

## Shorter Survival in Advanced Human Immunodeficiency Virus Type 1 Infection Is More Closely Associated with T Lymphocyte Activation than with Plasma Virus Burden or Virus Chemokine Coreceptor Usage

Janis V. Giorgi, Lance E. Hultin, Jane A. McKeating, Timothy D. Johnson, Bronwyn Owens,<sup>1</sup> Lisa P. Jacobson, Roger Shih, Julie Lewis, Dorothy J. Wiley, John P. Phair, Steven M. Wolinsky, and Roger Detels

*The Multicenter AIDS Cohort Study; Departments of Medicine and Biomathematics and Center for AIDS Research (School of Medicine) and Department of Epidemiology (School of Public Health), University of California, Los Angeles; School of Animal and Microbial Sciences, University of Reading, United Kingdom; Johns Hopkins School of Public Health and Hygiene, Baltimore, Maryland; Department of Medicine, Northwestern University Medical School, Chicago, Illinois*

To define predictors of survival time in late human immunodeficiency virus type 1 (HIV-1) disease, long- and short-duration survivors were studied after their CD4<sup>+</sup> T cells fell to  $\leq 50/\text{mm}^3$ . Immune activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as measured by elevated cell surface expression of CD38 antigen, was strongly associated with shorter subsequent survival ( $P \leq .002$ ). The naive CD45RA<sup>+</sup>CD62L<sup>+</sup> T cell reserve was low in all subjects and did not predict survival ( $P = .34$  for CD4<sup>+</sup> and  $.08$  for CD8<sup>+</sup> cells). Higher virus burden correlated with CD8<sup>+</sup> but not CD4<sup>+</sup> cell activation and, after correcting for multiple comparisons, was not associated with shorter survival ( $P = .02$ ). All of the patients' viruses used CCR5, CXCR4, or both, and coreceptor usage did not predict survival ( $P = .27$ ). Through mechanisms apparently unrelated to higher virus burden, immune activation is a major determinant of survival in advanced HIV-1 disease.

Immune dysfunction, increases of virus burden, and evolution of more pathogenic variants of human immunodeficiency virus type 1 (HIV-1) in the infected host are associated with advanced HIV-1 disease [1–6]. Clinically, the underlying cause of death for patients infected with HIV-1 is immune deficiency, and patients ultimately succumb to opportunistic infections or neoplastic diseases [7]. CD4<sup>+</sup> T cell numeric deficiency is a predictor of disease progression, and clinical AIDS develops at a median of  $\sim 50/\text{mm}^3$  CD4<sup>+</sup> T cells [8, 9]. The duration of survival after CD4<sup>+</sup> T cells reach this level is variable. Presumably, additional host or viral properties must contribute to outcome in advanced HIV-1 disease, but little is known about the relative contribution of each of these factors to the ultimate outcome.

T cell immune dysfunction is widely regarded as one of the most important additional immunologic determinants of disease progression [10–12]. Two competing models have been proposed for the mechanism underlying T cell dysfunction. One is that defects in immune response arise primarily due to clonal deletion as a consequence of the direct or proximal effects of HIV-1 infection on infected cells [13–15]. According to this view, immune dysfunction is actually a variation on numeric deficiency, except that the focus is on T cell clones with select reactivities. These clones could be lost over time as a result of selective targeting of HIV-1-infected T cells that subsequently become involved in immune responses. Alternatively, T cells could be lost because of host immune clearance mechanisms, including cytotoxic T lymphocyte activity or antibody-dependent cellular cytotoxicity [16]. An argument in favor of this view is the observation of reduced numbers of naive unprimed CD45RA<sup>+</sup>CD62L<sup>+</sup> T cells in advanced HIV-1 disease, a finding that supports the idea that a deficiency in T cell regeneration capacity may be implicated in the ultimate failure of host survival [17].

A second school of thought holds that immune dysfunction arises as a consequence of the host immune response to high levels of viral replication [1, 18–20]. Such operational immune suppression may also play a role during the acute phase of infection with other viruses, including Epstein-Barr and cytomegalovirus. Before latency is established in these herpes virus infections, excess T lymphocyte activation and diminished host response to other antigens has been demonstrated by our group

Received 27 May 1998; revised 13 November 1998.

Presented in part: 5th Conference on Retroviruses and Opportunistic Infections, Chicago, 1998 (abstract 110).

Informed consent was obtained from patients. Human experimentation guidelines of the US Department of Health and Human Services and of the University of California, Los Angeles, were followed.

Financial support: NIH (AI-35042, RR-00722, AI-35043, AI-37984, AI-35039, AI-35040, AI-37613, AI-35041); Medical Research Council and Lister Institute for Preventive Medicine (to J.M.).

<sup>1</sup> Present affiliation: Georgetown University Medical Center.

Reprints or correspondence: Dr. Janis V. Giorgi, UCLA School of Medicine, Dept. of Medicine/Cellular Immunology and Cytometry, 12-236 Factor Bldg., Los Angeles, CA, 90095-1745 (giorgi@mednet.ucla.edu).

The Journal of Infectious Diseases 1999;179:859–70

© 1999 by the Infectious Diseases Society of America. All rights reserved.  
0022-1899/99/7904-0013\$02.00

and others [21, 22]. T cell immune activation could result in functional anergy, because preactivated T cells are unable to respond to cognate antigen [23, 24]. Immune activation may also drive cells to premature apoptosis [25, 26]. Ultimately, excessive T cell turnover in response to chronic viral stimulation could lead to immunologic exhaustion, a possibility that is supported by the finding of T cell receptor repertoire perturbations in CD4<sup>+</sup> and CD8<sup>+</sup> T cells subpopulations [15] and shortened telomere length in CD8<sup>+</sup> T cells [27, 28]. Markers of T cell activation in HIV-1 infection include increased cell surface expression of CD38, HLA-DR, Fas, and CD45RO [26, 29–33].

Only a few studies have addressed determinants of survival in advanced HIV-1 disease [34–36]. In the current study, we address both immunologic and virologic determinants in late-stage disease. We use  $\leq 50$  CD4<sup>+</sup> cells/mm<sup>3</sup> of peripheral blood as a criterion for advanced disease. Because most patients with low CD4<sup>+</sup> T cells now are treated with potent antiretroviral therapies, which alter both virologic and immunologic parameters, we used stored specimens collected when participants in the Multicenter AIDS Cohort Study (MACS) were receiving less effective antiretroviral therapy. We focused on issues related to immune activation and immune system reserve, because distinguishing between these two possible mechanisms for immune system failure is central to our understanding of HIV-1 pathogenesis. We also measured virologic parameters that have been associated with survival in HIV-1 disease (i.e., virus burden and virus chemokine coreceptor usage) [4–6, 37, 38]. By simultaneously assessing the major immunologic and virologic parameters that have been associated with survival, we hoped to obtain a more accurate idea of the relative contribution of each of these factors to outcome in advanced HIV-1 disease.

## Methods

**Study groups.** We selected 37 HIV-1-infected men for study by the following criteria:  $\leq 50$  CD4<sup>+</sup> T cells/mm<sup>3</sup> at a visit between 1986 and 1994, subsequent survival  $< 6$  months or  $> 18$  months, and sufficient sample in the repository to allow testing. A confirmatory CD4<sup>+</sup> T cell count of  $\leq 50$ /mm<sup>3</sup> was required 6 months after the index visit in men who survived  $> 18$  months. A group of 12 randomly selected seronegative MACS participants whose samples were collected at the same visit and center as one of the infected men was used as a comparison group for immune parameters. We obtained cryopreserved peripheral blood mononuclear cells (PBMC) and plasma samples from the MACS repository (Biomedical Research, Rockville, MD) [39, 40]. The ethnicity of the study participants did not differ among the groups ( $P = .71$ ). Of the 49 study subjects, 38 were white; there was 1 Hispanic and 1 black/non-Hispanic each in the seronegative and  $< 6$  month survival groups and 3 Hispanics and 4 black/non-Hispanics in the  $> 18$  month survival group.

The 37 men in this study were selected from 684 HIV-1-infected MACS participants who had  $\leq 50$  CD4<sup>+</sup> T cells/mm<sup>3</sup> between 1986 (MACS visit 5) and 1994 (MACS visit 21). Of these 684 men, 51 survived  $< 6$  months and 11 of these had sufficient sample in the

repository to allow testing. These 11 men comprised the shorter survival group. In total, 366 men who reached a CD4<sup>+</sup> T cell count of  $\leq 50$ /mm<sup>3</sup> had a confirmatory CD4<sup>+</sup> T cell count. Of these, 103 had no antiretroviral therapy initiated for  $\geq 6$  months after the index visit, and 26 of these both survived  $> 18$  months and had sufficient sample in the repository to allow testing. These 26 men comprised the longer survival group.

Prior to the cutoff for this study in 1994, antiretroviral therapy in the MACS was primarily monotherapy or simultaneous use of 2 nucleoside analogues [41]. The criterion of no antiretroviral therapy initiated for  $\geq 6$  months after the index visit in the group with longer survival reduced the likelihood that men who would have died in  $< 6$  months without antiretroviral therapy would survive for  $> 18$  months. Like most MACS subjects, 23 of the 37 infected men in the study received either prophylaxis for opportunistic infections and/or antiretroviral medications by the time their CD4<sup>+</sup> T cell counts reached  $\leq 50$  cells/mm<sup>3</sup>. To evaluate the immunologic and virologic markers in the absence of any treatment, we compared marker values of the 6 men in the shorter survival group and the 8 men in the longer survival group who received neither prophylaxis for opportunistic infections nor antiretroviral medications before the index visit.

**Monoclonal antibodies (MAbs).** We obtained MAbs from Becton Dickinson Immunocytometry Systems (San Jose, CA). Those MAbs purchased as conjugates of fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC) included IgG2-FITC, CD4-FITC, CD8-FITC, HLA-DR-FITC, CD45RA-FITC, IgG1-PE, CD4-PE, CD38-PE, CD62L-PE, CD95-PE, IgG1-APC, and CD3-APC. We purchased purified CD45RO, CD4, and CD8 MAbs (Becton Dickinson) and then had them conjugated with APC by Molecular Probes (Eugene, OR). We enumerated T cell subsets using FITC/PE/APC MAb combinations of CD45RA/CD62L/CD4 or /CD8; HLA-DR/CD38/CD4 or /CD8; CD4/ or CD8/CD95/CD45RO; CD8/CD38/CD45RO and isotype controls.

**Immunophenotyping.** We thawed cryopreserved PBMC by standard methods [42]. Viability, assessed by trypan blue exclusion, was a median of 94%. We incubated  $5 \times 10^5$  cells with saturating amounts of fluorochrome-conjugated MAbs for each MAb combination. After being washed, cells were suspended in 7-amino actinomycin D (7-AAD; Calbiochem, La Jolla, CA) at a final concentration of 1  $\mu$ g/mL for dead cell discrimination [43]. We performed 4-color immunofluorescence on a dual-laser FACSCalibur flow cytometer (Becton Dickinson) and collected  $\geq 1000$  and usually 2500 events per sample for analysis. A gate was set on live cells, then on forward scatter versus side scatter, and finally on CD4<sup>+</sup> or bright CD8<sup>+</sup> T cell fluorescence. We measured HLA-DR and CD38 relative fluorescence intensities (RFI) as the median RFI using single-parameter histograms with no cursors set. We measured CD95 RFI as the mean RFI using a dot plot display of CD45RO versus CD95. We determined all percentage data from dot plot displays with fixed cursor settings as described previously [17, 44]. CD4<sup>+</sup> and CD8<sup>+</sup> T cell measurements on fresh whole blood on the day the samples were initially drawn (table 1) were made as described previously [45].

**Virion-associated HIV-1 RNA copies per milliliter.** Specimens anti-coagulated with heparin were pretreated with Heparinase 1 before testing by Amplicor HIV-1 Monitor assay (Roche Molecular Systems, Branchburg, NJ). In brief, a 200- $\mu$ L aliquot of the plasma

**Table 1.** Comparison of parameters in the study groups.

	Seronegative controls (n = 12)	Survival >18 months (n = 26)	Survival <6 months (n = 11)	P <sup>a</sup>
Time from study entry to index visit (years)	3.71 (1.88–9.11)	4.33 (1.97–9.55)	2.55 (1.94–9.35)	.14
CD4 T cells/mm <sup>3</sup> at study entry	901 (587–1341)	451 (197–933)	360 (244–1647)	.65
Survival after index visit (years)	NA	2.35 (1.51–6.47)	0.32 (0.05–0.46)	<.001
Parameters at index visit				
CD4 T cells/mm <sup>3</sup>	1032 (600–1686)	38 (16–50)	36 (17–48)	.36
CD8 T cells/mm <sup>3</sup>	665 (386–1191)	707 (229–1512)	546 (216–1454)	.44
CD4:CD8 ratio	1.6 (1.0–2.7)	0.05 (0.01–0.11)	0.05 (0.03–0.12)	.91
Age	36.3 (26–48)	38 (28.4–49.4)	36.8 (31.1–64.9)	.63

NOTE. Values are medians (ranges); NA, not applicable.

<sup>a</sup> Wilcoxon rank sum statistics were used to calculate *P* value for comparison between 2 seropositive groups with different survival times.

specimen was added to 50  $\mu$ L of a Heparinase 1 working solution (0.15 U Heparinase 1/ $\mu$ L in 225 mM NaCl, 75 mM Tris-HCl, 15 mM CaCl<sub>2</sub>, and 0.01% bovine serum albumin, pH 7.5), vortexed, and incubated at room temperature for 1 h. We added 90  $\mu$ L of 1.0 M EDTA to the vial of working lysis reagent. Next, each 250- $\mu$ L sample of Heparinase 1–treated plasma was added to 600  $\mu$ L of the working lysis reagent and analyzed in accordance with the Amplicor HIV-1 Monitor procedure. In the laboratory where HIV-1 RNA levels were determined for this study, intrapatient comparison of the numbers of copies of HIV-1 virion-associated RNA per milliliter of plasma for specimens anti-coagulated with heparin and EDTA showed a correlation coefficient of 0.85 ( $P \leq .001$ ). The method used to treat plasma specimens to remove heparin before the Amplicor HIV-1 Monitor polymerase chain reaction assay has been used in previous MACS studies [38, 46].

**Virus isolation and coreceptor usage.** On the day that we thawed the cryopreserved samples, we placed 10<sup>6</sup> viable PBMC into culture with 10<sup>6</sup> washed CD3 (Ortho Pharmaceuticals, Raritan, NJ)-stimulated PBMC from a pool of 3 normal participants. We maintained cultures in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; Omega Scientific, Tarzana, CA), 250 U/mL penicillin (Gibco), and 250 U/mL streptomycin (Gibco), 2 mM L-glutamine (Gibco), 25 mM HEPES (Gibco), and 5000 IU interleukin (IL)-2 (Chiron Therapeutics, Emeryville, CA) (RPMI-FCS-IL-2). We thawed the CD3-stimulated blasts and cultured the cells in RPMI-FCS-IL-2 before establishing the cultures to isolate virus. Pelleted CD3-stimulated blasts were resuspended in fresh media before use in cocultures. We used the same batch of cryopreserved, CD3-stimulated PBMC to isolate all of the primary viruses for this study, and these cells retained high levels of CCR5 and CXCR4 expression throughout the culture period. We harvested the culture supernatants from the cocultures every 1–2 days from days 2 through 14 and tested for p24 content by ELISA (Coulter, Opa Locka, FL). We completely replaced the culture medium with fresh RPMI-FCS-IL-2 each time we harvested the supernatant, and no new PBMC were added during the culture.

We determined HIV-1 chemokine receptor usage by use of U87/CD4 cells stably transfected with CXCR4 or CCR5 (provided by D. Littman, New York University School of Medicine) [47, 48]. Cells were plated at 5000/mL in 48-well plates 24 h before samples were added to test for virus entry. We evaluated all viruses under study in two independent experiments with HIV-1 strains SF-2, SF-162, and BAL as internal controls and independent markers for

infection efficiencies between assays. We obtained 1–3 isolates of primary viruses per individual 2–14 days after initiating the cocultures and used these to infect U87/CD4 cells expressing CXCR4 or CCR5. We kept the culture period to a minimum before testing viral chemokine receptor usage of the primary viruses to reduce potential artifacts due to extended in vitro culture. For the infections, stock containing the primary viruses was diluted 1:4 in culture medium and added to the transfected U87/CD4 cells. Seventy-two hours later, the cells were fixed with methanol/acetone (1:1 ratio, stored at –20°C) and incubated with MAbs specific for p24 antigen (EF7.1 and 38.6; Medical Research Council AIDS Directed Program Repository, London). We detected bound antibodies with a  $\beta$ -galactosidase–conjugated anti–mouse IgG and X-gal as described previously [49]. We quantified cells expressing p24 antigen as colored foci and viruses capable of inducing cytopathic effects as multinucleated foci.

**Statistical methods.** We used the two-sided exact Wilcoxon rank sum test [50] to test the power of differences between parameters at the index visit of the 2 seropositive groups (table 1). We use the one-sided Wilcoxon rank sum test to examine whether the detailed immunologic markers and virus burden (table 2) differed significantly between the two groups of HIV-1–infected men. We selected the one-tailed test for these later comparisons because we were testing immunologic and virologic parameters, which, on the basis of previous reports [17, 26, 32, 33, 38], we predicted at the outset would be more abnormal in the group with shorter survival. *P* values for the two-tailed tests are twice that for the one-tailed test. We used StatXact version 3.01 [51] to perform the exact tests. We made a total of 13 comparisons (table 2) and used a nominal significance level of  $P = .005$  to adjust for multiple comparisons. We calculated correlation coefficients using Spearman's rank correlation [50].

## Results

**Survival times for men with  $\leq 50$  CD4<sup>+</sup> T cells/mm<sup>3</sup>.** As summarized in table 1, we identified 2 groups of HIV-1–infected men with distinct subsequent survival times ( $P < .001$ ) after reaching  $\leq 50$  CD4<sup>+</sup> cells/mm<sup>3</sup>. One group survived >18 months (median, 2.35 years), and the other survived <6 months (median, 0.32 years). Immunologic and virologic parameters were measured at a single time point, called the index visit, when the CD4<sup>+</sup> T cells had first fallen to  $\leq 50$ /mm<sup>3</sup> of peripheral

**Table 2.** Comparison of T lymphocyte subset values and virus burden in the study groups at the index visit.

Markers measured <sup>a</sup>	Uninfected controls (n = 12)	Survival >18 mo (n = 26)	Survival <6 mo (n = 11)	>18 mo vs. <6 mo P	Correlation with virus burden (r <sup>2</sup> )
T lymphocyte reserve					
CD45RA <sup>+</sup> CD62L <sup>+</sup> (naive) CD4 T cell %	26 (18–43)	6 (0.5–38)	4 (1–36)	.338	.053
CD45RA <sup>+</sup> CD62L <sup>+</sup> (naive) CD8 T cell %	23 (10–43)	9 (0.2–26)	5 (2–17)	.078	.044
HLA-DR <sup>-</sup> CD38 <sup>-</sup> (resting) CD4 T cell %	78 (64–82)	36 (8–49)	20 (4–43)	.020	.003
HLA-DR <sup>-</sup> CD38 <sup>-</sup> (resting) CD8 T cell %	77 (58–84)	22 (5–51)	13 (2–29)	.010	.137
T lymphocyte activation					
CD4 T cell expression of CD38 (RFI)	16 (4–57)	87 (28–466)	221 (59–487)	<b>.002</b>	.048
CD4 T cell expression of HLA-DR (RFI)	3 (2–4)	10 (5–60)	20 (5–41)	.326	.008
CD4 T cell Fas expression (RFI)	36 (9–57)	60 (29–188)	63 (29–166)	.484	.063
CD45RO <sup>+</sup> CD4 <sup>+</sup> (memory) T cell %	70 (54–84)	87 (60–98)	85 (31–96)	.194	.005
CD8 T cell expression of CD38 (RFI)	14 (5–24)	190 (81–638)	411 (163–661)	<b>.001</b>	.240
CD8 T cell Fas expression (RFI)	20 (5–34)	37 (22–63)	47 (32–58)	.006	.260
CD45RO <sup>+</sup> CD38 <sup>+</sup> CD8 <sup>+</sup> "activated memory" cell %	2 (1–4)	34 (16–74)	60 (16–86)	.007	.160
HLA-DR <sup>+</sup> CD38 <sup>-</sup> CD8 <sup>+</sup> T cell %	15 (7–27)	7 (0.7–18)	1.9 (0.4–8)	<b>.002</b>	.240
Plasma HIV-1 copies/mL	NA	10 <sup>5.2</sup> (10 <sup>4.5</sup> –10 <sup>6.3</sup> )	10 <sup>5.6</sup> (10 <sup>4.9</sup> –10 <sup>6.1</sup> )	.02	—

NOTE. Except for P values and correlations (r<sup>2</sup>), data are medians (ranges).

<sup>a</sup> All differences between uninfected controls (n = 12) and combined seropositive groups (n = 37) were significant by Wilcoxon two-sided rank sum test at P = .005 level, except for CD45RO<sup>+</sup> CD4<sup>+</sup> T cell (memory) %, where P = .013; differences between values for groups with <6 month and >18 month survival were tested by Wilcoxon one-sided rank sum test. Values ≤ .005 (in bold) were considered significant after correction for multiple comparisons. NA, not applicable.

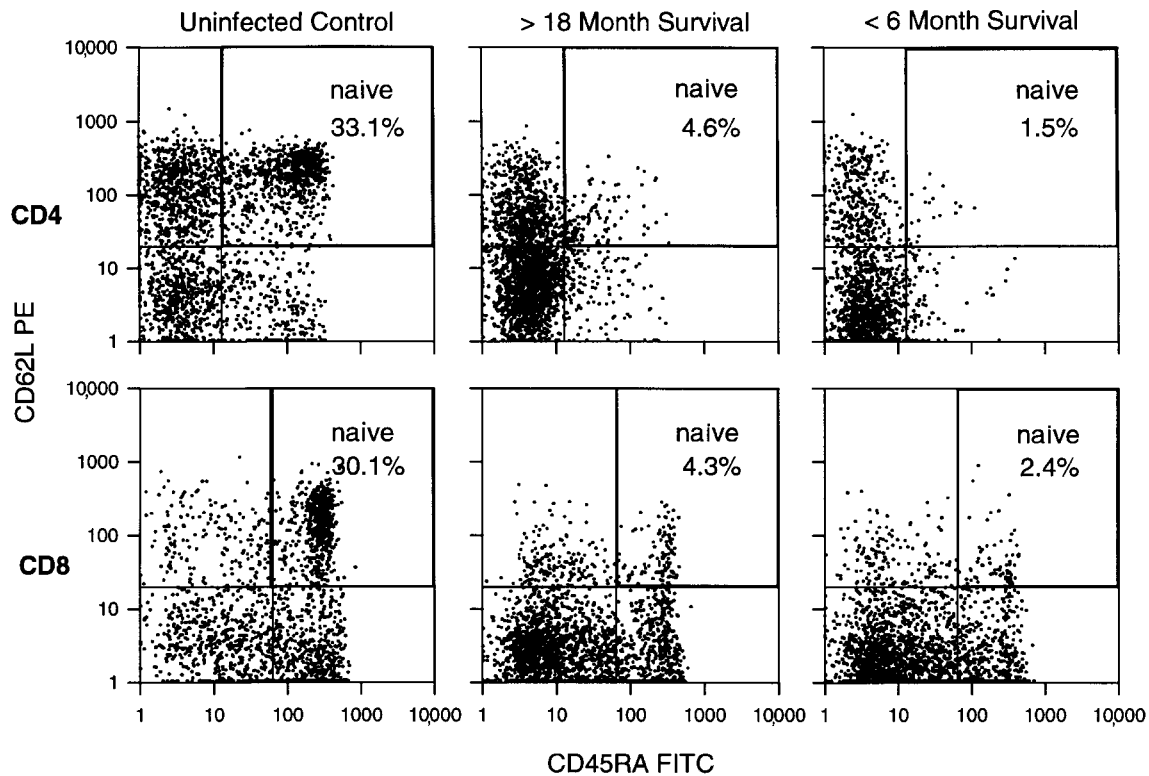
blood. No men displayed any overt manifestations of clinical disease at the index visit. The CD4<sup>+</sup> (P = .36) and CD8<sup>+</sup> T cell counts (P = .44), CD4:CD8 ratio (P = .91), and age (P = .63) were not different in the 2 groups. This was a seroprevalent cohort. However, as described previously [52], the similar CD4<sup>+</sup> cell counts of the 2 groups at entry into the study (P = .65), together with the similar time after study entry (P = .14), suggest that the duration of infection of the 2 seropositive groups prior to the index visit were similar.

*Immunologic differences between HIV-1-infected men with ≤50 CD4<sup>+</sup> T cells/mm<sup>3</sup> and uninfected controls.* Immunologic parameters of the 2 HIV-1-infected groups and the uninfected controls are summarized in table 2. We first documented that the immune abnormalities typically associated with HIV-1 infection were present in the HIV-1-infected men in our study. Like others, we found that the combined groups of HIV-1-infected men (n = 37) had fewer (P = .005) functionally unprimed naive cells than the uninfected men (n = 12). These naive cells were defined by CD45RA isoform usage plus L-selectin (CD62L) expression [17]. The levels of expression of the T cell activation antigens CD38, HLA-DR, and Fas were also increased (P = .005) as we and others have previously reported in chronic HIV-1 infection [20, 25, 26, 30, 31, 33, 53, 54]. Finally, although not significant after correcting for multiple comparisons (P = .013), we found higher numbers of primed CD45RO<sup>+</sup>CD4<sup>+</sup> T cells in HIV-1-infected compared with uninfected men as reported previously [13, 55]. Thus, our HIV-1-infected subjects had the profile of immunophenotypic abnormalities typically observed in advanced HIV-1 disease. We next compared the 2 infected groups with each other to determine whether the extent of any of these abnormalities was associated with differences in survival time.

*Immune cell reserve does not correlate with survival in advanced HIV-1 disease.* HIV-1 infection leads to increased rates of

apoptosis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells [25], and new T cells must be regenerated at an increased rate to keep pace with cell loss. Ultimately, the reserve may be depleted, leading to death of the HIV-1-infected host as a result of immune deficiency. We defined the immune cell reserve by measurement of the proportion of naive unprimed CD45RA<sup>+</sup>CD62L<sup>+</sup> T cells in the peripheral blood [17]. Median values in the uninfected men were 26% and 23%, respectively, for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Although percentages were significantly lower (P = .005) in both groups of HIV-1-infected men compared with uninfected men, the proportion of T cells that were of the naive phenotype did not differ between the 2 infected groups. The median values of CD45RA<sup>+</sup>CD62L<sup>+</sup> T cells among the CD4<sup>+</sup> T cells were 6% and 4%, respectively, in the groups with longer and shorter survival (P = .338). Among the CD8<sup>+</sup> T cells, the median values were 9% and 6%, respectively (P = .078). Figure 1 shows representative dot plots for each group of subjects. In agreement with others, we found that the size of the T cell reserve, as reflected through measurement of the CD45RA<sup>+</sup>CD62L<sup>+</sup> subset in the peripheral blood, is severely reduced in HIV-1-infected homosexual men who have ≤50 CD4<sup>+</sup> T cells/mm<sup>3</sup>. However, our results indicate that immune cell reserve does not correlate with survival time in advanced HIV-1 disease.

*Between group differences in resting HLA-DR<sup>-</sup>CD38<sup>-</sup>CD4<sup>+</sup> and CD8<sup>+</sup> T cells.* We define resting T cells as those that express neither HLA-DR nor CD38 [56]. We compared the percentage of resting CD4<sup>+</sup> and CD8<sup>+</sup> T cells at the index visit to determine whether these values correlated with survival. Figure 2 shows representative dot plots of these T cell subsets analyzed by multicolor flow cytometry for each group of subjects. The resting cells are in the lower left quadrant of the histograms. As listed in table 2, the median values in the uninfected men were 78% and 77%, respectively, for CD4<sup>+</sup> and CD8<sup>+</sup> T cells. After correcting for multiple comparisons, dif-



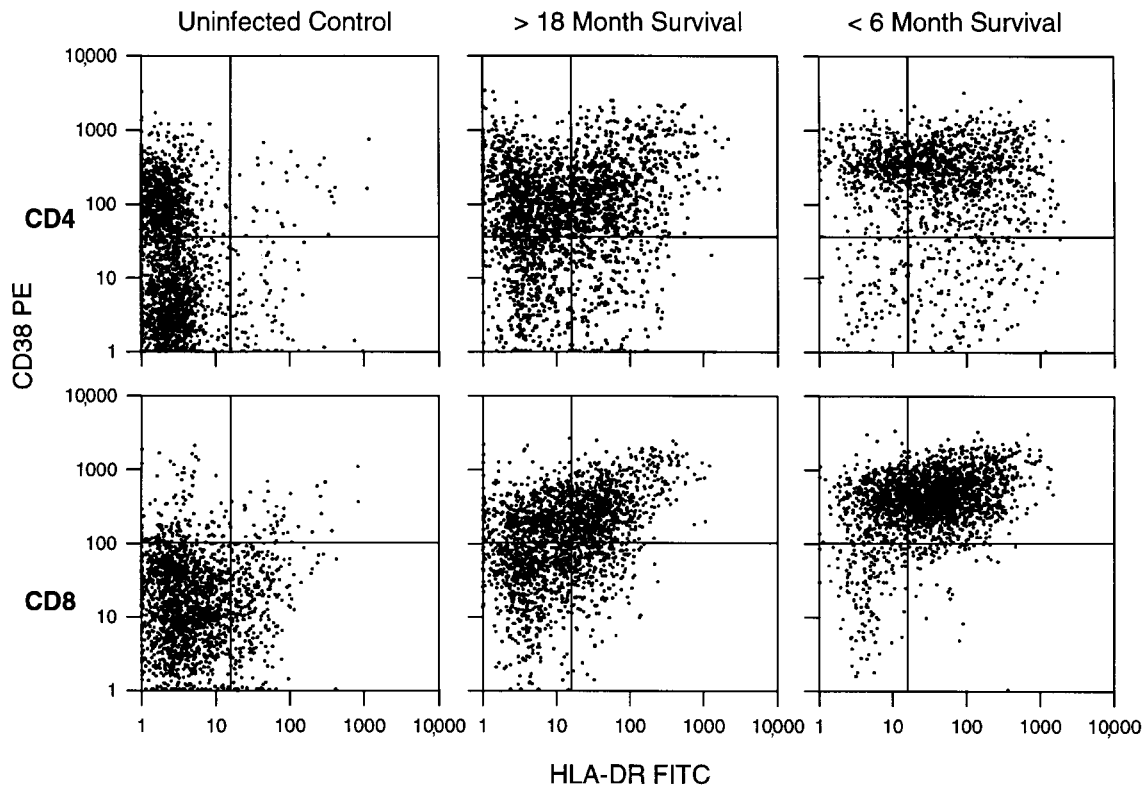
**Figure 1.** Enumeration of naive  $CD4^+$  and  $CD8^+$  T cells. Flow cytometry histograms for 3 representative participants: seronegative control and 2 subjects with different survival times after their  $CD4^+$  T cells fell to  $\leq 50/\text{mm}^3$ . % of naive  $CD4^+$  or  $CD8^+$  T cells among  $CD4^+$  or  $CD8^+$  T cells in each donor shown in upper right quadrants.

ferences were not significant between the percentage of resting  $CD4^+$  and  $CD8^+$  T cells for men in the 2 survival groups. For those with longer versus shorter survival, median values were 36% and 20%, respectively, for  $CD4^+$  T cells ( $P = .02$ ) and 22% and 13%, respectively, for  $CD8^+$  T cells ( $P = .01$ ). Although not statistically significant, this trend suggested that conversion of unprimed T cells to an activated state might correlate with shorter survival.

*T cell activation correlates with shorter survival in HIV-1-infected men with advanced disease.* We measured  $CD4^+$  and  $CD8^+$  T cell activation using several different antigens as markers of activation. Each was selected because it is associated with different potential aspects of immune dysfunction including enhanced tendency to undergo apoptosis. We found no association between shorter survival and increased expression on  $CD4^+$  T cells of HLA-DR or Fas antigen or the percentage of  $CD45RO^+$  memory T cells (table 2). However, we found a significant association between shorter survival and increased CD38 expression on  $CD4^+$  T cells (table 2). Figure 3 shows single-parameter histograms of the RFI of CD38 expression. The group with shorter survival had a median RFI of CD38 expression on  $CD4^+$  T cells of 221 U, whereas men with longer survival had a median RFI of 87 ( $P = .002$ ). As a comparison, the median RFI of CD38 expression on  $CD4^+$  T cells of

non-HIV-1-infected men was 16. By use of recently described methods [57], the number of molecules of CD38 per cell can be estimated by multiplying RFI values by 41. Thus, on our flow cytometer, an RFI of 221 represents  $\sim 9000$  molecules of CD38 expressed per cell. The 6 men with shorter survivals and the 8 men with longer survivals who did not receive either prophylactic treatment for opportunistic infections or antiretroviral medications prior to the index visit had RFI values on  $CD4^+$  T cells of 142 and 67, respectively ( $P = .03$ ). Thus, the relation between higher activation and shorter survival existed even in the untreated men.

We then gauged the levels of activation antigen expression displayed on  $CD8^+$  T cells. As for  $CD4^+$  T cells, we observed significantly higher levels of CD38 expression on  $CD8^+$  T cells obtained from men with shorter survival relative to men with longer survival (median RFIs: 411 and 190, respectively;  $P = .001$ ; table 2; figure 3). Parenthetically, these levels equate to  $\sim 17,000$  and  $\sim 8000$  molecules of CD38 expressed per cell, and are much higher than those typically observed during asymptomatic HIV-1 infection [32]. The uninfected control group had a median RFI of 14. The 6 men with shorter survival and the 8 men with longer survival who did not receive prophylactic treatment for opportunistic infections or antiretroviral medications before the index visit had RFIs of 433 and 145, re-



**Figure 2.** Enumeration of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets defined using HLA-DR (x-axis) and CD38 (y-axis) monoclonal antibodies. Flow cytometry histograms for 3 representative participants: seronegative control and 2 subjects with different survival times after their CD4<sup>+</sup> T cells fell to  $\leq 50/\text{mm}^3$  of peripheral blood. Resting HLA-DR<sup>-</sup>CD38<sup>-</sup>CD4<sup>+</sup> or CD8<sup>+</sup> T cells among all CD4<sup>+</sup> or CD8<sup>+</sup> T cells in each donor are in lower left quadrants; HLA-DR<sup>+</sup>CD38<sup>-</sup> T cells, which are associated with protection against disease progression, are in lower right quadrants.

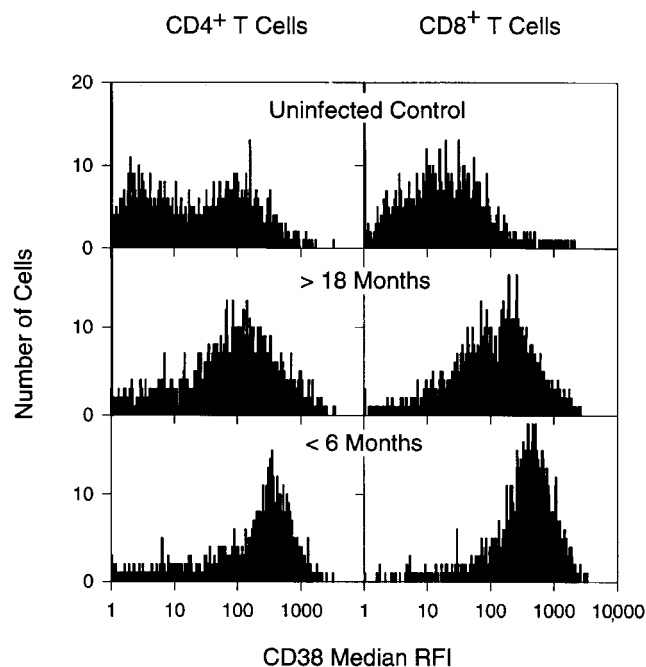
spectively ( $P = .02$ ). Again the relation between higher activation and shorter survival existed even in the untreated men.

Previous reports have focused on additional aspects of T cell activation, including higher proportions of CD45RO<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> cells and increased cell surface Fas expression [26, 33]. We found a higher percentage (table 2) of primed activated CD45RO<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> T cells in the group with shorter relative to longer survival ( $P = .007$ ). Likewise, Fas cell surface expression was also higher on CD8<sup>+</sup> T cells from men with shorter survival times ( $P = .006$ ). These differences approach the strict level of statistical significance set for the study and are likely to be biologically relevant. By use of a variety of cell surface markers that identify activated T cells, our results unequivocally establish that CD4<sup>+</sup> and CD8<sup>+</sup> T cell activation is associated with shorter survival in persons with advanced HIV-1 disease.

*Association between percentage of HLA-DR<sup>+</sup>CD38<sup>-</sup>CD8<sup>+</sup> T cells and survival in advanced HIV-1 disease.* We have identified the HLA-DR<sup>+</sup>CD38<sup>-</sup> phenotype of CD8<sup>+</sup> T cells as a marker of long-term nonprogression and future stable CD4<sup>+</sup> T cells in HIV-1 disease [54, 56, 58]. Therefore, we measured the percentage of HLA-DR<sup>+</sup>CD38<sup>-</sup>CD8<sup>+</sup> T cells to determine

if a higher percentage of cells with this phenotype correlated with longer survival in advanced HIV-1 disease. Figure 2 shows cells displaying this phenotype in the lower right quadrants of the immunofluorescent dot plots. As we would predict on the basis of our previous findings, the men who survived longer after the index visit had a higher percentage of HLA-DR<sup>+</sup>CD38<sup>-</sup>CD8<sup>+</sup> T cells compared with men with shorter survival (7% and 2%, respectively;  $P = .002$ ; table 2; figure 2). The HLA-DR<sup>+</sup>CD38<sup>-</sup>CD8<sup>+</sup> T cell proportions were 13% and 2%, respectively ( $P = .02$ ), in the 8 men with longer survival and the 6 men with shorter survival who were untreated prior to the index visit. Thus, higher levels of this potentially protective CD8<sup>+</sup> T cell subset are associated with longer survival.

*Virus burden in the 2 groups with different survival duration.* As shown in table 2, median numbers of virion-associated RNA copies in plasma were  $10^{5.2}/\text{mL}$  (range,  $10^{4.5}$ – $10^{6.3}$ ) and  $10^{5.6}/\text{mL}$  (range,  $10^{4.9}$ – $10^{6.1}$ ) in the groups with longer and shorter survival, respectively. These values were not significantly different ( $P = .02$ ) after adjusting for multiple comparisons. In untreated patients, there was also no difference between virus burden measurements ( $P = .66$ ). Since CD4<sup>+</sup> T cell immune activation is an in vitro correlate of target cell permissiveness for viral



**Figure 3.** Median relative fluorescence intensity (RFI) of CD38 expression on CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. Flow cytometry histograms for 3 representative participants: seronegative control and 2 persons with different survival times once their CD4<sup>+</sup> T cells fell to  $\leq 50/\text{mm}^3$ . Cursors were set as described previously [32].

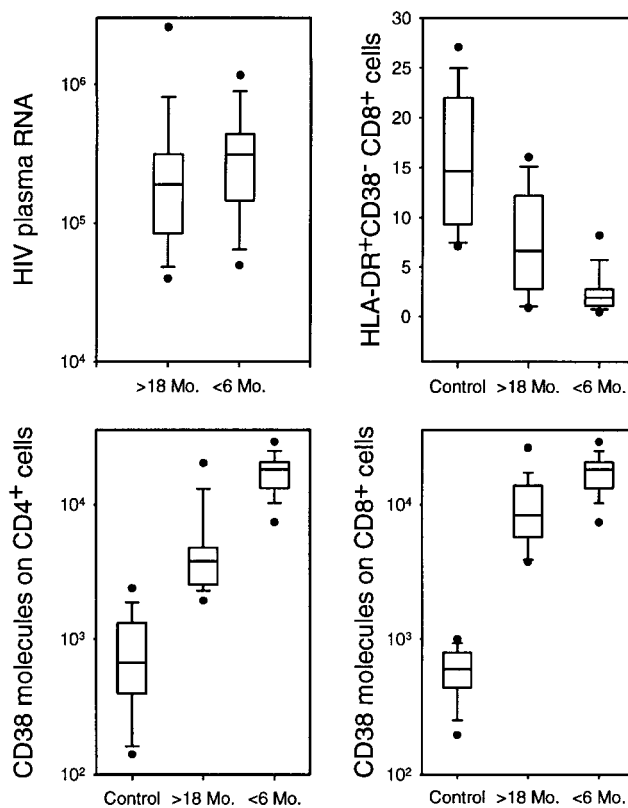
replication, we tested for an association between activation antigen expression on CD4<sup>+</sup> T cells and virus burden. We predicted that higher levels of CD4<sup>+</sup> T cell activation might fuel viral replication. Unexpectedly, we found no association between virus burden and the levels of any of the cell surface markers of CD4<sup>+</sup> cell immune activation (table 2, far right column:  $r^2 = .005-.063$ ). Most notably, CD38 expression on CD4<sup>+</sup> T cells, which we had found associated with survival ( $P = .002$ ), was not correlated with virus burden ( $r^2 = .048$ ;  $P = .18$ ). Thus, our data do not support the thesis that CD4<sup>+</sup> T cell activation is associated with shorter survival by providing a milieu that supports higher levels of viral replication. Meanwhile, 3 of the 4 markers of CD8<sup>+</sup> T cell activation were correlated with virus burden (table 2;  $r^2 = .240-.260$  [ $P = .003$ ]). Virus burden is thus correlated with CD8<sup>+</sup> but not with CD4<sup>+</sup> T cell activation *in vivo*.

Figure 4 summarizes the results of our significant immunologic findings and our findings with virus burden. HLA-DR<sup>+</sup>CD38<sup>-</sup>CD8<sup>+</sup> cells are expressed as percentages and CD38 expression is shown as the median number of molecules of CD38 on CD4<sup>+</sup> and CD8<sup>+</sup> cells, as described [57]. These box plots illustrate the considerable overlap in virus burden between the 2 groups of HIV-1-infected men and the divergence in the immune activation markers between the groups.

*Viruses from persons with advanced immune deficiency utilize*

*both CCR5 and CXCR4.* Viruses capable of utilizing the chemokine receptor CXCR4 are frequently isolated from patients with relatively rapid disease progression [6]. We reasoned that the enhanced lymphocyte activation observed in men with shorter survival might reflect infection by viruses utilizing CXCR4, leading to more rapid T cell turnover. Meanwhile, the men who survived longer might preferentially harbor viruses with CCR5 usage. We therefore examined chemokine receptor usage of viruses obtained from primary PBMC cultures and tested for their ability to infect U87/CD4 glioma cell lines stably expressing either CCR5 or CXCR4. The results in table 3 show the maximal infectivity values that we observed among the 1–3 samples tested for each person and represent the sample obtained on or within 3 days after we observed peak p24 production in the primary cultures.

Viruses were obtained and characterized from 10 of the 11 participants progressing to death in <6 months. Of these, 4 (40%) were only able to infect cells expressing CCR5, whereas 6 (60%) showed usage of both CCR5 and CXCR4 receptors with differing efficiencies (table 3). For participants who survived  $\geq 18$  months, viruses from 20 of 26 participants were



**Figure 4.** Box plots of virus burden and of 3 immunologic markers that showed significant difference between HIV-1-infected groups with different survival times after CD4<sup>+</sup> T cells fell to  $\leq 50/\text{mm}^3$ . Boxes, whiskers, and ● represent 25th–75th, 10th–90th, and 5th–95th percentiles, respectively; means, central line in each box.

**Table 3.** Chemokine coreceptor usage by primary viruses isolated from men with advanced HIV-1 disease and different lengths of survival.

Subject	Survival in years after index visit	Day of culture <sup>a</sup>	CCR-5 <sup>b</sup>	CXCR-4 <sup>b</sup>	Ratio CCR5/CXCR4 <sup>c</sup>
<b>&gt;18-month survival</b>					
1	6.47	6	4000	4200	0.9
2	5.66	10	600	1128	0.5
3	5.09	12	1632	0	—
4	4.33	6	>2400	0	—
5	4.16	8	60	140	0.4
6	3.94	12	120	60	2.0
7	2.84	8	1200	5600	0.2
8	2.83	10	2800	1140	2.4
9	2.52	10	0	620	—
10	2.43	8	264	72	3.7
11	2.40	8	>2400	48	>50
12	2.30	8	>2400	1820	>1.3
13	2.21	12	>7200	72	>100
14	2.13	8	456	1992	0.2
15	1.99	8	1800	0	—
16	1.80	12	1056	600	1.7
17	1.80	12	0	1560	—
18	1.72	12	2976	336	8.8
19	1.64	6	5600	600	9.3
20	1.51	6	>2400	2880	>0.8
<b>&lt;6-month survival</b>					
21	0.46	5	7000	10,400	0.7
22	0.45	6	3000	17,600	0.2
23	0.40	12	>2400	120	>20
24	0.39	12	840	0	—
25	0.37	8	2160	600	3.6
26	0.32	8	>2400	0	—
27	0.32	4	9800	360	27
28	0.31	12	260	0	—
29	0.23	8	>4800	0	—
30	0.05	6	>7200	120	>60

<sup>a</sup> Virus stocks were obtained from supernatants of cocultures of participant cells with anti-CD3-activated peripheral blood mononuclear cells from uninfected donors. Stock used to characterize coreceptor usage was harvested after indicated no. of days for each subject.

<sup>b</sup> U87/CD4 cells, transfected with indicated coreceptor, were incubated with coculture supernatant. Values indicate no. of multinucleated foci formed in assay as described in Methods.

<sup>c</sup> Ratio shown indicates relative no. of foci on CCR5- and CXCR4-expressing cells.

obtained and characterized. Three (15%) of these isolates used only CCR5, 2 (10%) replicated in cells expressing CXCR4 but not CCR5, and the remaining 15 (75%) used both receptors. We verified that expression levels of the two receptors were comparable on the U87/CD4 cells as described previously [47]. Furthermore, all viruses tested, whether they infected CCR5- or CXCR4-expressing U87/CD4 cells, induced multinucleated cytopathic effects (data not shown) as reported by Bjorndal et al. [48]. It is of interest that the replication rate of these viruses in PBMC cultures was independent of both coreceptor usage pattern (unpublished data) and progression group. Thus, we did not find an association between the particular subset of chemokine receptors used and the length of survival ( $P = .27$ ). These results indicate that although many people with advanced HIV-1 disease have virus populations capable of using both CCR5 and CXCR4, this was not a predictor of subsequent duration of survival.

## Discussion

Our results focus attention on the importance of understanding the role of immune activation in HIV-1 disease. We tested several of the mechanisms by which T cell activation might contribute to shortened survival. One leading hypothesis that we examined was that HIV-1-driven immune activation operates to decrease survival by depleting the reserve of naive lymphocytes. This thesis predicts that a decline in T cell regeneration capacity is associated with HIV-1 disease progression. We investigated whether a reduction in the pool of unprimed naive cells, defined by CD45RA isoform usage plus L-selectin (CD62L) expression, contributed to shortened survival after CD4<sup>+</sup> T cells were  $\leq 50/\text{mm}^3$  [17]. We found a lower proportion of the CD45RA<sup>+</sup> CD62L<sup>+</sup> T cell reserve in both groups of HIV-1-infected men compared with the uninfected control group. However, we did not find an association between



the extent of this depletion and survival time. Thus, our results do not support decline in the regenerative capacity of the immune system as a predominant mechanism leading to differences in survival during advanced HIV-1 disease.

A second hypothesis that we tested was that HIV-1-induced immune activation fosters a cellular environment more conducive to enhanced viral replication [59–62]. In our study, CD8<sup>+</sup> T cell activation correlated with HIV-1 virus burden, but unexpectedly, CD4<sup>+</sup> T cell activation did not (table 2). CD4<sup>+</sup> T cell activation thus does not seem to fuel enhanced viral replication in advanced HIV-1 disease. We are not aware of whether other studies have investigated an association between virus burden in vivo and CD4<sup>+</sup> T cell activation; however, the relationship between CD8<sup>+</sup> T cell activation and virus burden is well known [58, 63]. Whether high virus burden drives CD8<sup>+</sup> T cell activation, as we have suggested [58], or production of inflammatory cytokines drives viral replication [64] is unclear.

Our results here in advanced HIV-1 disease (table 2) extend previous observations that CD8<sup>+</sup> T cells displaying the HLA-DR<sup>+</sup>CD38<sup>-</sup> cell surface immunophenotype are associated with slower disease progression [54, 56, 58]. This CD8<sup>+</sup> subset is thus a potential source of inhibitory factors, especially the  $\beta$ -chemokines and MDC-1 [65, 66]. Elevated levels of serum markers of immune activation, including inflammatory cytokines that enhance viral replication, are associated with high levels of CD8<sup>+</sup> T cell activation, especially elevated CD38 levels [67]. It will be important to determine whether CD8<sup>+</sup> T cells are the major source of these factors. We are planning such studies, but were unable to test this in the current investigation because of insufficient cells. Notably, in the current study, virus burden did not correlate closely with survival. Therefore, even though CD8<sup>+</sup> cell activation was correlated with shorter survival as well as with higher virus burden, fostering an environment more favorable to enhanced viral replication does not seem to be the mechanism through which activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells contribute to shorter survival in advanced HIV-1 disease.

Recent findings from studies of antiretroviral therapies suggest an alternate mechanism, which we did not investigate directly, that could explain the association between immune activation and shortened survival. This thesis holds that HIV-1-driven immune activation precludes an appropriate immune response to cognate antigens, such as opportunistic pathogens, by impeding T cell recognition or signaling [23]. Activation-induced apoptosis of T cells, either by Fas-directed or other mechanisms, could be involved in this association [68, 69]. Support for this hypothesis derives from the observation that T cell immune functions such as phytohemagglutinin or anti-CD3 responses, which are not antigen-specific, are reversed within a few months when viral replication is effectively suppressed by antiretroviral drugs. This restoration of lymphocyte function is accompanied by decreased CD38 antigen expression [70, 71]. Meanwhile, restoration of lost T cell reactivity to recall antigens

is less often observed after antiretroviral therapy, possibly because selective clonal deletion may take longer to reverse [13, 70, 71]. Activation-induced anergy and apoptosis could thereby contribute to functional immune deficiency caused by clonal deletion.

Although the drugs used by these subjects were primarily monotherapy or simultaneous use of two nucleoside analogues, they undoubtedly had some influence on the overall survival of the men. Of interest, if antiretroviral drug use did affect survival in our study, the drugs did not seem to operate by effect on CD4<sup>+</sup> cell count or virus load, since these values were not different in the 2 groups. In an analysis of all MACS participants who reached 50 CD4<sup>+</sup> cells/mm<sup>3</sup> and had virus burden measured ( $n = 500$ ; L. Jacobson, unpublished data), we found that survival in those who initiated use of antiretrovirals after the index visit ( $n = 55$ ) was twice as long as for those who never used them ( $n = 56$ ). In the current study, men in the longer survival group who never used antiretroviral therapy ( $n = 3$ ) survived a median of 1.8 years; those who used antiretroviral therapy both before and after the index visit ( $n = 13$ ) survived a median of 2.2 years. Those who used antiretroviral therapy only after the index visit ( $n = 9$ ) survived a median of 2.8 years; these men began antiretroviral drug therapy a median of 1.3 years after the index visit. Taken together, this information supports the conclusion that use of antiretroviral therapy in the >18 month survival group (median, 2.35 years) does not explain the significantly longer survival for that group compared with the <6 month survival group (median survival, 0.32 years).

Our finding that virus burden did not associate with survival time is unlikely to be due to technical factors. Plasma specimens tested for this study, like most plasma specimens from MACS participants, were collected in heparin and cryopreserved within 6 h of collection. After thawing, the specimens were processed by addition of Heparinase 1 to the plasma to inactivate the heparin followed by addition of EDTA and measurement of HIV-1 RNA copy number by the Food and Drug Administration–approved Amplicor HIV-1 Monitor assay (Roche). In the laboratory where the current testing was done, we did an inpatient comparison of the numbers of copies of HIV-1 virion-associated RNA per milliliter of plasma detected by the Monitor assay in specimens collected in heparin and then treated as described above and also collected in EDTA and tested directly. Our results showed a high correlation coefficient of 0.85 ( $P < .001$ ). We have also compared results obtained using heparinase to prepare samples followed by the Monitor assay to results obtained on heparin-anticoagulated plasma specimens tested directly in the bDNA signal-amplification assay (Chiron), for which heparin is an acceptable anticoagulant. The results [38] showed a correlation coefficient of 0.93 ( $P < .001$ ) between the two sets of results. Stored plasma specimens from MACS participants have been used in a number of studies to establish that HIV-1 RNA levels from early during the course

of the disease are highly predictive of outcome [20, 38, 46]. Taken together, these results suggest that the method we used to measure virus burden was sufficiently accurate and robust to provide reliable measurements of plasma HIV-1 RNA. The difference between the current study and past work is that here we focus on patients with advanced HIV-1 disease, whereas earlier studies included subjects with a range of CD4<sup>+</sup> T cell counts and virus burden levels. Our results underscore the conclusion that virus load and CD4<sup>+</sup> T cell counts are not the only factors that influence the outcome of HIV-1 disease. Alternate pathologic influences clearly carry more weight in advanced disease [20].

The lack of predictive value of virus burden levels for survival in advanced HIV-1 disease was verified in an additional subset of MACS participants ( $n = 15$  lived <6 months and  $n = 45$  lived >18 months after the index visit). These men otherwise met the criteria for our study, but cryopreserved cells were not available. Again we found that the virus burden measurements for these 2 groups did not differ (median,  $10^{5.5}$  and  $10^{5.3}$  copies/mL in the <6 and >18 month groups, respectively;  $P = .24$ ). In another comparison, we identified 10 short-term survivors (<6 months; median survival, 0.27 years) and 15 long-term survivors (>18 months; median survival, 2.17 years) who did not report antiretroviral therapy up to the visit after the index visit. The 15 longer survivors did not report antiretroviral therapy through month 18 after the index visit. Again, we found no difference in the viral RNA levels at the index visit (median,  $10^{5.6}$  and  $10^{5.5}$  copies/mL in the <6 and >18 month survival groups, respectively;  $P = .13$ ). These additional sets of data convincingly validate our conclusion that there is no association of virus burden with survival time in subjects with advanced HIV-1 disease. Our findings are in agreement with another report that suggests that immunologic rather than virologic factors are predominant determinants of late HIV-1 disease progression [35].

We investigated chemokine coreceptor usage as a part of this study because we expected that differences in survival most likely came about because different people harbored viruses with differences in pathogenicity that arose from differences in coreceptor usage. Several reports have shown that the emergence of CXCR4-using viruses is associated with poor prognosis and declining CD4<sup>+</sup> T cells [72, 73]. We hypothesized that in our cohort of subjects with advanced HIV-1 infection, viruses that could use CXCR4 or were dual-tropic for both CXCR4 and CCR5 might be more pathogenic and lead to faster cell turnover, more activation, and shorter survival. However, in agreement with a previous report that examined syncytia-inducing phenotype [36], we did not see any difference between the coreceptor usage between the groups with different survival (table 3). Also, in agreement with a previous report that exclusive CXCR4 usage is unusual [6], we only observed exclusive CXCR4 usage for viral entry by primary viruses isolated from 2 men, both of whom were in the group with relatively prolonged survival. The evolution toward CXCR4 usage as an

entry cofactor for viruses isolated from these men at an advanced stage of disease suggests adaptation of the virus to a new environmental niche [74].

In summary, our results indicate that a number of virologic and immunologic parameters associate with profound CD4<sup>+</sup> T cell immunodeficiency as defined by  $\leq 50$  CD4<sup>+</sup> T cells/mm<sup>3</sup>. These include high virus burden, low naive T cell numbers, and marked T cell activation. Among these, T cell immune activation plays a predominant role in determining survival in advanced HIV-1 infection. We measured immune activation using a number of markers and found that shorter survival was associated with elevated cell surface expression of CD38 activation antigen on CD4<sup>+</sup> and CD8<sup>+</sup> T cells, elevated Fas expression on CD8<sup>+</sup> T cells, and an increased percentage of CD45RO<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> T cells. Of note, plasma virus burden and viral chemokine coreceptor usage did not associate with subsequent survival duration in these advanced patients. Thus, even though other variables certainly contribute to differences in rates of disease progression during the prolonged course of chronic HIV-1 infection, once the advanced stage of disease is reached, T cell immune activation is the predominate determinant of differences in survival time.

#### Acknowledgments

We gratefully acknowledge Sam Wu for performing the plasma RNA assays; Mary Ann Hausner, who assisted in the study coordination; and Jose Matud, Gayle Springer, and Traci Yamashita, who helped with data management and statistical analysis. Data were collected by MACS with centers (principal investigators) at Johns Hopkins School of Public Health (Alfred Saah, Alvaro Muñoz, Joseph Margolick); Northwestern University Medical School (John Phair); University of California, Los Angeles (Roger Detels, Janis V. Giorgi); and University of Pittsburgh (Charles Rinaldo). We thank Peter Balfe for critical review of the manuscript.

#### References

1. Fauci AS, Pantaleo G, Stanley S, Weissman D. Immunopathogenic mechanisms of HIV infection. *Ann Intern Med* **1996**;124:654–63.
2. Giorgi JV. Phenotype and function of T cells in HIV disease. In: Gupta S, ed. *Immunology of HIV infection*. NY: Plenum Press, **1996**:181–99.
3. Coffin JM. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. *Science* **1995**;267:483–9.
4. Schuitemaker H, Koot M, Kootstra NA, et al. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T cell-tropic virus populations. *J Virol* **1992**;66:1354–60.
5. Connor RI, Sheridan KE, Ceradini D, Choe S, Landau NR. Change in coreceptor usage correlates with disease progression in HIV-1 infected individuals. *J Exp Med* **1997**;185:621–8.
6. Scarlatti G, Tresoldi E, Bjorndal A, et al. In vivo evolution of HIV-1 coreceptor usage and sensitivity to chemokine-mediated suppression. *Nat Med* **1997**;3:1259–65.
7. Centers for Disease Control. Revision of the CDC surveillance case definition for acquired immunodeficiency syndrome. *MMWR Morb Mortal Wkly Rep* **1987**;36:3S–14S.

8. Taylor JM, Fahey JL, Detels R, Giorgi JV. CD4 percentage, CD4 number, and CD4:CD8 ratio in HIV infection: which to choose and how to use. *J Acquir Immune Defic Syndr* **1989**;2:114–24.
9. Taylor JMG, Sy JP, Visscher B, Giorgi JV. CD4<sup>+</sup> T-cell number at the time of acquired immunodeficiency syndrome. *Am J Epidemiol* **1995**;141:645–51.
10. Schellekens PTA, Roos MTL, De Wolf F, Lange JMA, Miedema F. Low T-cell responsiveness to activation via CD3/TCR is a prognostic marker for acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus-1 (HIV-1)-infected men. *J Clin Immunol* **1990**;10:121–7.
11. Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA, Fauci AS. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome: evidence for a selective defect in soluble antigen recognition. *N Engl J Med* **1985**;313:79–84.
12. Clerici M, Stocks NI, Zajac RA, et al. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients: independence of CD4<sup>+</sup> cell numbers and clinical staging. *J Clin Invest* **1989**;84:1892–9.
13. Connors M, Kovacs JA, Krevat S, et al. HIV infection induces changes in CD4<sup>+</sup> T-cell phenotype and depletions within the CD4<sup>+</sup> T-cell repertoire that are not immediately restored by antiviral or immune-based therapies. *Nat Med* **1997**;3:533–40.
14. Rosenberg ES, Billingsley JM, Caliendo AM, et al. Vigorous HIV-1-specific CD4<sup>+</sup> T cell responses associated with control of viremia. *Science* **1997**;278:1447–50.
15. Pantaleo G, Soudeyns H, Demarest JF, et al. Evidence for rapid disappearance of initially expanded HIV-specific CD8<sup>+</sup> T cell clones during primary HIV infection. *Proc Natl Acad Sci USA* **1997**;94:9848–53.
16. Wain-Hobson S. Virological mayhem. *Nature* **1995**;373:102.
17. Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA, Herzenberg LA. CD8 naive T cell counts decrease progressively in HIV-infected adults. *J Clin Invest* **1995**;95:2061–6.
18. Fahey JL, Taylor JMG, Detels R, et al. The prognostic value of cellular and serologic markers in infection with human immunodeficiency virus type 1. *N Engl J Med* **1990**;322:166–72.
19. Kestens L, Vanham G, Vereecken C, et al. Selective increase of activation antigens HLA-DR and CD38 on CD4<sup>+</sup>CD45RO<sup>+</sup> T lymphocytes during HIV-1 infection. *Clin Exp Immunol* **1994**;95:436–41.
20. Liu Z, Cumberland WG, Hultin LE, Kaplan AH, Detels R, Giorgi JV. CD8<sup>+</sup> lymphocyte activation in HIV-1 disease reflects an aspect of pathogenesis distinct from viral burden and immunodeficiency. *J Acquir Immune Defic Syndr Hum Retrovirol* **1998**;18:332–40.
21. Lynne JE, Schmid I, Matud JL, et al. Major expansions of select CD8<sup>+</sup> subsets in acute Epstein-Barr virus infection: comparison with chronic human immunodeficiency virus disease. *J Infect Dis* **1998**;177:1083–7.
22. Carney WP, Iacoviello V, Hirsch MS. Functional properties of T lymphocytes and their subsets in cytomegalovirus mononucleosis. *J Immunol* **1983**;130:390–3.
23. Lees O, Ramzaoui S, Gilbert D, et al. The impaired in vitro production of interleukin-2 in HIV infection is negatively correlated to the number of circulating CD4<sup>+</sup>DR<sup>+</sup> T cells and is reversed by allowing T cells to rest in culture: arguments for in vivo CD4<sup>+</sup> T cell activation. *Clin Immunol Immunopathol* **1993**;67:185–91.
24. Hofmann B, Nishanian P, Baldwin RL, Insixiangmay P, Nel A, Fahey JL. HIV inhibits the early steps of lymphocyte activation, including initiation of inositol phospholipid metabolism. *J Immunol* **1990**;145:3699–705.
25. Groux H, Torprier G, Monté D, Mouton Y, Capron A, Ameisen JC. Activation-induced death by apoptosis in CD4<sup>+</sup> T cells from human immunodeficiency virus-infected asymptomatic individuals. *J Exp Med* **1992**;175:331–40.
26. McCloskey TW, Oyaizu N, Kaplan M, Pahwa S. Expression of the Fas antigen in patients infected with human immunodeficiency virus. *Cytometry* **1995**;22:111–4.
27. Effros RB, Allsopp R, Chiu CP, et al. Shortened telomeres in the expanded CD28<sup>-</sup> CD8<sup>+</sup> cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. *AIDS* **1996**;10:F17–22.
28. Wolthers KC, Wisman GBA, Otto SA, et al. T cell telomere length in HIV-1 infection: no evidence for increased CD4<sup>+</sup> T cell turnover. *Science* **1996**;274:1543–7.
29. Malavasi F, Funaro A, Roggero S, Horenstein A, Calosso L, Mehta K. Human CD38: a glycoprotein in search of a function. *Immunol Today* **1994**;15:95–7.
30. Levacher M, Hulstaert F, Tallet S, Ullery S, Pocard JJ, Bach BA. The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol* **1992**;90:376–82.
31. Prince HE, Jensen ER. Three-color cytofluorometric analysis of CD8 cell subsets in HIV-1 infection. *J Acquir Immune Defic Syndr* **1991**;4:1227–32.
32. Liu Z, Cumberland WG, Hultin LE, Prince HE, Detels R, Giorgi JV. Elevated CD38 antigen expression on CD8<sup>+</sup> T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4<sup>+</sup> cell count, soluble immune activation markers or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* **1997**;16:83–92.
33. Bofill M, Mocroft A, Lipman M, et al. Increased numbers of primed activated CD8<sup>+</sup>CD38<sup>+</sup>CD45RO<sup>+</sup> T cells predict the decline of CD4<sup>+</sup> T cells in HIV-1-infected patients. *AIDS* **1996**;10:827–34.
34. Spino C, Kahn J, Dolin R, Phair JP. Predictors of survival in HIV-infected persons with 50 or fewer CD4 cells/mm<sup>3</sup>. *J Acquir Immune Defic Syndr Hum Retrovirol* **1997**;15:346–55.
35. Spijkerman IJB, Prins M, Goudsmit J, et al. Early and late HIV-1 RNA level and its association with other markers and disease progression in long-term AIDS-free homosexual men. *AIDS* **1997**;11:1383–8.
36. Richman DD, Bozzette SA. The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression. *J Infect Dis* **1994**;169:968–74.
37. Wolinsky SM, Korber BTM, Neumann AU, et al. Adaptive evolution of human immunodeficiency virus type 1 during the natural course of infection. *Science* **1996**;272:537–42.
38. Mellors JW, Muñoz A, Giorgi JV, et al. Plasma viral load and CD4<sup>+</sup> lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med* **1997**;126:946–54.
39. Kaslow RA, Ostrow DG, Detels R, Phair JP, Polk BF, Rinaldo CR Jr. The Multicenter AIDS Cohort Study: rationale, organization, and selected characteristics of the participants. *Am J Epidemiol* **1987**;126:310–8.
40. Chmiel JS, Detels R, Kaslow RA, et al. Factors associated with prevalent human immunodeficiency virus (HIV) infection in the Multicenter AIDS Cohort Study. *Am J Epidemiol* **1987**;126:568–77.
41. Detels R, Munoz A, McFarlane G, et al. Effectiveness of potent antiretroviral therapy on time to AIDS and death in men with known HIV infection duration. *JAMA* **1998**;280:1497–503.
42. Gjerset G, Nelson KA, Michael D. Methods for cryopreserving cells. In: Rose NR, deMacario EC, Fahey JL, Friedman H, Penn GM, eds. *Manual of clinical laboratory immunology*. 4th ed. Washington DC: American Society for Microbiology, **1992**:61–7.
43. Schmid I, Uittenbogaart CH, Giorgi JV. Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. *Cytometry* **1994**;15:12–20.
44. Yagi MJ, Chu FN, Jiang JD, et al. Increases in soluble CD8 antigen in plasma and CD8<sup>+</sup> and CD8<sup>+</sup>CD38<sup>+</sup> cells in human immunodeficiency virus type-1 infection. *Clin Immunol Immunopathol* **1992**;63:126–34.
45. Giorgi JV, Cheng HL, Margolick JB, et al. Quality control in the flow cytometric measurement of T-lymphocyte subsets: the Multicenter AIDS Cohort Study experience. *Clin Immunol Immunopathol* **1990**;55:173–86.
46. Mellors JW, Rinaldo CR Jr, Gupta P, White RM, Todd JA, Kingsley LA. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* **1996**;272:1167–70.

47. Deng H, Liu R, Ellmeier W, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **1996**;381:661–6.
48. Bjorndal A, Deng H, Jansson M, et al. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J Virol* **1997**;71:7478–87.
49. Clapham PR, McKnight A, Weiss RA. Human immunodeficiency virus type 2 infection and fusion of CD4-negative cell lines: induction and enhancement by soluble CD4. *J Virol* **1992**;66:3531–7.
50. Lehman EL. *Nonparametrics: statistical methods based on ranks*. San Francisco: Holden-Day, **1975**.
51. StatXact (version 3.01). Boston: Cytel Software, **1995**.
52. Muñoz A, Taylor JMG, Chmiel JS, Kingsley L, Van Raden M, Hoover DR. Estimation of time since exposure for a prevalent cohort. *Stat Med* **1992**;11:939–52.
53. Giorgi JV, Liu Z, Hultin LE, Cumberland WG, Hennessey K, Detels R. Elevated levels of CD38<sup>+</sup>CD8<sup>+</sup> T cells in HIV infection add to the prognostic value of low CD4<sup>+</sup> T cell levels: results of 6 years of follow-up. *J Acquir Immune Defic Syndr* **1993**;6:904–12.
54. Giorgi JV, Ho HN, Hirji K, et al. CD8<sup>+</sup> lymphocyte activation at human immunodeficiency virus type 1 seroconversion: development of HLA-DR<sup>+</sup>CD38<sup>+</sup>CD8<sup>+</sup> cells is associated with subsequent stable CD4<sup>+</sup> cell levels. *J Infect Dis* **1994**;170:775–81.
55. Chou CC, Gudeman V, O'Rourke S, et al. Phenotypically defined memory CD4<sup>+</sup> cells are not selectively decreased in chronic HIV disease. *J Acquir Immune Defic Syndr* **1994**;7:665–75.
56. Ho HN, Hultin LE, Mitsuyasu RT, et al. Circulating HIV-specific CD8<sup>+</sup> cytotoxic T cells express CD38 and HLA-DR antigens. *J Immunol* **1993**;150:3070–9.
57. Hultin LE, Matud JL, Giorgi JV. Quantitation of CD38 activation antigen expression on CD8<sup>+</sup> T cells in HIV-1 infection using CD4 expression on CD4<sup>+</sup> T lymphocytes as a biological calibrator. *Cytometry* **1998**;33:123–32.
58. Ferbas J, Kaplan AH, Hausner MA, et al. Virus burden in long-term survivors of human immunodeficiency virus (HIV) infection is a determinant of anti-HIV CD8<sup>+</sup> lymphocyte activity. *J Infect Dis* **1995**;172:329–39.
59. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen ISY. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **1990**;61:213–22.
60. O'Brien WA, Grovit-Ferbas K, Namazi A, et al. Human immunodeficiency virus type 1 replication can be increased in peripheral blood of seropositive patients after influenza vaccination. *Blood* **1995**;86:1082–9.
61. Staprans SI, Hamilton BL, Follansbee SE, et al. Activation of virus replication after vaccination of HIV-1-infected individuals. *J Exp Med* **1995**;182:1727–37.
62. Stanley SK, Ostrowski MA, Justement JS, et al. Effect of immunization with a common recall antigen on viral expression in patients infected with human immunodeficiency virus type 1. *N Engl J Med* **1996**;334:1222–30.
63. Bouscarat F, Levacher-Clergeot M, Dazza MC, et al. Correlation of CD8 lymphocyte activation with cellular viremia and plasma HIV RNA levels in asymptomatic patients infected by human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* **1996**;12:17–24.
64. Poli G, Fauci AS. Cytokine modulation of HIV expression. *Semin Immunol* **1993**;5:165–73.
65. Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 $\alpha$ , and MIP-1 $\beta$  as the major HIV suppressive factors produced by CD8<sup>+</sup> T cells. *Science* **1995**;270:1811–5.
66. Pal R, Garzino-Demo A, Markham PD, et al. Inhibition of HIV-1 infection by the  $\beta$ -chemokine MDC. *Science* **1997**;278:695–8.
67. Prince HE, Kleinman S, Czaplicki C, John J, Williams AE. Interrelationships between serologic markers of immune activation and T lymphocyte subsets in HIV infection. *J Acquir Immune Defic Syndr* **1990**;3:525–30.
68. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **1995**;373:123–6.
69. Finkel TH, Tudor-Williams G, Banda NK, et al. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat Med* **1995**;1:129–34.
70. Autran B, Carcelain G, Li TS, et al. Positive effects of combined antiretroviral therapy on CD4<sup>+</sup> T cell homeostasis and function in advanced HIV disease. *Science* **1997**;277:112–6.
71. Lederman MM, Connick E, Landay A, et al. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and zalcitabine: results of AIDS Clinical Trials Group Protocol 315. *J Infect Dis* **1998**;178:70–9.
72. Fouchier RAM, Groenink M, Kootstra NA, et al. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J Virol* **1992**;66:3183–7.
73. Koot M, Keet IPM, Vos AHV, et al. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4<sup>+</sup> cell depletion and progression to AIDS. *Ann Intern Med* **1993**;118:681–8.
74. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci USA* **1997**;94:1925–30.