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Kinetics of the Changes of Lymphocyte Subsets Defined by Cytokine Production at Single Cell Level During Highly Active Antiretroviral Therapy for HIV-1 Infection¹

Ana E. Sousa,* Ana F. Chaves,* Manuela Doroana,[†] Francisco Antunes,[†] and Rui M. M. Victorino^{2*‡}

The effects of highly active antiretroviral therapy on cytokine imbalances associated with HIV-1 infection have not been characterized. Using single cell analysis by flow cytometry, we show that a significant recovery in the frequency of IL-2-producing cells was only observed in patients with a sustained control of viral replication and that the overexpanded CD8 T cell population of CD28⁻ IFN- γ ⁺ cells was not significantly reduced after 1 yr of effective therapy. Moreover, a detrimental role of IL-4 is suggested by the association between an enhanced proportion of IL-4-producing cells within the CD4 and particularly the CD8 subset and viral load rebound. Finally, the kinetics of changes of cell subsets assessed for simultaneous production of different cytokines supports the view that cell reconstitution during highly active antiretroviral therapy is initially due to redistribution of terminally differentiated cells, followed by peripheral expansion of less differentiated ones and a late progressive increase of the proportion of functionally defined naive/memory precursor lymphocytes. These data bring new support for the role of cytokine imbalances in AIDS pathogenesis and may be relevant for the definition of immunointervention targets. *The Journal of Immunology*, 1999, 162: 3718–3726.

Different models have been proposed to explain the observed T cell recovery during highly active antiretroviral therapy (HAART)³ for HIV infection. Following the initial focus on the reduction of viral-induced cytolysis (1, 2), studies have stressed the importance of other possible mechanisms involved in T cell depletion in HIV immunodeficiency, namely altered lymphocyte traffic (3–5), limited renewal capacity of post-thymic cells (3–5), as well as reduced de novo lymphocyte production (6–8). The delineation of the relative contribution of these different pathophysiologic mechanisms and of the functional recovery obtained will further generate important insights on AIDS pathogenesis and lymphocyte homeostasis (9–11).

HIV infection is associated with significant dysregulation of the cytokine network that contributes to AIDS immunopathogenesis either as a result of cytokine modulation of viral replication or due to effects on the immune system, namely suppression of T cell

ability to mount appropriate immune responses, changes of cell susceptibility to apoptosis, and/or alterations of lymphocyte traffic (12, 13). In spite of the importance of delineating the effects of HAART on HIV-associated cytokine imbalances, these have not to date been characterized.

Flow-cytometric single cell analysis of lymphokine production represents a powerful approach to investigate such effects since it permits simultaneous surface immunophenotyping and thus overcomes the effects of the altered representation of the different cell subsets on the cytokine profiles (14–16). On the other hand, it circumvents the problem of cell selection during culture (14–16), which allows the study of cell populations that exhibit low proliferation rates and are prone to apoptosis in vitro, such as the CD8⁺CD28⁻ subset known to be markedly expanded in HIV-1 infection (17). Furthermore, the sequential study during HAART of the relative proportion of the different cytokine-producing subsets may bring insights on the factors determining the homeostasis of these populations.

This new approach has contributed to a better definition of cytokine imbalances in HIV-1 infection (18–20). Our previous studies documented major imbalances within the CD8 subset even in early stages of disease, with low IL-2 and a marked increased frequency of IFN- γ -producing cells mainly with a CD28-negative phenotype. Unexpectedly, there were limited alterations on the relative proportion of IL-2- and IFN- γ -producing cells within the CD4 subset in asymptomatic patients, although a significant decrease in the potential to produce IL-4 was documented in patients with more than 500/ μ l CD4 count (20).

In this study, we investigated sequentially at the single cell level by flow cytometry, the effects of HAART on ex vivo lymphokine production profiles as a means to further delineate the role of cytokine imbalances on AIDS pathogenesis and to try to identify putative targets for immunointervention that may be required to achieve full immunologic reconstitution after HAART.

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³ Abbreviations used in this paper: HAART, highly active antiretroviral therapy; AZ, sodium azide; BFA, brefeldin A; I, ionomycin; PE, phycoerythrin; TC, TriColor conjugated.

Table I. Mean values of viral RNA load and of CD4 and CD8 T cell subsets^a

Time	Viral Load (log copies/ml)	CD4 Counts/ μ l	% CD4	CD8 Counts/ μ l	% CD8
Baseline	4.8 (4.3 to 5.8)	378 (11 to 828)	22.1 (1.4 to 37.9)	747 (404 to 2027)	41.7 (28.2 to 55.8)
Follow-up					
Week 4	2.9 (2.2 to 4.7)***	507 (111 to 970)**	25.3 (8.25 to 43.2)	947 (319 to 2399)*	42.1 (29.3 to 51.9)
Week 8	2.6 (1.7 to 4.4)***	483 (68 to 860)**	23.9 (5.6 to 50.3)	868 (438 to 2096)*	41.8 (-12.7 to 8.9)
Week 16	2.3 (1.7 to 3.9)***	535 (101 to 1049)***	25.9 (8.0 to 43.1)*	869 (273 to 1926)*	40.1 (21.1 to 50.6)
Week 24	2.3 (1.7 to 4.5)***	594 (174 to 898)***	27.0 (11.7 to 45.4)***	924 (493 to 2484)*	40.2 (24.8 to 53.3)
Week 32	2.6 (1.7 to 5.0)**	602 (163 to 1053)***	28.4 (8.3 to 45.2)**	896 (482 to 1983)*	39.1 (28.5 to 50.3)*
Week 40	2.5 (1.7 to 5.1)**	567 (112 to 1179)***	25.8 (7.1 to 44.2)	855 (443 to 1295)	37.2 (22.3 to 51.9)**
Week 48	2.5 (1.7 to 5.1)**	626 (247 to 1224)**	28.8 (12.7 to 45.7)**	900 (409 to 1968)*	39.8 (28.5 to 57.3)

^a Values represent the mean value and the range. Cut off of the viral load test: 1.7 log copies/ml (50 RNA copies/ml). Significance in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Materials and Methods

Study population

Fourteen HIV-1-infected patients, six females and eight males, with a mean age of 32.6 ± 12.9 (range 20–61), were enrolled in the study. The mode of transmission was homosexual contact in five individuals, heterosexual contact in four, and i.v. drug addition in five patients who had stopped drug consumption at least 1 yr before the start of the study. Centers for Disease Control classification of HIV infection (1993) was as follows: eight patients in class A (2 A₁, 5 A₂, and 1 A₃), five in class B (2 B₁, 1 B₂, and 2 B₃), and one in class C₁ (previously treated tuberculosis). At start of treatment, the mean CD4 counts were $378 \pm 219/\mu$ l (range 11–828) with a documented decline in CD4 T cell counts during the previous year of the study (mean CD4 counts $503 \pm 160/\mu$ l 1 yr before). The mean viral load at time zero was 4.8 RNA log copies/ml (range 4.3–5.8), and the treatment protocol was lamivudine (150 mg bid), stavudine (40 mg bid), and nelfinavir (750–1000 mg bid). Eleven patients were virgin of previous antiretroviral therapy, and the other three had only received reverse-transcriptase inhibitors (zidovudine alone or in association with didanosine or zalcitabine). Immunologic and virologic studies were done at weeks 0, 2, 4, 8, 16, 24, 32, 40, and 48 of treatment.

mAbs and reagents

The following anti-human cytokine mAbs were used: anti-IL-2, clone MQ1-17H12, rat IgG2a (R-phycoerythrin-conjugated (PE)); anti-IFN- γ , clone 45.B3, mouse IgG1 (FITC conjugated); IL-4, clone 8D4-8, mouse IgG1 (purified and PE); mouse IgG1 (FITC and PE), rat IgG1 (PE), and rat IgG2a (PE) isotype controls; all were purchased from PharMingen (San Diego, CA). The final concentrations of the mAbs were determined by titration in the study conditions. Anti-human CD3 (TriColor conjugated (TC)), CD8 (FITC, PE, and TC), CD45RO (TC), CD69 (FITC and PE), and mouse IgG2a isotype control (FITC, PE, and TC) mAbs were obtained from Caltag (South San Francisco, CA). Anti-human CD4 (FITC), CD28 (PE), CD45RO (PE), and mouse IgG1 isotype control (FITC and PE) mAbs were purchased from Becton Dickinson (Mountain View, CA). PMA (Sigma, St. Louis, MO) and ionomycin (I; Calbiochem, La Jolla, CA) were used at 50 and 500 ng/ml final concentrations, respectively, and brefeldin A (BFA; Sigma) was used at 10 μ g/ml.

Immunofluorescent staining

Immunofluorescent staining was performed as previously described (20). Briefly, PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque (Life Technologies, Gaithersburg, MD) gradient centrifugation, immediately after venopuncture, and resuspended at 1×10^6 cells/ml in RPMI 1640 (Life Technologies), 10% pooled AB human serum (Sigma), 100 U/ml penicillin/100 μ g/ml streptomycin (Life Technologies), and 2 mM glutamine (Life Technologies). PBMCs were cultured with PMA + I in the presence of BFA for 4 h. After a wash in PBS (Sigma), cells were fixed with 2% formaldehyde (Sigma) by incubation for 20 min at room temperature, washed again in PBS, resuspended in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (AZ; Sigma) (PBS/BSA/AZ), and stained immediately for cytokines or stored at 4°C for up to 2 days. After surface staining with anti-CD8, anti-CD28, anti-CD69, anti-CD3, and anti-CD45RO mAbs, cells were washed with PBS/BSA/AZ and permeabilized with PBS/1% BSA/0.5% saponin. Intracellular staining was done with anti-IL-2, anti-IL-4, anti-IFN- γ , and anti-CD69 mAbs by 30-min incubation at

room temperature. After two washes in saponin buffer, cells were washed and resuspended in PBS/BSA/AZ.

Flow-cytometric analysis of intracellular cytokine

Ten thousand or fifty thousand events (samples stained for IL-4) were acquired in a FACSCalibur flow cytometer (Becton Dickinson), and five-parameter analysis was performed using Cellquest software. Analysis was done within a manual setting lymphogate, and thresholds were set according to isotype-matched negative controls, staining of unstimulated cells, and previous block of staining with unconjugated mAbs. Results were expressed as the percentage of cells of a particular T cell subset that stained positive for a given cytokine. Because of the previously documented rapid down-modulation of CD4 in response to phorbol esters (15), the CD4 subset was identified as the CD8⁺CD3⁺ cells. The presence of BFA in the culture protected the cell surface from activation-induced changes in CD45RO expression, allowing analysis within the ex vivo CD45RO⁺ subset (15). To exclude that differences in T cell activation could bias the cytokine profiles, CD69 was used to assess the level of PMA + I-induced activation of the cells and shown to be expressed in more than 95% of the T cells in all of the experiments. Lymphocyte surface phenotyping studies of noncultured PBMCs were done in parallel using the following mAb combinations: FITC-CD4/PE-CD8/TC-CD3, FITC-CD4/PE-CD28/TC-CD3, and FITC-CD4/PE-CD45RO/TC-CD3. Absolute numbers of lymphocyte subsets were found by multiplying their representation by the absolute lymphocyte count obtained at the clinical laboratory, and results are presented for the 14 patients at all of the different times of the study, except at week 2, in which absolute counts refer to nine patients.

Plasma viral RNA assay

Plasma viral RNA load was measured by reverse-transcriptase PCR (Ultra-sensitive Test; Roche Molecular Systems, Branchburg, NJ) and the threshold was 50 HIV-1 RNA copies/ml (1.7 log copies/ml).

Statistical analysis

The data are presented as arithmetic mean \pm SEM. Baseline and follow-up data were compared using paired *t* test or Wilcoxon-matched pairs test, according to the type of distribution of the variables, and for data of different groups unpaired *t* test and Mann-Whitney test were used; *p* values < 0.05 were considered significant. The Pearson's correlation coefficient was used to determine the correlation between two variables. For correlation with viral load, the Spearman's correlation coefficient was selected.

Results

Effects of treatment on viral load and on T cell subsets

HAART induced a marked decrease in the plasma viral load, which was kept below the limit of test detection (50 RNA copies/ml) after the sixteenth week of therapy in nine patients and a parallel increase in the absolute numbers of the peripheral blood CD4 and CD8 T cells in all of the 14 patients studied (Table I). Furthermore, a significant enhancement in the proportion of CD4 within the T cells was documented, leading to a progressive increase in the CD4/CD8 ratio that reached statistical significance at week 24 after start of treatment (0.58 ± 0.09 at baseline to 0.73 ± 0.11 , $p = 0.003$). No alterations were found in the frequency of

At start of treatment (576 CD4 cells/ μ l and 4.7 log RNA copies/ml)

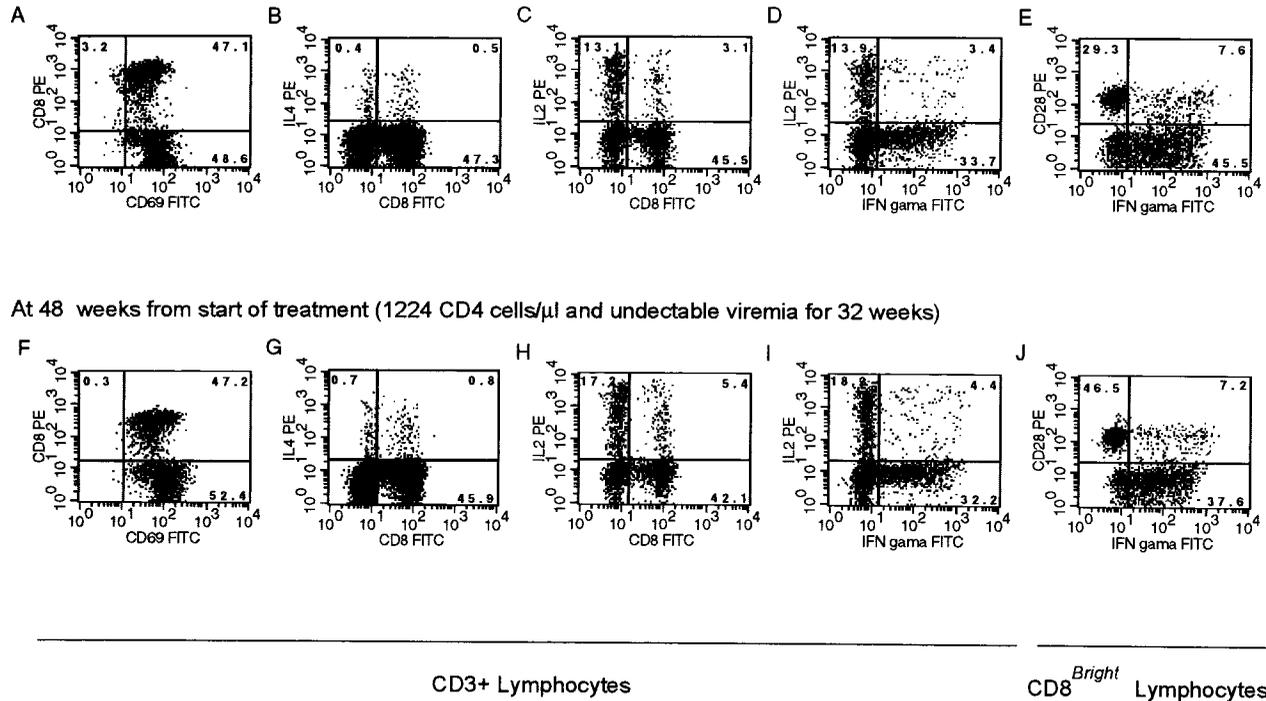


FIGURE 1. Flow-cytometric analysis of cytokine synthesis in a representative patient at baseline (A–E) and at 48 wk of follow-up (F–J). After a 4-h culture in the presence of PMA + I and BFA, PBMCs were stained for surface markers (CD3, CD8, and CD28) and for intracellular IL-2, IFN- γ , IL-4, and CD69. A total of 10,000 or 50,000 events (samples stained for IL-4) was acquired. Dot plots show CD3⁺ gated lymphocytes stained for CD8 and CD69 (A and F), IL-4 (B and G), or IL-2 (C and H), as well as for IFN- γ and IL-2 simultaneously (D and I). Analysis of CD8 bright lymphocytes, known to represent CD8 T cells, stained for IFN- γ and CD28, is shown in E and J. The percentage of events falling within a given quadrant is shown. CD4 T cells were defined as the CD8-negative cells within a CD3⁺ gate in view of the down-regulation on the CD4 molecule induced by PMA + I. CD69 was used as a marker of the PMA + I-induced activation to exclude biased cytokine profiles due to activation deficits.

double-negative T cells (CD4⁻CD8⁻) as well as of the double-positive T cells (CD4⁺CD8⁺). After a transient increase in the proportion of CD45RO⁺ cells at the second week of therapy, a progressive decline in the percentage of these cells was observed in the CD4 and CD8 subsets.

Analysis of IL-2-producing cells

A representative profile of the cytokine analysis is shown in Fig. 1. The mean percentage of IL-2-producing cells increased both in the CD4 (baseline value: $32.1 \pm 2.3\%$) and the CD8 subset (baseline value: $13.2 \pm 1.3\%$) after the second week of treatment, although the enhancement was most marked in the CD8 T cells (Fig. 2A). The analysis of absolute counts revealed an initial marked increase in the number of both IL-2-producing CD4 and CD8 T cells, although a sustained progressive increase after 24 wk of treatment was only observed in the IL-2-producing CD4 T cell counts (Fig. 2B). These alterations paralleled the increase in CD4 and CD8 counts, respectively. When the patients were divided according to the control of viral replication into a group of nine with sustained viremia below 50 RNA copies/ml (cutoff of the test) and another with rebound viremia after the initial drop induced by HAART, it was observed that the increase in the proportion of IL-2-producing cells within the CD4 subset only reached significance in the group with sustained suppression of viremia (Fig. 2C).

Analysis of IFN- γ -producing cells

There was a trend for an increase in the proportion of IFN- γ -producing CD4 T cells during therapy, although the comparison with base-

line mean value does not reach statistical significance. However, a significant increase in the number of peripheral blood CD4⁺IFN- γ ⁺ T cells was documented since the eighth week of treatment ($73 \pm 12/\mu$ l at the start of therapy to $104 \pm 13/\mu$ l, $p = 0.01$).

The change in the frequency of IFN- γ ⁺ cells within the CD8 T cell subset (baseline value: $52.8 \pm 4.8\%$) showed an initial slight increase until the sixteenth week of treatment, followed by a decrease that did not reach statistical significance (Fig. 3A), and similar results were documented for the IFN- γ ⁺CD8⁺CD3⁺ cell counts (baseline value: 477 ± 127 cells/ μ l), as shown in Fig. 3B. To investigate whether these relatively nonimpressive changes in IFN- γ production within the CD8 subset were the result of opposite effects of HAART on the IFN- γ -producing CD28⁺ and CD28⁻ populations, a further characterization of the phenotype of the CD8 subset was done. At time zero, there was a marked expansion of IFN- γ -producing CD28-negative cells within the CD8 subset ($32.9 \pm 10.4\%$ in patients versus $9.5 \pm 4.3\%$ in healthy controls, $p = 0.01$), which was only marginally reduced with treatment (no statistical significance; Fig. 3C). On the other hand, HAART induced an increase in the frequency of IFN- γ -producing cells with a CD28⁺ phenotype, statistically significant at weeks 8 and 16 (Fig. 3C). Moreover, a very significant decrease in the percentage of non-IFN- γ -producing CD28-negative cells was shown (Fig. 3E). Finally, a significant increase in the proportion of CD28⁺ IFN- γ ⁻ cells, a profile found in the majority of unprimed cells, was documented at week 24 of therapy and thereafter (Fig. 3E). Analysis of the absolute cell numbers revealed that the increase in CD8 counts was mostly due to cells with a CD28⁺

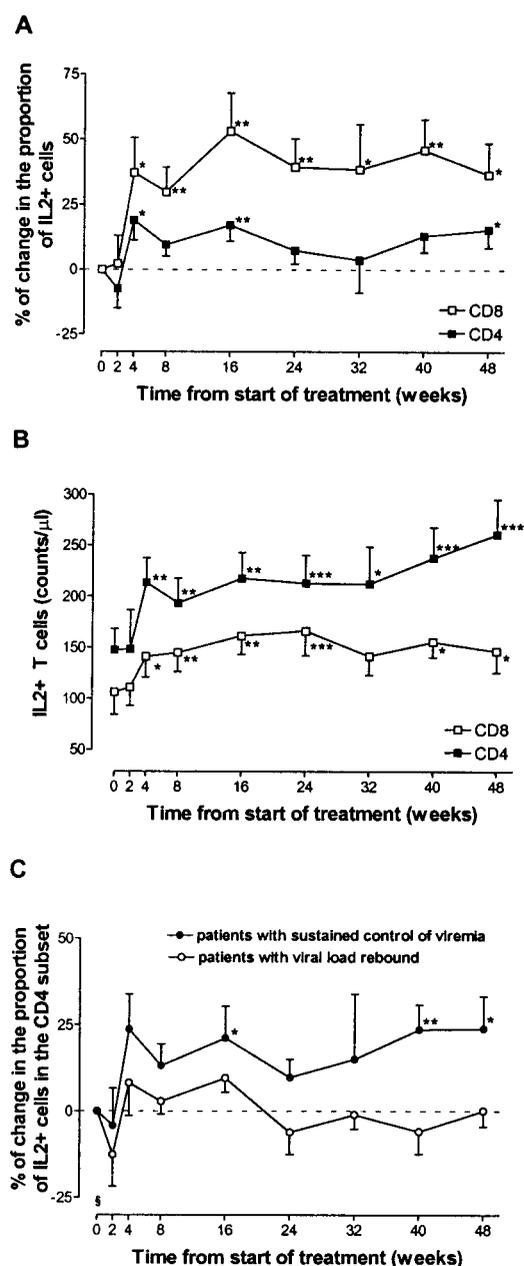


FIGURE 2. Kinetics of IL-2-producing cells. *A*, Mean percentage of change from baseline of the proportion of IL-2-producing cells within CD4 (■) and CD8 (□) T cell subsets. *B*, Mean absolute counts of IL-2-producing CD4 (■) and CD8 (□) T cells. *C*, Percentage of change from baseline of the proportion of IL-2-producing cells within the CD4 subset in the group of patients with sustained control of viremia (●) and in the group with viral load rebound (○). Statistic significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

phenotype, and that the number of circulating CD28-negative IFN- γ -producing cells did not diminish significantly with HAART (Fig. 3, *D*, *E*, and *F*).

Analysis of IL-4 production within T lymphocytes and within the CD45RO⁺ subset

At time zero, there was a negative correlation between the proportion of IL-4-producing CD4 cells and the CD4 counts ($r = -0.8223$, $p = 0.0003$) as well as with the percentage of peripheral

blood CD4 T cells ($r = -0.7499$, $p = 0.002$). The frequency of IL-4-producing CD8 T cells also correlated negatively with the degree of CD4 depletion at baseline ($r = -0.5545$, $p = 0.039$ for CD4 counts, and $r = -0.5975$, $p = 0.024$ for the percentage of CD4 T cells). No significant correlation was observed with the viral load.

The analysis of the distribution of individual values of IL-4-producing CD4 and CD8 T cells shows that 4 of 14 subjects have percentages clearly above normal range for healthy controls (Fig. 4, *A* and *B*). Viral load was not significantly different in these patients, although they exhibited lower CD4 counts (121 ± 66 versus 480 ± 47 cells/ μ l, $p = 0.001$). Interestingly, in two of these four patients, HAART induced a marked reduction in the percentage of IL-4-producing CD4 cells, although without reaching normal levels, and this was accompanied by a parallel decline in the hyper eosinophilia that these patients exhibited at baseline. The remaining 10 patients had percentages of IL-4-producing CD4 cells falling within the normal range, although their mean value was lower than that of healthy subjects. Curiously, in these patients, HAART induced an increase in the mean percentage of IL-4-producing T cells both within the CD4 and the CD8 subsets that reached higher degree of significance when the analysis was done within the memory/effector population (CD45RO-positive cells).

When patients were divided according to the level of control of the viral replication during HAART, two findings emerged. First, at baseline, the levels of IL-4-producing CD4 or CD8 T cells were very significantly higher in the group with rebound viremia ($6.9 \pm 2\%$ versus $1.9 \pm 0.5\%$ in patients with sustained suppression of viral load, $p = 0.018$ for CD4 T cells and $4.8 \pm 1.1\%$ versus $1 \pm 0.2\%$, $p = 0.0008$ for CD8 T cells). Second, the pattern of response to HAART was different in the two groups, as shown in Fig. 4, *C* and *D*.

When results were analyzed in terms of absolute counts of IL-4-producing CD4 and CD8 T cells, higher levels at baseline were found in patients with viral load rebound (13 ± 4.2 vs 8.8 ± 0.9 in the group with sustained control of viremia for IL-4⁺ CD4 counts, and 50 ± 21.7 vs 7.6 ± 1.6 , $p = 0.019$, for IL-4⁺ CD8 T cell counts), and an increase of IL-4-producing CD4 and CD8 counts was observed in both subgroups during therapy.

Analysis of simultaneous production of IL-2 and IFN- γ within T lymphocytes

Analysis of cells simultaneously stained for IL-2, IFN- γ , and CD3 (Fig. 5*A* in terms of relative proportions, and Fig. 5*B* in terms of absolute numbers) revealed that there was an increase in the proportion of IFN- γ ⁺IL-2⁻ T lymphocytes at the second week of treatment, followed by a progressive decline. This suggests that the early increase in T cell counts is predominantly due to terminally differentiated cells (IFN- γ ⁺IL-2⁻). The proportion of cells with a IFN- γ ⁺IL-2⁺ phenotype enhanced during therapy, and this enhancement reached statistical significance at weeks 4–24. Thus, within the Th1 cell population, therapy induced after the fourth week, a change from a terminally differentiated phenotype (IFN- γ ⁺IL-2⁻) to a less differentiated one (IFN- γ ⁺IL-2⁺) that is known to have higher proliferative responsiveness. Finally, the mean percentage of IFN- γ ⁺IL-2⁺ cells, a phenotype largely expressed by naive cells, showed a marked enhancement after week 24, which reached significance at week 40. Analysis according to the degree of viral load control showed that this increase in functionally defined naive cells only reached statistical significance in the group of patients with sustained suppression of viremia ($15.34 \pm 1.76\%$ at start of treatment to $19.73 \pm 2.16\%$ at week 40, $p = 0.005$, in

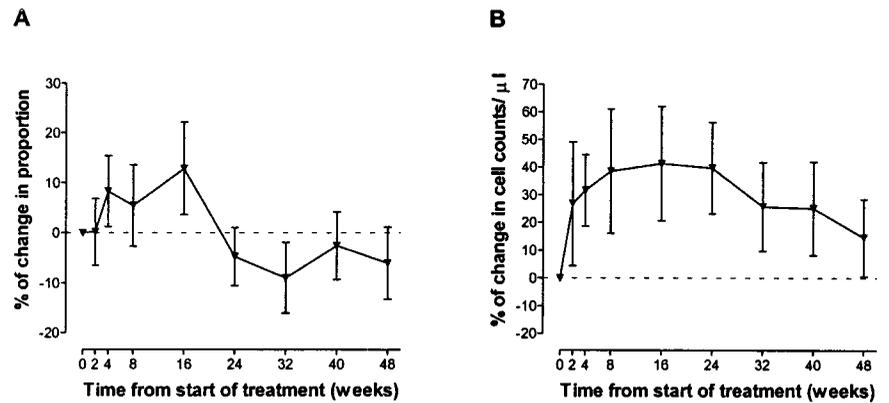
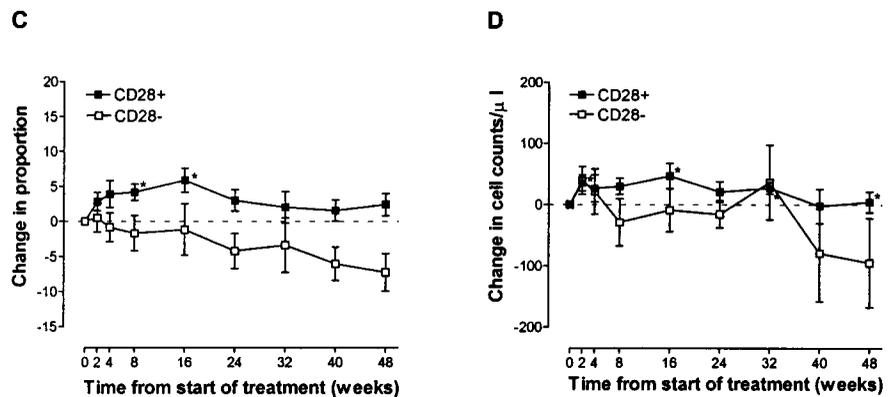
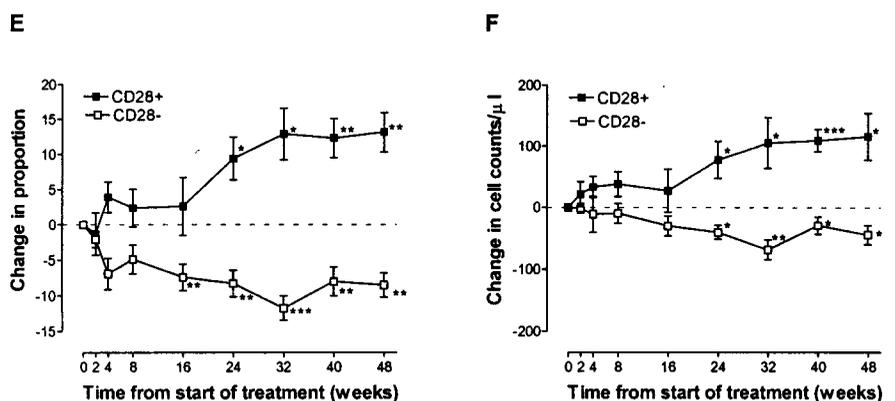
IFN γ producing CD8 T cells

FIGURE 3. Longitudinal analysis of IFN- γ production within the CD8 T cell population during triple therapy. The mean percentage of change from baseline in the proportion of IFN- γ^+ cells (A) and IFN- γ^+ cell counts (B) is shown. Further analysis was done according to the presence (■) or absence (□) of the surface expression of the costimulatory molecule CD28, and results are presented as mean change from baseline of the proportion of IFN- γ^+ cells (C), the number of IFN- γ^+ cells (D), the proportion of IFN- γ^- cells (E), and the number of IFN- γ^- cells (F). Statistic significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

IFN γ producing CD8 T cells according to CD28 phenotypeIFN γ non producing CD8 T cells according to CD28 phenotype

the group of patients with sustained control of viral replication versus $14.69 \pm 1.82\%$ at start of treatment to $14.69 \pm 1.69\%$ at week 40, in the group with rebound viremia.

Analysis of cells with a Th0 phenotype (IL-4⁺IFN- γ^+) within total T lymphocytes and within the CD45RO⁺ subset

The simultaneous analysis of IL-4 and IFN- γ production (Fig. 6, A and B) revealed a significant enhancement at the fourth week of treatment of the frequency of Th0 (IL-4⁺IFN- γ^+) cells both within the memory/effector subset (CD45RO⁺ gated cells) and within total T lymphocytes. An increase of IL-4⁺IFN- γ^-

cells was also documented, although later than for the Th0 population. No significant differences were found in the mean proportions of IL-4⁻IFN- γ^+ lymphocytes within both subsets during the follow-up. Similar findings were documented when the absolute counts of IL-4- and IFN- γ -producing subsets were analyzed (Fig. 7, A and B).

These patterns of alterations associated with therapy appear to be similar in the subgroup with viral load rebound and in the one with sustained control of viremia, although at baseline the proportion of Th0 and Th2 (IL-4⁺IFN- γ^-) cells was significantly higher in the subgroup with viral load rebound.

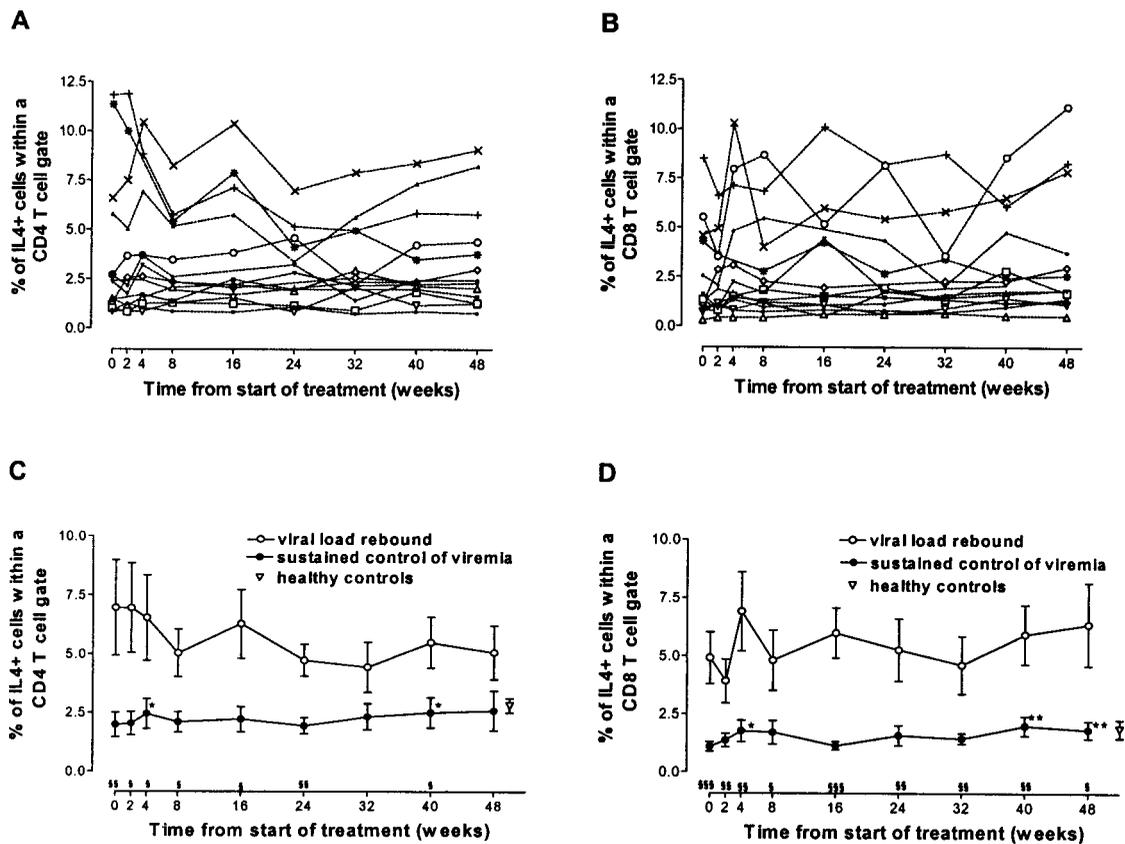


FIGURE 4. Kinetics of IL-4-producing cells. Individual proportions of IL-4⁺ cells within the CD4 subset (A) and within the CD8 subset (B). Comparison of the mean values of the group of patients with sustained suppression of viremia (●) and the group with viral load rebound after the initial drop in viremia (○) within the CD4 subset (C) and the CD8 subset (D). The symbol (▽) indicates mean values of healthy controls. Statistic significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. Statistic significance between the two groups of patients in each time of study: §, $p < 0.05$; §§, $p < 0.01$; and §§§, $p < 0.001$.

Discussion

In spite of the fact that cytokines are key regulators of cell proliferation, apoptosis, and lymphocyte traffic (21), the effects of HAART on HIV-associated cytokine imbalances have not been characterized. We investigated, at single cell level by flow cytometry, the potential for cytokine production of different T cell subsets during 48 wk of HAART. This approach permitted the analysis of the kinetics of changes of cell populations defined functionally by their cytokine production profile. An initial transitory increase in the representation of populations with a cytokine profile characteristic of Ag-experienced terminally differentiated cells was shown. This supports the view that the early increase of peripheral blood T cells is mainly due to alterations in the lymphocyte traffic and to the release of cells entrapped in the peripheral lymphoid organs in parallel with the major drop in viral load (3–5).

After the first 4 wk of therapy, there was a progressive increase in the proportion of IL-2-producing cells that was mostly due to a significant enhancement in the frequency of cells with the potential to simultaneously produce IL-2 and IFN- γ , a profile known to be associated with a population of primed cells with higher proliferative capacity (21, 22). Parallel studies in the same cohort of patients revealed that this is concomitant with the major recovery of in vitro lymphocyte proliferative responses to different mitogenic stimuli, namely anti-CD3 mAb, anti-CD3 plus anti-CD28 mAbs, PHA, pokeweed mitogen, and PMA + I (data not shown). This sequence of events would be consistent with a major role of pe-

ripheral cell expansion in cell recovery, after the initial effect of HAART on lymphocyte redistribution. Interestingly, this putative increase in renewal T cell capacity is further supported by the recent observation of an increase in the fraction of cells expressing the marker of proliferation Ki67 in the peripheral blood and lymph nodes during the same period after start of treatment (5).

After week 24, the increasing frequency of IL-2-producing T cells was mainly due to an enhancement of the proportion of cells producing IL-2, but no IFN- γ , a profile largely described in unprimed lymphocytes and possibly also present in memory precursor cells (15, 23–25). Thus, the increase in this population suggests an effective de novo generation of lymphocytes, possibly by thymopoiesis recovery, and an increase in the peripheral memory precursor pool, which may have important implications to the functional recovery during therapy. The prethymic stem cell differentiation is considered essential for the reconstitution of the perturbed TCR repertoire in HIV infection (6–9), and thus, it is important to note that only patients with sustained control of viremia showed a significant recovery of the frequency of IL-2⁺IFN- γ ⁻ T cells, which would be consistent with the view that an effective suppression of viral replication is important to achieve an immunologic reconstitution (6).

Defective production of IL-2 has long been described in HIV-associated immunodeficiency (12, 13) and represented the rationale for the trials of IL-2 immunotherapy aimed at improving Th and T cytotoxic immune responses (26). Our finding of an increase in the potential for IL-2 production after the fourth week of

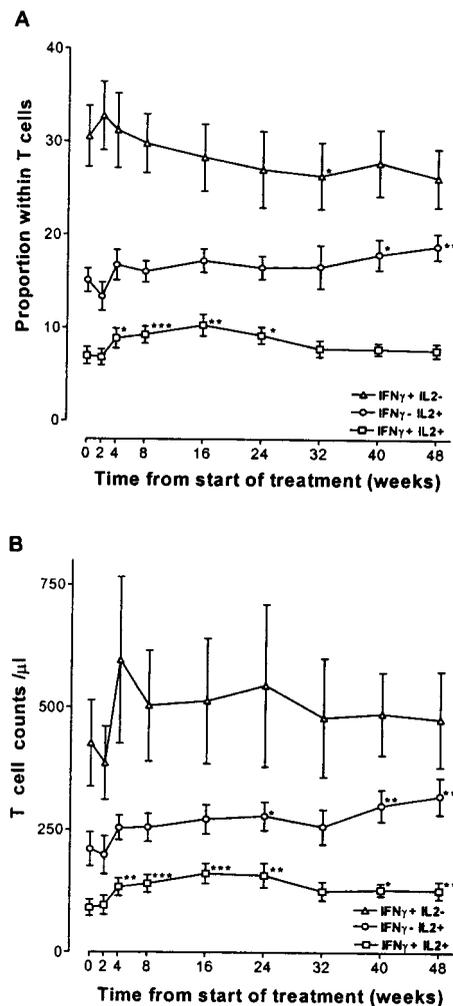


FIGURE 5. Longitudinal analysis of simultaneous production of IL-2 and IFN- γ during HAART. *A*, Results are expressed as the mean proportion of IFN- γ ⁺IL-2⁺ (□), IFN- γ ⁻IL-2⁺ (○), and IFN- γ ⁺IL-2⁻ (Δ) within CD3⁺ gated lymphocytes. *B*, Results are expressed as the mean absolute counts of IFN- γ ⁺IL-2⁺ (□), IFN- γ ⁻IL-2⁺ (○), and IFN- γ ⁺IL-2⁻ (Δ) CD3⁺ lymphocytes. Statistic significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

HAART represents a cellular basis for the functional T cell recovery, which until now had only been assessed in terms of in vitro lymphoproliferative responses (3, 8).

Another important issue in HIV immunodeficiency has been the origin and role of the major expansion of the CD8 subset observed during the primary infection and persisting throughout the natural course of disease (27–30). This expansion has been shown to be largely due to atypical CD8 T cells considered to be an effector terminally differentiated population on the basis of the increased cytolytic activity (31), the loss of the costimulatory molecule CD28 (17), the lower proliferative capacity with reduced telomere length (32) as well as on the cytokine production profile: IFN- γ ⁺, TNF- α ⁺, IL-2⁻ (20, 22). Although the generation of such cells is a subject of hot debate, most argue that they are Ag driven and include a large number of HIV-specific CTLs (30–33). Somehow unexpectedly, our study showed that 48 wk of sustained control of viral replication did not significantly reduce the expansion of the IFN- γ -producing CD8 T cells with a CD28-negative phenotype. One possible explanation is that even minor degrees of viral antigenic load are enough to maintain this expanded population. How-

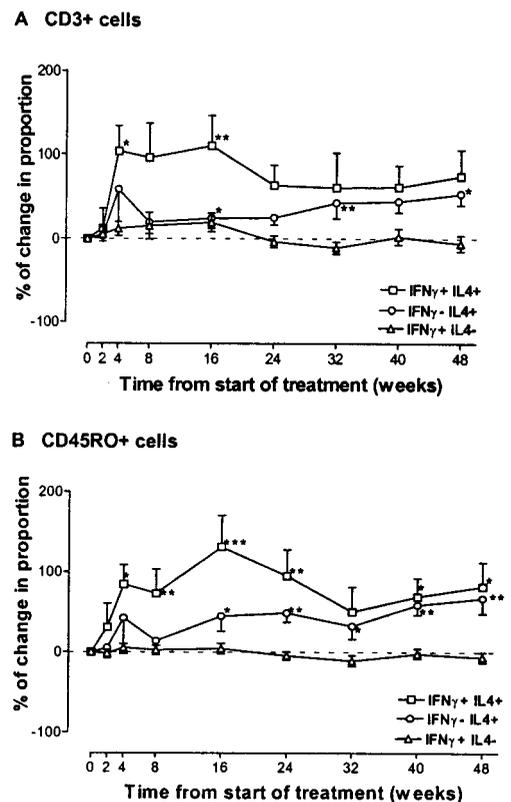


FIGURE 6. Longitudinal analysis of simultaneous production of IL-4 and IFN- γ during HAART. Results are expressed as the mean change of the proportion of IFN- γ ⁺IL-4⁺ cells (□), IFN- γ ⁻IL-4⁺ cells (○), and IFN- γ ⁺IL-4⁻ cells (Δ). *A*, Analysis within CD3⁺ gated lymphocytes (total T cells). *B*, Analysis within CD45RO⁺ gated lymphocytes (memory/effector subset). Statistic significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

ever, this hypothesis is not supported by recent data using direct staining of virus-specific CD8 cells with tetrameric complexes of MHC and HIV peptides, which showed a reduction of HIV-specific CD8 cells during HAART (33). Alternatively, this population might include large amounts of nonspecifically expanded CD8 T cells, which persistency, in spite of HAART, could result from distinct cell survival requirements and/or from a distinct array of adhesion molecules that leads to its persistence in the intravascular compartment (11, 34). In this respect, it is worth noting that our data showed that the observed increase in CD8 counts during HAART was largely due to CD28-positive cells.

In view of the role of IFN- γ in the regulation of cell recirculation (35), some authors have suggested that the alterations in lymphocyte traffic observed in HIV infection are due to the increase in IFN- γ production (11, 36). Our data showing a major increase in the absolute counts of peripheral blood cell populations in the absence of a reduction in the proportion of IFN- γ -producing cells do not favor a central role of IFN- γ in the traffic disturbances in HIV infection. However, a note of caution is required, since this approach measures the frequency of cells producing a given cytokine and, therefore, it is not possible to exclude that during HAART the amount of effective cytokine secretion per cell decreases, either as a result of the reduction of HIV viral Ag stimuli or due to the lower level of bystander lymphocyte activation.

The finding of an increased proportion of IL-4⁺ cells within the CD4 and, more significantly, within the CD8 subset of patients who did not achieve a sustained control of viral replication, favors a critical role of IL-4 in the disease outcome after HAART that

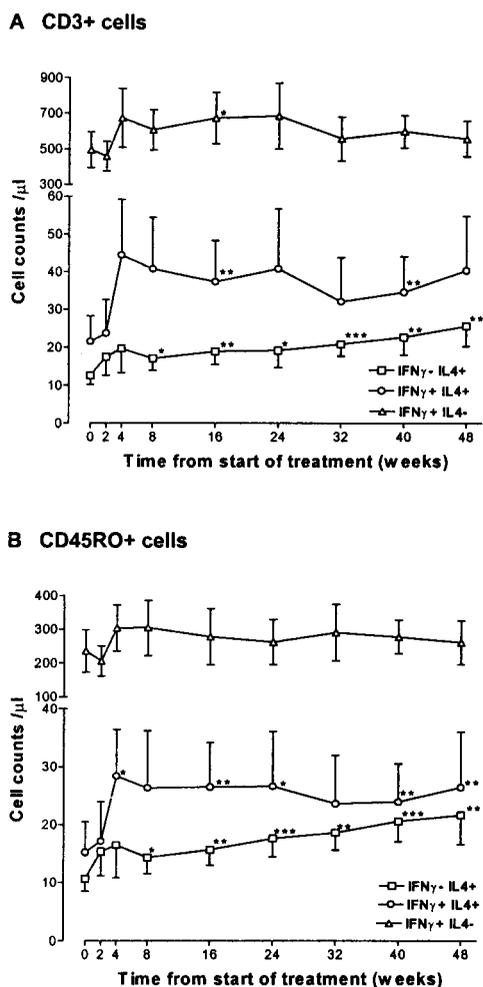


FIGURE 7. Longitudinal analysis of the absolute numbers of cells simultaneously producing IL-4 and IFN- γ during HAART. Results are expressed as the mean absolute counts of IFN- γ ⁺IL-4⁺ (□), IFN- γ ⁻IL-4⁺ (○), and IFN- γ ⁺IL-4⁻ (△) CD3⁺ lymphocytes (A) and CD45RO⁺ memory/effector lymphocytes (B). Statistical significance of the follow-up values in comparison with baseline values: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

warrants further investigation. Particularly interesting in this context are the recent data showing that IL-4 modulates the chemokine receptor expression on T cells (37, 38), which suggests a role of IL-4 in the phenotypic switch from nonsyncytia-inducing/CCR5 using virus to syncytia-inducing/CXCR4 using virus that has been associated with disease progression and an adverse response to treatment (38). Our findings also agree with previous observations by Maggi et al. suggesting a deleterious role of Th2-like CD8 T cells in HIV-1 infection (39).

The effects of HAART on the potential for cytokine production analyzed at single cell level by flow cytometry are not consistent with a simple Th1 to Th2 shift model (12). However, it is important to stress that a recovery of Th1 cells, as assessed by the production of IL-2 and IFN- γ , was documented, and moreover, that levels of IL-4 above the normal range seem to be associated with viral rebound during HAART, which represent observations fitting into the general cytokine shift model.

In conclusion, the sequence of recovery of T cell subsets defined by cytokine profiles during HAART is consistent with an early cell redistribution, followed by peripheral cell expansion and a late de novo lymphocyte production that is associated with an effective

suppression of viral replication. Moreover, these data bring new support for the role of cytokine imbalances in AIDS pathogenesis and are relevant for the definition of immunointervention targets.

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