Intracellular cytokines in the acute response to highly active antiretroviral therapy

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Objectives: Successful highly active antiretroviral therapy (HAART) is usually associated with a rapid decline in HIV plasma RNA levels and a gradual increase in CD4 T cells. We examined whether changes in cytokine production and profile precede other immunological changes and whether these might occur in temporal association with plasma HIV RNA changes.

Design and methods: Eleven HIV-1-infected patients were enrolled into a prospective cohort study; eight patients were naive to antiretroviral therapy. Blood samples were collected pre-therapy (week 0) and at 1, 2, and 3 weeks post-initiation of therapy.

Results: All 11 patients enrolled remained on triple HAART for 1 week, eight for 2 weeks, and six for $\geqslant 3$ weeks. When compared to week 0, these patients had a $\geqslant 2$-$\log_{10}$ decline in HIV plasma RNA levels and/or a decline to $\leqslant 400$ copies/ml by week 3 of therapy ($p = 0.004$). The numbers and percentages of CD4 and CD8 T cells, and the percentage of naive, memory, and activated T cells did not change significantly between weeks 0 and 1 or 0 and 3. Of all the immune parameters examined only: the percentage of CD4 T cells spontaneously producing tumor necrosis factor (TNF)-α (median, 2.4 versus 0.5% $P = 0.025$); the percentage of CD8 T cells spontaneously producing TNF-α (median, 0.6 versus 0.2% $P = 0.037$); and the percentage of CD3 T cells spontaneously producing interleukin-4 (median, 1.8 versus 0.8% $P = 0.004$) changed significantly between weeks 0 and 3.

Conclusions: In these patients, decreases in the percentage of T cells spontaneously producing TNF-α or interleukin-4 preceded changes in CD4 T cells. If confirmed by others, these observations may be useful as early predictors of response to and early failure of HAART.

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Introduction

Various cytokines affect every step in the HIV life cycle [1] and cytokine dysregulation may play an important role in HIV disease pathogenesis. In particular, the pro-inflammatory cytokine tumor necrosis factor (TNF)-α can accelerate HIV replication *in vitro* [2,3] and stimulate HIV expression via activation of the cellular transcription factor nuclear factor (NF)-κβ [1]. Enhanced expression and secretion of type 2 cytokines such as interleukin (IL)-4 may be associated with long term HIV disease progression [4–6].

Successful highly active antiretroviral therapy (HAART) rapidly reduces HIV-1 RNA levels in plasma, often to below detection limits [7,8]. This decline is usually followed by a more gradual increase in the number of circulating CD4 T cells [7–9]. Carcelain *et al.* [10] reported a very rapid and significant decrease in activation markers (CD38 and HLA-DR) on both CD4 and CD8 T cells after only 4 weeks of HAART. We wished to determine whether changes in cell-specific TNF-α or IL-4 production could be detected even earlier than this, preceding the increase in CD4 T-cell proportions and numbers, preceding the decrease in activation markers, and occurring concomitantly with the acute and profound decline in viral RNA levels.

Determining whether these cytokine changes occur – and occur acutely – was of interest for two reasons. First, acute changes in cytokine production by specific immune cells would suggest that they were related to direct effects of viral reduction on these particular immune cells. Second, these acute changes may be useful in early assessment of response to HAART and/or viral relapse.

Patients and methods

Study population

Between March and October 1999, we enrolled 11 HIV-1-infected male patients (median age 34 years, range 23–43 years). Patients were enrolled if they were clinically stable and qualified for recommended HAART based on CD4 cell counts (median, 116×10^6/l; range, $1–350 \times 10^6$/l) and/or HIV-1 plasma RNA level (median, 5.22 $\log_{10}$ copies/ml; range, 3.25–5.76 $\log_{10}$ copies/ml). Plasma HIV-1 viral RNA levels were assessed at the Centers for Disease Control and Prevention (CDC) using Roche Monitor versions 1.0 and 1.5 test kits (Roche Diagnostics, Indianapolis, Indiana, USA), which have a lower detection limit of 400 copies/ml. Eight patients were completely naive to HAART and three had been on therapy briefly but had not taken any antiretroviral medication for ⩾ 6 months before enrollment. All patients received HAART consisting of three of the following drugs: stavudine, lamivudine, nelfinavir, efavirenz, zidovudine, ritonavir, and indinavir. Blood samples were collected pre-therapy (week 0) and at weekly intervals for an additional 3 weeks. Informed consents were obtained from all patients, and the study was approved by the institutional review boards at CDC, Emory University, and the Veterans Affairs Medical Center in Decatur, Georgia, USA.

Reagents

Cell surface markers and intracellular cytokine production were analyzed using four-color cytofluorometry with the following commercially available murine and/or rat anti-human monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), or allophycocyanin (APC) obtained from: Becton Dickinson Biosciences, San Jose, California, USA (CD3–PE, CD3–PerCP, CD3–APC, CD4–FITC, CD4–PE, CD4–APC, CD8–FITC, CD8–PE, CD8–PerCP, CD14–PE, CD16–FITC, CD16–PE, CD19–APC, CD38–PE, CD45–FITC, CD45RA–FITC, CD45-RO–PE, CD56–FITC, CD56–PE, CD95–FITC, HLA-DR–FITC, and IL-2–FITC); DAKO, Capenteria, California, USA (CD30–FITC); Pharmingen, San Diego, California (CD62L–PE, CD86–PE, CD94–PE, IL3–FITC, IL4–PE, IL8–PE, IL13–PE, and IL-16–PE); Immune Source, Los Altos, California, USA [IL-4–APC, IL-5–FITC, IL-10–APC, interferon (IFN)-γ-APC, and TNF-α-FITC]; and R & D Systems, Minneapolis, MN, USA (IL6–PE).

Analyses of cell surface markers and intracellular cytokine production

Freshly obtained heparinized whole blood was aliquoted and incubated with 10 μg/ml brefeldin-A (Sigma, St. Louis, Missouri, USA) in an equal volume of RPMI 1640 (Gibco, Grand Island, New York, USA), with ('stimulated') or without ('spontaneous'/'unstimulated') 1 μg/ml ionomycin (Sigma) and 0.05 μg/ml phorbol 12-myristate 13-acetate (Sigma) at 37 °C for 5 h. After lysis of the red blood cells with ammonium chloride, the cells were stained for surface markers, then permeabilized and fixed with PermeaFix (Ortho, Raritan, New Jersey, USA) before intracellular staining for cytokines. Using this technique, we have found that $< 0.5\%$ of the unstimulated peripheral blood lymphocytes of healthy, HIV-negative US blood donors were positive for intracellular TNF-α [11] and a median of 0.6% of unstimulated peripheral blood CD3 cells of HIV-negative, African blood donors were positive for intracellular IL-4 (unpublished data, described in [12]). All cytokine reagents were used in combination with anti-CD4/anti-CD8, anti-CD16/56/anti-CD3, or anti-CD3. For assessments of CD8 T cells, gating was done on brightly fluorescing positive cells, to exclude

weakly fluorescing CD8 natural killer cells. Monocytes were defined on the basis of forward and side scatter of CD14-positive cells; intracellular IL-6, IL-8, IL-10, and TNF-α were assessed for these cells.

Additional fresh samples were stained without Permea-Fix treatment for CD3, CD4, CD8, CD38, CD45RA, CD62L, CD45RO, and HLA-DR. Cell data were collected and analyzed with a FACSCalibur flow cytometer (Becton Dickinson Biosciences) and Cell Quest software (Becton Dickinson Biosciences), with between 2×10^4 and $\geqslant 10^6$ events collected from each tube.

Statistical analysis

Non-parametric univariate analyses were performed by using Kruskal–Wallis tests. The significance level was set at $P \leqslant 0.05$. For analyses, patients with HIV plasma RNA levels of $\leqslant 400$ copies/ml were assigned a level of 200 copies/ml.

Results

HIV-1 Plasma RNA levels

Of the 11 patients enrolled, all remained on therapy for 1 week, eight for 2 weeks, and six remained on therapy for $\geqslant 3$ weeks. The six patients who remained on therapy for $\geqslant 3$ weeks had a $\geqslant 2$-$\log_{10}$ decline in HIV plasma RNA levels and/or a decline to $\leqslant 400$ copies/ml. No patient died, presented with an AIDS-defining event, or had an opportunistic infection during or at the conclusion of the study.

Acute changes in T-cell phenotype

Neither the absolute numbers nor the percentage of circulating CD4 or CD8 T cells changed significantly between weeks 0 and 3 (Table 1). Similarly, the percentage of circulating naive (CD4, CD45RA, CD62L), memory (CD4, CD45RO), and activated (CD4, CD38; CD4, HLA-DR; CD8, CD38; CD8, HLA-DR) T cells did not change significantly between weeks 0 and 1 or between weeks 0 and 3 (Table 1).

Acute changes in cell-specific cytokine production

Of the cytokines and T-cell types examined, T cell-specific production changed significantly between weeks 0 and 3 of HAART for only the following parameters: percentage of CD4 T cells spontaneously producing TNF-α; percentage of CD8 T cells spontaneously producing TNF-α; and percentage of CD3 T-cells spontaneously producing IL-4 (Fig. 1, Table 1). The percentage of CD4 T-cells spontaneously producing TNF-α had decreased in nine patients by week 1 of HAART and in the other two patients by week 2. Of the six patients who remained in the study through week 3, five had continued decreases in this percentage (Table 1, Fig. 2). Similar decreases between weeks 0

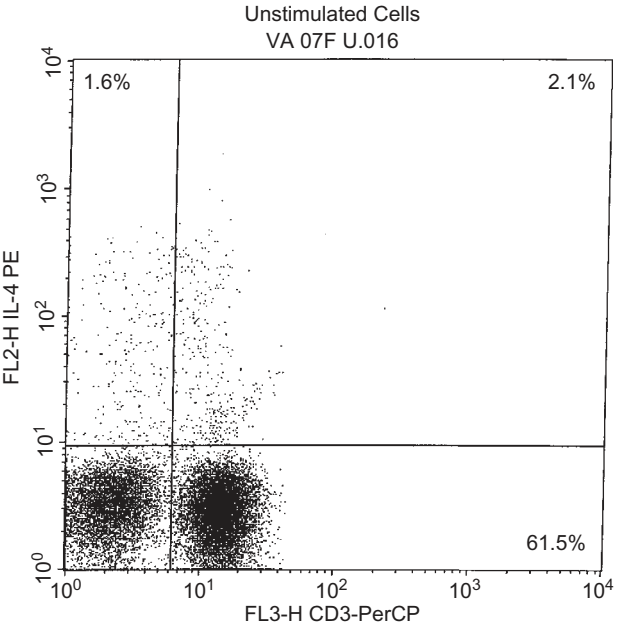

Fig. 1. Dot plot of intracellular IL-4 by surface CD3 staining, unstimulated peripheral blood lymphocytes of a representative patient.

Table 1. Median plasma HIV RNA levels, T-cell counts, and cytokine production at weeks 0 and 3 of triple antiretroviral therapy.

	Pre-therapy [median (range)] (n = 6)	3 Weeks post-initiation of therapy [median (range)] (n = 6)	*P* (Kruskal–Wallis)
HIV plasma RNA level ($\log_{10}$)	5.3 (3.3–5.5)	3.0 (2.3–3.2)	0.004
Percentage of CD4 cells	8.3 (2.3–17.8)	8.8 (1.8–10.6)	0.75
Absolute CD4 count ($\times 10^6$cells/l)	138 (9–350)	138 (33–256)	0.75
Percentage of CD8 cells	27.2 (10.4–42.8)	24.0 (3.0–34.4)	0.26
Absolute CD8 count ($\times 10^6$cells/l)	436 (70–776)	371 (56–934)	0.75
Percentage of CD4, CD45RO cells	64.9 (42.6–80.3)	70.6 (45.4–95.4)	0.42
Percentage of CD4, CD45RA, CD62L cells	17.4 (9.6–30.8)	17.6 (11.0–30.8)	0.87
Percentage of CD8, CD38	83.4 (75.4–92.8)	73.1 (65.1–90.3)	0.08
Percentage of CD4, TNF-α-positive (spontaneous)	2.4 (1.0–3.9)	0.4 (0.1–2.9)	0.025
Percentage of CD8, TNF-α-positive (spontaneous)	0.6 (0.3–1.1)	0.2 (0.1–0.8)	0.037
Percentage of CD3, IL-4-positive (spontaneous)	1.8 (1.4–2.3)	0.8 (0.4–1.4)	0.004

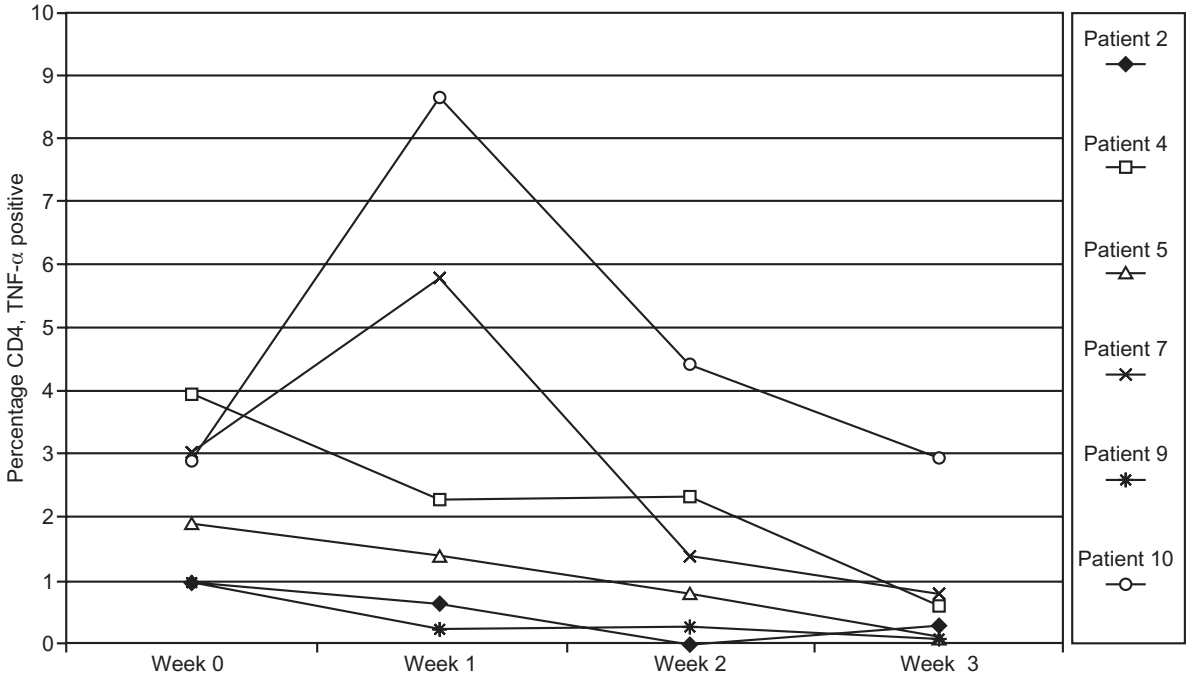

Fig. 2. Percentage of CD4 T cells producing TNF-α, by week, among patients remaining on therapy for 3 weeks.

and 3 occurred in the percentage of CD8 T cells spontaneously producing TNF-α (Table 1). Between weeks 0 and 1, the percentage of CD3 T cells spontaneously producing IL-4 decreased in seven out of the 11 patients; the percentage of CD4 and CD8 lymphocytes spontaneously producing IL-4 each decreased in six out of the 11 patients (data not shown). Of the four patients who did not show a CD3 cell-specific IL-4 decrease by week 1 of therapy, two were subsequently lost to follow-up, and the other two showed a decrease in production by week 3 of HAART (data not shown). The declines in the percentage of CD4 and CD8 T cells spontaneously producing IL-4 between weeks 0 and 3 were not statistically significant (data not shown). Of note, one patient (who had been followed for 3 weeks) stopped HAART after 24 weeks of therapy. After 2 weeks off therapy, he was given a revised regimen and an additional blood sample was drawn before re-initiation of HAART. In this sample, both the plasma HIV-1 viral load and the percentage of CD4 and CD8 T-cells spontaneously producing TNF-α had increased compared to these measurements from his week 3 sample (plasma RNA levels, 2.8 versus 5.2 $\log_{10}$ copies/ml; percentage of CD4 T cells spontaneously producing TNF-α, 0.8 versus 3.6%; percentage of CD8 T cells spontaneously producing TNF-α, 0.5 versus 0.9%). Changes were also seen in the percentage of CD3 T cells spontaneously producing IL-4 (0.9 versus 2.0%). Cytokine production by monocytes did not change significantly with therapy (data not shown). Cytokine production with stimulation was comparable to the levels found previously in this and other laboratories [11,12]; however, changes in these levels were not significant within the first 3 weeks of therapy. For example, with stimulation, a median of 2.1% CD3 cells produced IL-4 at week 0 and 1.8% at week 3, a median of 34.5% CD4 cells produced TNF-α at week 0 and 29.8% at week 3, and a median of 45.9% CD8 cells produced TNF-α at week 0 and 45.3% at week 3. Similarly, the percentage of cells producing IFN-γ with and without stimulation did not change significantly (e.g., at week 0, a median of 50.4% of stimulated CD3 cells produced IFN-γ versus 42.7% at week 3; at week 0, a median of 0.2% of unstimulated CD3 cells produced IFN-γ versus 0.4% at week 3).

Discussion

In this study, the earliest immune changes occurring after the initiation of HAART involved T lymphocyte production of two cytokines, TNF-α and IL-4. Of all the immune parameters studied, only the following showed statistically significant changes in the first 3 weeks of HAART: percentage of CD4 T cells spontaneously producing TNF-α; percentage of CD8 T cells spontaneously producing TNF-α; and percentage of CD3 T cells spontaneously producing IL-4. Of note, at 6 months post-therapy, six patients returned for additional evaluation; at that time, CD4 percentages and numbers had both increased significantly from the week 0 values and were correlated with the HIV viral

load (Spearman's correlation coefficient −0.68, $P = 0.016$; unpublished data).

Contrary to previous reports [7,8,13], we did not find significant changes in the absolute number or the percentage of circulating CD4 lymphocytes, memory T lymphocytes, or the percentage of CD8 T lymphocytes expressing CD38 during the first weeks of antiretroviral therapy. Rather, we found that down-regulation of the small proportions of T cells spontaneously producing TNF-α and IL-4 preceded any changes in the distribution or phenotype of the peripheral blood T-cell population. Cytokine production with stimulation was within normal ranges and changes in stimulated cytokine production with early therapy were not significant, suggesting that the declines in spontaneous production were related to *in vivo* stimulation.

TNF-α is an important factor in HIV disease pathogenesis. It can accelerate HIV replication *in vitro* [2,3] and also stimulate the release of other cytokines [14,15]. These, in turn, can accelerate HIV replication in T lymphocytes and macrophages [16]. In one study, an increase in serum TNF-α levels among HIV-1-infected patients was associated with virologic relapse and disease progression [17]. Our findings for the one patient who stopped medication after 6 months of antiretroviral therapy are consistent with this report and further show that the greatest TNF-α changes occur in CD4 T cells, the cellular target of HIV. In addition, we showed that down-modulation of TNF-α production by CD4 and CD8 T cells is the earliest and most consistent immune finding following initiation of HAART.

A shift from a type 1 toward a type 2 cytokine profile has been reported to be associated with long-term HIV disease progression [4–6,18]. Consistent with this, some authors have reported a shift from a type 2 to type 1 cytokine profile with successful HAART [19]. One study of *in situ* cytokine production by tonsillar cells of three asymptomatic, HIV-positive patients pre- and 4 weeks post-HAART therapy indicated that levels of both IL-4 and TNF-α were low in this lymphatic tissue, and remained low post-therapy [20]. This suggests that our findings are not due to differential cell recirculation but, rather, to a very early down-modulation of IL-4 production by T cells, occurring in parallel with the decreases in both HIV plasma RNA levels and TNF-α production by CD4 and CD8 T cells. Of note, the IL-4 changes were statistically highly significant but tended to occur later than the TNF-α changes, and both findings were specific for T cells. Changes in the percentage of natural killer and natural T cells producing TNF-α and in the percentage of B cells and monocytes producing IL-4 were not statistically significant at week 1 or week 3 of HAART.

In summary, we have shown that CD4 and CD8 T cell-specific cytokine changes occur very rapidly with HAART initiation. These changes preceded both the increase in CD4 T-cells and the decrease in surface activation antigens. These findings support the important role of cytokines – particularly TNF-α – in HIV infection and suggest that assessing the percentage of T cells spontaneously producing TNF-α and IL-4 might be useful in monitoring responses to HAART.

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