Limited CD4+ T-cell renewal in early HIV-1 infection: Effect of highly active antiretroviral therapy

Sylvain Fleury[1], Rob J. de Boer[5], G. Paolo Rizzardi[1], Katja C. Wolthers[6], Sigrid A. Otto[6], Craig C. Welbon[1], Cecilia Graziosi[1,8], Christian Knabenhans[1], Hugo Soudeyns[1], Pierre-Alexandre Bart[1], Serge Gallant[1], Jean-Marc Corpataux[3], Michel Gillet[3], Pascal Meylan[8], Pierre Schnyder[4], Jean-Yves Meuwly[4], William Spreen[7], Michel P. Glauser[1], Frank Miedema[6] & Giuseppe Pantaleo[1,2]

[1]*Laboratory of AIDS Immunopathogenesis, Department of Medicine, Division of Infectious Diseases,*
[2]*Division of Immunology,* [3]*Departments of General Surgery and* [4]*Radiology,*
Centre Hospitalier Universitaire Vaudois, University of Lausanne, Switzerland
[5]*Theoretical Biology, University of Utrecht, Utrecht, The Netherlands*
[6]*Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands*
[7]*Glaxo Wellcome, Research Triangle Park, North Carolina, USA*
[8]*Institute of Microbiology, University of Lausanne, Switzerland*
S.F., R.J.dB. & G.P.R. contributed equally to this study
Correspondence should be addressed to G.P.

We show that the fraction of proliferating CD4+ lymphocytes is similar in HIV-infected subjects in the early stage of disease and in HIV-negative subjects, whereas the fraction of proliferating CD8+ lymphocytes is increased 6.8-fold in HIV-infected subjects. After initiation of antiviral therapy, there is a late increase in proliferating CD4+ T cells associated with the restoration of CD4+ T-cell counts. These results provide strong support for the idea of limited CD4+ T-cell renewal in the early stage of HIV infection and indicate that after effective suppression of virus replication, the mechanisms of CD4+ T-cell production are still functional in early HIV infection.

It has been postulated that the turnover of CD4+ lymphocytes is increased up to 70-fold in HIV-infected subjects, and that about 10^9 cells are destroyed and replenished each day[1,2]. As a result of the destruction of large numbers of CD4+ T lymphocytes, the renewal system of CD4+ T cells will ultimately be exhausted. In favor of this hypothesis, an increase in the fraction of proliferating cells in different cell subsets (including CD4+ and CD8+ T cells, NK cells and B cells) was observed by *in vivo* BrdU-labeling in monkeys infected with simian immunodeficiency virus[3]. However, longitudinal analysis of changes in telomere length of peripheral blood CD4+ lymphocytes challenged the idea of increased turnover of CD4+ lymphocytes[4]. Furthermore, recent studies indicate that the increase in CD4+ T lymphocytes after highly active antiretroviral therapy (HAART) results from a combination of both redistribution and new production of cells[5].

Expression of Ki67 in blood and lymph nodes

We studied T-cell production by measuring expression of the Ki67 antigen, which is selectively expressed in proliferating cells[6–8]. Peripheral blood and lymph node mononuclear cells were isolated from eight HIV-negative donors who underwent vascular and general surgery, and from twenty-two HIV-infected subjects naive to antiviral therapy. The clinical histories of the HIV-infected subjects are in Table 1. The mean CD4+ T-cell level was 771 cells per μl in HIV-negative and 687 in HIV-positive subjects. Distribution of the nuclear antigen Ki67 was determined in blood and lymph node CD4+ and CD8+ T-cell subsets by flow cytometry. In HIV-negative subjects, the percentage of CD4+Ki67+ T cells was similar in blood (mean, 0.60%) and lymph nodes (mean, 0.54%) (Fig. 1*a* and Table 2). The percentage of CD8+Ki67+ T cells was higher in blood (mean, 0.41%) than in lymph nodes (mean, 0.18%), and this difference was statistically significant ($P = 0.037$) (Fig. 1*a* and Table 2). In contrast, in HIV-infected subjects both the percentages of CD4+Ki67+ and CD8+Ki67+ T cells were higher in blood (means, 0.80% for CD4 and 1.22% for CD8) than in lymph nodes (means, 0.39% for CD4 and 0.38% for CD8) (Fig. 1*b* and *c* and Tables 3 and 4). These differences were statistically significant (CD4+Ki67+ and CD8+Ki67+ T cells from blood versus lymph nodes: $P = 0.038$ and $P = 0.004$, respectively). Eleven of twenty-two HIV-infected subjects had a greater (at least twofold) percentage of CD4+Ki67+ T cells in blood than in lymph nodes (Fig. 1*b*). However, only two subjects (2066 and 2090) had a greater percentage of CD4+Ki67+ T cells in lymph nodes than in blood, whereas the remaining nine subjects had similar CD4+Ki67+ cell percentages in blood and lymph nodes (Fig. 1*b*). For CD8+ cells, fourteen of twenty-two subjects had higher percentages of Ki67+ T cells in blood whereas three (2055, 2066, and 2090) had higher percentages in lymph nodes; these percentages were similar in five subjects (Fig. 1*c*).

CD4/CD8 ratios in blood and lymph nodes

The CD4/CD8 ratios differed substantially between blood and lymph nodes in both HIV-negative and HIV-infected subjects. In

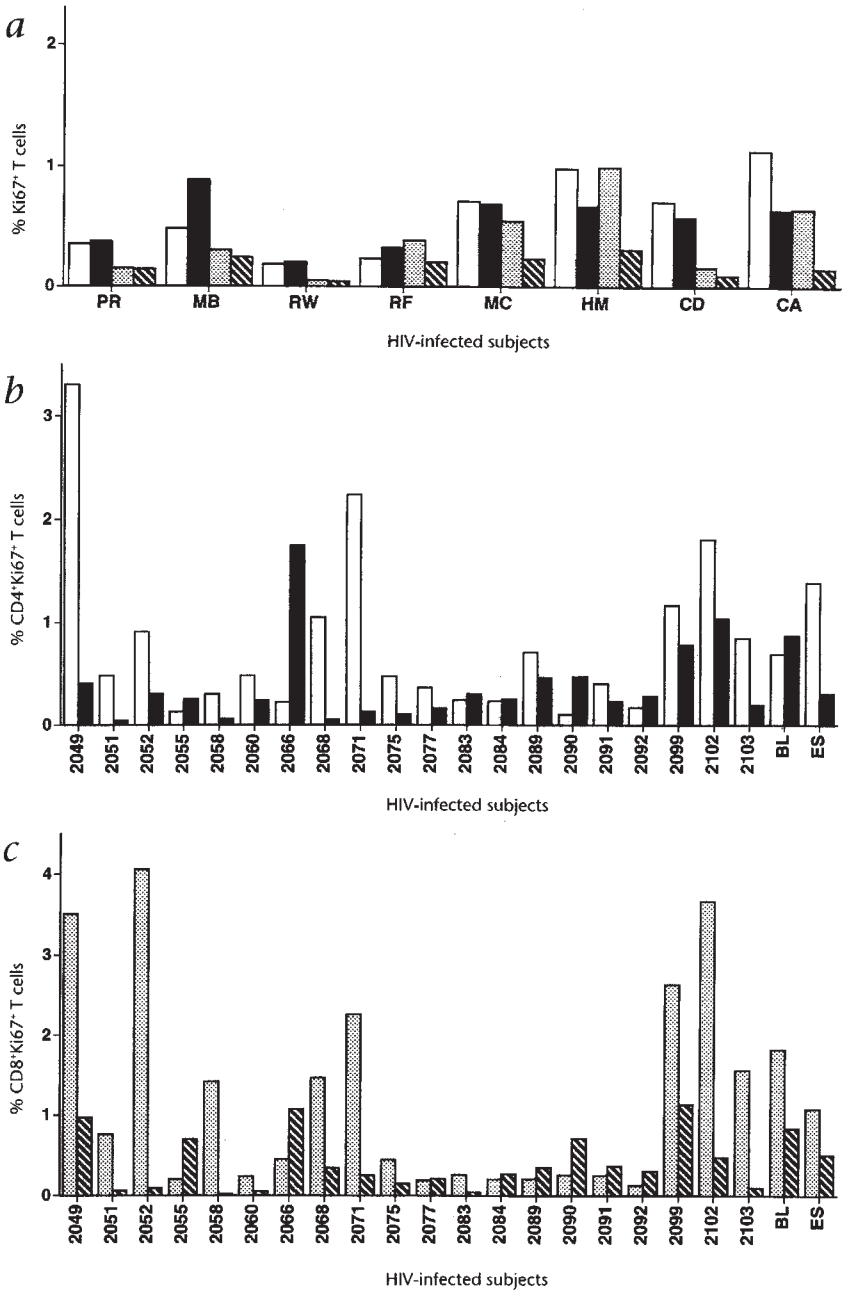

Fig. 1 Distribution of Ki67 nuclear antigen in T lymphocytes from blood and lymph nodes. ***a***, In eight HIV-negative subjects: $CD4^+$ T cells in blood (□) and LN (■); $CD8^+$ T cells in blood (▩) and LN (▨). ***b***, In twenty-two HIV-infected subjects: $CD4^+$ T cells in blood (□) and LN (■). ***c***, In twenty-two HIV-infected subjects: $CD8^+$ T cells in blood (▩) and LN (▨). Subject identification letters or numbers are on the horizontal axes.

HIV-negative subjects, the mean CD4/CD8 ratio was 1.23 in blood and 5.1 in lymph nodes, whereas the mean CD4/CD8 ratio was 0.77 in blood and 1.3 in lymph nodes in HIV-infected subjects.

Fractions of proliferating $CD4^+$ and $CD8^+$ lymphocytes

Here, we developed strategies to more directly assess the fraction of proliferating $CD4^+$ and $CD8^+$ T lymphocytes in HIV infection. We compared the fraction of proliferating $CD4^+$ and $CD8^+$ T lymphocytes between HIV-negative donors and HIV-infected subjects. We also calculated the fraction of proliferating T-lymphocyte populations from the numbers of proliferating T cells in lymph nodes, which represent the majority (50–60%) of the total lymphocyte population[9]; this allowed us to estimate more accurately the fraction of proliferating T cells in the total lymphocyte population. To eliminate the influence of events unrelated to HIV, such as antiviral therapy and opportunistic infections, on the actual fraction of proliferating T lymphocytes, we studied HIV-infected subjects who were naive to antiviral therapy and had high $CD4^+$ T cell counts (greater than 300 cells per μl).

We calculated the number of circulating T lymphocytes from hematological measures (that is, white blood cell [WBC] counts and percentage of lymphocytes), and assumed that the blood volume in an adult subject is about 5 L. The number of circulating $CD4^+$ and $CD8^+$ lymphocytes and the fraction of proliferating T cells, that is, $CD4^+Ki67^+$ and $CD8^+Ki67^+$ cells, were calculated from the percentages of these cell subsets as determined by flow cytometry. The number of lymphocytes in lymph nodes was calculated by multiplying the number of circulating lymphocytes (2% of total lymphocytes[9]) by a factor of 49. This provides an estimate of the total number of lymphocytes assuming that lymph node lymphocytes represent 98% of total lymphocytes. The total $CD4^+$ and $CD8^+$ T-lymphocyte populations and the fraction of proliferating T cells in these cell subsets were not extrapolated from blood but were calculated from the percentages of $CD4^+Ki67^+$ and $CD8^+Ki67^+$ T cells in lymph nodes as measured by flow cytometry.

In HIV-negative subjects ($n = 8$; Table 2), the mean numbers of circulating $CD4^+$ (3.9×10^9) and $CD8^+$ (3.2×10^9) T lymphocytes were similar, whereas the mean number of lymph node $CD4^+$ lymphocytes (2.5×10^{11}) was much greater than the mean number of lymph node $CD8^+$ lymphocytes (5.4×10^{10}) (Fig. 2 and Table 2). Similarly, the mean numbers of proliferating cells were 2.2×10^7 ($CD4^+Ki67^+$) and 1.2×10^7 ($CD8^+Ki67^+$) in blood, and 1.3×10^9 ($CD4^+Ki67^+$) and 9.5×10^7 ($CD8^+Ki67^+$) in lymph nodes (Fig. 2 and Table 2). Based on a 24-hour duration of the cell cycle[10], the total numbers of $CD4^+Ki67^+$ and $CD8^+Ki67^+$ T lymphocytes would represent estimates of the daily $CD4^+$ and $CD8^+$ T lymphocyte production.

These results provide the first estimate of the total number and of the fraction of proliferating $CD4^+$ and $CD8^+$ T lymphocyte populations in HIV-negative subjects. These data indicate that circulating $CD4^+$ and $CD8^+$ T lymphocyte subsets represent respectively 1.6% and 5.6% of the total (blood plus lymph node) lymphocyte population; that proliferating blood $CD4^+$ and $CD8^+$ T lymphocytes correspond to 1.7% and 10.9% of the total proliferating lymphocyte populations, respectively; that CD8 lymphocyte production is much lower (12-fold) than the $CD4^+$ lymphocytes production; and that the daily $CD4^+$ T lymphocyte production (mean, 1.3×10^9 cells) in HIV-negative sub-

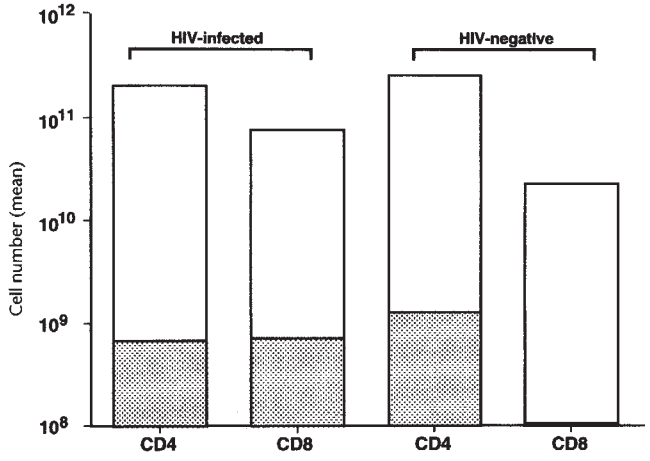

Fig. 2 Estimates of the total numbers of CD4⁺ and CD8⁺ lymphocyte populations and of the total numbers of proliferating CD4⁺ and CD8⁺ T cells in eight HIV-negative and 22 HIV-infected subjects. The data shown represent mean numbers of the total and proliferating CD4⁺ and CD8⁺ cell subsets, calculated from the analysis of LN lymphocytes that are representative of the total lymphocyte population. The fractions of proliferating CD4⁺ and CD8⁺ T lymphocytes were determined by analysis of Ki67 antigen in blood and LN CD4⁺ and CD8⁺ T cells. ▨, Total Ki67⁺ T cells; □, total number of T cells.

jects is in the same order of magnitude of that proposed for HIV-infected subjects (mean, 1.8×10^9)(ref. 2).

Using this calculation, we found that the mean numbers of CD4⁺ and CD8⁺ T lymphocytes in the blood of HIV-infected subjects were 3.4×10^9 and 5.7×10^9, respectively (Fig. 2 and Table 3); compared with HIV-negative subjects, a 1.8-fold increase was found in the number of circulating CD8⁺ lymphocytes ($P < 0.001$), whereas no significant difference was found for CD4⁺ lymphocytes ($P = 0.13$). In lymph nodes, the total CD4⁺ lymphocyte population was reduced 0.76-fold (mean, 1.9×10^{11}; $P = 0.05$), and the total CD8⁺ population was increased 3.1-fold (mean, 1.7×10^{11}; $P < 0.001$) (Fig. 2 and Tables 2, 3 and 4). The mean numbers of CD4⁺Ki67⁺ and CD8⁺Ki67⁺ cells in blood were 2.7×10^7 and 7.1×10^7, respectively. Compared with those of HIV-negative subjects, the mean numbers of proliferating cells were significantly higher for CD8⁺ (5.8-fold; $P = 0.016$) but not for CD4⁺ (1.2-fold; $P = 0.73$) lymphocytes (Fig. 2). In lymph nodes, the total number of CD4⁺Ki67⁺ lymphocytes (mean, 6.5×10^8) was significantly reduced (0.51-fold; $P < 0.001$) compared with that of HIV-negative subjects, whereas the total number of CD8⁺Ki67⁺ lymphocytes (6.5×10^8) was significantly increased (6.8-fold; $P = 0.0011$) (Fig. 2 and Table 4).

Changes in CD4⁺ T lymphocytes after HAART

As in other studies[2,11,12], we found a rapid decrease of viremia and increase in CD4⁺ T-cell counts in sixteen subjects after initiation of HAART with inhibitors of protease (Amprenavir/141W94) and reverse transcriptase (Abacavir/1592U89) (Fig. 3*a* and *b*). The increase in CD4⁺ T cells might reflect the number of cells destroyed and replenished each day[2], and thus the reduction in the number of CD4⁺ proliferating lymphocytes in HIV-infected subjects could reflect HIV-mediated destruction of these dividing cells. If so, the increases in CD4⁺ lymphocytes should result from newly produced cells and the increase in cell numbers should correlate with the percentages of proliferating cells at baseline, that is, before therapy. However, no significant correlations were found between the percentages of CD4⁺Ki67⁺ cells at baseline and the increases in CD4⁺ lymphocytes during the first two weeks of therapy (Fig. 3*c*). These observations provide support for the redistribution model to explain the early increase in CD4⁺ T cells[5].

Kinetics of proliferating CD4⁺ lymphocytes after HAART

We compared the early and late increases in CD4⁺ T-cell counts with the changes in the fraction of proliferating CD4⁺ cells in twelve of sixteen HIV-infected subjects who completed 24 weeks of therapy. A rapid increase in CD4⁺ T cell counts was found at week 2, followed by a slight decrease at week 4 and then a progressive and sustained increase until week 24 (Fig. 4*a*). In eight of the twelve patients, the fraction of proliferating CD4⁺ cells in blood was re-assessed in six at week 4 and in the remaining two at week 8. At 4–8 weeks, compared with baseline, the fraction of proliferating CD4⁺ cells in blood was not increased (mean, 2.3×10^7 CD4⁺Ki67⁺ cells at baseline versus 1.0×10^7 CD4⁺Ki67⁺ cells at 4–8 weeks)(Fig. 4*b*).

However, the late increase in CD4⁺ cell counts in the subjects ($n = 12$) who completed 24 weeks of therapy was always associated with a late increase in the fraction of proliferating CD4⁺ cells in both blood and lymph nodes (Fig. 4*c*). The mean number of blood CD4⁺Ki67⁺ cells was 6.9×10^7 after 24–36 weeks of therapy, compared with 2.6×10^7 at baseline (p = 0.01) (Fig. 4*d*). More importantly, compared with baseline, the fraction of proliferating CD4⁺ cells significantly increased in lymph nodes (means, 3.5×10^9 CD4⁺Ki67⁺ cells after 24–36 weeks of therapy versus 6.0×10^8 at baseline; $P < 0.0001$) (Fig. 4*c* and *d*). In contrast, after HAART the fraction of proliferating CD8⁺ cells did

Table 1 Immunologic and virologic measures, and clinical histories of 22 HIV-infected subjects

HIV-infected subjects	Age	Sex	Risk factor	CD4⁺ T Cells/ µl	CD8⁺ T Cells/ µl	HIV-1 RNA (copies/ml)
2049	28	F	iv drug user	612	538	39260
2051	36	M	iv drug user	449	1428	137550
2052	28	M	homosexual	554	1300	10237
2055	71	M	homosexual	1213	452	98490
2058	39	F	heterosexual	1060	1293	90730
2060	55	F	heterosexual	443	555	43180
2066	30	M	homosexual	534	620	23695
2068	33	M	heterosexual	1255	1490	19790
2071	25	F	heterosexual	711	867	35910
2075	29	F	heterosexual	822	820	8764
2077	55	M	heterosexual	547	784	18205
2083	29	M	homosexual	537	904	18015
2084	32	M	homosexual	579	1709	22235
2089	44	F	iv drug user	1088	573	107335
2090	33	M	iv drug user	862	3154	67590
2091	34	M	homosexual	623	1045	7390
2092	30	M	homosexual	524	1594	14120
2099	36	M	iv drug user	408	1545	11163
2102	40	M	iv drug user	654	1209	48620
2103	37	M	bisexual	972	1328	4525
BL	23	M	homosexual	355	1408	797*
ES	41	F	iv drug user	315	645	107873

*HIV-1 subtype non-B.

Table 2 Estimates of the number of proliferating Ki67⁺CD4⁺ and Ki67⁺CD8⁺ lymphocytes in blood and lymph node of HIV-negative subjects

BLOOD

HIV negative subjects	*Abs number WBC	% Lymph	CD4 cells/μl	% CD4⁺ T cells	CD8 cells/μl	% CD8⁺ T Cells	% CD4⁺ Ki67⁺	% CD8⁺ Ki67⁺	Number of CD4⁺Ki67⁺ T cells	Number of CD8⁺Ki67⁺ T cells	Number of CD4 T cells in the blood	Number of CD8 T cells in the blood
PR	6.6	28.0	680	36.8	676	36.6	0.35	0.15	1.2×10^7	5.1×10^6	3.4×10^9	3.4×10^9
MB	4.4	36.0	616	38.9	665	42.0	0.48	0.30	1.5×10^7	1.0×10^7	3.1×10^9	3.3×10^9
RW	5.4	31.0	939	56.1	499	29.8	0.18	0.05	8.5×10^6	1.2×10^6	4.7×10^9	2.5×10^9
RF	7.1	27.0	912	47.6	696	36.3	0.23	0.38	1.0×10^7	1.3×10^7	4.6×10^9	3.5×10^9
MC	4.3	38.0	696	42.6	653	39.9	0.71	0.55	2.5×10^7	1.8×10^7	3.5×10^9	3.3×10^9
HM	6.3	22.0	565	40.8	481	34.7	0.99	1.00	2.8×10^7	2.4×10^7	2.8×10^9	2.4×10^9
CD	6.4	35.0	900	40.2	907	40.5	0.71	0.16	3.2×10^7	7.3×10^6	4.5×10^9	4.5×10^9
CA	5.5	33.0	862	47.5	583	32.1	1.14	0.65	4.9×10^7	1.9×10^7	4.3×10^9	2.9×10^9
Mean			772		645		0.60	0.41	2.2×10^7	1.2×10^7	3.9×10^9	3.2×10^9
± s.d.			±148		±134		±0.35	±0.32	$\pm 1.4 \times 10^7$	$\pm 7.7 \times 10^6$	$\pm 7.4 \times 10^8$	$\pm 6.7 \times 10^8$

LYMPH NODE

HIV negative subjects	*Abs number WBC	% Lymph	% CD4⁺ T cells	% CD8⁺ T cells	% CD4⁺ Ki67⁺	% CD8⁺ Ki67⁺	Number of CD4⁺Ki67⁺ T cells	Number of CD8⁺Ki67⁺ T Cells	†Number of CD4 T cells in LN	†Number of CD8 T Cells in LN
PR	6.6	28.0	70.4	10.3	0.37	0.14	1.2×10^9	6.5×10^7	3.2×10^{11}	4.7×10^{10}
MB	4.4	36.0	44.2	7.3	0.89	0.24	1.5×10^9	6.8×10^7	1.7×10^{11}	2.8×10^{10}
RW	5.4	31.0	61.5	7.3	0.20	0.04	5.0×10^8	1.2×10^7	2.5×10^{11}	3.0×10^{10}
RF	7.1	27.0	57.9	10.6	0.32	0.20	8.7×10^8	1.0×10^8	2.7×10^{11}	5.0×10^{10}
MC	4.3	38.0	53.5	21.4	0.69	0.23	1.5×10^9	2.0×10^8	2.1×10^{11}	8.6×10^{10}
HM	6.3	22.0	43.2	15.1	0.67	0.31	9.8×10^8	1.6×10^8	1.5×10^{11}	5.1×10^{10}
CD	6.4	35.0	64.4	15.9	0.58	0.09	2.0×10^9	7.9×10^7	3.5×10^{11}	8.7×10^{10}
CA	5.5	33.0	54.1	12.3	0.64	0.15	1.5×10^9	8.2×10^7	2.4×10^{11}	5.5×10^{10}
Mean					0.54	0.18	1.3×10^9	9.5×10^7	2.5×10^{11}	5.4×10^{10}
± s.d.					±0.23	±0.09	$\pm 4.8 \times 10^8$	$\pm 5.8 \times 10^7$	$\pm 7.0 \times 10^{10}$	$\pm 2.2 \times 10^{10}$

*Abs: absolute number; WBC: white blood cells; Lymph: lymphocytes.
†Numbers of CD4 and CD8 T cells in lymph node have been considerated to represent 98% of the total lymphocyte population.

not significantly increase late (6.5×10^8 at baseline versus 9.1×10^8 at 24–36 weeks), consistent with a reduction over time of CD8⁺ T-cell numbers in both blood and lymph nodes (data not shown). The fraction of CD4⁺Ki67⁺ cells (both percentages and/or numbers) in both blood and lymph nodes after 24–36 weeks of therapy was significantly increased (about 3 fold) compared with that of HIV-negative subjects (mean blood CD4⁺Ki67⁺ cells, 2.2×10^7 in HIV-negative subjects versus 6.9×10^7 in HIV-infected subjects, $P = 0.0031$; mean lymph node CD4⁺Ki67⁺ cells, 1.3×10^9 in HIV-negative subjects versus 3.5×10^9 in HIV-infected subjects, $P = 0.0014$)(Fig. 4*d*).

The late increase in CD4⁺ T-cell numbers and the increased fraction of proliferating CD4⁺ cells in blood and lymph nodes were associated with an increase in the percentage of CD4⁺ cells in both blood (means, 33.3 at baseline and 38.7 at weeks 24–36; $P = 0.017$) and lymph nodes (means, 35.8 at baseline and 47.6 at weeks 24–36; $P = 0.009$). There was a normal distribution of CD4⁺Ki67⁺ cells between blood and lymph nodes, and four patients (2049, 2058, 2071 and 2077) had increased percentages of CD4⁺Ki67⁺ cells in blood compared with lymph nodes; however, after HAART, the distribution of CD4⁺Ki67⁺ cells in both the blood and the lymph node compartments was similar to that measured in HIV-negative subjects (Fig. 1 and data not shown).

Discussion

In previous studies[2,13–16], three assumptions have been made to calculate the CD4⁺ T lymphocyte turnover: blood CD4⁺ T cells are representative of the total CD4⁺ lymphocyte population[2,13–15]; the increase in CD4⁺ T cell numbers after antiviral therapy reflects the proportion of CD4⁺ T cells produced and destroyed each day[2] before treatment; and estimates of the fraction of proliferating cells in advanced disease can be extrapolated to the early stages of disease.

As for the hypothesis that the fraction of proliferating CD4⁺ T cells is increased in HIV infection[1,2], our results for the fraction of proliferating CD4⁺Ki67⁺ T cells in lymph nodes, which are presumably representative of the total lymphocyte population, fail to show an increase in the fraction of proliferating CD4⁺ T cells in HIV-infected subjects in early disease, compared with HIV-negative individuals. Therefore, the approach of extrapolating from the 2% of blood lymphocytes is not valid for calculating the total fraction of proliferating CD4⁺ T cells. However, the fraction of proliferating CD8⁺Ki67⁺ T cells in the lymph nodes of HIV-infected subjects is significantly increased, compared with that of HIV-negative subjects.

If there was increased destruction of CD4⁺ T lymphocytes, the rapid increase in blood CD4⁺ T cell counts after HAART (at early time points) should correlate with the fraction of proliferating CD4⁺Ki67⁺ T cells at baseline and therefore should be associated with a rapid increase in the fraction of proliferating CD4⁺Ki67⁺ T cells. However, neither a correlation between the fraction of proliferating CD4⁺Ki67⁺ T cells at baseline and the early increase in blood CD4⁺ T cell counts (Fig.3*c*) nor an increase in the fraction of proliferating CD4⁺ T cells during the first 4–8 weeks of therapy (Fig. 4*b*) was observed despite effective suppression of

Table 3 Estimates of the numbers of proliferating Ki67⁺CD4⁺ and Ki67⁺CD8⁺ lymphocytes in blood of HIV-infected subjects

HIV infected subjects	*Abs number WBC	% Lymph	CD4 cells/ µl	% CD4⁺ T cells	CD8 cells/µl	% CD8⁺ T Cells	% CD4⁺ Ki67⁺	% CD8⁺ Ki67⁺	Number of CD4⁺Ki67⁺ T cells	Number of CD8⁺Ki67⁺ T cells	Number of CD4 T cells in blood	Number of CD8 T cells in blood
2049	4.4	30	612	46.4	538	40.8	3.30	3.51	1.0×10^8	9.5×10^7	3.1×10^9	2.7×10^9
2051	4.7	49	449	19.5	1428	62.0	0.48	0.76	1.1×10^7	5.4×10^7	2.2×10^9	7.1×10^9
2052	5.6	43	554	23.0	1300	54.0	0.91	4.06	2.5×10^7	$2.6. \times 10^8$	2.8×10^9	6.5×10^9
2055	6.3	35	1213	55.0	452	20.5	0.13	0.02	7.9×10^6	4.5×10^5	6.1×10^9	2.3×10^9
2058	7.7	34	1060	40.5	1293	49.4	0.30	1.42	1.6×10^7	9.2×10^7	5.3×10^9	6.5×10^9
2060	4.7	28	443	33.7	555	42.2	0.48	0.24	1.1×10^7	6.7×10^6	2.2×10^9	2.8×10^9
2066	5.7	24	534	39.0	620	45.3	0.22	0.45	5.9×10^6	1.4×10^7	2.7×10^9	3.1×10^9
2068	9.9	33	1255	38.4	1490	45.6	1.05	1.47	6.6×10^7	1.1×10^8	6.3×10^9	7.4×10^9
2071	4.5	47	711	33.6	867	41.0	2.24	2.26	8.0×10^7	9.8×10^7	3.6×10^9	4.3×10^9
2075	4.6	45	822	39.7	820	39.6	0.47	0.45	1.9×10^7	1.8×10^7	4.1×10^9	4.1×10^9
2077	3.8	41	547	35.1	784	50.3	0.36	0.19	9.8×10^6	7.4×10^6	2.7×10^9	3.9×10^9
2083	5.1	40	537	26.3	904	44.3	0.24	0.26	6.4×10^6	1.2×10^7	2.7×10^9	4.5×10^9
2084	6.2	43	579	21.7	1709	64.1	0.23	0.20	6.7×10^6	1.7×10^7	2.9×10^9	8.5×10^9
2089	4.8	39	1088	58.1	573	30.6	0.71	0.21	3.9×10^7	6.0×10^6	5.4×10^9	2.9×10^9
2090	9.3	61	862	15.2	3154	55.6	0.10	0.26	4.3×10^6	4.1×10^7	4.3×10^9	1.6×10^{10}
2091	4.4	50	623	28.3	1045	47.5	0.40	0.25	1.3×10^7	1.3×10^7	3.1×10^9	5.2×10^9
2092	7.6	30	524	23.0	1594	69.9	0.17	0.13	4.5×10^6	1.0×10^7	2.6×10^9	8.0×10^9
2099	4.5	56	408	16.2	1545	61.3	1.17	2.64	2.4×10^7	2.0×10^8	2.0×10^9	7.7×10^9
2102	4.1	55	654	29.0	1209	53.6	1.81	3.68	5.9×10^7	2.2×10^8	3.3×10^9	6.0×10^9
2103	7.4	37	972	35.5	1328	48.5	0.85	1.57	4.1×10^7	1.0×10^8	4.9×10^9	6.6×10^9
BL	2.9	68	355	18.3	1408	71.4	0.69	1.83	1.2×10^7	1.30×10^8	1.8×10^9	7.0×10^9
ES	4.8	34	315	19.3	645	39.5	1.39	1.08	2.2×10^7	3.5×10^7	1.6×10^9	3.2×10^9
Mean ± s.d.	—	—	687 ± 276	—	1148 ± 598	—	0.80 ± 0.79	1.22 ± 1.27	2.7×10^7 ± 2.7×10^7	7.1×10^7 ± 7.7×10^7	3.4×10^9 ± 1.4×10^9	5.7×10^9 ± 3.0×10^9

*Abs: absolute; WBC, white blood cells; Lymph, lymphocytes.

virus replication (Fig. 3*a*). Therefore, these results do not support the hypothesis of extensive destruction of CD4⁺ T lymphocytes in the early stages of HIV disease. There is insufficient data at present to exclude the thymus as a site of ongoing T-cell production in HIV infection, and thus the possibility that increased destruction of CD4⁺ T lymphocytes occurs in the thymus should be considered. However, our results have excluded the possibility that there is increased destruction of CD4⁺ T lymphocytes in secondary lymphoid organs.

Collectively, these results provide strong support for the hypothesis that, at least in HIV-infected subjects in early disease, limited cell renewal rather than destruction[4,17,18] may represent an important mechanism for the progressive loss of CD4⁺ T cells. The limited CD4 renewal may result from an effect of HIV on hematological lymphoid progenitors[19], on T-lymphocyte precursors in the thymus[18] and/or on the clonogenic potential of mature T cells[20,21].

Lymphocyte destruction may become important in the advanced stages of the disease, however, particularly in HIV-infected subjects experiencing rapid loss of CD4⁺ lymphocytes, as seems the case in syncytium-inducing variants of HIV (refs. 22,23). Furthermore, in the advanced stages of the disease, opportunistic pathogens may increase the fraction of activated CD4⁺ and CD8⁺ T cells and thus potentially influence the fraction of proliferating cells. Therefore, estimates of the fraction of proliferating T cells cannot be extrapolated from advanced to early stages of the disease.

We have shown that the rapid increase in blood CD4⁺ T-cell counts after HAART was not associated with an increase in the fraction of proliferating CD4⁺Ki67⁺ T cells at the beginning of the treatment. This observation provides strong evidence that this initial increase in blood CD4⁺ T-cell counts is the result of redistribution of CD4⁺ T cells from other lymphoid compartments to peripheral blood. More importantly, CD4⁺ T-cell numbers in blood and lymph nodes normalized by weeks 24–36 after therapy, and this was associated with a late increase in the number of proliferating CD4⁺ T cells. Late increases in the fraction of CD4⁺Ki67⁺ proliferating T cells after HAART have been also shown by using deuterated glucose *in vivo* labeling in adult humans (Cesar, D. *et al.* Abstract, 5th Conference on Retroviruses and Opportunistic Infections, Chicago, 1–5 February 1998). Therefore, these results indicate that the mechanism of renewal of CD4⁺ T cells is still intact in HIV-infected subjects in the early stages of the disease, as shown by the significant increase in proliferating CD4⁺Ki67⁺ T cells with therapy. These results advance the understanding of HIV pathogenesis and provide a rationale for the development of immune-based interventions to compensate the lack of CD4⁺ T cells production.

Methods

Study populations. Eight HIV-negative subjects and twenty-two HIV-infected subjects were enrolled in this study. HIV-negative subjects (four males and four females) underwent vascular (n = 3, varicose vein stripping) and general (n = 5, uncomplicated bilateral inguinal herniorraphy and osteosynthesis for ankle fracture) surgery, and lymph nodes were collected during surgery. HIV-infected subjects (fifteen males and seven females) were screened for a therapeutic clinical trial with two newly developed drugs (an inhibitor of reverse transcriptase [1592U89] and an inhibitor of protease [141W94] from GlaxoWellcome). The inclusion criteria for this trial were no previous history of antiviral therapy, CD4 cell count > 400 per µl, and plasma viremia > 5,000 HIV RNA copies per ml. Twenty of twenty-two subjects fulfilled the inclusion criteria for entering the study. Sixteen of twenty HIV-infected subjects completed the first two weeks of therapy and twelve of twenty have completed 24 or 36 weeks of therapy. Collection of lymph nodes in the group of HIV-negative subjects and excision and ultrasound-guided lymph node biopsies in the group of HIV-infected subjects were done in studies approved by the Institutional Review Board of the Centre Hospitalier Universitaire

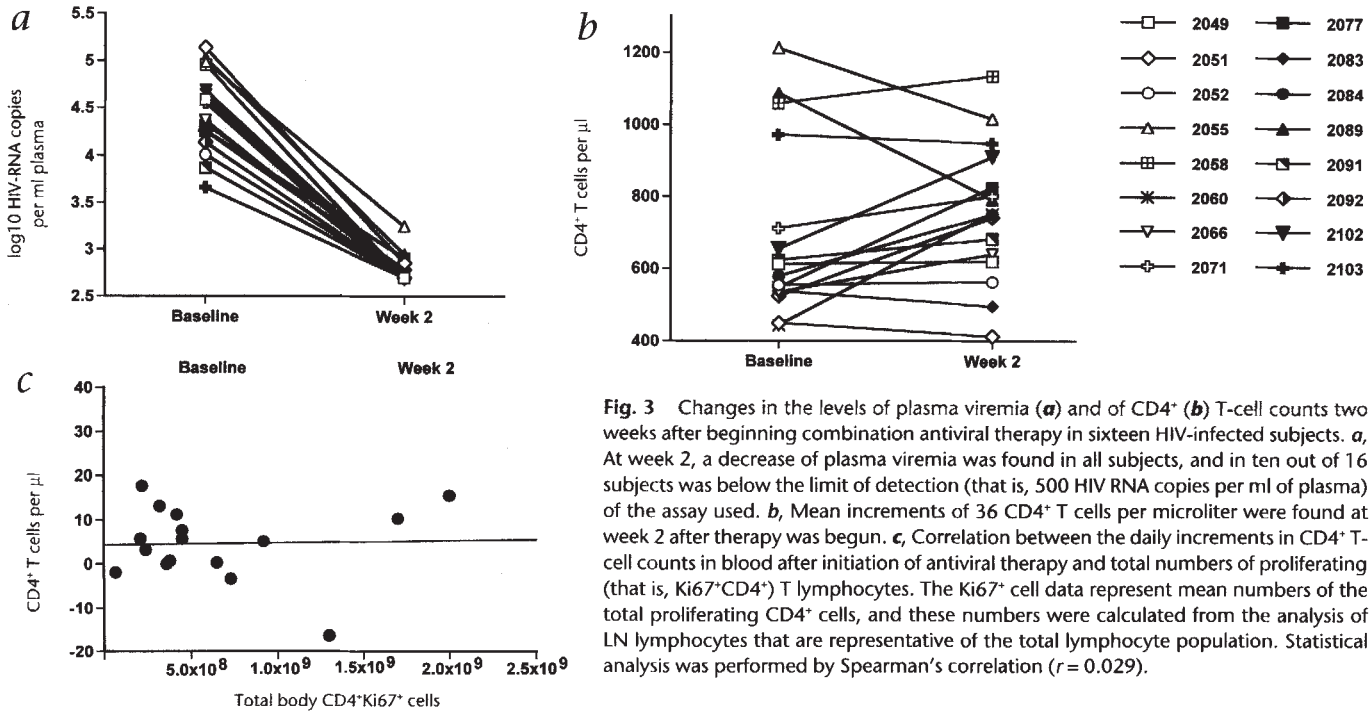

Fig. 3 Changes in the levels of plasma viremia (***a***) and of CD4⁺ (***b***) T-cell counts two weeks after beginning combination antiviral therapy in sixteen HIV-infected subjects. ***a***, At week 2, a decrease of plasma viremia was found in all subjects, and in ten out of 16 subjects was below the limit of detection (that is, 500 HIV RNA copies per ml of plasma) of the assay used. ***b***, Mean increments of 36 CD4⁺ T cells per microliter were found at week 2 after therapy was begun. ***c***, Correlation between the daily increments in CD4⁺ T-cell counts in blood after initiation of antiviral therapy and total numbers of proliferating (that is, Ki67⁺CD4⁺) T lymphocytes. The Ki67⁺ cell data represent mean numbers of the total proliferating CD4⁺ cells, and these numbers were calculated from the analysis of LN lymphocytes that are representative of the total lymphocyte population. Statistical analysis was performed by Spearman's correlation ($r = 0.029$).

Vaudois. Inguinal lymph nodes were obtained from both HIV-negative and HIV-infected subjects.

Isolation of mononuclear cells from blood and lymph nodes. Blood mononuclear cells were isolated in VACUTAINER CPT tubes containing sodium citrate, Ficoll Hypaque density fluid and a polyester gel barrier (Becton Dickinson, San Jose, California). Mononuclear cells from lymph nodes were isolated as described[24]. Tissue specimens were placed into tissue culture dishes (60 × 15 mm) containing RPMI 1640 plus 10% fetal bovine serum (FBS) and minced with a scalpel, and the cells were extricated. After being centrifuged at 400 *g* for 10 min, the cells were washed with PBS, counted and prepared for FACS analysis.

Flow cytometry. All flow cytometric analyses were done on freshly isolated blood and lymph node mononuclear cells. Cell suspensions of about 5×10^5 blood and lymph node mononuclear cells were resuspended in 100 µl of PBS containing 2% FBS and stained with anti-human phycoerythrine (PE)-conjugated CD4 and/or anti-CD8 monoclonal antibody (mAb) (PharMingen, San Diego, California). As a negative control, an isotype-matched Ig-PE antibody was used. Cells were incubated for 30 min at 4 °C. After being washed in 2 ml of PBS plus 2% FBS, cells were spun down at 4 °C for 5 min at 400*g*. Cell pellets were resuspended in 100 µl of PBS without FBS. For permeabilization, 1 ml of Ortho PermeaFix solution (Ortho Diagnostic, Raritan, New Jersey) was added and cells were incubated for 30 min at room temperature. After this incubation, permeabilized and fixed cells were spun down at 4 °C for 5 min at 400*g*, and then washed with 2 ml of PBS containing 2% FBS. After being washed, cell pellets were resuspended in 100 µl of PBS containing 2% FBS, and anti-human Ki67-FITC mAb (Immunotech, Westbrook, Maine) was added for intracellular staining. As a negative control, an isotype-matched IgG-FITC was used. After being incubated for 30

Table 4 Estimates of the numbers of proliferating Ki67⁺CD4⁺ and Ki67⁺CD8⁺ lymphocytes in lymph nodes of HIV-infected subjects

HIV Negative Subjects	*Abs WBC	% Lymph	% CD4⁺ T cells	% CD8⁺ T cells	% CD4⁺ Ki67⁺	% CD8⁺ Ki67⁺	Number of CD4⁺Ki67⁺ T cells	Number of CD8⁺Ki67⁺ T Cells	Number of CD4 T cells in LN	Number of CD8 T Cells in LN
2049	4.4	30	49.0	29.3	0.40	0.97	6.3×10^8	9.2×10^8	1.6×10^{11}	9.5×10^{10}
2051	4.7	49	25.5	39.0	0.04	0.06	5.8×10^7	1.3×10^8	1.4×10^{11}	2.2×10^{11}
2052	5.6	43	35.7	28.0	0.30	0.09	6.3×10^8	1.5×10^8	2.1×10^{11}	1.7×10^{11}
2055	6.3	35	67.2	13.7	0.25	0.07	9.1×10^8	5.2×10^7	3.6×10^{11}	7.4×10^{10}
2058	7.7	34	58.9	26.1	0.06	0.02	2.3×10^8	3.3×10^7	3.8×10^{11}	1.7×10^{11}
2060	4.7	28	27.4	35.0	0.24	0.05	2.1×10^8	5.6×10^7	8.8×10^{10}	1.1×10^{11}
2066	5.7	24	29.0	24.0	1.75	1.07	1.7×10^9	8.6×10^8	9.7×10^{10}	8.0×10^{10}
2068	9.9	33	44.8	27.8	0.05	0.34	1.8×10^8	7.6×10^8	3.6×10^{11}	2.2×10^{11}
2071	4.5	47	35.8	20.3	0.13	0.25	2.4×10^8	2.6×10^8	1.9×10^{11}	1.1×10^{11}
2075	4.6	45	33.9	32.1	0.10	0.15	1.7×10^8	2.4×10^8	1.7×10^{11}	1.6×10^{11}
2077	3.8	41	32.0	25.8	0.16	0.21	2.0×10^8	2.1×10^8	1.2×10^{11}	9.8×10^{10}
2083	5.1	40	23.8	22.8	0.30	0.04	3.6×10^8	4.6×10^7	1.2×10^{11}	1.1×10^{11}
2084	6.2	43	25.5	26.1	0.25	0.27	4.2×10^8	4.6×10^8	1.7×10^{11}	1.7×10^{11}
2089	4.8	39	58.1	30.6	0.46	0.35	1.3×10^9	5.3×10^8	2.7×10^{11}	1.4×10^{11}
2090	9.3	61	15.6	20.0	0.47	0.71	1.0×10^9	2.0×10^9	2.2×10^{11}	2.8×10^{11}
2091	4.4	50	35.4	25.3	0.23	0.37	4.4×10^8	5.0×10^8	1.9×10^{11}	1.4×10^{11}
2092	7.6	30	28.5	30.6	0.28	0.31	4.5×10^8	5.3×10^8	1.6×10^{11}	1.7×10^{11}
2099	4.5	56	22.3	26.2	0.78	1.14	1.1×10^9	1.8×10^9	1.4×10^{11}	1.6×10^{11}
2102	4.1	55	33.3	33.8	1.04	0.48	1.9×10^9	9.0×10^8	1.8×10^{11}	1.9×10^{11}
2103	7.4	37	26.7	53.3	0.19	0.10	3.4×10^8	3.6×10^8	1.8×10^{11}	3.6×10^{11}
BL	2.9	68	32.1	58.0	0.87	0.84	1.3×10^9	2.4×10^9	1.6×10^{11}	2.8×10^{11}
ES	4.8	34	47.0	53.1	0.30	0.51	5.6×10^8	1.1×10^9	1.9×10^{11}	2.1×10^{11}
Mean ± s.d.					0.39 ± 0.40	0.38 ±0.35	6.5×10^8 ±5.2×10^8	6.5×10^8 ±6.6×10^8	1.9×10^{11} ±8.1×10^{10}	1.7×10^{11} ±7.1×10^{10}

*Abs, absolute; WBC, white blood cells; Lymph, lymphocytes.

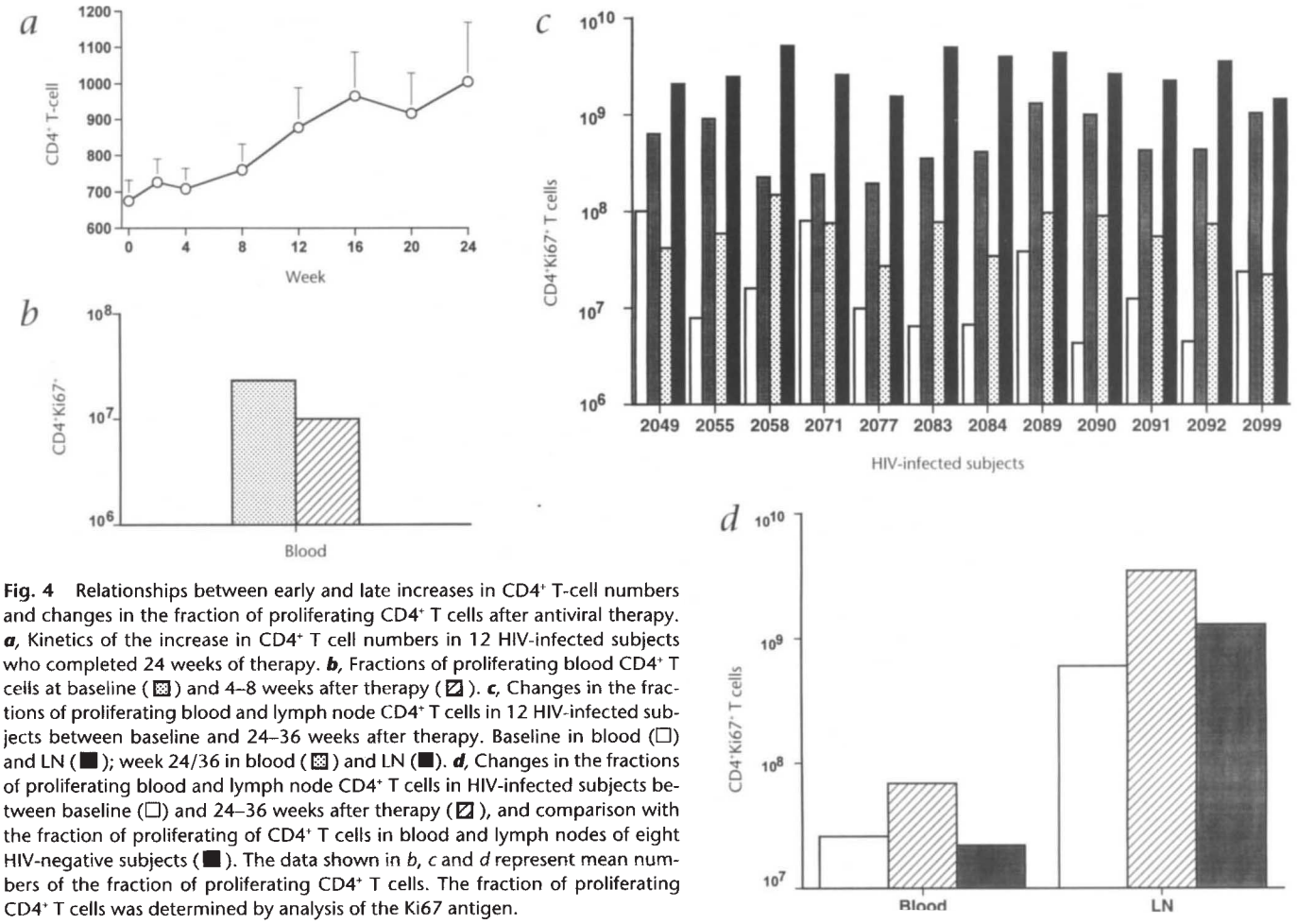

Fig. 4 Relationships between early and late increases in CD4⁺ T-cell numbers and changes in the fraction of proliferating CD4⁺ T cells after antiviral therapy. ***a***, Kinetics of the increase in CD4⁺ T cell numbers in 12 HIV-infected subjects who completed 24 weeks of therapy. ***b***, Fractions of proliferating blood CD4⁺ T cells at baseline (▨) and 4–8 weeks after therapy (▨). ***c***, Changes in the fractions of proliferating blood and lymph node CD4⁺ T cells in 12 HIV-infected subjects between baseline and 24–36 weeks after therapy. Baseline in blood (□) and LN (■); week 24/36 in blood (▨) and LN (■). ***d***, Changes in the fractions of proliferating blood and lymph node CD4⁺ T cells in HIV-infected subjects between baseline (□) and 24–36 weeks after therapy (▨), and comparison with the fraction of proliferating of CD4⁺ T cells in blood and lymph nodes of eight HIV-negative subjects (■). The data shown in *b*, *c* and *d* represent mean numbers of the fraction of proliferating CD4⁺ T cells. The fraction of proliferating CD4⁺ T cells was determined by analysis of the Ki67 antigen.

minutes at room temperature and washed twice with PBS containing 2% FBS, cells were resuspended in 300 µl of cold PBS without FBS and stored at 4 °C in the dark until flow cytometry analysis.

Calculation of fractions of proliferating CD4⁺ and CD8⁺ T lymphocytes. The total number of circulating CD4⁺ and CD8⁺ T lymphocytes and of the fraction of proliferating cells within these cell subsets were calculated as follows. Based on hematological measures (that is, white blood cell [WBC] counts and percentage of lymphocytes), the number of total circulating lymphocytes was calculated assuming that the blood volume in an adult subject is about 5 L. Therefore, by multiplying the number of lymphocytes per µl by 5×10^6, we obtained estimates of the total lymphocyte population. The total numbers of circulating CD4⁺ and CD8⁺ T cells and of proliferating cells (that is, Ki67⁺CD4⁺ and Ki67⁺CD8⁺ cells) were calculated from the percentages of these cell subsets in the total lymphocyte population as determined by flow cytometry. The total number of lymphocytes in lymph nodes was calculated by multiplying by 49 the number of circulating lymphocytes, which represent 2% (ref. 9) of the total lymphocyte population, assuming that lymphocytes in lymph nodes are representative of 98% of total lymphocytes. The total numbers of CD4⁺ and CD8⁺ T cells and of proliferating cells (that is, Ki67⁺CD4⁺ and Ki67⁺CD8⁺ cells) were calculated from the percentages of these cell subsets in lymph nodes as assessed by flow cytometry.

Plasma viremia. Quantification of HIV RNA from plasma was performed by the branched-DNA assay[25]. The lower limit of detection of the assay was 500 HIV RNA copies per ml of plasma.

Statistical analysis. The differences in the means of the percentages from the flow cytometry of HIV-infected and HIV-negative subjects were compared by the F-test and Student's *t*-test as described[26]. Total cell numbers were computed by multiplication of the corresponding percentages with the estimated peripheral blood lymphocyte count. Thus, for comparing the means of the total cell numbers and of the fractions of proliferating T cell subsets at baseline and after therapy between the two groups of data, the same tests were used after taking the logarithm of the data. CD4 and CD8 recovery slopes during the first two weeks of treatment were calculated by subtracting the average of two pre-treatment counts and dividing by the precise number of days of treatment. This gives the linear increase per day. Correlation between the daily increments in CD4 T-cell counts in blood after initiation of antiviral therapy and total numbers of proliferating cells was evaluated by Spearman's correlation.

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