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Kinetics of CD4+ T cell repopulation of lymphoid tissues after treatment of HIV-1 infection

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ABSTRACT **Potent combinations of antiretroviral drugs diminish the turnover of CD4+ T lymphocytes productively infected with HIV-1 and reduce the large pool of virions deposited in lymphoid tissue (LT). To determine to what extent suppression of viral replication and reduction in viral antigens in LT might lead correspondingly to repopulation of the immune system, we characterized CD4+ T lymphocyte populations in LT in which we previously had quantitated viral load and turnover of infected cells before and after treatment. We directly measured by quantitative image analysis changes in total CD4+ T cell counts, the CD45RA+ subset, and fractions of proliferating or apoptotic CD4+ T cells. Compared with normal controls, we documented decreased numbers of CD4+ T cells and increased proliferation and apoptosis. After treatment, proliferation returned to normal levels, and total CD4+ T and CD45RA+ cells increased. We discuss the effects of HIV-1 on this subset based on the concept that renewal mechanisms in the adult are operating at full capacity before infection and cannot meet the additional demand imposed by the loss of productively infected cells. The slow increases in the CD45RA+ CD4+ T cells are consistent with the optimistic conclusions that (*i*) renewal mechanisms have not been damaged irreparably even at relatively advanced stages of infection and (*ii*) CD4+ T cell populations can be partially restored by control of active replication without eradication of HIV-1.**

In the lymphatic tissues of HIV-1-infected individuals, ongoing cycles of productive infection generate large numbers of virions, most of which are stored like other antigens in immune complexes associated with follicular dendritic cells (1, 2). However, unlike conventional antigens, the HIV-1 follicular dendritic cell pool represents a large and persistent antigenic stimulus of $>10^{11}$ virions that presumably drives activation, proliferation, and programmed cell death, processes that, along with the loss of productively infected cells, are directly or indirectly responsible for immune depopulation and depletion (3–10). We recently investigated viral replication and load in lymphoid tissue (LT) in a cohort of previously untreated HIV-1-infected individuals given a combination of antiretroviral drugs and found that, by the end of the third week of treatment, the cells that account for most of the production of virus were barely detectable and that, by 6 months, the pool of HIV-1 associated with follicular dendritic cells in LT was reduced 3,000-fold (2). This suppression of active replication of HIV-1 and reduced antigenic mass in LT might provide a milieu in which some of the abnormalities in CD4+ T cell populations might be reversed, and indeed Autran *et al.* (11) recently showed that antiretroviral therapy does have positive effects on CD4+ T cells in blood. To directly examine the treatment-associated effects on CD4+ T cell populations in LT where most of the cells reside, we devised a quantitative image analysis technique to directly measure LT CD4+ T cell subsets, proliferation, and apoptosis in small samples of LT before and after treatment. From comparable measurements in the LT of HIV-1-seronegative individuals, we also determined and describe the effects of HIV-1 infection on CD4+ T cell populations. Based on these analyses, we advance hypotheses about the mechanisms of depletion and repopulation of CD4+ T cells in LT.

MATERIALS AND METHODS

LT Biopsies. We obtained biopsies of palatine tonsil and a lymph node from normal volunteers and participants in a LT substudy of triple therapy (2). The biopsies were fixed in Streck's tissue fixative for at least 24 h and then processed and embedded in paraffin.

Immunohistochemical Staining of CD4+ T Cells in LT. Sections of 5 mm were cut, attached to silanized slides, and then deparaffinized, rehydrated, and immersed in 1 mm EDTA (pH 8.0). Antigen reactivity was enhanced by microwaving. Nonspecific reactions were blocked by immersion in 0.3% H_2O_2 in methanol and then 5% nonfat milk. To detect CD4+ T cells, the sections were reacted overnight at 4°C with anti-human CD4+ mAb (NCL-CD4–1F6, NovoCastra, Newcastle, U.K.), subsequently washed in PBS, and incubated at room temperature with biotinylated anti-mouse IgG (Vector Laboratories) and then an avidin–biotin complex (Vector Laboratories). CD4+ T cells were stained by reaction with Vector red substrate.

Proliferating and Apoptotic CD4+ T Cells. The proportion of CD4+ T cells undergoing proliferation or apoptosis was determined by double labeling CD4+ T cells. Proliferating cells were stained immunohistochemically with antibody to a proliferating cell antigen Ki67. CD4+ T cells were stained first. Sections were blocked in a solution of 5% nonfat milk and avidin D solution (Avidin–Biotin Blocking Kit, Vector Laboratories), rinsed in PBS, and reacted overnight at 4°C with monoclonal anti-Ki67 (NCL-Ki67-MM, NovoCastra) and then biotinylated secondary antibody and fluorescein isothiocyanate–streptavidin antibody (Zymed). For CD4+ and terminal

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Abbreviations: LT, lymphoid tissue; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.
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deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL), the sections were pretreated with proteinase K (20 mg/ml in 10 mM Tris·HCI, pH 7.4) for 15 min at 37°C. CD4+ T cells were stained as described above. Cells with fragmented DNA were detected with a commercial kit (*in situ* Cell Death Detect Kit, Fluorescein, Boehringer Mannheim) following the manufacturer's instructions.

Quantitative Image Analysis of CD4+ T Cell Populations. Stained cells were counted by computer-assisted quantitative image analysis (QIA) of tissue sections. Bright-field (for CD4+ T cells) or dark-field (for fluorescein isothiocyanate Ki67 or TUNEL+ cells) video images were captured with a low light, cooled charge-coupled device camera (Optronics International model TEC-470, Chelmsford, MA) and IMAGES/METAMORPH (Universal Imaging, Media, PA) software. The more darkly staining CD4+ T cells were distinguished from background with the METAMORPH "threshold" tool (see Fig. 1), and the number of cells was measured from the standard area (in square pixels) of a positive cell as described in the text. Doubly labeled CD4+ Ki67+ or TUNEL+ cells were counted by first converting the images into binary form. Using the metamorph process tool, the binary images were multiplied (see Fig. 2) to identify and count the number of double-positive cells.

Total Body Estimates. We multiplied the measured mean of CD4+ T cells/mg LT (Tables 1 and 2) $\times$ 700 $\times$ 10^6 μg to estimate CD4+ T cells in LT of a hypothetical 70-kg individual in which LT is 1% of the total body weight (13). We assumed that, at the stage of HIV-1 infection we studied, LT is still $\approx$1% of total body weight. The mean CD4+ T cell count/mm^3 (Tables 1 and 2) was multiplied by 5×10^6 mm^3 (the total blood volume of 5 liters) to estimate CD4+ T cells in circulation. Repopulation rates were estimated from the mean extrapolated total body increases, e.g., of 1.4×10^{10} CD4+ CD45RA+ cells $\div$ 168 days = 7×10^7 cells/day.

RESULTS AND DISCUSSION

Quantitative Image Analysis of CD4+ T Cell Populations in Small Samples of Lymphoid Tissue. Tonsils can be biopsied repeatedly (12) and provide an accessible source of LT representative of other secondary LT for measuring tissue viral load (1). We previously evaluated changes in the frequency of productively infected LT cells and the size of the pool of virions in tonsil biopsies after combination antiretroviral treatment (2) and had suitably preserved portions of many of the biopsies to evaluate the effects of treatment on CD4+ T cells in LT. Because we had insufficient tissue to analyze by FACS, we adopted a QIA method (1) to directly count CD4+ T cells in tissue sections. To count CD4+ T cells by QIA, we first stained the cells (Fig. 1*A*) and then acquired video images (Fig. 1*B*). We used the METAMORPH computer program to distinguish in the video images CD4+ T cells whose intensity of staining exceeded a threshold value of staining. There are highlighted in red (Fig. 1*C*). The area of the highlighted cells in square pixels is converted to cell number from a predetermined standard mean area of a stained cell. We determined this conversion factor by manually counting a number of cells, measuring their area, and finding a value for the standard number of pixels that would give the correct cell count.

In comparisons of this method with FACS analysis of mouse lymph nodes, we found that, for FACS analysis from lymph nodes of biopsy size, many cells were not recovered and viability was reduced. In larger pooled preparations, the FACS- and QIA-derived estimates of the number of CD4+ T cell counts/mg agreed within 50–75% (data not shown), thus providing justification for the use of this technique for assessing lymphocyte populations in our small samples of LT.

The chronic state of immune activation (3, 4) in HIV-1 infection is accompanied by increased rates of proliferation (5) and apoptosis of CD4+ T cells in blood (6–9) and LT (10). Because we expected that reductions in viral load in LT might diminish proliferation and/or apoptosis of CD4+ T cells in LT, we also developed double-label QIA methods to evaluate directly these changes. For proliferation, we stained CD4+ T cells immunohistochemically so that they were only visible in bright-field images. We reacted the same section with an

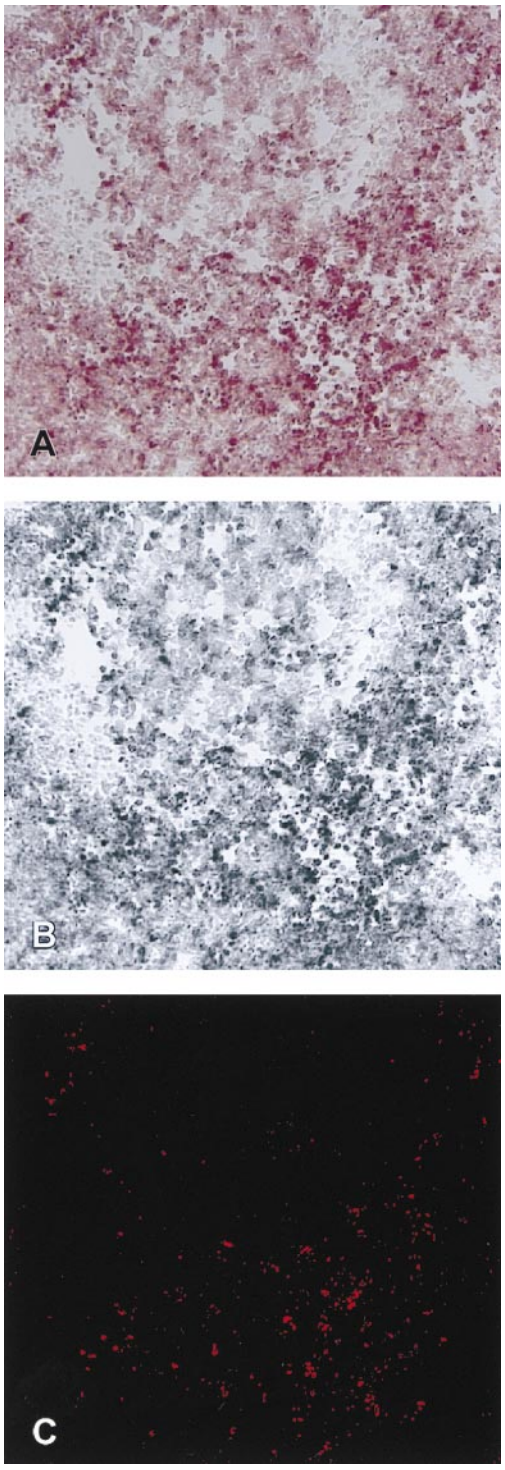

FIG. 1. Quantitative image analysis of CD4+ T cells in lymphoid tissue. (*A* and *B*) Color and black and white video images of CD4+ T cells in a tissue section stained immunohistochemically with antibody to CD4 and vector red substrate. (*C*) The darker stained cells in *B* are identified with a red overlay. The number of CD4+ T cells in the field is computed automatically from the measured area of the objects highlighted in red and the standard area of a cell.

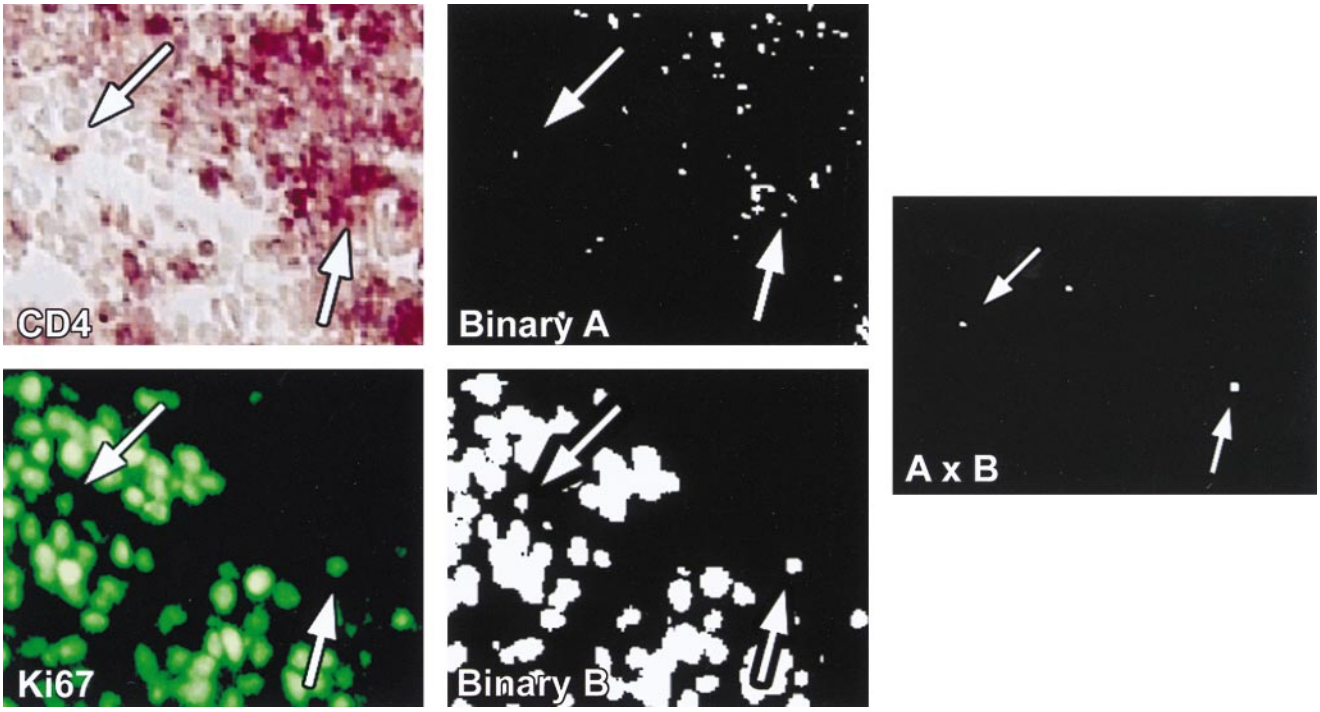

FIG. 2. Quantitative image analysis of doubly labeled CD4+ T cells in LT. The upper panels display the color images of immunohistochemically stained CD4+ T cells and the binary derivative (A) of the digitized information. All cells with a binary value of 1 are white. The arrow points to two CD4+ T cells that are Ki67+. In the lower panels, proliferating cells, mainly the B lymphocytes concentrated in the germinal center, have been identified by staining with antibodies to Ki67 and fluoresceinated secondary antibody. Positive cells are green in the color image and white in the binary derivative image (B). The arrow again points to two double-positive cells, identified in the panel at the right by multiplying the binary derivatives A × B to locate and count the CD4+ Ki67+ cells.

antibody (anti-Ki67) that marks proliferating cells and subsequently with a fluorescein isothiocyanate-labeled secondary antibody so that proliferating cells were only visible in fluorescent images. We show in Fig. 2 a bright-field image of CD4+ T cells and an image of fluorescent Ki67+ cells in the same field. We used the METAMORPH computer program to obtain binary derivatives of the separate images. When these are multiplied, they give the number of Ki67+ CD4+ T cells in the field (Fig. 2). We similarly obtained minimal estimates of the number of CD4+ T cells undergoing apoptosis by using the TUNEL assay and fluoresceinated reagents as the second label.

Because the CD45RA isoform has been used extensively to investigate lymphocyte regeneration and to monitor changes in "naive" CD4+ T cells in blood after antiretroviral treatment (14–18), we also evaluated changes in the CD45RA+ subset of CD4+ T cells. For the CD45RA subset, we found that we could not reliably count doubly labeled cells by QIA when both antigens were in the cell membrane because the computer program could not discriminate between colocalized signals in one or more adjacent cells. However, because we could distinguish CD45RA+ CD4+ cells visually, we manually counted doubly labeled cells, and we show in Tables 1 and 2 the percentage of CD45RA+ CD4+ cells. We estimated the total number of cells in this subset by multiplying the fraction of CD45RA+ CD4+ cells times the total CD4+ T cell count obtained by QIA.

Normal CD4+ T Cell Counts in LT. We first used these methods to determine CD4+ T cell counts in normal tonsils.

Table 1. CD4+ T cell profiles in HIV-1-negative individuals

Patient	Sample	CD4+ T cell, count/mm^3 blood	CD4+ T cell count, LT/μg	CD45 RA+ CD4+ T cells in LT, %	Ki67+ CD4+ T cells in LT, %	TUNEL+ CD4+ T cells in LT
1	Lymph node	NT	314	41	0.32	0.3
2	Tonsil	NT	357	38	0.1	0.02
3	Tonsil	1,097	371	54	0.35	0.2
4	Tonsil	1,192	286	48	0.42	0.15
5	Tonsil	621	271	NT	0.6	0.5
	Mean	970	320	45	0.4	0.2

The number of CD4+ T cells/mm^3 in blood was determined by FACS analysis (NT, not tested). The CD4+ T cell count per microgram in LT was determined by QIA of sections in which CD4+ T cells had been stained immunohistochemically. At least 20 sections distributed throughout the tissue specimen were examined at a magnification of ×160, and the number of CD4+ T cells was determined at least twice, with values that agreed to within ±5%, in representative fields that had well defined lymphoid follicles and paracortical regions. The microgram of LT was determined from the area examined (3.75×10^{-3} cm^2 at ×160) the section thickness (5×10^{-4} cm) × the previously determined (1) density of ≈1 gm/cm^3. The percentage of CD45RA+ CD4+ T cells was determined by manually counting ≈1,000 double-labeled cells. The percentage of CD4+ T cells undergoing proliferation or apoptosis was determined by double labeling with first anti-CD4+ and then anti-Ki67 or TUNEL assay, respectively, as described in *Materials and Methods*. Single- and double-positive CD4+ T cells were quantitated by QIA and binary multiplication. An average of 3,680 CD4+ cells was counted to determine the percentage of double-positives.

In Table 1, the total number of cells is expressed per milligram of LT calculated from the areas in which the cells were counted, the section thickness, and previously determined tissue density (1). Densities of CD4+ T cells in tonsillar tissue and one lymph node from a normal individual were equivalent. From the number of CD4+ T cells/mg of tonsil, we extrapolated estimated total numbers of cells per patient, as described in *Material and Methods*, to compare our estimates for CD4+ T cells with prior analyses of infected cell turnover in this cohort (2), as well as other reports of CD4+ T cell turnover and regeneration (17, 19–21). In normal, uninfected individuals, we estimate that there are 2.2×10^{11} CD4+ T cells in LT and 4.9×10^{9} CD4+ T cells in blood, in agreement with the cited figure of 2% of the total CD4+ T cells in circulation (22). The fraction of CD45+ CD4+ T cells (45%) is also similar to the fraction determined by Janossy *et al.* (23) in LT. The new determinations in this report of proportions of proliferating and apoptotic CD4+ cells in normal individuals had means of 0.4% and 0.2%, respectively. On the reasonable assumption that the time course of these processes is ≈1 day (24), to maintain steady-state, an additional fraction of the CD4+ T cell population must be removed to equal proliferation and influx of cells from the thymus or other renewal sources. This fraction represents cells at earlier stages of apoptosis that would not be counted in the TUNEL assay. We will return shortly to this steady-state model in assessing the impact of the loss of productively infected cells.

CD4+ T Cell Subsets in HIV-1-Infected Individuals Before Treatment. In the tonsils of nine individuals in which we already had measured viral replication and load (2), we found that, before treatment, the apparent extent of CD4+ T cell depletion differed in the blood and LT compartments. If the extent of immune depletion were judged by the number of CD4+ T cells in circulation, we might expect that the total number of CD4+ T cells would be reduced by 75%, but instead we found that the total CD4+ T cell count in LT was reduced in the infected group to only ≈44% of normal (calculated from the means in Table 2). Preservation of CD4+ T cells in LT compared with blood also has been suggested by the observation that the ratios of CD4+ T cells to CD8+ T cells or B cells is higher in LT than in blood (25). We conclude that the magnitude of immune depletion in HIV infection previously may have been overestimated from CD4+ T cell counts in blood and speculate that lymphocyte activation and trapping (26) account for the relative maintenance of the CD4+ T cell population in LT.

Disproportionate Impact of HIV Infection on the CD45RA+ CD4+ T Cell Population. In one major respect, changes in CD4+ T cell subsets parallel well documented changes in the blood (27). We also found the greatest depletion

Table 2. CD4+ T cell profiles in HIV-1-positive individuals before and after treatment

Patient	Age	Biopsy	CD4+ T cell counts/mm^3 in blood	CD4+ T cell count/μg in LT	CD45RA+ CD4+ T cells in LT, %	Ki67 CD4+ T cells in LT, %	TUNEL+ CD4+ T cells in LT, %
1	41	Baseline	40	84	23	1.8	0.3
		Rx day 2	30	81	–	1.9	0.3
		Rx week 24	170	174	29	0.6	0.7
2	43	Baseline	170	95	25	2	0.5
		Rx week 3	260	120	24	1.9	0.3
		Rx week 24	260	142	32	0.5	0.3
		Rx week 60	194	204	36	–	–
3	36	Baseline	233	142	23	1.5	0.4
		Rx week 3	350	186	36	1.1	0.2
		Rx week 24	560	196	34	0.6	0.2
		Rx week 52	–	239	31	–	–
4	41	Baseline	217	147	27	0.5	0.5
		Rx day 2	270	139	–	0.5	–
		Rx week 24	350	175	30	0.1	0.5
5	38	Baseline	393	185	25	1.1	0.4
		Rx day 2	330	187	–	1.2	–
		Rx week 3	470	200	25	0.3	–
		Rx week 24	500	200	36	0.2	0.3
		Rx week 60	–	220	40	–	–
6	43	Baseline	600	158	26	1.3	0.4
		Rx day 2	530	129	–	–	–
		Rx week 3	970	143	29	0.7	–
		Rx week 24	730	161	36	0.3	0.5
		Rx week 60	–	206	35	–	–
7	26	Baseline	163	112	21	0.4	0.3
		Rx day 2	210	108	–	0.4	–
		Rx week 24	530	141	26	0.1	0.4
8	36	Baseline	117	154	20	0.9	NT
		Rx week 24	290	166	31	0.1	NT
9	43	Baseline	220	151	22	–	0.6
		Rx week 3	240	171	22	–	0.3
		Rx week 24	210	176	30	–	0.2
Mean	39	Baseline	239	136	24	1.2	0.4
		Rx day 2	274	129	–	1.0	–
		Rx week 3	458	162	27	1.0	0.3
		Rx week 24	400	170	32	0.4	0.4
		Rx week 52–60	–	217	36	–	–

See Table 1 legend.

in the CD45RA+ subset of CD+ T cells in LT. In this cohort of individuals at moderately advanced stages of infection, the proportion of CD45RA+ CD4+ T cells had been reduced from 45% in normal tonsil to 24% (Tables 1 and 2; see Fig. 4).

Proliferation, Apoptosis, and the Possibly Paradoxically Large Contribution of Productive Infection to Immune Depletion. The sustained antigenic stimulus of HIV-1 associated with follicular dendritic cells (1) would be expected also to sustain activation, proliferation, and apoptosis in LT. We, in fact, found that the proportion of proliferating CD4+ T cells was about threefold higher than in the tonsils and lymph node of the uninfected controls ($P < 0.02$, Student's t test), in good agreement with previous determinations of threefold increases in proliferation in blood CD4+ T cells (5). The proportion of TUNEL+ CD4+ T cells also was increased by twofold ($P < 0.05$; Tables 1 and 2). Again in a steady-state model, we assumed that the proliferation of ≈109 CD4+ T cells/day (calculated from the proportion of K167 CD4+ T cells; Table 2) is balanced by equivalent losses to programmed cell death and removal. If this is the case, the measured turnover of productively infected cells of 7×10^7/day in this cohort, although relatively small in absolute numbers, could make a large contribution to depletion of CD4+ T cells. We refer to this as a "trickle down" model to contrast it with immune depletion by exhaustion of proliferative capacity (20).

Initial Response to Treatment. There was little immediate change in the CD4+ T cell counts, but by 3 weeks, we measured an increase in CD4+ T cells at a mean rate of ≈8×10^8/day. Changes in proliferation or apoptosis were insignificant (Fig. 3; Table 2). Under the same steady-state assumptions, the CD4+ T cells that were added because the treatment largely stems the loss of 7×10^7 cells to productive infection cannot account for the initially rapid expansion of CD4+ T cells in LT; nor can the changes in the other measured parameters account for similar rapid increases in CD4+ T cells in blood. Additional mechanisms such as redistribution (11) therefore likely are involved in the initial increases of CD4+ T cells in both blood and tonsillar tissue.

Long Term Response to Treatment. After 6 months of treatment, the increases in the total CD4+ T cell population averaged 1.1×10^8 cells/day over the entire period and continued at this rate to 12–14 months. Similar sustained increases in CD45RA+ CD4+ T cells at a rate of ≈8×10^7/day account for approximately two-thirds of the total and the increased relative proportion of CD45RA CD4+ T cells (Fig. 4). Both of the increases in total CD4+ T cells and CD45+RA subset were significant by 6 months of treatment ($P < 0.05$ and $P < 0.001$, respectively, Student's t test) and highly significant at 12–14 months (both $P < 0.001$). Over the course of 6 months of treatment, the fraction of Ki67+ CD4+ T cells declined to a mean value equivalent to HIV-1-seronegative controls, and, with one exception, the fraction of TUNEL+ cells was unaffected or continued to decrease slightly (Fig. 3; Table 2).

These observations on the treatment response over several months produce evidence for one of the predicted effects of reducing one major antigenic stimulus in HIV-1 infection, namely HIV-1 itself. After 6 months of treatment, the fraction of proliferating, CD4+ T cells fell to normal levels in parallel with reduction of virions in LT by 99.9%. The continued elevation in the proportion of TUNEL+ CD4+ T cells in the context of decreased proliferation and increased numbers of CD4+ T cells is puzzling but is compatible with a decline in the rate of removal of cells at earlier stages of apoptosis.

Speculations About Mechanisms of Depletion and Renewal of CD45RA+ CD4+ T Cells and the Red Queen Hypothesis. The increases in the CD45RA+ subset of CD4+ T cells we measured at ≈8×10^7 cells/day were equivalent to the regeneration rates in young adults after intensive chemotherapy for cancer (17) and thus are consistent with the conclusion that, even at relatively late stages of infection, the immune system retains the capability of renewing this subset. The mechanisms of renewal and functional capacities of this subset are, for the most part, speculative at this time because of the limitations in the kinds of studies that were possible in small samples of fixed tissues. The linear increases in this subset are consistent with replenishment of naive CD4+ T cells by thymopoiesis or other peripheral mechanisms (28, 29), and this interpretation is supported by the recent report that apparently naive CD4+ T cells can be detected in blood after ≈ 1 year of combination antiretroviral therapy (11). The linear increase also is consistent with a conversion of CD45RO+ to CD45RA+ CD4+ T cells as the antigenic stimulus subsides with treatment. Such a conversion process has been documented, albeit over a longer period (3.5 years) (30), and would not by itself account for both an increase in the total number of CD4+ T cells and the total number of the CD45RA+ subset.

Why is the CD45RA+ subset of CD4+ T cells disproportionately affected by HIV-1 infection and treatment? HIV-1 infection drives differentiation and activation of naive and memory cells. Some of the activated cells will become productively infected and die, imposing an additional drain on the immune system over preinfection steady-state cell losses, perhaps compounded by infection and injury to the thymus (31–33). The documented decrease in regenerative capacity

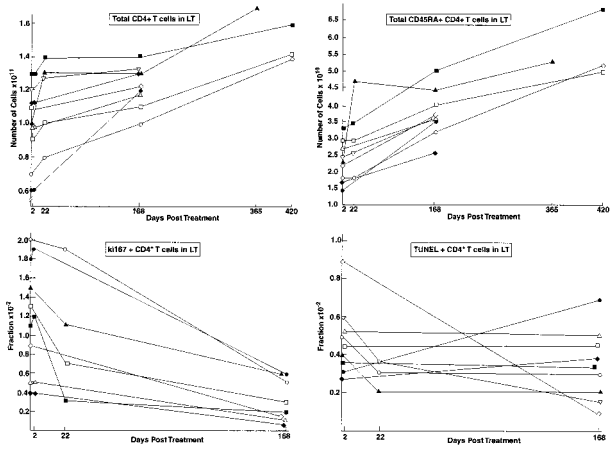

FIG. 3. Treatment-induced changes in LT in total body CD4+ T cells and naive CD45RA+ cells, were determined as described in this report. Total CD4+ T cell body counts were calculated by extrapolation as described; this number times the percentage of RA+ cells equals the total CD45RA+ CD4+ T cell count. The fractions of Ki67+ and TUNEL+ CD4+ T cells in tonsillar biopsies were determined as described in this report, before and at designated times after treatment.

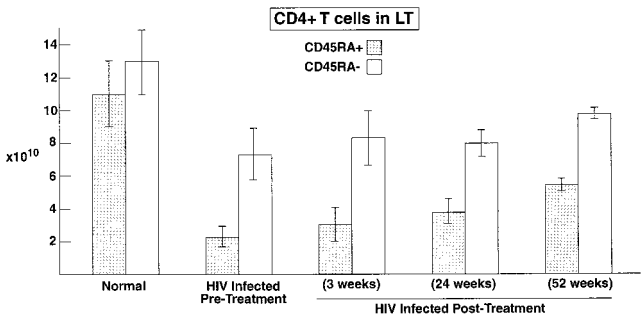

FIG. 4. CD4+ T cell subsets in LT were counted as described, and the mean body totals ± 1 SD (indicated by I) from Tables 1 and 2 are shown for uninfected and infected individuals before and after 3 weeks, 6 months, and 12–14 months of triple antiretroviral therapy.

from 10^9 CD45RA+ CD4+ T cells/day in infants to $<10^8$/day in adults is consistent (17) with the notion that T cell replacement by thymopoiesis or other peripheral mechanisms normally operates in adults at close to maximum capacity just to maintain steady-state populations. We refer to this model as the Red Queen Model by analogy to the Red Queen's response to Alice in Lewis Carroll's *Through the Looking Glass* (''it takes all the running you can do, to keep in the same place'') (34). The net result is that the number of naive CD45RA+ CD4+ T cells will decrease at a rate equivalent to their representation in the population of productively infected cells. When treatment curtails these additional losses, the CD45RA+ CD4+ T cell population can grow at a rate set by the renewal capacity in the infected individual. The pace and eventual extent of repopulation will, in general, be slow and variable, depending in any particular individual on the stage of infection, the pre-existing depletion, and the residual renewal capacity at the time treatment is initiated.

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