A Low Level of CD4$^+$CD28$^+$ T Cells Is an Independent Predictor of High Mortality in Human Immunodeficiency Virus Type 1–Infected Patients

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This study investigated coexpression of CD28, CD45RA, and CD45RO on CD4$^+$ and CD8$^+$ cells in 107 human immunodeficiency virus (HIV) type 1–infected patients, who were followed-up prospectively and were not treated with highly active antiretroviral therapy, and 65 control subjects. The most important novel finding was that a 50% reduction in CD4$^+$CD28$^+$ cells predicted increased mortality (relative hazards [HR], 1.6; 95% confidence interval [CI], 1.0–2.6; \( P = .04 \)), even after adjusting for the CD4$^+$ cell counts, virus load, \( \beta_2 \)-microglobulin and hemoglobin levels, and HIV disease stage. Patients with progressed HIV infection had decreased concentrations of all studied cell subsets. Concerning the proportions of cells, only CD4$^+$CD28$^+$, CD4$^+$CD45RA$^+$, and CD8$^+$CD45RO$^+$ cells decreased with HIV progression. Low proportions of CD4$^+$CD45RA$^+$, CD8$^+$CD45RA$^+$, and CD8$^+$CD45RO$^+$ cells predicted mortality only in univariate but not in multivariate Cox analyses. If our results are confirmed in other studies, coexpression of CD28 on CD4$^+$ cells may be a useful marker to evaluate HIV progression.

The natural progress of human immunodeficiency virus (HIV) type 1 infection causes specific changes in the proportions of most T cell subsets [1–10]. HIV disease progression leads to a profound depletion of CD4$^+$ cells coexpressing CD28 [1–4]. Lack of CD28 expression on T cells has functional consequences, because CD28 is the primary costimulatory molecule for naive T cells [11], which, upon engagement, enhances long-term T cell survival and prevents anergy induction [12]. Consequently, the majority of the CD4$^+$ cell pool (CD28$^+$) may become apoptotic or anergic after activation in advanced HIV infection [13–15]. HIV disease progression also is associated with depletion of naive T cells (CD45RA$^+$CD62L$^+$) [5–7] and a relative expansion of immature and activated CD4$^+$ cells (CD38$^+$ and HLA-DR$^+$) [1, 8], whereas early HIV infection is associated with depletion of memory T cells (CD45RO$^+$) [5, 6, 9] and expansion of CD8$^+$ immature and activated cells (CD45RA$^+$CD62L$^-$, CD28$^-$, CD38$^+$, CD27$^+$, and CD45RO$^+$) [2, 7, 9, 10]. The above described changes in T cell phenotypes probably reflect a combination of appropriate antiviral immune defense mechanisms and inappropriate immune activation and immunosuppression [16, 17]. The characteristic changes in T cell subsets during HIV progression make it obvious to investigate whether specific changes have stronger prognostic value than changes in the CD4$^+$ cell count when evaluating pro-
gression to relevant endpoints. Such knowledge provides insight into HIV pathogenesis and may potentially also improve monitoring of HIV-infected patients.

It has been demonstrated that low levels of CD4+CD28+ cells and a low T cell reactivity to CD3/CD28 costimulation predict AIDS [3, 18]. Furthermore, the CD4+CD28+ cell level correlates negatively with various HIV disease progression markers [4]. The CD4+CD45RA+ cell level has been shown to predict death independent of the total CD4+ cell count [6] and high levels of CD38+ T cells or expression of CD38 on T cells was recently shown to predict fast progression to AIDS [19–23], loss of CD4+ cells [24, 25], and death [22].

Several studies have investigated the association between specific T cell phenotypes and progression to AIDS [3, 19–23], whereas only few studies have investigated these T cell phenotypes and progression to death [6, 22]. Because of the progressive nature of HIV-1 infection, we investigated the proportion and concentration of costimulatory (CD28+), naive (CD45RA+), and memory (CD45RO+) CD4+ and CD8+ cells in HIV-1–infected patients, who were categorized according to the Centers for Disease Control and Prevention (CDC) criteria [26], and in healthy individuals. Furthermore, we investigated whether these T cell subsets could predict mortality in HIV-1–infected patients. We hypothesized that a reduction in CD28+ and CD45RA+ CD4+ cells would be associated with increased mortality in HIV-1–infected patients.

SUBJECTS, MATERIALS, AND METHODS

Study population. The HIV-1–infected patients and control subjects described in the present study were enrolled at the Department of Infectious Diseases, Rigshospitalet (Copenhagen, Denmark), from November 1994 to August 1995, as described elsewhere [27]. A total of 107 HIV-1–infected patients and 65 control subjects were included in the study. The HIV-1–infected patients (90% men) had a mean age of 44 years (95% confidence interval [CI], 42–46), and their mean time from the first positive HIV-antibody test was 7.3 years (95% CI, 6.8–7.8). The control group (91% men; mean age, 44 years; 95% CI, 42–46) matched the patient group with respect to sex and age. Of the 107 patients, 83 were infected through homosexual/bisexual contact and 16 through heterosexual contact, 2 were injection drug users, 2 were infected by blood transfusions, and 4 had unknown risk factors. The patients were categorized according to the CDC criteria clinical stages [26]: 37 in CDC category A (A1, 5 patients; A2, 22 patients; and A3, 10 patients), 45 in CDC category B (B1, 2 patients; B2, 19 patients; and B3, 24 patients), and 25 in CDC category C (C1, 1 patient; C2, 2 patients; and C3, 22 patients). The geometric (G) mean CD4+ cell count and virus load were 0.267 × 10^3 cells/L (95% CI, 0.210–0.340) and 90.034 copies/mL (95% CI, 0.018–0.065) in CDC category A patients, 0.132 × 10^3 cells/L (95% CI, 0.093–0.188) and 4041 copies/mL (95% CI, 2174–7510) in CDC category B patients, and 0.034 × 10^3 cells/L (95% CI, 0.018–0.065) and 10,796 copies/mL (95% CI, 4174–27,925) in CDC category C patients. At enrollment, 41 (38%) patients received antiretroviral treatment with 1 (n = 29) or 2 (n = 12) nucleoside reverse-transcriptase inhibitors (NRTIs), whereas no patients received protease inhibitors or 3 NRTIs.

Blood sampling and isolation of blood mononuclear cells (BMNCs). BMNCs were isolated from heparinized blood by density gradient centrifugation (Lymphoprep Nycomed Pharma AS) on LeukoSep tubes (Greiner) and were washed 3 times with Stock solution I ad HANK (Bie & Berntsen A/S). Cells were frozen in freezing medium (50% RPMI 1640 medium, 30% fetal calf serum [FCS; Gibco BRL], 20% dimethyl sulfoxide [DMSO; Bie & Berntsen A/S]) and were kept in liquid nitrogen until thawed for analyses.

Clinical chemistry tests and virus load. The concentration of lymphocytes and hemoglobin were analyzed by a cell counter (SE-9000; Toa-Sysmex). Serum β₂-microglobulin was measured with a quantitative competitive enzyme assay (Pharmacia Diagnostic), according to the manufacturer’s recommendations. HIV-1 RNA was quantified retrospectively, in serum stored at −80°C, by a standardized reverse-transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor test; Roche Diagnostic Systems), according to the manufacturer’s recommendations. The detection limit of the assay was an HIV RNA level of 20 copies/mL. Values below this level were recorded as 20 copies/mL.

Flow cytometry. The flow cytometry analyses were performed shortly after inclusion of the last subject in the study. To reduce the day-to-day variation in the assays, thawed cells from patients and control subjects were analyzed simultaneously. Direct cell surface immunofluorescent staining was performed on 10^5 thawed BMNCs, according to the manufacturer’s recommendations. The monoclonal antibodies (MAbs) used were directly conjugated to fluorescein-isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP). The following MAbs were obtained: CD4, IgG 1 (DAKO); CD3, CD8, and CD45RO, IgG 1 (Becton-Dickinson); and CD45RA, CD28 (Immunotech). Labeled cells were analyzed by use of a flow cytometer (Epics XL-MCL; Coulter), and the subsequent computer analyses were performed with PC-Lysys software from Becton-Dickinson (Immunochemistry Systems). Lymphocytes were distinguished from monocytes on the basis of their forward–versus–right-angle light scatter. Lymphocyte gates with <2% CD14+ monocytes were used in all the analyses. The absolute number (10^9 cells/L) of CD4+ or CD8+ cells coexpressing CD28, CD45RA, or CD45RO was calculated by multiplying the proportions with the concentration of lymphocytes, which were obtained from hematology analysis.
We have previously investigated whether freezing of BMNCs changes the composition of lymphocyte subsets in HIV-1–infected patients and control subjects and found that freezing did not change the composition of lymphocyte subsets systematically in HIV-1–infected patients or control subjects [27].

Statistical analysis. The effect of HIV infection and HIV progression (CDC category) on the proportion and concentration of CD28+, CD45RA+, and CD45RO+ T cell phenotypes were analyzed with 1-way analysis of variance (ANOVA) and Tukey posthoc tests with 4 grouping variables: control subjects and CDC categories A–C. The residuals were investigated for a normal distribution, and, to fulfill this assumption, the T cell phenotype data were log2 transformed. Data are presented as mean or geometric mean with 95% CIs.

Survival analysis was performed with death as the main endpoint. The prognostic value of the proportion and concentration of each of the CD4+ or CD8+ phenotypes was evaluated only in subjects with complete data (n = 84). Follow-up times were calculated from the date of enrollment to the date of death or censorship on 1 May 1996, just before the first introduction of protease inhibitor–based antiretroviral therapy. Patients who were lost during follow-up (n = 2) were censored from the last day they were known to be alive. The progression was evaluated by use of the Kaplan-Meier method by comparing survival times in patients stratified by the 50% percentiles of each CD4+ and CD8+ phenotypes with the log-rank test. Univariate and multivariate Cox proportional hazards models were done to determine the ability of the proportion or concentration of each of the different T cell phenotypes to predict mortality unadjusted, as well as after adjusting for the CD4+ cells, virus load, β2-microglobulin and hemoglobin levels, and CDC category. Variables not predictive for survival in the univariate Cox models—that is, age and NRTI treatment at enrollment—were not included in the multivariate models, but each multivariate model was subsequently reanalyzed in a model that included age and NRTI treatment. The variables in the models were log transformed, if necessary, to provide the best fit determined by the Wald χ² value: log⁡_2 for CD4 cell count and β₂-microglobulin and log₂ for virus load. The hazards ratio or relative hazards (HR) are presented with 95% CIs and p values. α < 0.05 was considered to be significant. Statistical calculations were performed by use of SAS 8.2 (SAS Institute) and SYSTAT 8.0 (SSPS).

RESULTS

CD4+ phenotypes. The concentration of CD4+ cells was, as expected, markedly reduced in all stages of HIV infection and most pronounced in the advanced stages (figure 1A). The proportions of CD4+ cells coexpressing CD28 or CD45RA were not significantly reduced in the asymptomatic stage of HIV infection but decreased with HIV progression and were markedly reduced in CDC category C patients, compared with those in control subjects and CDC category A and B patients (figure 1B and 1C). The proportion of CD4+ cells coexpressing CD45RO was comparable in patients and control subjects and did not change with HIV progression (figure 1D). The concentrations of CD4+CD28+, CD4+CD45RA+, and CD4+CD45RO+ cells were reduced in patients and continued to decrease with HIV progression (data not shown).

CD8+ phenotypes. In accordance with the results of previous studies the concentration of CD8+ cells was increased in the asymptomatic stage of HIV infection but decreased with HIV progression to a level below the control subjects in CDC C patients (figure 1E). The proportion of CD8+ cells coexpressing CD28 was markedly reduced in all stages of HIV infection and did not change with HIV progression (figure 1F). The proportion of CD8+ cells coexpressing CD45RA was not significantly different in patients and control subjects although these cells tended to decrease with HIV progression (figure 1G). The proportion of CD8+ cells coexpressing CD45RO was increased in the earlier stages of HIV infection but decreased slightly to a level equal to the control subjects in CDC C patients (figure 1H). As for the concentration of CD8+ cells, the concentrations of CD8+CD45RA+ and CD8+CD45RO+ cells were increased in the asymptomatic stage of HIV infection and decreased with HIV progression to levels below (CD45RA+) or equal to (CD45RO+) the control subjects in CDC C patients (data not shown). The concentration of CD8+CD28+ cells was unchanged in CDC A patients and decreased with HIV progression to a level below the control subjects in CDC C patients (data not shown).

Survival analysis. The Kaplan-Meier survival curves for high versus low concentrations of CD4+ and CD8+ cells showed, as expected, increased mortality in patients with low concentrations (figure 2A and 2F). Kaplan-Meier analysis also showed increased mortality in patients with low proportions of CD28+, CD45RA+, and CD45RO+ cells within the CD4+ cell subset (figure 2B–2D). Kaplan-Meier analysis of the CD8+ phenotypes showed increased mortality in patients with low proportions of CD45RA+ and CD45RO+ cells (figure 2G and 2H), whereas the Kaplan-Meier curves for the proportion of CD28+ cells within the CD8+ cell subset did not differ (figure 2F). The Kaplan-Meier survival curves for high versus low concentrations of CD4+ or CD8+ cells coexpressing CD28, CD45RA, or CD45RO all differed, with the highest mortality in patients with low concentrations (all P < .001; data not shown).

Univariate Cox regression analyses. When the proportions of the CD4+ and CD8+ phenotypes were fitted as continuous variables in univariate Cox analyses, reduced proportions of CD28+ and CD45RA+ cells within the CD4+ cell subset and reduced proportions of CD45RA+ and CD45RO+ cells within the CD8+ cell subset predicted increased mortality rates (table
Figure 1. Concentration of CD4+ and CD8+ T cells and proportions of cells coexpressing CD28, CD45RA, and CD45RO within the CD4+ and CD8+ T cell subsets in healthy individuals (△, n = 65) and Centers for Disease Control and Prevention (CDC)–categorized human immunodeficiency virus type 1 (HIV-1)–infected patients (●, n = 107). The concentration of CD4+ T cells (A), percentage of CD28+ of CD4+ T cells (B), percentage of CD45RA+ of CD4+ T cells (C), percentage of CD45RO+ of CD4+ T cells (D), concentration of CD8+ T cells (E), percentage of CD28+ of CD8+ T cells (F), percentage of CD45RA+ of CD8+ T cells (G), and percentage of CD45RO+ of CD8+ T cells (H) are shown for healthy individuals and CDC-categorized patients (CDC A [n = 37], CDC B [n = 45], and CDC C [n = 25]). The geometric mean values with 95% confidence intervals are shown together with P values for 1-way analysis of variance (ANOVA) and Tukey post-hoc tests.
Figure 2. Kaplan-Meier curves showing overall survival in human immunodeficiency virus (HIV) type 1–infected patients with high or low levels of CD4+ and CD8+ T cells and proportions of CD28+; CD45RA+; and CD45RO+ T cells. To generate high-level and low-level groups of equal size (n = 42), the patients were stratified according to the 50% percentile of the concentration of CD4+ or CD8+ T cells or the proportions of CD28+, CD45RA+ or CD45RO+ T cells within the CD4+ and CD8+ T cell subset. Follow-up times (days) were calculated from the date of enrollment to the date of death or censored on 1 May 1996, just before the first introduction of protease inhibitor–based antiretroviral therapy. Survival times for high-level and low-level groups are shown for the concentration of CD4+ T cells (A; median, 0.167 × 10^6 cells/L), percentage of CD28+ of CD4+ T cells (B; median, 65%), percentage of CD45RA+ of CD4+ T cells (C; median, 29%), percentage of CD45RO+ of CD4+ T cells (D; median, 38%), the concentration of CD8+ T cells (E; median, 0.510 × 10^6 cells/L), percentage of CD28+ of CD8+ T cells (F; median, 36%), percentage of CD45RA+ of CD8+ T cells (G; median, 45%), and percentage of CD45RO+ of CD8+ T cells (H; median, 28%). P values for the log-rank test are shown, as well as the total no. of subjects in each group and the no. who died or were censored.
The most important novel finding was that a reduction in the proportions of CD45RO+ cells within the CD8+ cell subset did not predict mortality in univariate Cox analyses (table 1). Reduced concentrations of CD4+ or CD8+ cells coexpressing CD28, CD45RA, or CD45RO all predicted increased mortality in univariate Cox analyses (all \( P < .0001 \); data not shown). Other independent prognostic variables were the concentration CD4+ cell counts and \( \beta_2 \)-microglobulin levels, whereas virus load did not predict mortality in the adjusted model (table 1).

### DISCUSSION

The present study evaluated different T cell phenotypes as markers of disease progression and as predictors of mortality. The most important novel finding was that a reduction in the proportion or concentration of CD4+ cells coexpressing CD28 independently predicts increased mortality in HIV-1–infected patients. Because several different multivariate Cox models were fitted in this study, the results should be interpreted with some caution due to the risk of introducing a type 1 error. Consequently, the prognostic value of CD28 coexpression on CD4+ cells needs to be confirmed in other studies. If these findings are confirmed in an independent cohort, they may have important implications both in the understanding of HIV pathogenesis and in the monitoring of HIV-infected patients.

Concerning HIV pathogenesis, this study reemphasizes that the HIV-associated CD4+ cell defect is not only quantitative, because low levels of CD4+CD28+ cells may be an additional marker of immune incompetence. This is supported by the finding that reduced levels of CD4+ and CD4+CD28+ cells result in the concentration of CD4+CD28+ cells coexpressing CD28, CD45RA, or CD45RO all predicted increased mortality in univariate Cox analyses (all \( P < .0001 \); data not shown). Other independent prognostic variables were the concentration CD4+ cell counts and \( \beta_2 \)-microglobulin levels, whereas virus load did not predict mortality in the adjusted model (table 1).

### Table 1. Cox univariate and multivariate regression analyses of survival.

<table>
<thead>
<tr>
<th>Percentage of CD4+ T cells</th>
<th>Percentage of CD8+ T cells</th>
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<tbody>
<tr>
<td>CD28( ^a )</td>
<td>CD45RO( ^a )</td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>( P )</td>
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<tr>
<td>Cox models</td>
<td></td>
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<tr>
<td>Univariate( ^b )</td>
<td>2.6 (1.8–3.8)</td>
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<tr>
<td>Multivariate( ^c )</td>
<td>1.6 (1.0–2.6)</td>
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<tr>
<td>CD4+ cells (10(^6) cells/L, 50% reduction)</td>
<td>1.6 (1.1–2.2)</td>
</tr>
<tr>
<td>Virus load (copies/ml, 10( ^{x} ) increase)</td>
<td>1.0 (0.5–1.8)</td>
</tr>
<tr>
<td>( \beta_2 )-microglobulin (( \mu )mol/L, 2( x ) increase)</td>
<td>3.3 (1.3–8.1)</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L, 1 mmol/L reduction)</td>
<td>1.3 (0.8–2.1)</td>
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<tr>
<td>CDC category( ^d ) A vs. B vs. C</td>
<td>1.5 (0.6–4.2)</td>
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**NOTE.** \( P \) values are shown for each of the 6 univariate and multivariate Cox models and are in boldface type for variables with \( P < .05 \). CDC, Centers for Disease Control and Prevention; CI, confidence intervals; HR, relative hazards or hazard ratio.

\( ^a \) HR associated with a 50% reduction in the proportion of each of the 6 different T cell phenotypes

\( ^b \) Univariate Cox models only included subjects who were included in the multivariate Cox models (\( n = 84 \))

\( ^c \) Multivariate Cox models showing the HRs are associated with a 50% reduction in the proportions of each of the different T cell phenotypes

\( ^d \) CDC criteria [26].

1. Reductions in the proportions of CD45RO+ cells within the CD4+ cell subset or reductions in the proportions of CD28+ cells within the CD8+ cell subset did not predict mortality in univariate Cox analyses (table 1). Reduced concentrations of CD4+ or CD8+ cells coexpressing CD28, CD45RA, or CD45RO all predicted increased mortality in univariate Cox analyses (all \( P < .0001 \); data not shown). Clinical and serological variables providing significant prognostic value in univariate Cox analyses were the concentration of CD4+ cells (HR, 1.7; 95% CI, 1.4–2.1; \( P < .0001 \); virus load (HR, 1.9; 95% CI, 1.1–3.5; \( P = .03 \)), \( \beta_2 \)-microglobulin (HR, 4.3; 95% CI, 2.0–9.1; \( P < .0001 \)), hemoglobin (HR, 2.4; 95% CI, 1.6–3.6; \( P < .0001 \)), and CDC category (HR, 6.8; 95% CI, 2.8–16.6; \( P < .0001 \)), whereas higher age and treatment with NRTIs at enrollment did not.

**Multivariate Cox regression analyses.** In the multivariate Cox model the concentration of CD4+ cells counts, virus load, \( \beta_2 \)-microglobulin and hemoglobin levels, and CDC category were fitted with the proportions of CD28+, CD45RA+, or CD45RO+ cells within the CD4+ or CD8+ cell subset. After this adjustment, a reduced proportion of CD28+ cells within the CD4+ cell subset still predicted increased mortality, whereas the other T cell phenotypes did not (table 1). Similar conclusions were reached when the concentrations of the different T cell phenotypes were analyzed in the multivariate Cox model. Thus, the concentration of CD4+CD28+ cells still provided independent prognostic information (HR, 1.6 [1.0–2.6]; \( P = .04 \)), whereas the other T cell phenotypes did not (data not shown). Other independent prognostic variables were the concentration CD4+ cell counts and \( \beta_2 \)-microglobulin levels, whereas virus load did not predict mortality in the adjusted model (table 1).
in comparable HRs in our multivariate Cox model. CD28 is a constitutively expressed transmembrane homodimeric glyco-protein on 95% of CD4+ cells and 50% of CD8+ cells [12, 28]. CD28-B7 interaction transduces intracellular signals that synergize with T cell receptor (TCR)-derived signals [12, 28] to enhance T cell proliferation and cytokine secretion, to sustain the T cell response and enhance long-term T cell survival, and to prevent anergy induction [12, 28]. Consequently, TCR engagement in the absence of CD28 ligation, results in either apoptosis or a state of anergy [13–15]. Besides the important role of CD28 costimulation in T cell activation it is not known whether the strong negative prognostic value of low levels of CD4+CD28+ cells is directly or indirectly associated with CD28 expression. A direct association could be explained by an impaired T cell response in CD4+CD28− cells resulting in accelerated loss of CD4+ cells and a poor clinical outcome. An indirect association could be explained by increased expression of activation markers (CD38, HLA-DR, or CD95) on CD28− cells [14, 29–31], which is associated with a poor clinical outcome in HIV−1−infected patients [19–25].

Because CD4+CD28+ cells independently predict mortality in HIV−1−infected patients, this study may suggest that CD4+CD28− measurements could be used to monitor patients, since the level of CD4+CD28− cells could have a potential value to assess when highly active antiretroviral therapy (HAART) should be initiated. However, since this study investigated a patient cohort with fairly progressed disease, it cannot be concluded from these data whether low levels of CD4+CD28− cells have the same negative prognostic value in patients with less progressed disease. Nor does this study indicate whether changes in CD28 expression during HAART are equally clinically relevant.

In accordance with the results of previous studies, the proportions of CD4+CD28− [1–4], CD4+CD45RA− [5–7, 9], and CD8+CD45RO− [9] cells decreased with HIV progression. The loss of quiescent CD4+ cells (CD45RA+ and CD28+) in advanced HIV infection seems paradoxical, because HIV preferentially infects activated CD4+ cells [32, 33]. However, it was recently proposed [34] that the HIV-induced chronic immune activation leads to a high outflow of naive T cells into the memory T cell compartment [35, 36]. Because the number of naive T cells recently emigrated from the thymus is markedly reduced in HIV infection [37, 38], the replenishing capacities for naive T cells are quickly exceeded [36, 38], resulting in depletion of naive T cells. It should be noted that the present study defined naive cells (CD45RA+) without using CD62L as an additional marker for true naive cells (CD45RA−CD62L−), which enables revertant cells (CD45RA−CD62L−) to be included in the naive cell population. Thus, the level of true naive cells in advanced HIV infection may have been even lower than that described in our study. The proportions of CD28+ cells in the control subjects in this study were lower than previously described elsewhere [12, 28], which could be caused by differences in laboratory procedures. However, one partial explanation also could be that the CD4+CD28+ and CD8+CD28+ cells were analyzed in lymphocyte gates without using CD3 to gate on T cells. Thus, inclusion of CD4+ or CD8+ non−T cells (monocytes and NK cells) in the lymphocyte gates may have reduced the proportion of CD28+ cells. Because the proportions of CD28+ T cells in the patients were within the expected ranges, according to the CD4+ T cell level [1], the relatively low proportion of CD28+ cells in the control subjects, however, did not affect the conclusions from the present study.

Some of the changes in the proportions of T cells coexpressing CD45RO or CD45RA across the clinical patient groups were notable. In apparent contrast with previous studies [5, 6, 9], this study found neither a selective reduction of CD4+CD45RO+ cells in the asymptomatic stage of HIV infection nor an increased proportion of CD4+CD45RO+ cells in patients with advanced HIV infection. These findings probably most reflect that the patient cohort had advanced HIV infection—even the asymptomatic CDC category A patients were immunologically fairly progressed, despite lack of clinical signs of HIV disease progression in their files. It was also notable that the proportions of CD4+ or CD8+ cells coexpressing CD45RA or CD45RO did not change in opposite directions but rather changed in the same directions with HIV progression. However, this phenomenon was previously described elsewhere [7, 39], which suggested that increased proportions of double negative (CD45RA−CD45RO−) or CD45RA+CD45RO− T cells account for this finding.

In univariate survival analysis, we demonstrated that, besides CD4+CD28+ cells, low proportions of CD4+CD45RA−, CD8+CD45RA−, and CD8+CD45RO− cells also predicted mortality in HIV−1−infected patients, whereas low proportions of CD4+CD45RO− and CD8+CD28+ cells did not. The finding that low levels of CD4+CD45RA−, but not CD8+CD45RO− cells, univariately predict increased mortality in HIV−1−infected patients has been described elsewhere [6], whereas the association between mortality and the proportion of CD8+CD45RA− and CD8+CD45RO− T cells has not. However, because no additional phenotype markers were used in the definition of cytotoxic T cell phenotypes (CD27 and CCR7) [39,40], the changes in the studied CD8− phenotypes and the association to mortality may be difficult to interpret. Cytotoxic T cells are, according to their phenotypes, described as naive [CD28+, CD45RA+, CD27+, and CCR7+], early [CD28−, CD45RA−(−), CD27+, and CCR7+], intermediate [CD28+−, CD45RA−(−), CD27+, and CCR7+], or late differentiated [CD28−, CD45RA−(−), CD27−, and CCR7−] [40]. Because CD45RA is reexpressed on late differentiated cells and intermediate− and late-differentiated cytotoxic T cells both lack CD28 coexpression, CD45RA or CD28 coexpression alone do not distinguish between cytotoxic T cell differentiation and functional characteristics [40]. Thus, the negative prognostic value

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associated with loss of CD8\(^+\)CD45RA\(^-\) cells may reflect loss of both early- and late-differentiated cytotoxic T cells. Furthermore, because both intermediate–(limited cytotoxicity) and late (highly cytotoxic)–differentiated cytotoxic T cells are CD28\(^-\), this may explain why changes in the proportion of CD8\(^-\)CD28\(^+\) cells did not predict mortality. However, the notable early reduction and the unchanged proportion of the CD8\(^-\)CD28\(^+\) cells during HIV progression also may explain why the CD8\(^-\)CD28\(^+\) cells did not predict mortality. Finally, the recent finding that late declines in the frequency of Gag/Pol tetramer-binding cells are associated with preferential loss of CD27\(^+\)CD45RO\(^-\) cells may explain the association between loss of CD8\(^-\)CD45RO\(^-\) cells and mortality [39].

In multivariate survival analysis, the CD45RA\(^-\) and CD45RO\(^-\) T cell phenotypes did not predict mortality. This finding emphasizes that analyzing CD45RA or CD45RO expression alone is of limited use for evaluating HIV progression. Besides low CD4\(^-\)CD28\(^-\) cell levels, reduced CD4\(^+\) cell count and increased serum levels of \(\beta_2\)-microglobulin also were independent predictors of increased mortality, whereas virus load was not. This finding probably reflects that this study investigated a patient cohort with progressed HIV disease, because the prognostic value of virus load is markedly reduced in advanced HIV disease [41–43]. In advanced HIV disease, the CD4\(^+\) cell counts and markers of immune activation (\(\beta_2\)-microglobulin, neopterin, soluble interleukin–2r, and hemoglobin) are better predictors of survival [41–44].

In summary, we found that a reduced proportion or concentration of CD4\(^+\) cells coexpressing CD28 were independent predictors of increased mortality after adjusting for CD4\(^+\) cells, virus load, \(\beta_2\)-microglobulin and hemoglobin levels, and CDC category. It is not known whether the poor clinical outcome associated with low levels of CD4\(^-\)CD28\(^+\) cells reflect an impaired T cell response in CD4\(^-\)CD28\(^+\) cells or a close association between lack of CD28 coexpression and high expression of immune activation markers. As expected, the concentration of all investigated T cell subsets decreased with HIV progression. However, concerning the proportions of cells, only CD4\(^+\) CD28\(^-\), CD4\(^+\)CD45RA\(^+\) and CD8\(^+\)CD45RO\(^-\) cells decreased with HIV progression. Low proportions of CD4\(^+\)CD45RA\(^-\), CD8\(^-\)CD45RA\(^-\), and CD8\(^+\)CD45RO\(^-\) cells predicted mortality only in univariate but not in multivariate Cox analyses. In conclusion, if our results are confirmed in other studies, coexpression of CD28 on CD4\(^+\) cells may be a useful marker to evaluate HIV progression.

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References

1. Choi BS, Park YK, Lee JS. The CD8/HLA-DR expressions on CD4\(^+\) T cells but not CD8\(^-\)T cells are significant predictors for progression to AIDS. Clin Exp Immunol 2002; 127:137–44.
8. Ramzouzi S, Jouen BF, Gilbert D, et al. During HIV infection, CD4\(^+\) CD38\(^+\) T-cells are the predominant circulating CD4\(^+\) subset whose HLA-DR positivity increases with disease progression and whose V beta repertoire is similar to that of CD4\(^+\) CD38\(^+\) T-cells. Clin Immunol Immunopathol 1995; 77:33–41.
20. Carbone J, Gil J, Benito JM et al. Increased levels of activated subsets
of CD4 T cells add to the prognostic value of low CD4 T cell counts in a cohort of HIV-infected drug users. AIDS 2000; 14:2823–9.