Survival and Proliferation of CD28\(^{-}\) T Cells During HIV-1 Infection Relate to the Amplitude of Viral Replication

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**Background.** CD28\(^{-}\) T lymphocytes progressively increase during aging, autoimmunity, and HIV-1 infection. Expansion of these cells stands in contrast with their senescent phenotype described by several studies. Understanding the functional properties and phenotype of CD28\(^{-}\) T cell during HIV-1 infection is important, because this subset incorporates T cells specific for HIV-1 and other chronic pathogens.

**Methods.** Blood samples were obtained from 23 healthy and 43 HIV-1–infected individuals: 26 receiving antiretroviral therapy and 17 naive to treatment. The phenotype of CD28\(^{-}\) and CD28\(^{+}\) T cells was determined by flow cytometry. T cells were activated through T-cell receptor before apoptosis and proliferation measurements. Interleukin (IL)–2, tumor-necrosis factor, interferon-\(\gamma\), and perforin production were analyzed using enzyme-linked immunosorbent assay.

**Results.** CD28\(^{-}\) T cells from patients receiving antiretroviral therapy exhibited a low sensitivity to apoptosis and enhanced proliferation after TCR stimulation, compared with T cells of uninfected individuals. On the contrary, CD28\(^{-}\) T cells from viremic patients showed a decreased Bcl-2 expression, a high sensitivity to apoptosis, and poor proliferative ability, compared with treated patients and control subjects. T cells from untreated patients produced less IL-2, possibly underlying their decreased proliferative abilities.

**Conclusions.** The level of HIV-1 replication and associated immunoactivation represent a critical factor in regulating survival and activation of CD28\(^{-}\) T cells.
conditions stands in contrast with the impaired proliferation of these cells suggested by previous reports [10, 12, 16].

T cell–based immunity against pathogens that are able to establish long-term infections, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), or HIV-1, may be largely maintained by chronically activated CD28+ T cells [1, 4, 19–22], and the functionality of these cells may have a strong impact on the level of immune control of these infections. Paradoxically, the increasing number of antigen-experienced T cells during HIV-1 infection, including the CD28+ T cells, coexists with immunodeficiency reflected by poor immune responses toward vaccine or recall antigens [23, 24] and impaired control of malignancies [25].

The present study aims at further understanding the regulation of survival and proliferative abilities of CD28− T cells, which are accumulated in chronic HIV-1 infection, compared with CD28+ T cells found in healthy individuals. We show that, although loss of CD28 expression is associated with acquisition of a senescent and pro-apoptotic phenotype, the fate of the CD28− T cells is strongly influenced by the presence of active viral replication. In fact, CD28− T cells from HIV-1–infected patients with high levels of viremia showed a high sensitivity to apoptosis and poor proliferative ability after activation. Of interest, the same population of CD28− T cells from patients receiving antiretroviral therapy (ART) exhibited a lower sensitivity to apoptosis and strongly proliferated after TCR triggering. The control of HIV-1 replication may thus improve immunity against persistent infections by converting the apoptosis-sensitive and weakly proliferating CD28− T cells to highly functional effector cells.

METHODS

Cell Cultures and Flow Cytometry
Blood samples were obtained from healthy donors (n = 23) and from HIV-1–infected patients (n = 43). Twenty-six patients were receiving combination therapy (mean treatment time, 5.5 years; mean CD4+ T-cell count, 521 cells/mm³ [range, 237–804 cells/mm³])3), with no detectable level of viremia except for 2 individuals (viral load, 49 and 634 copies/mL). Seventeen HIV-1–infected patients were naive to treatment (CD4+ T-cell count, 459 cells/mm³ [range, 227–879 cells/mm³]; mean viral load, 41,682 copies/mL [range, 1230–338,000 copies/mL]). The ethical committee at Karolinska Institutet approved the study, and informed consent was obtained from all individuals included.

Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll gradient centrifugation (Lymphoprep). T lymphocytes were separated using the Pan T cell Isolation Kit (Miltenyi Biotech). Separation of CD28+ and CD28− T cells was done using CD28 PE and anti-PE MicroBeads (Miltenyi). Cells were cultured in RPMI-1640 containing l-glutamine, 10% FCS, and antibiotics. For flow cytometric measurements, the following fluorochrome-conjugated antibodies were used: Amcyan anti-CD3, Alexa-Fluor700 anti-CD8, FITC anti-CD57 and Annexin V, PE anti-CD127 (IL-7Rα), anti-Fas (CD95), anti-CD25, anti-Bcl-2, APC anti-CD28, PE-Cy7 anti-CCR7, PE anti-PD-1 (Clone EH12.1), and FITC anti-CD27 (all from BD Pharmingen). PerCP-Cy5.5 anti-CD45RA, and PE-Texas Red-conjugated anti-CD4 were from eBioscience and AbCam. FITC anti-Ki67 was from Dako. Quantification of relative telomere length (RTL) was performed on purified CD28+ and CD28− T cells using Telomere PNA Kit/FITC (Dako). Fluorescence intensities were measured with FACS LSR II (Becton Dickinson), and data were analyzed using FlowJo, version 8.4.4 (Tree Star).

T-Cell Apoptosis and Proliferation
T cells were cultured at a density of 0.5 x 10⁶ cells/mL in 96-well plates coated with anti-CD3 antibody (clone UCHT1; BD Pharmingen) at concentrations of 1 and 10 μg/mL. At 24 h, apoptosis was assessed by Annexin V-binding. T-cell proliferation was studied using carboxyfluorescein diacetate succinimidyld ester (CFSE) labeling according to the manufacturer (Invitrogen) after 4 days of culture.

Measurement of Cytokines and Perforin Production
Interleukin (IL)–2, tumor necrosis factor (TNF), and interferon (IFN)–γ production was analyzed in culture supernatants with use of enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen). Intracellular staining was performed on T cells activated with precoated anti-CD3 (10 μg/mL) for 72 h with the last 6 h in the presence of 50 ng/mL of PMA and 1μM ionomycin (Sigma), using FITC-labeled anti-IL-2 antibodies. Perforin production was assessed using an ELISA kit from MabTech.

Statistical Analysis
For the phenotype characterization, apoptosis, and proliferation assays, statistical analyses were performed using 2-way analysis of variance (ANOVA), followed by Bonferroni posttests. RTL comparison and Fas co-stimulation effects were assessed using Wilcoxon sign-rank test. All statistical tests were performed using Prism (GraphPad Software).

RESULTS

The CD28− T-Cell Population Comprises Highly Differentiated Effector Cells
Chronic HIV-1 infection is characterized by increased ration of T cells with effector or memory phenotype [1–3]. Accordingly, we detected a higher frequency of CD28− T cells in ART-treated or untreated HIV-1–infected patients, compared with control subjects, with the higher frequency among CD8− T cells in all 3 groups (Figure 1A, B). Furthermore, on the basis of CD45RA and CCR7 expression [26], we found that CD28− T cells mostly comprised highly differentiated CD45RA−CCR7− (effector
memory) and CD45RA+CCR7- (effector effector) T cells in all groups, whereas the CD45RA+CCR7- phenotype was rare among CD28+ T cells (Figure 1C, D). The TNF receptor family member CD27 is a differentiation marker that is present in naive and typical memory T cells but progressively downregulated in parallel with the acquisition of an effector phenotype [26–28]. This marker was expressed at low levels on CD28+ T cells (Figure 1E), whereas its expression was consistently high in CD28+ T cells. Of note, there was no difference between HIV-1–infected patients and control subjects for those markers, suggesting that CD28+ T cells that accumulate in HIV-1–infected patients display a similar phenotype of differentiated effector cells to that found in healthy individuals.

CD28- T Cells From Healthy and HIV–1–Infected Individuals Display a Senescent Phenotype and Altered Apoptosis Regulation

In view of previous findings showing impaired proliferative responses and altered regulation of apoptosis among CD28- T cells [5, 29, 30], we compared the expression of markers associated with senescence and survival regulation on CD28- T cells from healthy individuals and HIV–1–infected patients, ART treated or naive.

In agreement with previous reports [10, 31], CD28- T cells presented significantly shorter telomeres than does the CD28+ subpopulation (Figure 2A). The expression of CD57, a marker of senescence, was detected at higher levels on CD28+ T cells, compared with CD28+ T cells, in all 3 groups (Figure 2B). The expression of another CD28 family member, the programmed death-1 (PD-1) molecule, implicated in T-cell exhaustion during HIV–1 infection [7], was also significantly increased in CD28+ T cells, compared with CD28- T cells (Figure 2C). Of interest, we found that PD-1 expression was higher in CD28+ T cells from HIV–1–infected patients naive to treatment, compared with cells from ART-treated patients, consistent with the reported association between PD-1 expression on HIV–1–specific T cells and viral load [32].

Conversely, the nuclear antigen Ki67, a marker specific for cell cycling, was present at significantly higher levels in CD28+ T cells, compared with CD28- T cells, with the highest percentages observed among CD28+ T cells from untreated HIV–1–infected patients (Figure 2D).

The death receptor Fas was expressed at very high levels (>90%) on CD28- T cells without differences between HIV–1–infected patients and control subjects (Figure 2E). We found a higher percentage of Annexin V-binding cells among the CD28- T cells isolated from HIV–1–infected patients, compared with control subjects (Figure 2F), especially on T cells from untreated patients. The percentage of cells expressing low levels of the anti-apoptotic molecule Bcl-2 was significantly higher in

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**Figure 1.** Differentiation stage of CD28+ T cells. T cells from healthy controls (n = 10) and HIV–1–infected patients with (n = 7) or without ART treatment (n = 10) were assessed by multicolour flow cytometry. Percentages of CD28+ among total T cells (A), CD4+ and CD8+ T cells (B) are determined. In addition effector and memory subsets were measured among the CD28+ (C) and CD28- (D) T-cell populations using CD45RA and CCR7 markers. CD27 expression (E) was measured on CD28+ and CD28- T-cell subsets. Data expressed in percentages represent median with 25th–75th interquartile range with means displayed as +, except for (C–D) where data represent mean ± SD. In panels B and E the statistical analyses between CD4+ and CD8+ T cells or CD28+ and CD28- T cells (for all included subjects, both patients and control individuals) were performed by two-way ANOVA, whereas the analysis between individual groups was calculated by Bonferroni post-tests. *P < .05; **P < .01; ***P < .001.
CD28− T cells, compared with CD28+ T cells (Figure 2G), with the highest percentage found on CD28− T cells from untreated patients (>50%). The receptor for IL-7, a survival factor for peripheral T cells, was expressed at lower levels in the CD28− subpopulation, compared with their CD28+ counterparts, without differences between cohorts (Figure 2H).
Taken together, our results indicate that CD28− T cells from HIV-1–infected and healthy individuals display a similar senescent and pro-apoptotic phenotype; however, a major difference was observed for Bcl-2 expression.

**Apoptosis Sensitivity and Proliferation of CD28− T Lymphocytes**

CD28− T cells displayed a senescent phenotype with a potentially impaired proliferative ability and altered regulation of survival in both HIV-1–infected and uninfected individuals (Figures 1–2). Therefore, we analyzed activation-induced apoptosis and proliferation of CD28− T cells isolated from naive or ART-treated patients and control subjects. Levels of spontaneous apoptosis were higher for T cells isolated from viremic HIV-1–infected patients, particularly in case of CD28+ T cells (Figure 3A), which after TCR stimulation, also showed a higher sensitivity to apoptosis (P < .001). In the case of ART-treated patients, CD28+ T cells displayed low levels of apoptosis, although still at rates higher than among uninfected donors. CD28+ T cells from all 3 groups responded with low levels of activation-induced apoptosis.

Next, we evaluated the levels of proliferation under the same conditions (Figure 3B). Surprisingly, CD28− T cells proliferated more than did their CD28+ counterparts in all groups of donors. The CD28− subset from ART-treated patients showed the strongest proliferation after TCR triggering. Of note, when suboptimal concentration of anti-CD3 was used, CD28− T cells from the majority of ART-treated donors maintained high level of proliferation, whereas only weak or no proliferation was detected in case of CD28+ T cells from untreated patients or control subjects. When analyzing survival and proliferation in the CD4+ or CD8+ T-cell subsets, we found that the differential effect of TCR triggering on CD28− T cells from treated and untreated patients occurred irrespective of whether the T cells were CD4+ or CD8+ (Figure 3C, D).

**Apoptosis of CD28− T Cells Correlates With Viral Load During HIV-1 Infection**

A significant correlation was found between the levels of spontaneous and activation-induced apoptosis of CD28− T cells and viral loads (P < .05, Figure 4). On the contrary, the levels of CD28+ T-cell apoptosis were not associated with viral loads. Our results indicate that the level of HIV-1 replication strongly determines whether CD28− T cells undergo apoptosis or proliferate upon TCR-mediated activation.

**Fas-Mediated Apoptotic and Proliferative Signals on CD28− T Lymphocytes**

The Fas receptor has been extensively implicated in the progressive depletion of T cells occurring through apoptosis during HIV infection [33–35]. In addition, we previously showed that Fas molecules can transmit strong proliferative signals to weakly activated T cells isolated from HIV-1–infected individuals [36]. Because CD28− T cells are characterized by a higher Fas expression, compared with CD28+ cells (Figure 2E), we studied whether HIV-1 viremia may influence the balance between apoptotic and stimulatory Fas signals on CD28− T cells. Cross-linking of the Fas molecules on suboptimally activated T cells had a marginal effect on Fas signals on CD28− T cells; however, T cells both CD28+ and CD28− benefited from Fas costimulatory signals independently of HIV-1 infection or the introduction of ART (Figure S1B), suggesting that viremia has no major influence on the proliferative role of Fas on T cells.

**Decreased CD28− T-Cell Proliferation Is Associated With Decreased IL-2 Production in Viremic Patients**

We analyzed whether the differential ability of CD28− T cells of viremic and aviremic patients to proliferate after TCR triggering could result from differences in IL-2 production or expression of the high affinity IL-2 receptor (CD25). In line with their enhanced proliferative ability, T cells isolated from ART-treated patients produced the highest levels of IL-2 after activation with anti-CD3 antibodies (Figure 5A). To discriminate the source of IL-2 production among the activated T-cell subpopulations, we measured intracellular IL-2 and found that CD28+ T cells were the main source of IL-2 (Figure 5B). The higher levels of cytokine production were found among persons receiving ART (n = 5; mean ± standard deviation [SD], 80.98% ± 17.97%), compared with viremic patients (n = 5; mean ± SD, 44.8% ± 9.27%; P < 0.001) and control individuals (n = 5; mean ± SD, 37.71% ± 17.97%; P < 0.001).

CD28− T cells up-regulated CD25 expression after activation with similar kinetics in ART-treated or untreated patients, reaching a comparably high level by day 3 that exceeded CD25 expression observed in control individuals (Figure 5C). On the contrary, CD25 induction among activated CD28+ T cells was significantly higher in patients receiving ART, compared with untreated patients or aviremic individuals. On the other hand, the sensitivity of CD28+ T cells to IL-2 may be similar in both viremic and nonviremic patients, as indicated by their comparable CD25 expression.

**T Cells Preserve Effector Functions Despite Impaired Regulation of Survival and Proliferation**

To further evaluate the effector functions of T cells from the 3 groups, we measured TNF, IFN-γ, and perforin levels in response to TCR activation (Figure 6). Compared with control subjects, total T cells from HIV-1–infected individuals produced higher levels of both cytokines and perforin. Of note, TNF, IFN-γ, and perforin were mainly produced by CD28− T cells after TCR triggering, with no difference between the groups of patients and control subjects. These results indicate that some of the effector functions of CD28− T cells may be preserved independently of the level of viremia during HIV-1 infection.
It is widely assumed that T-cell responses are compromised in HIV-1–infected patients and that several T cell–inhibitory mechanisms have been associated with the disease, acting mostly on HIV-1–specific T cells [7, 37]. Impaired development and maintenance of an antigen-specific T-cell repertoire against HIV-1 during the course of infection may represent a major obstacle for efficient therapies that aim at targeting and improving T-cell responses.

A differential expression of CD28 was reported among T cells of different specificities: very low in HIV-1 specific or CMV-specific T cells and high in EBV-specific T cells and hepatitis C virus–specific T cells [4]. Of interest, the expression of another CD28 family member, the PD-1 molecule, strongly implicated in the blockade of T-cell proliferation in HIV-1–infected individuals, also showed a virus-specific expression pattern. In fact, HIV-1–specific T cells were characterized by high PD-1 expression, EBV-specific T cells contained a mixture of PD-1 positive and PD-1 negative T cells, and CMV-specific T cells were mostly PD-1 negative [38], further indicating that the phenotypic and functional differentiation of pathogen-specific T cells may be strongly influenced by the properties of the pathogen during chronic infections.

Plasma viral load has been found to be a major factor determining functional abilities of HIV-1–specific CD8+ T cells and, in parallel, PD-1 and CD28 expression levels [32, 38]. Although the inhibitory receptors PD-1 and CTLA-4 have been
implicated in decreased functionality of HIV-1–specific T cells [7], other markers associated with impaired proliferation or altered apoptosis sensitivity, such as down-regulation of CD28 or increased CD57 expression, characterize a very high proportion, often the majority, of peripheral T cells [1, 5, 9, 10, 39]. CD57 expression was suggested as a better marker for T-cell senescence in HIV-1–infected individuals than the loss of CD28 [39]; CD28 down-regulation and CD57 expression, however, widely overlapped at the cellular level. The increased apoptosis sensitivity of T cells described in HIV-1–infected individuals [18] stands in contrast with the apoptosis resistance proposed for CD28− T cells [15, 17, 40]. Similarly, the replicative senescence associated with the CD28− subset is in contrast with their increased prevalence in circulation [12, 16]. In addition, reports on the apoptosis sensitivity of CD57+CD8+ T cells from HIV-1–infected patients are also contradictory [39, 41]. These opposing data may suggest that although a very high number of antigen-experienced T cells acquire markers associated with decreased functionality, the regulation of their survival and the ability of these cells to proliferate in response to TCR stimulation may not be simply determined by their differentiation stage. In fact, we hypothesized that CD28− T lymphocytes, representing an important source for memory responses in chronic infections, may function differently in uninfected individuals and in HIV-1–infected patients with different levels of viral replication and the associated inflammatory conditions. Consistent with previous reports [10, 39], we found that CD28− T cells isolated from either HIV-1–infected or uninfected individuals displayed features of senescence, such as shortened telomeres and high CD57 expression, compared with their CD28+ counterparts. In addition, the PD-1 molecule, implicated in T-cell exhaustion during HIV-1 infection [7], was increased in CD28− T cells of HIV-1–infected and control individuals, compared with CD28+ T cells, with the highest rate among CD28− T cells from untreated HIV-1–infected patients. These findings may explain the low rates of proliferation observed among CD28− T cells from untreated patients.

On the other hand, in contrast to the reported apoptosis resistance of CD28− T cells [15, 17], we observed that CD28− T cells displayed a pro-apoptotic phenotype, with very high levels of Fas expression and lower levels of IL-7Rα and Bcl-2 in comparison with CD28+ T cells in both HIV-1–infected individuals and control subjects. The lowest Bcl-2 expression was found among CD28− T cells from untreated patients, indicating a decreased survival ability of these cells in viremic conditions. Indeed, the pro-apoptotic phenotype found in CD28− T cells translated into an increased sensitivity to spontaneous and activation-induced apoptosis only in the case of HIV-1–infected patients naive to treatment. Because few T cells are infected in relation to the high numbers of CD28− T cells, these cells are likely to be affected by indirect consequences of HIV-1 replication, including the exacerbated immune activation. We found that CD28− T cells, despite a senescent phenotype, retain the capacity to proliferate after TCR triggering. These results differ from previous studies showing that, compared with the CD28+ counterparts, CD28− T cells proliferated weakly in response to TCR triggering [12, 16]. Of interest, CD28− T cells from HIV-1–infected patients who received ART had an enhanced proliferation in response to TCR signaling, compared with untreated patients and control subjects.

Here, we show that the ability of T cells to produce IL-2 after activation was decreased in untreated patients. Because CD25 expression, a prerequisite for high affinity IL-2 binding, was induced similarly on activated CD28− T cells of HIV-1–infected individuals with and without therapy, the higher production of IL-2 in patients receiving ART may underlie the increased CD28− T-cell proliferation in these individuals. CD28− T cells from patients naive to treatment maintained
effector functions, as shown by their similar capacity to secrete TNF, IFN-γ, and perforin after TCR triggering. Although TNF was produced similarly by activated CD28− T cells in all 3 cohorts, the sensitivity of T cells to this cytokine may vary according to the level of immunoactivation [42]. In combination with the low Bcl-2 expression that we detected in CD28− T cells of viremic patients, TNF may contribute to apoptosis [42]; TNF may also counteract the increased IL-2 production that we found in patients receiving ART [43].

The differential sensitivity of CD28− T cells to apoptotic and proliferative signals indicates a different origin of these cells in viremic and nonviremic conditions. As suggested by our results on differential expression of Ki67 expression on CD28− T cells (lower) and CD28+ T cells (higher), particularly among untreated patients, it is likely that CD28− T cells arise continuously from the activation of CD28+ T cells in the presence of viremic conditions. Alternatively, the high proliferative ability of CD28− T cells in patients receiving ART suggests that accumulation of these cells might be the result of antigen-specific expansions coupled with a better preservation of the expanded clones. Whether persisting infections provide continuous antigenic stimuli to CD28− T cells or these cells obtain survival strategies that are independent of the original antigens is a question yet to be studied [44].

CD28− T-cell expansion mostly involving CMV-specific T-cell clones has been associated with decreased life expectancy in older individuals. Such observation raises the question of whether CD28− T-cell expansions may contribute to immunodeficiency because of the impaired functionality of a high number of effector T cells, many of them specific for pathogens that establish persistent infections. On the other hand, our results showed that the expanded CD28− T-cell population can include potent antigen-specific T cells with the ability to proliferate and effector functions provided that HIV-1 replication is controlled by ART. In favor of this scenario is the improved response to vaccination observed in patients receiving ART [23]. Thus, the increased ratio of peripheral CD28− T cells may not obviously lead to a compromised immunity against pathogens, because the functionality of these cells is influenced by HIV-1 replication rather than their stage of differentiation.

High levels of HIV-1 replication and the associated generalized immune activation induce a pronounced susceptibility of CD28− T cells to apoptosis, which in turn, may contribute to the impaired immune responses exhibited by HIV-1–infected individuals. Therefore, therapeutic approaches considering early ART intervention, together with therapy that directly limits immune activation [45], might prevent the accumulation of dysfunctional CD28− T cells observed during HIV-1 infection.
Figure 6. Production of TNF, INF-γ and perforin by T Cells isolated from HIV-1–infected patients and from noninfected individuals. Total T cells (left panels), CD28+ (middle panels) or CD28- T cells (right panels) were isolated from treatment naive HIV-1 infected patients (n = 5), from patients undergoing ART (n = 5) as well as from noninfected individuals (n = 5) and cultured in the presence of anti-CD3 antibodies used at the indicated concentrations. Concentrations of TNF (upper panels) and perforin (lower panels) were measured by ELISA in the culture supernatants after 24 hours. INF-γ concentrations (middle panels) were assessed after 4 days.

Supplementary Data

Supplementary data are available at http://jid.oxfordjournals.org online.

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