, Staphylococcal enterotoxin B.

levels of PCD in vitro in $CD4^+$ and $CD8^+$ T cells from HIV-infected persons (5–11), as well as a relationship between HIV infection and PCD in mature $CD4^+$ T cells, thymocytes, and hematopoietic progenitor cells (12–20). Candidate mechanisms for T cell apoptosis in response to TCR stimulation include the ligation of the CD95 (Fas/Apo-1) cell-surface molecule (21–23), involving the expression of CD95, the expression of the CD95 ligand, and the induction of a differentiation stage that renders T cells sensitive to CD95 ligation (24–29).

In a model that addressed the pathogenesis of cell-mediated immunity defects independently of the question of T cell death, Clerici and Shearer proposed that, as in other chronic parasite bacterial or viral infections (30–33), the progression of HIV infection to disease may result from a progressive shift of $CD4^+$ T cells from the Th1 to the Th2 phenotype, leading to a loss of IL-2 and IFN-γ production, concomitant with increases in IL-4 and IL-10 secretion (34). Consistent with this idea, in vitro addition of IL-12, a cytokine that favors the induction of a Th1 cell response (35), or of antibodies to IL-10 or IL-4, two cytokines that inhibit Th1 cell responses and favor the induction of a Th2 cell response (36), were shown to restore, in HIV-infected persons, the proliferative response of T cells in response to recall antigens and mitogens (37, 38). In this study, we have explored the possibility that cytokines may affect T cell functions by regulating apoptosis in T cells from HIV-infected persons. Our results indicate that the defective $CD4^+$ Th1 cell response in HIV infection is not related to a Th1 to Th2 cytokine switch, but to a process of activation-induced $CD4^+$ Th1 cell death by apoptosis in response to normal levels of Th2 cytokines and to CD95 (Fas/Apo1) ligation, which can be prevented by the addition of IL-12.

Materials and Methods

Reagents, Antibodies, and Cytokines. Murine anti-human mAb used were CD4 (Leu 3a, Becton Dickinson & Co., Mountain View, CA); CD8 (Leu 2a; Becton Dickinson); CD14 (IOM2); CD19 (IOB4); CD56 (IOT56); CD95 (UB_2); HLA class II (IOT2a); and CD3 (IOT3b) (Immunotech, Marseille, France). Purified azide-free rat anti-human cytokine neutralizing IgG1 mAb included anti-IL-10 (JES3-9D7), anti-IL-4 (MP4-25D2), anti-IL-5 (TRFK5) (PharMingen, San Diego, CA), and purified azide-free murine anti-human CD95 IgM mAb (CH_{11}) and control IgM mAb (GC_{323}) (Immunotech). Other reagents were Staphylococcal enterotoxin B (SEB) (Toxin Technology Inc., Madison, WI); PHA, PWM, cycloheximide (CHX) (Sigma, La Verpillière, France); cyclosporin A (CSA) (Sandimmum, Sandoz, Rueil Malmaison, France), acridine orange dye (Immunotech), and YO-PRO-1 dye (Molecular Probes, Inc., Eugene, OR). Cytokines included rIL-12 and rIL 10 (R&D Systems, Abingdon, UK), and rIL-2, kindly provided by Roussel-UCLAF (Paris, France).

Cells and Culture Conditions. Heparinized venous peripheral blood was obtained from HIV-1–seropositive adults with $CD4^+$ T cell counts ranging from 100 to 1,500/mm^3 (mean = 560 ± 363) and from HIV-seronegative healthy controls. PBMC were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation, and were cultured in RPMI 1640 (Gibco, Courbevoie, France) supplemented with 10% heat-inactivated FCS (Boehringer Mannheim, Meylan, France), 2 mM L-glutamine, 1 mM sodium pyruvate (Gibco), and gentamicin (Gentalline; 8 μg/ml, Schering-Plough, Levallois-Perret, France). In some experiments, PBMC were depleted of B cells and of either $CD4^+$ or $CD8^+$ T cells by negative selection using CD19, CD56, CD4, or CD8 mAb, and magnetic beads coated with anti-mouse IgG (Dynal, Great Neck, NY), as described (11). Contaminating $CD4^+$ or $CD8^+$ T cells were <5%, as assessed by flow cytofluorometry (Epics Profile; Coulter Coultronics, Margency, France). Cells were cultured in 96-well culture plates (Falcon; Becton Dickinson) at 2.5×10^5/ml, or at 10^6/ml in 24-well plates for cytokine detections (Nunc, Roskilde, Denmark). For T cell activation, stimuli used were PWM (10 μg/ml), SEB (1 μg/ml), PHA (5 μg/ml), and CD3 mAb (0.5 μg/ml). When added to the cultures, mAbs against the IL-4, IL-5, and IL-10 cytokines were used at 5 μg/ml, rIL-12 at 20 ng/ml, rIL-10 at 10 ng/ml, and rIL-2 at 20 IU/ml, CSA at 0.5 μg/ml, and CHX at 0.2 μg/ml. The effect of CD95 cross-linking was explored by incubating cells with the CD95 mAb CH_{11} (1.5 μg/ml) (28) or with the control IgM mAb GC_{323} (1.5 μg/ml).

Apoptosis Measurement and Sorting of Living Cells. Percentages of apoptotic cells were measured in duplicate under the light microscope by two different investigators. Cells counted as apoptotic included cells with characteristic nuclear chromatin condensation and fragmentation, as well as already dead cells that had lost trypan blue exclusion capacity, as previously described (11). Percentage measurement variation between the two investigators was <1%. Percentages of cell death prevention, when so mentioned, were expressed as follows:

$$\frac{\left(\begin{array}{c}\text{apoptosis in}\\ \text{stimulated cells}\end{array}\right) - \left(\begin{array}{c}\text{apoptosis in}\\ \text{stimulated cells with}\\ \text{mAb or cytokines}\end{array}\right)}{\left(\begin{array}{c}\text{apoptosis in}\\ \text{stimulated cells}\end{array}\right) - \left(\begin{array}{c}\text{apoptosis in}\\ \text{unstimulated cells}\end{array}\right)} \times 100$$

Percentages of apoptotic cells counted under the light microscope were confirmed by flow cytofluorometry analysis after incubation with the nuclear dye acridine orange as described (11), or with the nuclear dye YO-PRO-1, which does not affect the functional properties of living cells (50). After incubation with YO-PRO-1 (5 μM for 10^6 cells) for 10 min, lymphocytes were gated under forward and side light scatter, apoptotic cells representing two peaks of high fluorescence intensity, and reduced forward scatter. After stimulation with PWM, living lymphocytes were separated from apoptotic lymphocytes with >98% efficiency, using cell sorting coupled with flow cytofluorimetry analysis (Epics Elite cell sorter; Coulter Coultronics). In contrast to the acridine orange or Hoechst 33342 (Sigma) nuclear dyes, YO-PRO-1 only stains apoptotic cells and is excluded by living cells, allowing further functional studies to be performed on living cells (50). In an experiment, autologous adherent accessory cells (2×10^5/ml) were added to the living lymphocytes (10^6/ml) sorted 36 h after stimulation with PWM, and cells were restimulated by PHA for an additional 36 h culture.

Cytokine Detection. Immunoassays used for the determination of cytokine secretion were ELISA Kits for IL-2, IL-4, and IL-12 (R&D Systems), IFN-γ (Genzyme, Cambridge, MA), and IL-10 (Biosource, Camarillo, CA). IL-5 was determined using a pair of rat anti-human IL-5 mAb (TRFK5, and JES1-5A10) (PharMingen) in a two-site sandwich ELISA.

Quantification of Cytokine mRNA by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total cellular RNA was ex-

tracted from 5 × 10^6 frozen cells at 10 h of culture, using the RNAzol technique according to manufacturer's recommendations (Bioprobe, Montreuil, France) and then extracted by phenol/chloroform procedure. cDNAs were synthesized by adding to the mRNA preparation 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Cergy Pontoise, France) in the presence of oligo-dT_{12-18} (Gibco-BRL) and dNTP. Samples were incubated at 42°C for 60 min.

cDNA (50 ng) was incubated with β-actin sense (5′-GGGT-CAGAAGGATTCCTATG-3′) and antisense (5′-GGTCTC-AAACATGATCTGGG-3′) primers, Taq polymerase (Bioprobe), the 4 dNTP, 0.1 μCi of dTTP ^{32}P (Amersham, Les Ulis, France), 25 mM $MgCl_2$, and graded concentrations (0.01, 0.1, and 1 pg) of a competitor (pQB2) (39, 40). PCR was then performed, each cycle consisting of 94°C, 1 min, 57°C, 1 min, and 72°C, 1.5 min. The products of 31 cycles were electrophoresed on a 2% agarose gel that was dried and autoradiographed for 3 h. The intensities of the 237- and the 388-bp bands (corresponding to the amplified β-actin cDNA and to the amplified competitor, respectively) were determined using an image analyzer (IMSTAR, Paris, France), and their ratio was calculated. A curve of the ratios was established according to the competitor concentration for which a similar amount of competitor and cDNA were present. Results were expressed as the number of β-actin cDNA molecules present in each sample.

Quantitation of cytokine cDNAs was then performed as follows. For IFN-γ (sense, 5′-GCAGAGCCAAATTGTCTCCT-3′; antisense, 5′-ATGCTCTTCGACCTCGAAAC-3′), IL-10 (sense, 5′-AAATTTGGTTCTAGGCCGGG-3′; antisense, 5′-GAGTACAGGGGCATGATATC-3′), IL-4 (sense, 5′-TGCCTC-CAAGAACACAACTG-3′; antisense 5′-AACGTACTCTGG-TTGGCTTC-3′), and IL-12 p40 chain (sense, 5′-ATTGAG-GTCATGGTGGATGC-3′; antisense, 5′-AATGCTGGCATT-TTTGCGGC-3′) cDNAs, a competitive PCR was performed by adding to a volume of cDNA preparation containing 10^5 molecules of β-actin cDNA and graded concentrations of competitor (39, 40). For IL-12 p35 cDNA (sense, 5′-GCCCTGTGCCTT-AGTAGTAT-3′; antisense, 5′-GCTCGTCACTCTGTCAATAG-3′), a semiquantitative PCR was performed: fourfold dilutions of cDNA were amplified in the same conditions as described above except that no competitor was added. To express results, an arbitrary value of 1 was attributed to the amount of IL-12 p35 chain cDNA present in the sample from unstimulated PBMC from the healthy individual.

Statistical Analyses. Statistical significance was assessed by either Student's *t* test or paired Student's *t* test, as mentioned.

Results and Discussion

Activation-induced T Cell Death Is Associated with a Th1 Cytokine Secretion Profile. T cells from HIV-infected persons undergo in vitro death by apoptosis, a process that is markedly enhanced by TCR stimulation with PWM (Fig. 1 *A*), bacterial superantigens, or the CD3 mAb (5–11). In the present study, secretion of IL-2, IFN-γ, IL-10, IL-4, and IL-5 was compared in PBMC from HIV-infected persons with those from uninfected controls in the absence of any stimulation and after stimulation with PWM. In the absence of stimulation, the only cytokine secreted was IL-10, but not significant differences were observed between levels of spontaneous IL-10 released for 24 h by PBMC from HIV-infected and -uninfected persons (not shown). 24 h after activation, the secretion of IL-2, IFN-γ, and IL-10 by PBMC from HIV-infected persons and from controls was at similar levels, with no significant differences (Fig. 1 *B*). 24 and 40 h after stimulation, IL-4 and IL-5 secretion was very low in PBMC from both HIV-infected persons and controls, except for elevated IL-4 secretion in PBMC from only 1 of the 16 HIV-infected persons (Fig. 1 *C*). Quantitative measurements of IFN-γ, IL-4, and IL-10 mRNA expression by competitive RT-PCR in PBMC from two HIV-infected persons and one control confirmed the absence of elevated Th2 cytokine expression in the activated T cells from HIV-infected persons (Table 1).

Analysis of cytokine secretion by PBMC depleted either of $CD4^+$ or of $CD8^+$ T cell subsets showed that the Th1 cytokine IL-2 was secreted exclusively by the $CD4^+$ T cells (Fig. 2 *A*). IL-2 secretion levels, although not significantly different in HIV-infected and -uninfected persons, were more variable among individuals in the HIV-infected group, with some values being very low (Fig. 1 *B*). We explored

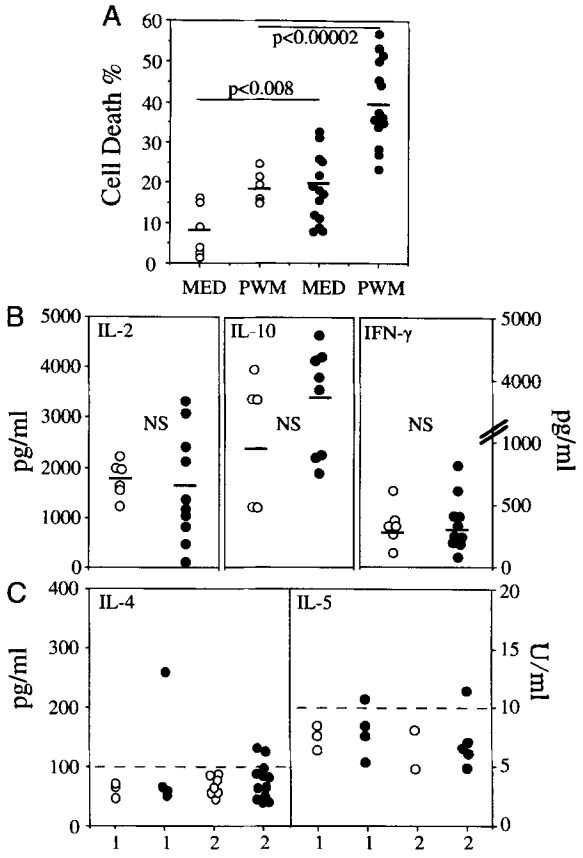


Figure 1. Activation-induced cell death and cytokine secretion in PBMC from HIV-1–infected individuals (●) or controls (○). (*A*) Apoptosis was assessed under the light microscope 36 h after incubation with medium or PWM. (*B*) IL-2, IL-10, and IFN-γ secretions were measured 24 h after PWM stimulation. (*C*) IL-4 and IL-5 secretion were measured 24 h (*1*) or 40 h (*2*) after PWM stimulation. *Dotted line* represents significant detection limit. Each circle represents results from a different study subject. Bars represent the mean value in each group. Statistical significance was assessed using Student's *t* test. *NS*, no significant differences between groups.

Table 1. *Quantitative Analysis of Cytokine mRNA Expression by Competitive RT-PCR*

	Stimulus	IFN-γ	IL-4	IL-10
CTR	Med	0.4	0.7	125
	PWM	392	3.2	546
HIV	Med	2.8	0.09	275
	PWM	348	0.25	175
HIV	Med	1.6	0.1	39
	PWM	640	0.7	129

PBMC from two HIV-infected persons (*HIV*) and one control (*CTR*) were incubated for 10 h in the absence (*Med*) or presence of PWM. Results represent picograms of IL-10, 10^{-3} pg of IFN-γ, and 10^{-5} pg of IL-4 cytokine mRNA expression for 10^5 molecules of β-actin mRNA.

whether this heterogeneity could be related to the heterogeneity of CD4+ T cell counts in the HIV-infected persons. As shown in Fig. 2 *B*, when levels of IL-2 were expressed per 4 × 10^5 CD4+ T cells/ml, after correcting for the variation in CD4+ T cell counts, IL-2 secretion by T cells from HIV infected persons, although still heterogenous, was found to be significantly higher than that of T cells from controls. In additional experiments, living lymphocytes from one HIV-infected person and from a control were separated from apoptotic lymphocytes 36 h after in vitro stimulation with PWM, using cell sorting coupled to flow cytofluorometry analysis (Fig. 3). After restimulation of the surviving cells with PHA for an additional 36 h, only IL-2 and IFN-γ were secreted, although at low levels, in cells from the HIV-infected persons, and no IL-4 secretion was observed (Fig. 3). Together, these data suggest the sole presence in HIV-infected persons of CD4+ T cells with a Th1 cell phenotype rather than the existence of a Th1 to Th2 cytokine switch, as previously proposed (34). Our findings of a lack of increased Th2 cytokine secretion are consistent with recent results from cytokine messager RNA analysis in vivo in the lymph nodes of HIV-infected persons (41).

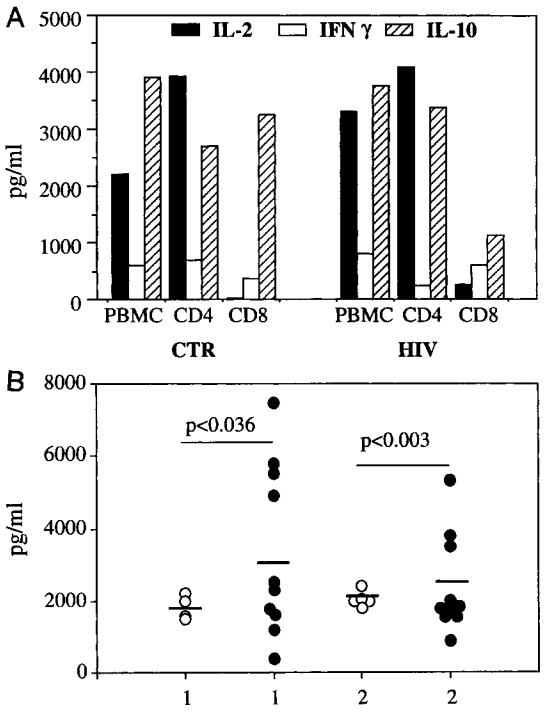


Figure 2. Cellular origin and kinetics of cytokine secretion. (*A*) Cytokine secretion in PBMC depleted either of CD8+ T cells (*CD4*) or of CD4+ T cells (*CD8*) 24 h after PWM stimulation. (*B*) IL-2 secretion expressed per 4 × 10^5 CD4+ T cells, 24 h (*1*) or 40 h (*2*) after stimulation with PWM of PBMC from HIV-infected persons (●) or controls (○). Each circle represents a different individual.

Preventive Effect of IL-12 and of Antibodies to IL-10 or IL-4 on Activation-induced Apoptosis of CD4+ T Cells. Despite the fact that the levels of the Th2 cytokines IL-10 and IL-4 expressed by activated PBMC from HIV-infected persons were not superior to those expressed by activated PBMC from controls, the addition of antibodies against the Th2 cytokines IL-10 or IL-4 had a statistically significant preventive effect on activation-induced apoptosis in PBMC from HIV-infected persons (Fig. 4), while antibodies to the Th2 cytokine IL-5 had no preventive effect (not shown). IL-10 and IL-4 have been shown to downregulate Th1 cell function by several means (36), including an inhibitory effect of IL-10 on monocyte/macrophage secretion of IL-12 (42), a cytokine that upregulates Th1 cell function and whose production has been reported to be impaired in HIV-infected persons (43). As shown in Fig. 4, addition of rIL-12 also prevented activation-induced apoptosis in PBMC from HIV-infected persons. Addition of the Th1 cytokine rIL-2 had a different effect, preventing spontaneous apoptosis, but not activation-induced apoptosis (not shown), a situation in which IL-2 was already secreted (Fig. 1 *B*). Further experiments performed in PBMC depleted of B cells, NK cells, and either CD4+ T cells or CD8+ T cells indicated that, in contrast to drugs such as CSA and the protein synthesis inhibitor cycloheximide, which showed some preventive effect on apoptosis in both CD4+ and CD8+ T cells from HIV-infected persons, the addition of IL-12 or of antibodies to IL-10 or IL-4 had a preventive effect on activation-induced apoptosis in CD4+ T cells, but not in CD8+ T cells, from HIV-infected persons (Figs. 5 *A* and 6).

The preventive effect of anti–IL-4 antibodies on CD4+ T cell death was less frequently observed than that of rIL-12 or of anti–IL-10 antibodies (Fig. 5 *A*). In instances in which anti–IL-4 antibodies did not prevent apoptosis, they did not enhance the preventive effect of anti–IL-10 antibodies (Fig. 5 *B*), suggesting that IL-4 was involved in CD4+ T cell apoptosis induction in only some HIV-infected persons.

Cytokines have been shown to regulate T cell differentiation and function by two different means: (*a*) cytokines such as IL-2 and IL-12, or IL-4, act by inducing the differentiation and expansion of the Th1 or Th2 CD4+ cell population, respectively; (*b*) cytokines such as IL-10, IL-4, or IFN-γ, which can be secreted by accessory cells, play an indirect role in Th1 or Th2 expansion by downregulating

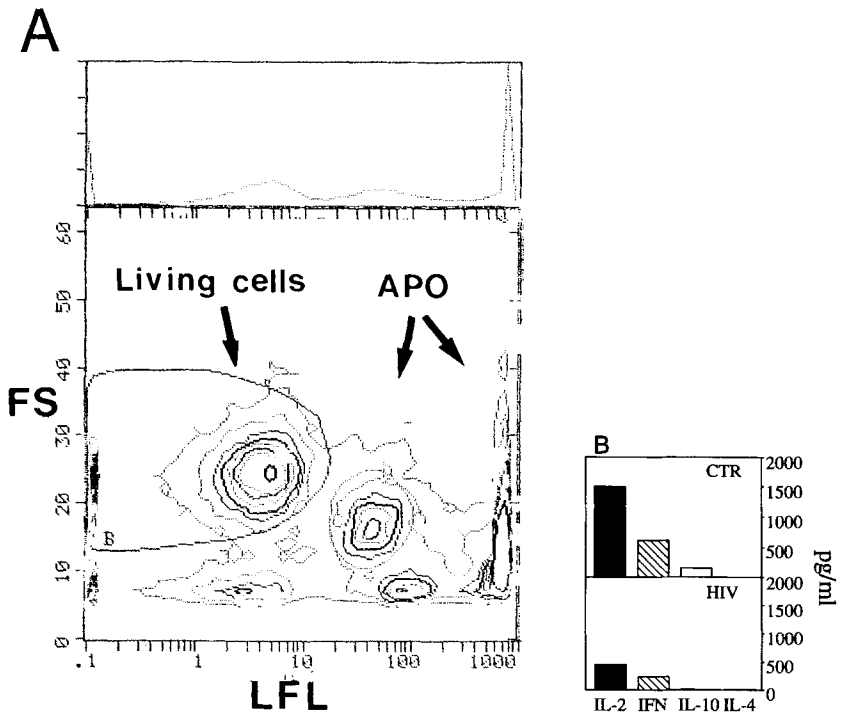
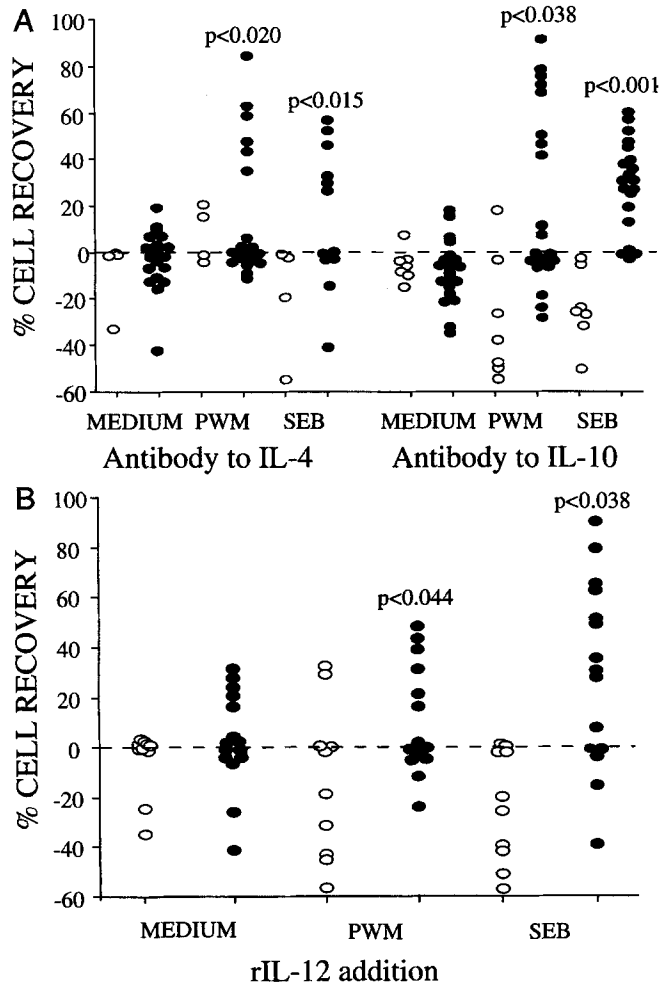


Figure 3. Cytokine secretion after restimulation of sorted surviving cells. 36 h after PWM-mediated apoptosis induction, PBMC from an HIV-infected person and a control were incubated with the nuclear dye YO-PRO-1 and living cells were sorted from apoptotic cells using cell sorting coupled to flow cytofluorometry. (*A*) Flow cytofluorometry analysis of living and apoptotic cells from the HIV-infected person. *F.S.*, forward scatter; *L.F.L.*, log fluorescence intensity. (*B*) After cell sorting, autologous adherent accessory cells (2×10^5/ml) were added to the sorted living lymphocytes (10^6/ml) and cytokine secretion was measured 36 h after restimulation with PHA.



the converse Th cell population; IL-10 and IL-4 by inhibiting Th1 cell expansion, and IFN-γ by inhibiting Th2 cell expansion (36, 44). Our findings suggest that cytokine-mediated regulation of the Th1 cell response could also operate through the control of T cell PCD, and that Th2 cytokine-mediated Th1 cell PCD may represent a mechanism of Th1 cell downregulation that is prevented by IL-12.

Our findings that IL-10 and IL-4 secretion (Fig. 1) and IL-10 mRNA expression (Table 1) were similar in PBMC from HIV infected persons and from controls, and that IL-4 mRNA expression was even lower in PBMC from HIV-infected persons (Table 1), indicate that additional factors act together with IL-10 and IL-4 in inducing apoptosis in activated $CD4^+$ Th1 cells from HIV infected persons. An obvious possibility suggested by our findings of a preventive effect of exogenous IL-12 is that such an additional factor is represented by the previously reported defect in IL-12 secretion of PBMC from HIV-infected persons (43). Quantitative measurement of the IL-12 p40 chain mRNA by competitive RT-PCR showed a similar expression in PWM-stimulated PBMC from two HIV-infected persons and one control (Table 2). Semiquantitative RT-PCR of the IL-12 p35 chain mRNA revealed great variations, but

Figure 4. Preventive effect of anti–IL-4 mAb, anti–IL-10 mAb, and rIL-12 on activation-induced cell death. Apoptosis was measured in PBMC from HIV-1–infected persons (●) and from controls (○) 24 h after incubation with medium, PWM, or SEB, in the absence or presence of anti–IL-4 mAb, IL-10 mAb, or rIL-12. Each circle represents a different individual. Percentages of cell death prevention (cell recovery) were calculated as described in Materials and Methods. Statistical significance was assessed using paired Student's *t* test.

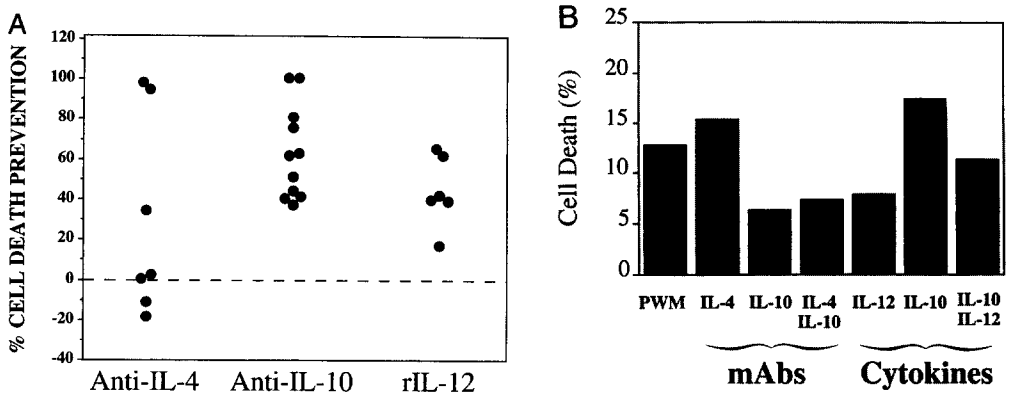


Figure 5. (*A*) Preventive effect of anti–IL-4 mAb, anti–IL-10 mAb, and of rIL-12 on activation-induced death of CD4 cells from HIV-infected persons. PBMC from HIV-1–infected persons were depleted of B, NK, and CD8 T cells, and were incubated for 36 h with PWM in the absence or presence of anti–IL-4 mAb, anti–IL-10 mAb, or IL-12. Each circle represents a different individual. Statistical significance was assessed using paired Student's *t* test. (*B*) Comparative effect of cytokines on activation-induced death of $CD4^+$ T cells. PBMC from an HIV-infected person were depleted of B, NK and $CD8^+$ T cells, and were incubated for 24 h with PWM in the absence or the presence of either mAb directed against IL-4 or IL-10 (or a mixture of both), or with rIL-10 or IL-12 cytokines (or a mixture of both). Background of apoptosis in the absence of PWM was <15%, and it was subtracted from the results.

with opposite patterns in the two HIV-infected persons (Table 2). Finally, in the conditions of our T cell activation assays, we could not detect secretion of the active form of the IL-12 p70 heterodimer in PWM-stimulated PBMC from the two HIV-infected persons and the control. Therefore, we do not know at this stage whether normal amounts of the active form of IL-12 are secreted (and taken up) by PBMC from HIV-infected persons, but are unsufficient to prevent IL-10– or IL-4–mediated apoptosis, or whether PBMC from HIV-infected persons indeed present a defect in the secretion of the active form of IL-12 (43).

Concerning the preventive effect of the addition of exogenous rIL-12 on apoptosis, our findings suggest the involvement of two synergistic mechanisms: a direct protective effect of rIL-12 on IL-10–mediated apoptosis (Fig. 6), and a modest inhibitory effect of rIL-12 on IL-10 mRNA expression (Table 2). Together, our findings suggest that IL-12 may play a major role in $CD4^+$ Th1 cell survival by preventing IL-10– and IL-4–mediated apoptosis in response to TCR stimulation. After our manuscript was sub-

Table 2. *Analysis of Cytokine mRNA Expression by RT-PCR*

Patients	Stimulus	IL-10	IL-12 P40	IL-12 P35
CTR	Med	125	8.8	1
	PWM	546	40	16
	PWM rIL-12	265	39	3
	PWM mAb IL-10	134	40	64
HIV	Med	275	1.6	64
	PWM	175	35	20
	PWM rIL-12	74	37	0.5
	PWM mAb IL-10	68	37	14
HIV	Med	39	0.3	0.01
	PWM	129	62	0.5
	PWM rIL-12	62	73	14
	PWM mAb IL-10	121	59	14

Quantitative analysis of IL-10 and IL-12 p40 mRNA expression was performed by competitive RT-PCR; IL-12 p35 mRNA expression was measured by semiquantitative RT-PCR (see Materials and Methods). PBMC from two HIV-infected persons (*HIV*)and one control (*CTR*) were incubated for 10 h in the absence (*Med*) or presence of-PWM (*PWM*), or they were incubated simultaneously with PWM and rIL-12, or PWM and anti–IL-10 antibodies. Results represent picograms of IL-10, 10^{-4} pg of IL-12 p40 cytokine mRNA expression for 10^5 molecules of β-actin mRNA, and IL-12 p35 values are expressed according to the amount of IL-12 p35 chain cDNA present in the sample from unstimulated PBMC from the control individual.

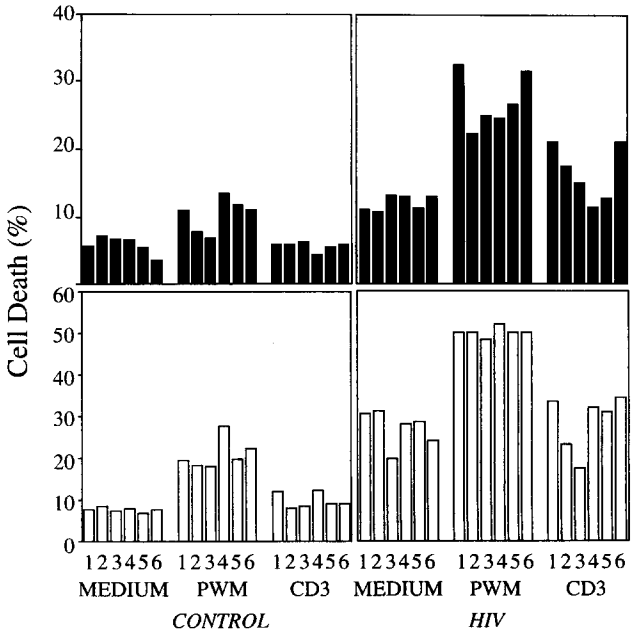


Figure 6. Comparative effect of cytokines and drugs on activation-induced death of $CD4^+$ and $CD8^+$ T cells. PBMC from an HIV-1–infected person and a control were depleted of B, NK, and either $CD8^+$ (■) or $CD4^+$ T cells (□), and were incubated for 36 h with medium, PWM, or CD3 mAb in the absence (*1*) or presence of CSA (*2*), CHX (*3*), rIL-12 (*4*), anti–IL-10 mAb (*5*), or anti–IL-4 mAb (6). These results are from one representative experiment out of four.

mitted for publication, a recent publication confirmed that apoptosis of activated T cells from HIV-infected persons can be prevented by the addition of antibodies to IL-10 or IL-4, or by the addition of rIL-12 (45). This publication differs, however, from our findings in several important ways. First, it reports that anti–IL-10 or anti–IL-4 antibodies and rIL-12 treatment have the same preventive effect on apoptosis of both $CD4^+$ and $CD8^+$ T cells; second, the complexity of the stimuli used to induce T cell apoptosis (a mixture of PWM and SEB) renders interpretation of the results difficult; third, it describes an additional preventive effect of rIL-2 on activation-induced T cell apoptosis; finally, it attributes the preventive effect of Th1 cytokines and of anti-Th2 cytokine antibodies on T cell apoptosis to the existence of a Th1 to Th2 cytokine switch in HIV-infected persons. Our results clearly indicate that the progressive loss of cell-mediated Th1 function in HIV-infected persons can occur in the absence of a Th1 to Th2 cytokine switch, and is related to the fact that the stimulation of $CD4^+$ Th1 cells induces their rapid death by apoptosis in response to normal levels of IL-10 and IL-4. A progressive loss of Th1 $CD4^+$ cells in the absence of a compensatory expansion of Th2 $CD4^+$ cells could participate in the progressive $CD4^+$ T cell dysfunction and depletion that lead to AIDS. The recent finding that HIV-1 may preferentially replicate in Th2 and Th0 $CD4^+$ T cell clones, and not in $CD4^+$ Th1 cell clones (46), provides a possible mechanism by which expansion of $CD4^+$ Th2 cells may be impaired in HIV-1–infected persons.

Preventive Effect of IL-12 on CD95 (Fas/Apo-1)–mediated Apoptosis of $CD4^+$ T Cells. TCR activation–induced death of T cell hybridomas and of preactivated nontransformed T cells has been recently shown to involve the CD95 cell-surface receptor (21–23). CD95-mediated apoptosis of T cells does not only depend on the expression of CD95 and of the CD95 ligand, but also on the acquisition of a sensitive phenotype to CD95-mediated apoptosis (28, 29, 47). As shown in Fig. 7 *A*, flow cytofluorometry analysis indicated that the percentage of $CD4^+$ and $CD8^+$ T cells expressing cell-surface CD95, in the absence of in vitro TCR stimulation, was significantly higher in HIV-infected persons than in controls. Antibody-mediated ligation of CD95 induced high levels of apoptosis in PBMC and in both $CD4^+$ and $CD8^+$ T cells from HIV-infected persons, but not from controls (Fig. 7, *B* and *C*). The percentage of apoptosis induced by CD95 ligation in T cells from HIV-infected persons was similar to that induced by TCR stimulation (11). The addition of rIL-12 had the same preventive effect (~50%) on apoptosis induced by CD95 ligation (Fig. 7 *D*) and by TCR stimulation (Fig. 5 *A*) in $CD4^+$ T cells from HIV-infected persons, but had only a very slight preventive effect on $CD8^+$ T cell apoptosis in response to CD95 ligation (Fig. 7 *D*). These findings suggest that IL-12 directly interferes with intracellular messengers, leading to apoptotic death of $CD4^+$ T cells from HIV-infected persons. In T cells from controls, it has been shown that TCR stimulation upregulates CD95 expression within 24 h, but that the T cells are resistant to apoptosis in response to CD95

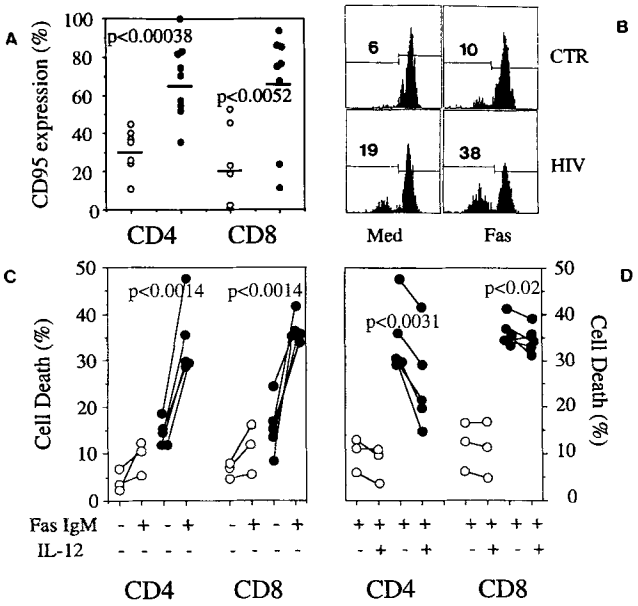


Figure 7. (*A*) Percentages of $CD4^+$ and $CD8^+$ T cells expressing the CD95 (Fas) molecule. Flow cytofluorometry analysis was performed in PBMC from HIV-infected persons (●) and from controls (○) by using FITC-labeled CD95 mAb and with phycoerythrin-labeled CD4 or CD8 mAb. *CD4*, percentages of $CD4^+$ T cells expressing CD95; *CD8*, percentages of $CD8^+$ T cells expressing CD95. Each circle represents a different individual. Bars represent the mean value in each group. Statistical significance was assessed using Student's *t* test. (*B*) Apoptosis in response to CD95 (*Fas*) ligation. PBMC from an HIV-infected person (*HIV*) and a control (*CTR*) were incubated for 18 h with medium (*Med*) or CD95 IgM mAb (*Fas*) 18 h after incubation with medium (*left*) or CD95 IgM mAb (*right*). PBMC from an HIV-infected person (*bottom*) and a control (*top*) were stained with the nuclear dye acridine orange, and apoptosis was assessed using flow cytofluorometry analysis. The percentage of apoptotic cells corresponding to the peak of reduced fluorescence intensity is indicated in each experimental condition. (*C*) Apoptosis of $CD4^+$ and $CD8^+$ T cells in response to CD95 (*Fas*) ligation. PBMC from HIV-infected persons (●) and from controls (○) were depleted of either $CD8^+$ T cells (*CD4*) or of $CD4^+$ T cells (*CD8*), and were incubated for 18 h in the presence (+) or absence (−) of the CD95 mAb (*Fas IgM*). Each circle represents a different individual. Statistical significance was assessed using paired Student's *t* test. (*D*) Preventive effect of rIL-12 on CD95 (Fas)-mediated apoptosis of $CD4^+$ and $CD8^+$ T cells. PBMC from HIV-infected persons (●) and from controls (○) were incubated for 18 h with the CD95 mAb (*Fas IgM*) in the presence (+) or absence (−) of IL-12. IL-12 was added 30 min before the CD95 mAb. Each circle represents a different individual. Significance was assessed using paired Student's *t* test. Mean cell death prevention percentage (as in Figs. 4 and 5 *A*) of IL-12 on CD95-mediated apoptosis was 48.8% in $CD4^+$ T cells from HIV-infected persons, and 8.6% in $CD8^+$ T cells from HIV-infected persons.

ligation (28, 47). Sensitivity to TCR- and CD95-mediated apoptosis, which may involve a downregulation of the expression of Fas-associated tyrosine phosphatase FAP-1 (29), occurs only after several days of culture and after several steps of TCR restimulation (28, 47, 48). It is possible, therefore, that the increased susceptibility of $CD4^+$ Th1 cells from HIV-infected persons to TCR- and CD95-mediated apoptosis is related to a chronic in vivo activation of these $CD4^+$ T cells. The preventive effect of anti–IL-10 and anti–IL-4 antibodies on TCR activation-induced apoptosis of $CD4^+$ T cells from HIV-infected persons suggests that these Th2

cytokines may participate in T cell death either by inducing CD95 ligand expression or by maintaining CD95 sensitivity in TCR-activated $CD4^+$ T cells in the absence of IL-12. The precise mechanisms by which IL-12, IL-10, and IL-4 regulate apoptosis of CD4 T cells from HIV-infected persons, and the possibility that FAP-1 represents one of the intracellular targets of such regulation, are currently under investigation. In normal B cells, it has been recently shown that defined activation signals can modulate the sensitivity to CD95-mediated apoptosis, CD40 ligation by inducing susceptibility to CD95 ligation, surface Ig ligation by exerting a dominant protective effect (49). Our results suggest that IL-12 plays a similar dominant protective effect in $CD4^+$ Th1 cells from HIV-infected persons against apoptosis mediated both by CD95 ligation, and by TCR stimulation in the presence of the Th2 cytokines IL-10 and IL-4. Our findings, therefore, suggest that IL-12 may represent an important candidate for therapeutic strategies aimed at the prevention of $CD4^+$ T cell death and the promotion of $CD4^+$ Th1 cell functions in HIV-infected persons.

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