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Differential Susceptibility to Activation-Induced Apoptosis Among Peripheral Th1 Subsets: Correlation with Bcl-2 Expression and Consequences for AIDS Pathogenesis

Eric Ledru,* Hervé Lecoeur,* Sylvie Garcia,* Thierry Debord,† and Marie-Lise Gougeon2*

It has been proposed that HIV infection is associated with an imbalance in Th1 and Th2 subsets. Recent reports indicate that Th1 and Th2 effectors differ in their susceptibility to activation-induced apoptosis. To determine whether increased T cell apoptosis in HIV-infected patients contributes to alterations in cytokine production, we performed single-cell analysis of type 1 and type 2 cytokine production by CD4 and CD8 T cells, simultaneously with detection of apoptosis. We demonstrate that a differential alteration in representation of Th1 subsets, rather than commitment of T cells to secrete Th2 cytokines, occurs throughout HIV infection. A significant decrease in the number of IL-2- or TNF-α-producing T cells was observed, whereas those producing IFN-γ remained preserved. Furthermore, there is a gradient of susceptibility to activation-induced apoptosis (IL-2 < IFN-γ < TNF-α) among the different Th1 subsets. This gradient was detected in both CD4 and CD8 subsets, as well as in control donors and HIV-infected patients, in whom the susceptibility to apoptosis of IL-2- and IFN-γ producers was increased compared with controls. This differential intrinsic apoptosis susceptibility of Th1 effectors was found to be tightly regulated by Bcl-2 expression. In HIV-infected persons, disappearance of IL-2-producing T cells was a good indicator of disease progression and was correlated with the progressive shrinkage of the CD4+CD45RA+ T cell compartment and a gradual increased susceptibility to activation-induced apoptosis of the IL-2-producing subset. This close relationship between the CD45RA/CD45RO ratio, the level of type 1 cytokine production, and susceptibility to apoptosis should be considered in HIV-infected patients under antiviral or immune-based therapies. The Journal of Immunology, 1998, 160: 3194–3206.

T cells can be divided into different functional subsets on the basis of their expression of the CD45 isoforms (1). Upon activation, naïve T cells down-regulate CD45RA expression and differentiate into memory/effector CD45RO+ cells, which are able to proliferate following subsequent exposure to recall Ags. CD45RA+ and CD45RO+ T cells differ in the requirement for Ag-dependent activation and in the pattern of cytokine production (2–4). Furthermore, according to the cytokine environment in which they develop and the nature of the costimulatory signals delivered by the APCs, naïve T cells can differentiate into effector cells with polarized patterns of cytokine production. Th1 cells secrete IL-2, IFN-γ, and TNF-α, which are important mediators of cellular immunity and are involved in activation of macrophages; whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, which provide help to B cells for Ig synthesis, mediate eosinophilia, and antagonize the macrophage-activating action of Th2 cytokines. In addition, a third subset of helper T cells has been identified, Th0 cells, which produce both Th1 and Th2 cytokines (5). Some pathologic situations are associated with an imbalance in Th1 and Th2 subsets, but the mechanisms underlying this imbalance remain unclear (5). Recent reports indicate that Th1 effectors differ from Th2 effectors in their susceptibility to activation-induced apoptosis, with Th1 T cells being highly sensitive to Fas-induced apoptosis (6–8).

Activation-induced apoptosis is involved in peripheral T cell deletion and has been implicated in the loss of CD4 Th cells in AIDS (9–12). HIV infection is characterized by a persistent immune activation responsible for the continuous recruitment of CD45RO+ effectors and the concomitant decline of the CD45RA+ naive compartment in both CD4 and CD8 subsets (13). This is associated with an increased rate of spontaneous and activation-induced apoptosis in both subsets, leading to the progressive alteration in T helper functions (14). This activation-induced deletion of Th cells is accompanied by early impairment of cytokine synthesis. It was proposed several years ago that a shift from Th1 to Th2-type secretion during HIV infection might perturb the immune system and lead to progression to AIDS (15). Since that time, many studies reported alteration in cytokine production by T cells of HIV-infected patients but discrepant results were found. A decrease in IL-2 production by patients’ peripheral T cells in response to Ag or mitogen stimulation or to TCR ligation, or a decrease in constitutive IL-2 mRNA expression, has been uniformly reported (16–21). In contrast, IFN-γ production was found to increase (18, 22–25), decrease (20, 21), or remain unchanged as well (26). Furthermore, analysis of CD4+ T cell clones derived from AIDS patients revealed a reduced number of clones producing IL-2 and IFN-γ, together with an increased number of Th0-type T cell clones when compared with healthy controls (27–29). The pattern of cytokines produced by an effector population can now be...
analyzed using a single-cell analysis method that allows enumeration of Th1/Th2 subsets derived from peripheral T cells stimulated in short-term cultures and provides information on the number and the phenotype of cells that are potentially capable of producing a given cytokine. To evaluate whether a specific commitment to secrete certain Th1 or Th2 cytokines is found at the T cell level in HIV-infected patients and to investigate the basis of alterations in the cytokine pattern, we have performed a single-cell analysis of Th1 (IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-5, IL-13) CD4- and CD8-producing cells and have determined whether perturbations in the proportions of CD45RA/CD45RO T cells influence the cytokine pattern in HIV-infected patients. In addition, we have asked whether alterations in the representation of some Th1 subsets was the consequence of a differential susceptibility to activation-induced apoptosis. Our results indicate that there is no commitment of T cells to secrete Th2 cytokines in HIV-infected patients, but rather, a differential alteration in the representation of Th1 subsets. In particular, a significant decrease in the number of IL-2-producing T cells with a preserved production of IFN-γ was observed throughout HIV infection, and this was directly related to the shrinkage of the CD45RA T cell compartment as the infection progressed. In addition, we report that among the different Th1 subsets, there is a gradient of susceptibility to activation-induced apoptosis, which is regulated by Bcl-2 expression and which contributes to the dysregulation in type 1 cytokine production in AIDS.

Materials and Methods

Human blood samples and lymphocyte isolation

Peripheral blood samples were obtained from 64 HIV-1-infected patients at the Service for Infectious Diseases, Bégin Military Hospital, St. Mandé, France. Clinical characteristics of these patients are shown in Table I. None of the patients studied received anti-HIV protease inhibitor, but some of the patients studied received anti-HIV nucleoside reverse transcriptase inhibitors (RTI, association of two reverse transcriptase inhibitors). Clinical characteristics of the HIV-infected patients studied are shown in Table I. None:13 None:12 None:1

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* Patients were grouped on the basis of their ex vivo CD4 percentage, according to the reference values of the CDC proposal (84).

PBMC from healthy donors or from HIV-infected patients were stimulated for 16 h with PPI (50 ng/ml PMA (Sigma Chemical Co., St. Louis, MO), 100 ng/ml PHA-A (Murex Diagnostic, Paris, France), and 300 ng/ml ionomycin (Sigma Chemical Co.), Brefeldin A (Sigma Chemical Co.) was added at a final concentration of 10 μg/ml during the last 12 h to inhibit cytokine secretion. Enumeration at the single-cell level of cytokine-producing peripheral T cells was performed as previously described (30). Briefly, stimulated PBMC were washed in PBS containing 1% BSA and 0.1% sodium azide (PBS-BSA-NaNO3) and incubated in the same buffer with FITC-conjugated anti-CD3 or anti-CD8 mAbs and 20 μg/ml of 7-amino-actinomycin D (DAPI) (7-AAD) (Sigma Chemical Co.). 7-AAD staining was performed to discriminate between living and apoptotic cell (including early apoptotic cells), as previously shown (31, 32). Stained cells were further washed in PBS-BSA-NaNO3 containing 20 μg/ml of actinomycin D (DA; Sigma Chemical Co.). fixed in 1% paraformaldehyde for 15 min at +4°C. Fixed cells were then permeabilized by incubation for 15 min with 0.05% (w/v) SAP (Sigma Chemical Co.) diluted in PBS-BSA-NaNO3 at room temperature. Intracellular cytokine staining was then performed with PE-conjugated anti-cytokine mAbs in SAP buffer containing 20 μg/ml of PE-20 μg/ml of 7-AAD, and the simultaneous labeling of cell surface Ags (FL-1 and FL-2) (31, 32). For each sample, 20,000 stained cells were acquired and analyzed with LYSYS II software (Becton Dickinson). Cytokine production was evaluated on both living and apoptotic T cells.

**Combined analysis in peripheral T lymphocytes of their susceptibility to activation-induced apoptosis in relation to the cytokine produced**

To analyze in blood T cells the relationship between the synthesis of a given cytokine and the propensity to undergo apoptosis, PBMC were stimulated 16 h with PPI and triple stained with FITC-conjugated anti-CD3 or anti-CD8 mAbs, PE-conjugated anti-cytokine mAbs, and the nuclear dye 7-AAD, as detailed above. The spectral properties of 7-AAD allow the staining of apoptotic cells by fluorescence emission in the red channel FL-3 (650 nm < wavelength < 850 nm), and the simultaneous labeling of cell surface Ags (FL-1 and FL-2) (31, 32). For each sample, 20,000 events were immediately placed on a FACScan flow cytometer, and apoptotic cells were quantified according to their 7-AAD staining. The combination of intracellular staining with anti-cytokine mAbs and nuclear staining with 7-AAD on stimulated T cells permitted analysis of the propensity of a given type 1 subset to undergo activation-induced cell death.

**Bcl-2 intracellular staining and its relation to apoptosis**

Intracellular detection of Bcl-2 protein within T cell subsets was performed as described previously (33). 5 × 10^5 PBMCs stimulated with PPI for 16 h were washed in PBS-BSA-NaNO3 and incubated with PE- and/or PerCP-conjugated mAbs specific for T cell surface Ags, and in some experiments, with the nuclear dye 7-AAD to detect apoptosis, as described above. The stained cells were further washed in PBS-BSA-NaNO3, fixed in 1% paraformaldehyde (Sigma Chemical Co.) for 20 min at room temperature, washed in PBS, and then incubated for 15 min at +4°C in PBS-BSA-NaNO3.
containing 0.05% (w/v) SAP (Sigma Chemical Co). They were then intracellularly stained with FITC-conjugated anti-Bcl-2 mAbs and, in some experiments, with PE-conjugated anti-cytokine mAbs, in SAP buffer for 30 min at RT. Finally, the labeled cells were washed in SAP buffer, fixed with 1% paraformaldehyde in PBS, and immediately applied to a FACScan flow cytometer (Becton Dickinson). The relation between Bcl-2 expression and the percentage of apoptotic (7-AAD<sup>+</sup>) T cells was analyzed for each cytokine-positive subset with LYSYS II software (Becton Dickinson).

**Statistical analyses**

Statistical analyses included the Mann-Whitney test, Wilcoxon matched pairs test, Spearman regression analysis, and χ²-test. A p value < 0.05 was considered significant.

**Results**

**HIV infection is associated with early alterations in the proportions of T cells producing type 1 cytokines rather than an enrichment in T cells producing type 2 cytokines**

To measure accurately the Th1 and Th2 populations and to determine their respective proportions at different stages of HIV infection, we used a method of single-cell analysis by flow cytometry. PBMC from control donors or HIV-infected persons were stimulated for 16 h with PPL and cells were surface stained with anti-CD3 mAbs and intracellularly stained with mAbs against the Th1 cytokines IL-2, IFN-γ, and TNF-α and the Th2 cytokines IL-4, IL-5, and IL-13. Figure 1A represents representative stainings for IL-2, TNF-α, and IFN-γ in stimulated PBMC of a control donor and of an HIV-infected patient belonging to group 2 (13% < CD4 < 28%). The proportion of T cells synthesizing IL-2 was significantly decreased in the HIV-infected donor (18.5% of CD3 T cells were IL-2<sup>+</sup> compared with 56% in the control donor), whereas the percentage of T cells synthesizing IFN-γ was slightly increased (30.0% of CD3 T cells were IFN-γ<sup>+</sup> in the patient compared with 20% in the control donor). The proportion of T cells synthesizing TNF-α was decreased in the patient (14.0% of CD3 T cells were TNF-α<sup>+</sup> vs 35.0% in the control donor).

These alterations in type 1 cytokine production were confirmed when a panel of 62 HIV-infected patients was analyzed (Fig. 1B). Indeed, the proportion of CD3 T cells producing IL-2 was significantly lower in HIV-infected persons compared with control donors (median value 21.0% in patients vs 37.0% in controls, p < 0.0003). A decrease was also found in the proportion of CD3 T cells synthesizing TNF-α (median value 27.9% in patients vs 33.4% in controls, p < 0.01). In contrast, the percentage of T cells producing IFN-γ was not significantly modified when the whole patient population (median value 17.2% in patients vs 16.4% in controls) was taken into consideration, although an increase in IFN-γ-producing T cells was observed in patients in groups 2 and 3 vs group 1 (Fig. 1B). The decreased proportion of T cells synthesizing IL-2 and TNF-α in HIV-infected persons was an early phenomenon, detected in patients with mild immune deficiency (group 2, 13% < CD4 < 28%). It is noteworthy that, both in control donors and in patients, T cells that simultaneously produced two type 1 cytokines were hardly detected (data not shown).

A similar approach was used to quantify T cells producing type 2 cytokines. The detection of IL-4, IL-5, or IL-13<sup>+</sup> T cells was performed in the PBMC of controls (n = 8) or patients (n = 18) stimulated for 16 h with PPL. The proportion of IL-4 or IL-5<sup>+</sup> CD3 T cells was very low in control donors (median values 1.7% for IL-4 and <1% for IL-5) and remained unchanged in patients (median values 1.6% for IL-4 and <1% for IL-5). IL-10 production was always < 1%, in control donors as well as in HIV-infected patients. T cells producing IL-13 were not detected in controls or in patients from groups 1 or 2 (median value <1%). In contrast, in some advanced patients suffering from a hyper-IgE syndrome, IL-13<sup>+</sup> T cells were detected and their proportion could represent 26% of total CD3 T cells. Type 2-producing T cells were mainly of the CD8 phenotype (data not shown).

Taken together, these observations indicate that, under these conditions of stimulation, the previously reported perturbations in Th1/Th2 balance throughout HIV infection (34) are not detected at the peripheral T cell level at early stages of HIV infection. The only type 2 cytokine detected was IL-13, but only in some AIDS patients with hyper-IgE syndrome.

**Differential alteration in CD4 and CD8 subsets from HIV-infected persons in their synthesis of IL-2, IFN-γ, and TNFα**

For each type 1 cytokine synthesized, the respective contribution of the CD4 and CD8 T cell subsets in their synthesis was assessed. Because of the down-modulation of the CD4 molecule on PPI-stimulated T cells, the percentage of CD4 T cells producing a given cytokine was considered as the percentage of CD3+CD4<sup>+</sup> cells producing this cytokine. Figure 2 shows the proportion of CD4 and CD8 T cells synthesizing IL-2, IFN-γ or TNF-α in 16h-PPI-stimulated PBMC. In control donors, IL-2 is mainly produced by the CD4 T cell subset whereas IFN-γ and TNF-α are synthesized in similar proportions by both CD4 and CD8 subsets. In HIV-infected patients, the proportion of CD4 T cells producing IL-2 strongly decreased, particularly at advanced stages of the infection. In contrast, the ability of the CD8 subset to synthesize IL-2 was unchanged in HIV-infected persons (Fig. 2). The global decrease in the proportion of IL-2-synthesizing T cells observed in patients therefore appears to be the consequence of both a reduced number of peripheral CD4 T cells and a reduced capacity of the remaining CD4 T cells to synthesize IL-2 upon activation.

IFN-γ was produced in similar proportions by CD4 and CD8 T cells in controls’ PBMC whereas the CD8 subset generally contributed in a greater proportion to IFN-γ production in patients’ PBMC (Fig. 2). As reported above, IFN-γ production by T cells appeared therefore to be preserved and sometimes enhanced throughout HIV infection, due to both an increase in the number of CD8 T cells and an increased capacity of these cells to produce IFN-γ. A different situation was found for TNF-α. Both CD4 and CD8 subsets equally contributed to TNF-α synthesis in control donors. In patients, a decreased proportion of CD8 T cells producing TNF-α was observed (Fig. 2). The reduction in the proportion of T cells able to produce TNF-α throughout HIV infection therefore appears to be the consequence of both a reduced capacity of CD8 T cells to synthesize TNF-α and a reduced number of peripheral CD4 T cells.

**Heterogeneity in the synthesis of type 1 cytokines among CD45RA<sup>−</sup> and CD45RA<sup>+</sup> T cell subsets**

Although the CD45RA compartment exhibits a lower proliferative activity in response to recall Ags or TCR ligation, a recent study showed that, following PMA + ionomycin activation, CD45RA<sup>+</sup> T cells have a low but significant capacity to secrete several cytokines, mainly IL-2, TNF-α and GM-CSF (4, 35). The flow cytometric analysis at the single-cell level allows identification of a possible commitment to cytokine production of a given subset. To define whether the CD45RA<sup>−</sup> and CD45RA<sup>+</sup> subsets showed a differential ability to produce type 1 cytokines, PBMC from healthy donors were stimulated with PPI and the synthesis of IL-2, IFN-γ and TNF-α was measured in the CD45RA<sup>−</sup> subset (Fig. 3). All three cytokines were produced by both CD45RA<sup>−</sup> and CD45RA<sup>+</sup> subsets, but a distinct pattern was observed for each subset. The majority of IL-2-producing CD3 T cells was detected...
in the CD45RA+ compartment whereas IFN-γ producers were mainly detected in the CD45RA- compartment. The TNF-α producers were found equally distributed among CD45RA+ and CD45RA- subsets (Fig. 3A).

The important contribution of the CD45RA+ subset in the production of IL-2 was observed in both the CD4 and CD8 subsets. As shown in Figure 3B, more than 50% of IL-2 CD4 T cell producers and more than 60% of IL-2 CD8 T cell producers were CD45RA+ in most of the healthy donors tested. This is in striking contrast with the situation observed for the two other cytokines, IFN-γ and TNF-α, which appeared to be mainly synthesized by the CD45R0 compartment in both CD4 and CD8 subsets (Fig. 3B). Surprisingly, in spite of some alterations in the proportions of CD4 and CD8 T cells producing those cytokines in HIV-infected patients (Fig. 1), their distribution in the CD45RA and CD45R0 compartments were unchanged. In particular, IL-2 was still primarily produced by CD45RA+ cells in both CD4 and CD8 subsets (Fig. 3B).

It was reported that CD45RA molecule could be re-expressed on T cell clones following stimulation with PMA and ionomycin (36) and that phenotypic conversion from CD45R0 to a CD45RA could occur after PHA long-term in vitro stimulation (3). To verify the stability of CD45RA expression on peripheral T cells activated by...
PPI, the experiments reported on Figure 3 were also performed on PBMC selectively depleted of CD45RA or CD45RO T cells. Both CD45RA and CD45RO phenotypes were found to be quite stable after 16 h of PPI stimulation (data not shown).

The decrease in IL-2 producers throughout HIV infection is directly related to the loss of naive CD45RA⁺ CD4⁺ T cells

Recent studies demonstrated that HIV disease involves the loss of naive CD4 and CD8 T cells leading to an altered naive/memory T cell representation (13, 30). Since we observed that IL-2 was preferentially produced by CD45RA⁺ T cells, we tested whether the decrease in IL-2 producers throughout HIV infection was related to the loss of CD45RA⁺ T cells. As shown in Figs. 4A, the loss of the CD45RA compartment detected in HIV-infected persons paralleled the decrease in the proportion of CD3 T cells synthesizing IL-2. Analysis of the composition in cytokine-secreting cells following PPI stimulation of the CD45RA compartment in both CD4 and CD8 subsets is shown in Figure 4B. The most striking difference in the composition of the CD45RA subset in patients vs controls was observed for the production of IL-2 by CD4 T cells. Indeed, the reduction in the size of the CD4⁺ CD45RA⁻ compartment in HIV disease was associated with the loss of IL-2 producers which represented only a mean of 39% of the CD4⁺ CD45RA⁺ subset in patients vs 56% in controls (Fig. 4B). However, a reduction in the relative proportions of IFN-γ and TNF-α-producing T cells in the CD4⁺ CD45RA⁻ compartment, although less dramatic, was also observed in patients (3% vs 6% for IFN-γ and 25% vs 34% for TNF-α in patients vs controls respectively). Surprisingly, although decreased in size, the CD8⁺ CD45RA⁻ compartment in patients was not modified with respect to its composition in cytokine-producing subsets (Fig. 4B). The proportion of IL-2 producers represented 24% of the CD8⁺ CD45RA⁺ subset in patients vs 27% in controls, and that of IFN-γ and TNF-α was 16% vs 18% and 19% vs 21% in patients and controls respectively. It is noteworthy that a high proportion of CD45RA⁺ T cells in patients did not secrete either IL-2 or IFN-γ or TNF-α (Fig. 4B). This nonsecreting population was particularly important in the CD4 subset of patients where it represented 33% of the CD4⁺ CD45RA⁺ T cells compared with 3% in controls (p < 0.0001). Although large in the CD8⁺ CD45RA⁺ subset, this population was not significantly different in patients and controls (Fig. 4B). In the CD45RO⁺ populations, the nonsecreting population was only slightly increased in HIV-infected patients (data not shown).

To determine the relationship between the loss of CD45RA⁺ T cells and the alterations in cytokine production throughout HIV infection, the proportion of CD45RA⁺ T cells was plotted against the percentages of cytokine-producing cells in CD4 or CD8 T cells (Fig. 5). As expected from the previous observations, the shrinking of the CD45RA⁺ T cell compartment has a direct consequence on the production of IL-2 by CD4 T cells, since a statistically significant correlation was found between the percentage of CD45RA⁺ T cells and the percentage of IL-2-producing CD4 T cells. The production of IFN-γ and TNF-α were not dependent on the alteration of the CD45RA subset (Fig. 5). Altogether, these observations indicate that the dramatic decrease in IL-2 production throughout HIV infection might be primarily a consequence of the loss of naive CD45RA⁺ T cells, which does not affect the production of other type 1 cytokines predominantly synthesized by the CD45RO⁻ subset.

**Differential susceptibility to activation-induced apoptosis of Th1 subsets**

CD4 and CD8 T lymphocytes of HIV-infected persons show an increased susceptibility to spontaneous and activation-induced apoptosis (37, 38), which is correlated with the in vivo activation state of peripheral lymphocytes and with disease progression (14). Because the fragility of CD45RA⁺ T cells was found to be greater in patients compared with controls, although less than in the CD45RO⁺ subset (14), we asked whether the disappearance of IL-2-producing T cells throughout HIV infection was related to...
their susceptibility to activation-induced apoptosis. The rate of apoptosis among the different cytokine-producing T cell subsets was quantified by flow cytometry. PBMCs were activated with PPI for 16 h and triple stained with surface CD3-specific mAbs, intracellular cytokine-specific mAbs, and with the nuclear dye 7-AAD for the detection of apoptotic cells. The median rate of apoptosis in CD3 T cells under these conditions of activation was 40.6% in patients compared with 18.8% in controls \((p < 0.0001)\). Moreover, this rate of apoptosis in CD3 T cells was found to correlate in HIV-infected donors with disease progression, as assessed by the ex vivo CD4 percentage \((r = 0.53, p < 0.0001, n = 58)\). A representative analysis of apoptosis among cytokine-producing CD3 T cells from control donors and HIV-infected patients is shown on Figure 6A. Interestingly, we found an important heterogeneity in the susceptibility to activation-induced apoptosis of Th1 CD3 T cells in control donors. A low level of apoptosis was detected in IL-2 producers, whereas IFN-\(\gamma\) and TNF-\(\alpha\) producers showed a higher propensity to undergo apoptosis following activation \((p < 0.05\) and \(p < 0.001\) vs IL-2, respectively; Table II). This differential susceptibility to apoptosis of Th1 CD3 T cells was similarly observed in HIV-infected patients. However, the rate of apoptosis in each subset was significantly higher for IL-2 producers \((p < 0.0001\) vs controls); for IFN-\(\gamma\) producers \((p < 0.0001\) vs controls); and for the TNF-\(\alpha\)-producing cells \((p < 0.0001\) vs controls) (Table II). When this same analysis was performed in the CD4 and CD8 T cell subsets, the gradient in the degree of susceptibility to apoptosis observed among cytokine-producing CD3 T cells (IL-2 < IFN-\(\gamma\) < TNF-\(\alpha\)) was also found at the level of the two T cell subsets (Fig. 6B). In control donors, IL-2 producers appeared very resistant to activation-induced apoptosis in both CD4 and CD8

**FIGURE 3.** Contribution of the CD45RA\(^+\) T cell population to cytokine production. PPI-stimulated PBMCs were dual stained with anti-CD3 (or anti-CD8) mAbs and anti-CD45RA mAbs. Cells were then permeabilized and intracellularly stained with anti-cytokine mAbs. A, Histograms from one representative control donor. The proportion of CD3 T cells synthesizing IL-2, TNF-\(\alpha\), and IFN-\(\gamma\) was 39, 23, and 11%, respectively. The percentages of CD45RA\(^+\) T cells among each cytokine-producing subset are indicated. B, Similar analysis performed on CD4 T cells and on CD8 T cells from 7 controls \(\bigcirc\) and 11 HIV-infected patients (\(\bullet\)). Horizontal bars show median values. Arrows indicate statistical differences between the percentages of cytokine-producing subsets (Wilcoxon matched pairs test, \(p < 0.05\)). No significant difference was observed between patients and controls for each cytokine subset (Mann-Whitney test).

**FIGURE 4.** Relationship between the decrease in the CD45RA\(^+\) compartment and the proportion of IL-2 producers in HIV infection. A, Percentages of CD45RA\(^+\) cells among total PBMCs or IL-2 T cells among CD3 T cells from 24 controls or 63 patients. Histograms represent the mean values and SDs of the ex vivo percentage of CD45RA\(^+\) cells among PBMCs. Numbers represent the clinical groups of HIV-infected patients, and C represents control donors. B, Vertical axis compares the percentages of CD45RA\(^+\) cells among CD4 or the CD8 T cells from 7 controls or 11 patients. The proportion of IL-2-, IFN-\(\gamma\)-, or TNF-\(\alpha\)-producing cells among the CD45RA T cells is indicated. T cells secreting none of these three cytokines are indicated as Other. Arrows indicate significant statistical comparisons calculated by the Mann-Whitney test. *, \(\chi^2\) test, \(p < 0.0001\).
subsets. In contrast, TNF-α producers were highly susceptible to apoptosis; this was particularly evident in the CD8 subset. CD4 and CD8 cells producing IFN-γ were in an intermediate position, with marked fragility of these cells in several healthy donors (Fig. 6B). In HIV-infected patients, this differential fragility of type 1 cytokine-producers among CD4 and CD8 T cells followed the same order, but the rate of activation-induced apoptosis was significantly higher in some of these producers as compared with their counterparts in control donors. In the CD4 subset, the proportion of apoptotic cells was increased in IL-2- and IFN-γ-synthesizing cells, while not significantly different from controls in TNF-α-producing cells. In the CD8 subset, only the IFN-γ producers exhibited a significantly higher level of apoptosis as compared with controls (Fig. 6B).

The differential susceptibility to activation-induced apoptosis of Th1 subsets is related to variable levels of Bcl-2 expression

Following activation, T lymphocytes expand and differentiate into effectors despite the expression of death factors. Signal transduction pathways exist, therefore, which inhibit apoptosis through the expression of survival factors such as proteins from the Bcl-2 family (39). To determine whether differential Bcl-2 expression may account for the heterogeneity in the susceptibility to apoptosis between the different cytokine producers, we compared the intracellular expression of Bcl-2 in these different subsets following PPI activation. We previously reported that three levels of Bcl-2 expression were detected on freshly isolated peripheral T cells, associated with differential susceptibility to spontaneous and Fas-mediated apoptosis (30, 33). Figure 7A shows the analysis of intracellular Bcl-2 expression on the three different cytokine-producing subsets following PPI activation of PBMCs. The representative profiles in Figure 7 were obtained with T cells from an HIV-infected donor, but were quite similar for T cells of control donors. Striking differences in the proportions of low (L), normal (N), and high (H) Bcl-2-expressing T cells were detected among the three subsets. Interestingly, IL-2 producers expressed a very high level of Bcl-2 (89% of them were Bcl-2 H). In contrast, IFN-γ and TNF-α producers were equally distributed in Bcl-2 N and H subsets (Fig. 7A). The relation between Bcl-2 expression and the susceptibility to activation-induced apoptosis was analyzed by quantifying, with the 7-AAD dye, the proportion of apoptotic cells within Bcl-2 L, N, and H subsets. Similar to the situation observed for spontaneous and Fas-mediated apoptosis of peripheral T cells (30, 33), a correlation was found between the level of Bcl-2 expression and the propensity of PPI-activated T cells to undergo apoptosis: 94% of Bcl-2 L cells were apoptotic after 16 h of PPI activation vs only 14% in the Bcl-2 H cells (Fig. 7B). When analyzing the three Th1 subsets after PPI activation, a similar correlation was found between the level of Bcl-2 expression and the rate of apoptosis, the Bcl-2 (L + N) being more susceptible to apoptosis than the Bcl-2 H cells (Fig. 7C). Consequently, as shown in Table II, the gradient of susceptibility to activation-induced apoptosis among the cytokine producers (IL-2 < IFN-γ < TNF-α) was found correlated with a gradient in
Bcl-2 expression: the resistance to apoptosis of IL-2 producers was due to the very low proportion of Bcl-2 (L + N) cells, whereas the greater susceptibility of IFN-γ and TNF-α producers was related to an increased proportion of Bcl-2 (L + N) cells (Table II). In HIV infection, a global decrease in Bcl-2 expression was detected in the three Th1 subsets, and consequently, an increased rate of apoptosis was observed for each Th1 subset compared with its counterpart in control donors (Table II).

FIGURE 6. Differential susceptibility to activation-induced apoptosis of Th1 subsets. PPI-stimulated PBMC were analyzed after a combination of intracellular staining with anti-cytokine mAbs and nuclear staining with 7-AAD. A, Representative dot plots of the staining on CD3T cells from a control and an HIV-infected donor of group 2 are shown. The number in the left quadrant indicates the percentage of corresponding cytokine-positive cells among living cells; the number in the right quadrant indicates the percentage of cytokine-positive cells among apoptotic cells. The number in brackets indicates the proportion of apoptotic cells among the corresponding cytokine-positive subset. B, Analysis was performed on CD4 and CD8 subsets from 9 controls and 19 HIV-infected donors; the percentage of apoptotic cells in each cytokine-positive subset is shown. Horizontal bars show median values. Arrows indicate statistical differences between the percentages of cytokine-producing subsets (Wilcoxon matched pairs test, \( p \leq 0.05 \)). The \( p \) values at the bottom of the figure indicate the statistical comparisons between controls and HIV-infected donors (Mann-Whitney test).
The rate of apoptosis in IL-2-producing T cells is associated with disease progression

To assess whether increased apoptosis in cytokine-producing subsets was correlated with the observed defects in cytokine production throughout HIV infection, the percentage of activation-induced apoptosis in a given Th1 subset was plotted against the percentage of this Th1 subset among CD3 T cells. As shown in Figure 8 (upper panel), the decrease in the number of IL-2 producers was significantly associated with increased apoptosis in this subset. A similar trend was observed for TNF-α, whereas no correlation could be observed between the rate of apoptosis in IFN-γ producers and their proportion. To define whether the increased priming for apoptosis of Th1 subsets in patients was related to disease progression, the percentage of activation-induced apoptosis in each subset was plotted against the ex vivo percentage of CD4 T cells. As shown in Figure 8, lower panel, the increased rate of apoptosis in CD3 T cells synthesizing IL-2 or TNF-α significantly correlated with the ex vivo drop of CD4 T cells, whereas no correlation was found between the rate of apoptosis in IFN-γ producers and disease progression.

Discussion

HIV infection is characterized by the progressive disappearance of the CD45RA<sup>+</sup> naive compartment and a relative increase in numbers of activated/memory CD45R0<sup>+</sup> cells (13). In the present study, we first investigated how perturbations in the proportions of these two populations would contribute to the modified pattern of cytokine production during HIV infection. To analyze the actual capacity of peripheral cells to produce a given cytokine, without any in vitro selection by multiple restimulation, we enumerated cytokine-producing cells by single-cell analysis after a short period

| Table II. Susceptibility to apoptosis and Bcl-2 expression by CD3<sup>+</sup> T cells according to cytokine production<sup>a</sup> |
|----------------|--------------------------|--------------------------|
|                | Controls                 | HIV-Infected             |
|                | % Apoptosis<sup>b</sup>  | % Apoptosis<sup>b</sup>  |
|                | (n = 20)                 | (L + N) Bcl-2<sup>c</sup> (n = 6) | (L + N) Bcl-2<sup>c</sup> (n = 8) |
| IL-2           | 8.7 ± 1.4                | 4.6 ± 1.8                |
| IFN-γ          | 16.7 ± 3.3<sup>c</sup>   | 16.8 ± 8.0<sup>c</sup>   |
| TNF-α          | 21.5 ± 3.8<sup>d</sup>   | 24.9 ± 4.7<sup>d</sup>   |

PBMCs, stimulated for 16 h with PMA, ionomycin, and PMA (in the presence of brefeldin A during the last 12 h of culture), were stained with anti-CD3 mAbs (and 7-AAD for apoptosis quantification), then permeabilized and stained intracellularly with anti-cytokine mAbs (and anti-Bcl-2 mAbs for study of Bcl-2 expression). Analysis was performed on the CD3<sup>+</sup> subset.

<sup>a</sup> Mean ± SEM. L, low; N, normal.

<sup>b</sup> p < 0.05 vs IL-2-producing cells (Wilcoxon matched pairs test).

<sup>c</sup> p < 0.05 vs IL-2- and IFN-γ-producing cells.

FIGURE 7. Relationship between the differential susceptibility to activation-induced apoptosis of Th1 subsets and their level of Bcl-2 expression. A, Intracellular detection of Bcl-2 protein within CD3<sup>+</sup> T cells according to their pattern of cytokine production. Three subsets were defined according to their level of Bcl-2 expression: L, low Bcl-2 cells; N, normal Bcl-2 cells; H, high Bcl-2 cells. Numbers indicate the percentage of cytokine-positive cells in each Bcl-2 subset. Data are from a representative experiment with PBMC from an HIV-infected donor from group 2. Similar results are observed with T cells from control donors. B, Relationship between the susceptibility to apoptosis of CD3<sup>+</sup> T cells, as determined by 7-AAD incorporation, and the level Bcl-2 expression. Numbers indicate the percentage of apoptotic cells within L, N, or H Bcl-2 subsets. C, Relationship between Bcl-2 expression and the susceptibility to apoptosis of each cytokine-producing subset. Numbers indicate the percentages of apoptotic cells within the H or (L + N) Bcl-2 subsets.

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of polyclonal stimulation. We report that significant alterations in the proportions of T cells producing type 1 cytokines was observed in patients upon HIV infection, rather than a shift from Th1 to Th2 subsets as proposed by Clerici et al. (34). Using this approach, we found that peripheral T cells from control donors are not primed for Th2 cytokines, since a very low proportion produce IL-4, IL-5, or IL-13. Similar low proportions of Th2 cells were found in HIV-infected donors. The only exceptions were observed for several AIDS patients with hyper-IgE or Job-like syndrome, who showed an increased proportion of peripheral IL-13 producers. When detected, Th2 cytokines were mainly produced by CD8 T cells (data not shown); our data are compatible with the reports of CD8 T cells with a type 2 phenotype in HIV-infected persons with hyper-IgE syndrome (40, 41) and with other reports failing to detect increased IL-4 production in stimulated PBMC, purified CD4 T cells, or in CD4 T cell clones from HIV-infected patients (21, 28, 42–44). Nevertheless, our study does not rule out the possibility of an increased type 2 cytokine synthesis by non-T cells during HIV infection.

Single-cell analysis of IL-2-, IFN-γ-, and TNF-α-producing cells at the CD4 and CD8 T cell levels revealed that IL-2 was the only cytokine in which synthesis was reduced at the CD4 T cell level in HIV-infected patients (Fig. 2). Interestingly, our study reveals that IL-2 is, for the most part, produced by CD4+CD45RA+ T cells (Fig. 3). The reduction in the percentage of IL-2-producing cells throughout HIV infection appears to be a consequence of the shrinkage of the CD4+CD45RA+ compartment and is also associated with the impairment in cytokine synthesis of the remaining CD4+CD45RA+ cells (Fig. 4). The progressive reduction of CD45RA+ T cells had no consequence on IFN-γ synthesis. Indeed, our approach indicated that IFN-γ is mainly produced by CD45R0 CD8 T cells, a subset that is expanded throughout HIV infection (13). As a consequence, the proportion of IFN-γ-producing cells in HIV-infected patients was found unchanged and sometimes increased as compared with healthy donors (Figs. 1 and 2). Although these data are discrepant with a few reports on CD4 T cell clones or peripheral T cells (21, 29), they are in agreement with others showing an increased capacity of patients’ T cells to produce IFN-γ or to express IFN-γ mRNA (18, 22, 24, 25). In fact, in agreement with a previous report on cytokine gene expression (18), our data show that HIV infection is associated with a clear dissociation at the T cell level in the patterns of IL-2 and IFN-γ synthesis, indicating that HIV disease is associated with alterations among Th1 subsets. Such alterations appeared to be the consequence of modifications in the proportions of CD45RA and CD45R0 subsets. The polarization of CD45RA T cells to IL-2 synthesis and of CD45R0 T cells to IFN-γ synthesis has already been described in healthy donors (2, 3), and recent studies have confirmed that cytokine polarization was an intrinsic characteristic of these subsets (4). In HIV infection, impairment of IL-2 synthesis is due to a quantitative and qualitative alteration of the CD45RA subset. The influence of the size of the CD45RA compartment on the type of cytokine secreted during diseases was also reported in atopic patients in whom IgE and IgA

![Figure 8](http://www.jimmunol.org/)

FIGURE 8. The decrease in the percentage of IL-2 producers and their susceptibility to apoptosis is associated with disease progression. A, The percentage of activation-induced apoptosis in a given Th1 subset was plotted against the percentage of this Th1 subset among CD3 T cells. B, The percentage of activation-induced apoptosis in each subset was plotted against the ex vivo percentage of CD4 T cells. Correlations were determined using the Spearman regression analysis. 63 patients were tested. NS, not significant.
production was found to be associated with an increase in the CD45RA+ cell number (45).

The powerful proinflammatory cytokine TNF-α, produced by monocytes/macrophages and also by T cells, may play an important role in activating HIV replication in patients (46, 47). Contradictory results on TNF-α production in HIV infection have been reported and may be attributable to different methodologic approaches (18, 23, 48–50). However, an increase in TNF-α production by PBMCs appears to be associated, in advanced stages of the disease, with the occurrence of AIDS-associated pathologies and coinfections (51, 52). Using for the first time the approach of single-cell analysis, we show a reduction in the percentage of TNF-α-producing T cells in patients compared with controls. This reduction, independent of the shrinkage of the CD45RA compartment, was the consequence of the decreased capacity of CD8 T cells to synthesize TNF-α combined with the reduction in the number of CD4 T cells producing this cytokine (Figs. 1 and 2). Since the patients did not present with active coinfections at the time of our study, this decreased TNF-α production at the T cell level is probably the direct consequence of HIV infection. The reduced synthesis of TNF-α in HIV-infected patients could be detrimental, in view of reports in various models of viral infection showing that TNF-α can synergize with IFN-γ to express a potent antiviral activity (53) and that TNF-α favors the leukocyte infiltration in vi-rally infected tissues (54). This hypothesis is compatible with the significant correlation we found between the reduction in the percentage of TNF-α-producing T cells and the progression of disease (Fig. 8).

Because lymphocytes from HIV-infected individuals were shown to die by apoptosis upon stimulation in vitro (14, 37, 38, 55), we asked whether the alterations in the representation of the Th1 subsets was the consequence of their differential susceptibility to activation-induced apoptosis. Exogenous cytokines can modulate the susceptibility of lymphocytes from HIV-infected patients to the apoptotic process (56). Our data suggest that the intrinsic capacity of lymphocytes to produce a given cytokine upon activation can also influence their survival. We observed that lymphocytes committed to IFN-γ or TNF-α production were more sensitive to activation-induced apoptosis than lymphocytes committed to IL-2 production. A copriming for apoptosis and IFN-γ production has been previously shown to occur during the intrathymic process of negative selection, since double-positive thymocytes undergoing apoptosis were shown to express in situ high level of IFN-γ mRNA (57). This was also suggested to occur in single-positive thymocytes (58). In human PBMC stimulated by cross-linking of the CD4 molecule, TNF-α and IFN-γ production were associated with the induction of apoptosis, which could be prevented by a cytokine synthesis inhibitor (59). Other studies proposed a role for IFN-γ in the promotion of activation-induced death, which was the consequence of an autocrine process on Th1 clones (60, 61) and a paracrine process on Th2 clones via the up-regulation of Fasl expression (61). This copriming for apopto-sis and IFN-γ production has not been observed in all cases (62). Nevertheless, a recent study has shown that the regulation of IFN-γ synthesis was controlled by a cytokine protease, caspase-1, which is involved in the apoptotic pathway (63). The copriming of lymphocytes for apoptosis and IFN-γ synthesis in HIV infection could therefore represent a regulatory mechanism involved in the selective removal of specific Th effectors. In addition, the preferential clearance by apoptosis of TNF-α-secreting cells might contribute to limit the inflammatory process.

Several mechanisms could account for the gradient of susceptibility to activation-induced apoptosis of the Th1 subsets (TNF-α > IFN-γ > IL-2), which we found in control donors as well as in HIV-infected persons, in CD4+ as well as CD8+ subsets (Fig. 6), and which was independent of the CD45RA+ or the CD45RO+ nature of the producing cells (data not shown). This gradient of susceptibility to activation-induced apoptosis tightly correlated with the expression level of the Bcl-2 molecule (Fig. 7). This control, by Bcl-2 molecule regulation, of the survival of cytokine-producers is in agreement with recent data demonstrating that apoptosis induced by cytokines preferentially involves the ceramide pathway, regulated by Bcl-2, rather than by the Fas-induced ICE (IL-1-βconverting enzyme) pathway, independent of Bcl-2 (64, 65). The influence of the Fas pathway on the control of the differential susceptibility to apoptosis of Th1 subsets could not been determined in our study, but several reports suggested that the control of apoptosis by Fas pathway or by molecules from the Bcl-2 family were independent (66–68). Discordant data were recently reported concerning the possible involvement of the Fas system in the survival of Th1 vs Th2 cells. The report that Th1 clones are more susceptible to apoptosis than Th2 clones was found to be related to increased levels of Fasl expression on Th1 clones (6, 61, 69). However, other studies did not confirm these observations (70). In a recent report, Zhang et al. (7) found that Th1 and Th2 effectors express comparable levels of Fas and Fasl, but only Th2 effectors express high levels of FAP-1, which may act to inhibit Fas signaling. Other members of the TNF family can also transduce the death signal (71), and the high level of apoptosis we observed in TNF-α+ lymphocytes, especially in CD8+ T cells from healthy donors, could be the consequence of an autocrine suicide of these cells (72–74).

IL-2 has been shown to exhibit pro- or antiapoptotic properties according to the cellular activation status and to the cellular mi-croenvironment. Lenardo et al. have shown that preincubation of mouse T cells with IL-2 increased the susceptibility of the cells to apoptosis after TCR stimulation (75), and the requirement for IL-2 was confirmed in human T cells in a Fas-Fasl-dependent model of apoptosis (76). In contrast, IL-2 can rescue cells from apoptosis induced by growth factor withdrawal (38); this rescue was shown to be controlled by molecules from the Bcl-2 family (77–79). In this study, we showed that the intrinsic property of IL-2+ cells to overexpress Bcl-2, as compared with IFN-γ- or TNF-α-producing cells (Fig. 7), enabled them to be resistant to activation-induced apoptosis in normal donors. In HIV-infected patients, an increased susceptibility to apoptosis was observed in IL-2 producers, which was related to a down-regulation of Bcl-2 expression. The progressive decrease in the proportion of IL-2-synthesizing T cells was found to be correlated with their susceptibility to activation-induced apoptosis and disease progression. On the other hand, IFN-γ synthesis was found preserved in patients despite an increased rate of apoptosis in the IFN-γ-producing subset. Indeed, the increased cell death of IFN-γ producers is probably counter-balanced by the increased percentage of CD8+ T cells, the main source of IFN-γ producers. Altogether, these data confirm the important relationship between apoptosis in peripheral T cells and the functional collapse of the immune system in AIDS (12, 14, 30).

Taking into consideration the powerful efficacy of the new antiretroviral drugs, important questions are raised concerning the functional restoration of the immune system in treated HIV-infected patients. Because the functional alteration of untreated patients' T lymphocytes was associated with their defective IL-2 production (34), the recovery of a normal proportion of IL-2 producers should be monitored during the therapy. Preliminary observations on the restoration of the immune system of advanced patients under tri-therapies, including an anti-HIV protease, indicate that recovery of the CD45RA T cell population was not
observed for most of the patients, at least after 1 year (80). Consequently, and according to recent data showing that the recovery of a normal proportion of CD4+ IL-2 producers was not observed in the absence of an increase in the CD45RA subset (E. Ledru, H. Lecoeur, and M. L. Gougeon, unpublished observations), it may be necessary to combine IL-2 therapy with antiretroviral therapy in patients showing very low levels of CD45RA T cells before treatment. Recent reports on in vivo administration of IL-2 in antiretroviral-treated HIV-infected patients showed the efficacy of such a strategy in restoring or maintaining low levels of CD4+ T cells in HIV-infected individuals, at least after 1 year (80). Consequently, the combined follow-up of these parameters by single-cell analysis should be included in the monitoring of the immune system of patients submitted to antiviral or immune-based therapies.

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References

2. Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, A. Springer, H. A. Young, and S. Shaw. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CD29, and Pgp-1) and have enhanced IFN-γ production. J. Exp. Med. 168:1401.
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. J. Exp. Med. 182:1759.


