CD4⁺CD28null T lymphocytes resemble CD8⁺CD28null T lymphocytes in their responses to IL-15 and IL-21 in HIV-infected patients

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ABSTRACT

HIV-infected individuals suffer from accelerated immunologic aging. One of the most prominent changes during T lymphocyte aging is the accumulation of CD28null T lymphocytes, mainly CD8⁺ but also CD4⁺ T lymphocytes. Enhancing the functional properties of these cells may be important because they provide antigen-specific defense against chronic infections. The objective of this study was to compare the responses of CD4⁺CD28null and CD8⁺CD28null T lymphocytes from HIV-infected patients to the immunomodulatory effects of cytokines IL-15 and IL-21. We quantified the frequencies of CD4⁺CD28null and CD8⁺CD28null T lymphocytes in peripheral blood from 110 consecutive, HIV-infected patients and 25 healthy controls. Patients showed increased frequencies of CD4⁺CD28null and CD8⁺CD28null. Both subsets were positively correlated to each other and showed an inverse correlation with the absolute counts of CD4⁺ T lymphocytes. Higher frequencies of HIV-specific and CMV-specific cells were found in CD28null than in CD28⁺ T lymphocytes. Activation of STAT5 by IL-15 and STAT3 by IL-21 was higher in CD28null compared with CD28⁺ T lymphocytes. Proliferation, expression of CD69, and IFN-γ production in CD28null T lymphocytes were increased after treatment with IL-15, and IL-21 potentiated most of those effects. Nevertheless, IL-21 alone reduced IFN-γ production in response to anti-CD3 stimulation but increased CD28 expression, even counteracting the inhibitory effect of IL-15. Intracytoplasmatic stores of granzyme B and perforin were increased by IL-15, whereas IL-21 and simultaneous treatment with the 2 cytokines also significantly enhanced degranulation in CD4⁺CD28null and CD8⁺CD28null T lymphocytes. IL-15 and IL-21 could have a role in enhancing the effector response of CD28null T lymphocytes against their specific chronic antigens in HIV-infected patients. J. Leukoc. Biol. 98: 373–384; 2015.

Introduction

Aging is associated with phenotypic and functional alterations in the immune system, which particularly affect the T lymphocyte compartment. These changes, associated with morbidity and mortality, are often referred to as immunosenescence. Increasing evidence suggests that HIV-infected individuals experience similar immunologic changes as uninfected elderly persons [1, 2]. Therefore, HIV-infected individuals suffer from accelerated aging, probably from immune activation and inflammation from chronic infection [3–5]. The persistent infection leads to constant stimulation of the immune system. HIV-infected individuals also lose CD4⁺ T lymphocytes by activation-induced cell death. On the other hand, thymus involution reduces the capacity to produce new cells, resulting in a reduction of circulating, naive T lymphocytes [6]. These events lead to impaired T lymphocyte homeostasis, resulting in progressive accumulation of highly differentiated T lymphocytes, one of the hallmarks of early aging in HIV infection [7].

The increase in the proportion of highly differentiated T lymphocytes limits T lymphocyte diversity, altering the overall immune response to pathogens by their reduced susceptibility to apoptosis and their oligoclonal expansions against chronic infection [8]. Loss of CD28 expression in highly differentiated T lymphocytes is a key predictor of immune incompetence in the elderly and HIV-infected individuals [9]. These subpopulations are thought to show the propensity to secrete proinflammatory cytokines, such as TNF-α and IFN-γ, and to lose the capacity to produce IL-2 [10]. Moreover, they showed a decline in proliferative capacity associated with short telomere length and partial activation, and even an anergic state by the absence of costimulatory signal during immune activation. Other characteristics of CD28null T lymphocytes are an altered capacity to help B cell proliferation and antibody production, thus affecting vaccine responses [11]. Nevertheless, these CD28null cells are able to be activated, maybe through the expression of...
several NKR-s. Among NKG2 receptors, only NKG2D has been shown to express in CD4+CD28null-aged T lymphocytes. In this way, augmented costimulation through NKG2D is effective in rescuing CD4-unhelped CD8+ and CD28null T lymphocytes from their pathophysiological fate [12, 13].

Despite the accumulation of CD28null T lymphocytes being more marked within the CD8 subset, the increase of the percentage of CD4+ T lymphocytes that lack CD28 expression is common in elderly persons and in patients with chronic infections and autoimmune diseases [14–17]. CD4+CD28null T lymphocytes are phenotypically and functionally distinct from CD4+CD28+ T lymphocytes but exhibit similarities with CD8+CD28null T lymphocytes, for example, in relation to activation and cytotoxic function [18]. In HIV-infected patients, high levels of CD4+ and CD8+ T lymphocytes lacking expression of CD28 have been reported [19, 20]. Low levels of CD4+CD28+ T lymphocytes, but not CD8+CD28+ T lymphocytes, are independent predictors for progression to AIDS and of high mortality in HIV-infected patients, whereas effective antiretroviral therapy reduces the proportion of those subsets [21–23]. Moreover, nadir CD4+ T lymphocyte count and numbers of CD4+CD28null T lymphocytes predict functional responses to immunizations in chronic HIV-1 infection [24].

Generation of highly differentiated T lymphocytes is dependent on antigen stimulation, but their survival is antigen-independent and requires peripherally produced cytokines, particularly those that use the common γ-chain for signaling, such as IL-15 [25–27]. IL-15 not only stimulates the proliferation of CD8+CD28null and CD4+CD28null T lymphocytes, but also promotes both their effector function and their cytotoxic properties [28–30]. Meanwhile, IL-21 could be useful for up-regulating the cytotoxic effector function of CD8+ T lymphocytes without inducing HIV-1 replication in vitro [29, 31]. This fact makes it very interesting as a therapeutic option for HIV-infected patients.

As the accumulation of CD28null T lymphocyte is one of the most prominent changes in HIV-infected individuals, enhancing the functional properties of these cells with the above-described cytokines may be important as they provide antigen-specific defense against chronic infections. Therefore, our objectives were to evaluate the effects of IL-15 and IL-21 on CD4+CD28null and CD8+CD28null T lymphocytes and verify if the 2 CD28null T lymphocyte subsets showed similar patterns in response to these activating cytokines.

MATERIALS AND METHODS

Study subjects
Blood samples were obtained from 110 consecutive patients with documented HIV infection (30 females) followed at the Infectious Diseases Unit of Hospital Universitario Central de Asturias (Oviedo, Spain). Samples from 25 healthy controls (7 females), matched in age, were obtained from the Centro de Transfusiones del Principado de Asturias (Oviedo, Spain). Informed consent was obtained from patients and controls before participating in the study. The study was approved by the Hospital Central de Asturias ethics committee. The individuals were selected in agreement with the following criteria: age ≤65 yr, absolute values of CD4+ T lymphocytes >150 cells/μl; HIV RNA load <50 copies/ml, and HAART based on medical criteria and patient preferences. The mean age was 45.9 ± 9.5 yr (range 24–64 yr) for the 110 HIV-infected individuals and 43.6 ± 5.7 yr (range 25–62 yr) for the healthy control subjects. The different experiments to analyze the effects of IL-15 and IL-21 cytokines on CD28null T lymphocytes were performed with those HIV-infected individuals whose percentage of CD4+CD28null T lymphocytes was >10%.

Quantification of CD4+CD28null and CD8+CD28null T lymphocytes
The percentages of CD4+CD28null and CD8+CD28null T lymphocytes were determined in peripheral blood from the participants by staining with anti-CD3 (FITC), anti-CD28 (PE) (eBioscience, San Diego, CA, USA), anti-CD8 (PerCP), and anti-CD4 (APC) (Immunostep, Salamanca, Spain). One hundred microliters of whole blood was stained with the combination of labeled mAbs for 30 min at room temperature. Red blood cells were lysed with FACS Lysing Solution (BD Biosciences, San Jose, CA, USA), washed in PBS, and analyzed with CellQuest software in the FACSCalibur Cytometer (BD Biosciences). Appropriate isotype-control mAbs were used for marker settings. CaliBRITE Beads (BD Biosciences) were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity. BD Multisec Check Control and Multisec Check CD4 Low Control were used as quality controls.

Isolation of PBMCs and cell culture
Peripheral blood mononuclear cells were isolated from peripheral blood anticoagulated with EDTA by centrifugation on Ficoll-Hypaque gradients (Lymphoprep, Nycomed, Oslo, Norway). Cultures were performed in RPMI 1640 medium containing 2 × 10−3 M l-glutamine and HEPES (BioWhitaker, Verviers, Belgium) and supplemented with 10% FCS (ICN Flow, Costa Mesa, CA, USA) and antibiotics. Cells were incubated at 37°C under 5% carbon dioxide.

Antigen-specific stimulation
Antigen-specific responses were analyzed in PBMCs from HIV-seropositive individuals. CMV-infected cell lysate was prepared as previously described [14] by infecting human embryonic lung fibroblasts with the AD169 strain of CMV. Viral titers in the supernatant were determined by standard plaque assays. The virus was inactivated by repeated freeze-thaw cycles. PBMCs were stimulated with 15-mer peptides overlapping by 11 amino acids and spanning the entire gag protein of HIV (10 μg/ml) (JPT Peptide Technology, Berlin, Germany) and with CMV (105 PFU/ml) in 15 ml conical polypropylene tubes in a humidified 37°C incubator for 18 h. Activation was assessed by surface staining with anti-CD28 (FITC) (eBioscience). The cells were also stained with anti-CD8 (PE), anti-CD3 (PerCP), and anti-CD4 or anti-CD8 (APC). red blood cell samples were lysed with FACS lysing solution, washed in PBS, and analyzed with CellQuest software.

To quantify IFN-γ production, PBMCs (4 × 106 cells/ml) were cultured in medium with CMV (104 PFU/ml) and with gag peptides for 18 h. Cultures were treated after the first 2 h with the secretion-inhibitor breklikin A (10 μg/ml) (Cambiochem, Darmstadt, Germany). After 16 additional hours, cells were fixed using BD Fixation Buffer (10 min at 37°C), stained for 37°C incubator for 18 h. Activation was assessed by surface staining with anti-CD28 (FITC) (eBioscience). The cells were also stained with anti-CD8 (PE), anti-CD3 (PerCP), and anti-CD4 or anti-CD8 (APC), and with 2 mM EDTA for 15 min at room temperature, washed, and stained with antibodies against anti-CD28 (PE), anti-CD3 (PerCP), and anti-CD4 (APC) at 4°C and then stained with anti-IFN-γ (BD Biosciences) for 30 min at room temperature. Cells were washed and resuspended in 1% paraformaldehyde until FACS analysis.

STAT3 and STAT5 quantification
To isolate CD4+ and CD8+ T cells negatively, cells were separated by centrifugation on Ficoll-Hypaque gradients after 20 min of incubation with the RosetteSep Human CD8+ or CD4+ T cell Enrichment Cocktail (STEMCell Technologies, Gnebnoile, France). These isolated CD4+ and CD8+ T lymphocytes were stimulated with IL-15 (50 ng/ml) (PeproTech Inc., Rocky Hill, NJ, USA), IL-21 (50 ng/ml) (PeproTech), or both recombinant proteins for 5 min at 37°C. Cells were fixed using BD Cytofix Fixation Buffer (10 min at 37°C), stained on the surface with anti-CD28 (PE), and permeabilized in BD.
Phosflow Perm Buffer III (30 min on ice). They were washed twice in BD Pharmingen Stain Buffer and stained with 20 μl Alexa Fluor 488-conjugated phospho-STAT5 (BD Biosciences) or Alexa Fluor 647-conjugated phospho-STAT3 antibody (BD Biosciences) for 1 h at room temperature. The cells were analyzed on a BD FACScalibur flow cytometer.

**Proliferation assay**

PBMCs were resuspended in PBS at a final concentration of 5–10 × 10⁶ cells/ml and incubated with 1.5 μM CFSE (Invitrogen, Paisley, Scotland, UK) for 10 min at 37°C, and washed with RPMI 1640 medium containing 2 × 10⁻³ M l-glutamine and HEPES twice. Resting cells and those stimulated with anti-CD5 (1 μg/ml) (eBioscience) were then cultured at 2 × 10⁶ cells/ml in the presence of human recombinant IL-15 (50 ng/ml), IL-21 (50 ng/ml), or a combination of both cytokines. The proliferative responses of CD4⁺ and CD8⁺ T lymphocytes were analyzed on day 7 after staining with anti-CD28 (PE), anti-CD8 (PerCP), and anti-CD4 (APC). The cells were analyzed on a BD FACScalibur flow cytometer.

**Stimulation with IL-15 and IL-21**

PBMCs (4 × 10⁶ cells/ml), resting and activated by anti-CD3 (1 μg/ml), were cultured in the presence and absence of IL-15 (50 ng/ml), IL-21 (50 ng/ml), and a combination of both cytokines for different times. To analyze the activated phenotype, cells were cultured for 18 h and then stained with anti-CD69 (FITC), anti-CD28 (PE), anti-CD3 (PerCP), and anti-CD4 or anti-CD8 (APC). Frequencies of cells with intracytoplasmic stores of granzyme B, CD69 (FITC), anti-CD28 (PE), anti-CD3 (PerCP), and anti-CD4 (APC). The cells were analyzed on a BD FACScalibur flow cytometer.

**Lysosomal degranulation assay**

CD107a LAMP-1 expression was used to measure CD4⁺CD28null and CD8⁺CD28null T lymphocyte degranulation. PBMCs were incubated for 5 h in medium or stimulated with anti-CD5 (1 μg/ml) in the absence or presence of IL-15 (50 ng/ml), IL-21 (50 ng/ml), and IL-15 plus IL-21. After the first hour of culture, monensin (2 μM) (eBioscience), a protector of cell degranulation, was added. Cells were then stained with anti-CD28 (FITC), anti-CD8 (PE), anti-CD4 (PerCP), and anti-CD107a antibodies (eBioscience). All samples were analyzed with CellQuest software in the FACScalibur Cytometer.

**Statistical analysis**

Frequencies of CD4⁺CD28null and CD8⁺CD28null T lymphocytes were compared using the nonparametric Wilcoxon signed-rank test when data were not normally distributed, or with Student’s t test for paired data. Analyses were performed using the SPSS 15.0 statistical software package (SPSS Inc., Chicago, IL, USA). P values of 0.05 or less were considered significant.

**RESULTS**

**CD28null T lymphocytes in peripheral blood of HIV-infected patients**

Characteristics of the 135 individuals enrolled in the study are shown in Table I. We quantified the frequencies of CD4⁺CD28null and CD8⁺CD28null T lymphocytes in peripheral blood from 110 consecutive HIV-infected patients. Absolute counts of CD4⁺ T lymphocytes, adjusted by age, showed an inverse correlation with the frequencies of CD4⁺CD28null and CD8⁺CD28null subsets (Fig. 1A: linear regression, P = 0.01 and P = 0.001, respectively). Absolute counts of CD4⁺ T lymphocytes showed a positive correlation with both absolute counts of CD8⁺ and CD8⁺CD28⁺ T lymphocytes (data not shown). Meanwhile, the frequency of CD4⁺CD28null T lymphocytes displayed a positive correlation with CD8⁺CD28null T lymphocytes in these patients (Fig. 1B; linear regression, P < 0.001). HIV-infected patients showed increased frequencies of both CD28null subsets when compared with healthy controls (n = 25; data not shown). In fact, healthy controls showed significant lower frequencies not only compared with patients with <500 CD4⁺ T lymphocytes/μl but also with patients with >500 CD4⁺ T lymphocytes/μl (Fig. 1C).

CD4⁺CD28null T lymphocytes have been described as antigen-specific cells against chronic viral antigens, mainly in some autoimmune diseases [14]. To investigate this point in HIV-infected individuals, we compared the induction of CD69 expression and IFN-γ production in response to HIV gag peptides and to CMV antigens in CD4⁺ and CD8⁺ T lymphocytes. All studied individuals were seropositive for CMV. Figure 2A shows the antigen-specific responses in CD28null T lymphocytes from a representative HIV-infected patient. The responses to HIV and to CMV were significantly higher in CD28null with respect to CD28⁺ T lymphocytes in both CD4⁺ and CD8⁺ T lymphocytes (Fig. 2B).

Frequencies of CD28null T lymphocytes were related to the absolute counts of CD4⁺ T lymphocytes in HIV-infected patients. The frequency of HIV- and CMV-specific cells was greater in CD28null T lymphocytes than it was in CD28⁺ T lymphocytes.

**STAT5 and STAT3 activation by IL-15 and IL-21**

IL-15 and IL-21 are cytokines implicated in the proliferation and activation of NK cells and cytotoxic T lymphocytes, properties also shown by CD28null T lymphocytes. When we compared intracellular effects of STAT5 and STAT3, we found that phosphorylation of these signal factors was strongly induced by IL-15 and IL-21, respectively, in both CD4⁺ and CD8⁺ subsets (Fig. 3). However, CD28null T lymphocytes showed significantly higher levels of pSTAT5 in response to IL-15 (Fig. 3A and B) and pSTAT3 in response to IL-21 (Fig. 3C and D) with respect to CD28⁺ T lymphocytes. Simultaneous treatment with IL-15 and IL-21 did not have an enhancing effect on STAT5 or STAT3 activation in any of these subsets. Levels of pSTAT5 showed significant differences between CD4⁺ and CD8⁺ T lymphocytes in both CD28⁺ (median 33.3% [IR: 39.7] vs. 50.1% [IR: 23.1], respectively; Wilcoxon test, P = 0.042) and CD28null subsets (48.5% [IR: 41.6] vs. 77.3% [IR: 27], respectively; P = 0.0042). MFI comparisons between CD28⁺ and CD28null T lymphocytes showed no differences in the percentage of positive cells (data not shown). Activation of STAT5 by IL-15 and STAT3 by IL-21 was greater in CD28null with respect to CD28⁺ T lymphocytes.

**Increased levels of CD28null T lymphocytes in response to IL-15 and IL-21 treatment**

IL-15 and IL-21 have a role in the proliferation of specific subsets of T lymphocytes. Proportions of CD28null with respect to CD28⁺ T lymphocytes, in both CD4⁺ and CD8⁺ T lymphocytes, were
significantly increased after treatment with IL-15 alone or in combination with IL-21 for 7 d (Fig. 4A). When cells were stimulated with anti-CD3, IL-15 enhanced the CD28null/CD28+ ratio. CD28null T lymphocytes might have increased because of an enhanced proliferative response to IL-15 compared with that of CD28+. To analyze the effects of IL-15 and IL-21 on the proliferation of CD28− and CD28+ T lymphocytes, PBMCs were stained with CFSE and cultured in medium alone or with anti-CD3 and stimulated with different combinations of the cytokines are represented in Fig. 4A. Treatment with IL-15 alone or in the presence of IL-21 enhanced CD69 expression in both CD28null T lymphocyte subsets with respect to cells cultured in medium. IL-21 did not exert any effect on CD69 expression when it was added alone to the culture, but it significantly enhanced the inductor effect of IL-15 in CD4+CD28null and CD8+CD28null T lymphocytes. The observed effect of these cytokines on the CD69 expression was the same in cells treated with anti-CD3.

We then wanted to evaluate whether IL-15 and IL-21 also had a similar role in IFN-γ production in CD4+CD28null and CD8+CD28null T lymphocytes (Fig. 6B). The cells were precultured for 18 h in medium alone or in medium containing IL-15, IL-21, or both. Cultures were maintained in these conditions or stimulated with anti-CD3 for 5 additional hours. Treatment with the cytokines did not produce measurable levels of IFN-γ (data not shown); nevertheless, IL-15 with or without IL-21 significantly enhanced the IFN-γ production in response to anti-CD3 stimulation. IL-21 did not increase this stimulatory effect of IL-15 but displayed an inhibitory effect in both CD28null subsets in response to anti-CD3, with significant differences in CD4+CD28null T lymphocytes. IL-21 enhanced IL-15 activation of CD28null T lymphocytes; however, IL-21 alone reduced IFN-γ production.

The potentiating effect of IL-21 on IL-15-induced activation was slight; it was always reproducible, even when experiments were performed at early time points (data not shown).

| TABLE 1. Selected demographic, clinical, and laboratory parameters of the cohort |
|---------------------------------|-----------------|-----------------|
|                                | HIV+            | HIV−            |
|                                | (n = 56)        | (n = 54)        |
| Age, yr                        | 46.1 (24–61)    | 45.4 (26–66)    |
| Male                           | 37 (66.7)       | 43 (79.6)       |
| Duration of HIV infection, yr  | 11.3 (25–23.6)  | 9.5 (10–21.6)   |
| CD4 T cell count, cells/μl     | 334 (51–492)    | 846 (511–1482)  |
| CD4 T cell percentage          | 21.2 (13.1–34.9)| 31.2 (18.5–46.1)|
| Nadir CD4 T cell count (cells/μl)| 248 (49–459) | 454 (98–976) |
| CD8 T cell count (cells/μl)    | 862 (230–1678)  | 707 (353–1423)  |
| CD8 T cell percentage          | 54.8 (37–71)    | 42.4 (32–65)    |
| HIV RNA load, copies/ml        | <50             | <50             |
| HAART duration, yr             | 46.1 (24–61)    | 45.4 (26–66)    |
| <3.0                           | 12 (21.4)       | 11 (20.4)       |
| 3.0–5.0                        | 13 (23.2)       | 8 (14.8)        |
| 5.0–7.0                        | 16 (28.6)       | 16 (33.3)       |
| >7.0                           | 15 (26.8)       | 17 (31.3)       |

Continuous variables are expressed as median (interquartile range); categorical variables are expressed as the number (%) of cases. HAART, highly active antiretroviral treatment; NA, not applicable. *Fisher’s exact test for categorical variables, and Kruskal-Wallis test for continuous variables.
**DISCUSSION**

It is well known that CD4+ T lymphocytes acquire typical properties of cytotoxic cells when they become highly differentiated cells and lose expression of the CD28 coreceptor. Nevertheless, to our knowledge, this is the first study demonstrating that CD4+CD28null responses to IL-15 and IL-21 are nearly equal to those of CD8+CD28null T lymphocytes in HIV-infected patients. IL-21 counteracts the reduction in CD28 expression induced by IL-15; however, it potentiates IL-15-induced proliferation, activation, and cytotoxic properties in CD4+CD28null and CD8+CD28null T lymphocytes, although at low level. In contrast, IL-21 alone displays inhibitory effects on anti-CD3-stimulated cells with IL-21. CD4+CD28null and CD8+CD28null T lymphocytes showed similar increases in their cytotoxic properties in response to IL-15, IL-21, or both treatments.

**IL-15 and IL-21 up-regulate granzyme B and perforin expression and degranulation in CD4+CD28null and CD8+CD28null T lymphocytes**

We compared the influence of IL-15 and IL-21 on granzyme B and perforin expression in CD4+CD28null and CD8+CD28null T lymphocytes. After 5 h of culture with IL-15, IL-21, or both, percentages of granzyme B+ and perforin+ cells were significantly increased in both CD28null subsets (Fig. 7), and only IL-21 alone showed no effect on perforin expression in CD4+CD28null T lymphocytes (Fig. 7B and D). IL-15 up-regulated intracytoplasmic stores, measured as MFI, of both granzyme B in CD4+CD28null and CD8+CD28null T lymphocytes (Fig. 7A and C) and perforin in CD4+CD28null T lymphocytes (Fig. 7B and D). Costimulation with IL-21 induced significant increases compared with IL-15 alone in all cases (Fig. 7). Granzyme B levels were greater in CD8+ than they were in CD4+ T lymphocytes in response to IL-15 and to IL-15 plus IL-21. In all conditions of culture, perforin expression was greater in CD8+ T lymphocytes.

LAMPS are not usually present on the surface of T lymphocytes but are exposed only during active degranulation. CD107a (LAMP-1) expression has been described as a good marker for cytotoxic cellular activity [32]. Isolated PBMCs were cultured in medium or stimulated for 5 h with anti-CD3 alone or in combination with IL-15, IL-21, or both, and levels of surface CD107a were assessed in CD4+CD28null and CD8+CD28null T lymphocytes. Culture in medium alone (Fig. 7E) or medium containing the cytokines (data not shown) displayed no differences in degranulation. However, anti-CD3 stimulation induced expression of CD107a, which was significantly increased by the addition of IL-15 or IL-21 (Fig. 7E). Again, simultaneous treatment with the 2 cytokines enhanced degranulation significantly with respect to the single cytokines. CD4+CD28null T lymphocytes showed reduced CD107a expression compared with CD8+CD28null T lymphocytes; nevertheless, the differences only reached statistical significance after treatment of anti-CD3-stimulated cells with IL-21. CD4+CD28null and CD8+CD28null T lymphocytes showed similar increases in their cytotoxic properties in response to IL-15, IL-21, or both treatments.
CD8^+^CD28null T lymphocytes have been defined as a useful marker to evaluate HIV progression and also to predict the response to immunization in HIV-infected patients [21, 23, 24]. Progression of accumulation of CD28null T lymphocytes is a common feature of aging that is also described in patients with autoimmune diseases and chronic infections, such as HIV infection [1, 8, 15, 16]. The data suggest that loss of CD28 expression may be attributed to repeated antigenic stimulation by continued exposure to the same chronic antigens [4].

Accordingly, levels of CD4^+^CD28null correlated positively with CD8^+^CD28null T lymphocytes, indicating a possible common origin. We found more HIV- and CMV-specific cells in CD4^+^CD28null and CD8^+^CD28null than in CD28^+^ T lymphocytes. CMV infection could have a nonspecific effect on the differentiation state of other antigen-specific CD4^+^ T lymphocytes [34–37]. Accordingly, its profound effect on CD27 and CD28 expression of CMV-specific cells and non-CMV-specific populations of CD4^+^ T lymphocytes has been demonstrated [22, 38].

It is striking that CD8^+^ T lymphocytes differentiate faster than CD4^+^ T lymphocytes do, although they principally undergo the same changes. CD4^+^ T lymphocytes are more resistant to phenotypic and functional changes than are CD8^+^ T lymphocytes because homeostatic control of the CD4 compartment is more robust than that of CD8^+^ T lymphocytes. This fact is evidenced not only by the stability of the naive and central memory compartments but also by the finding that the TCR diversity of naive CD4^+^ T lymphocytes is well maintained for decades after thymic function has ceased and that oligoclonal expansion of CD4^+^ memory T lymphocytes is infrequent and, if it occurs, the expansion is small [15, 39].

Most of these cells without CD28 expression have short telomeres and might be poorly functional, suggesting that these populations have undergone a process of end-stage differentiation [40–43]. They have an effector memory or effector phenotype and show expression of NK-associated receptors, including the expression of killer lectin receptor G1 and...
(KLRG-1), CD57, CD158, CD85j, and even NKG2D in CD4+ T lymphocytes [15, 44–47].

Some members of the γc family of cytokines, such as IL-2, IL-7, IL-15, and IL-21, with functions in regulation of lymphocyte development, homeostasis, reconstitution, expansion of pathogen-specific lymphocytes, and vaccine responses, have been contemplated as therapeutic candidates for patients with AIDS. Several studies have shown that IL-15 mainly acts on HIV-specific CD8+ T lymphocytes, enhancing survival, activation, IFN-γ production, and cytotoxic ability [48, 49]. Furthermore, higher serum IL-15 levels have been correlated with better control of infection in HIV-infected patients [50], and a combination of IL-15 and HAART could increase the percentage of proliferating effector memory CD4+ and CD8+ T lymphocytes [51].

However, it has been recently demonstrated in nonhuman primate models that IL-15 treatment contributes to an increase viral load and acceleration of disease progression [52, 53]. In spite of these serious concerns regarding the utility and efficacy of IL-15 as an adjuvant therapy for HIV infection, its effects on CD28null T lymphocytes must be taken into account because IL-15 is produced in vivo during chronic, active infections, including HIV-infection [54]. Moreover, IL-15 has been implicated in the stable loss of CD28 expression in CD8+ memory T lymphocytes, in part through the induction of TNF-α secretion [28], and it may be responsible for the survival, the expansion, or both in CD28null T lymphocytes.

Similarly, we have found that IL-15 increases the proliferation and frequency of CD4+CD28null and CD8+CD28null T lymphocytes compared with their CD4+CD28+ T lymphocyte counterparts. Moreover, IL-15 clearly activates CD28null T lymphocytes, as shown by both expression of CD69 and IFN-γ production and an enhancing effect on their cytolytic properties. Similarly, we have recently demonstrated that IL-15 displays a striking effect on CD4+ T lymphocytes in elderly people, with a greater capacity for activating CD28null than CD28+ cells [30]. So far, the effects of IL-15 are thought to be more pronounced in the CD8+ than in the CD4+ T lymphocyte compartment; nevertheless, acquisition of properties typical of CD8+ cells may make CD4+CD28null cells more responsive to this cytokine.

Figure 3. Activation of STAT5 and STAT3 by IL-15 and IL-21. Comparison of STAT5 (A) and STAT3 phosphorylation (C) in response to stimulation with IL-15 (50 ng/ml), IL-21 (50 ng/ml), or both in CD4+ and CD8+ T lymphocytes grouped by their expression of CD28 (n = 6). (B and D) Histogram plots showing representative experiments. Paired t test was used to compare paired means and P values are depicted in the panels.
IL-21 is produced mainly by CD4+ T lymphocytes. This cytokine appears to primarily modulate the function of mature lymphocytes, having little effect on in vitro CD8+ T lymphocyte proliferation. Studies have shown that IL-21 production is impaired during acute infection, whereas HIV elite controllers have serum IL-21 levels comparable to those of uninfected controls [55]. The in vitro data and preclinical investigations have justified the interest in IL-21 as a therapeutic option for HIV-infected patients [29, 31, 56]. A study published by White et al. [29] demonstrated that culturing PBMCs from HIV-infected individuals with IL-21 resulted in up-regulation of perforin in CD8+ T lymphocytes, including memory and effector subsets and virus-specific T lymphocytes. However, this cytokine did not lead to changes in the activation and proliferation of T lymphocytes or promote viral replication.

IL-15 and IL-21 have been shown to activate the Jak/STAT signaling pathway upon receptor binding and to preferentially induce pSTAT5 and pSTAT3 transcription factors, respectively. Data about activation levels of STAT5 and STAT3 in HIV-infected patients, compared with healthy people, in response to IL-15 and IL-21 are controversial [29, 57]. Nevertheless, both cytokines exerted an increased activating effect on CD28null cells relative to the counterpart CD28+ populations. Similarly, we have previously described that IL-15 displays a prominent activating effect on CD24+CD28null T lymphocytes in healthy, elderly people [30]. The slight differences we found between CD4+CD28null and CD8+CD28null T lymphocytes were mainly in response to IL-15 and may have been due to increased phosphorylation of STAT5 in CD8+ T lymphocytes. In the short times in which we assayed STAT phosphorylation, we observed a minimal activating effect by IL-21 on STAT 5. This or other downstream regulatory mechanisms or both may be responsible for the enhancing effect of this cytokine on IL-15-induced proliferation and cytotoxicity.

**Figure 4.** Proliferation in CD4+CD28null and CD8+CD28null populations in response to IL-15 and IL-21 cytokines in resting and anti-CD3-activated T lymphocytes. PBMCs were labeled with CFSE (1.5 μM) and cultured for 7 d in the presence of IL-15 (50 ng/ml), IL-21 (50 ng/ml), or both (n = 10). Percentages of dividing cells were analyzed on day 7 of culture. (A) The CD28null/CD28+ ratio in CD4+ and CD8+ T lymphocytes in response to IL-15, IL-21, or both in resting and anti-CD3-activated cells. * a significant difference compared with the baseline (P < 0.05). (B) Proliferative capacity of CD4+CD28null and CD8+CD28null T lymphocytes in response to the studied cytokines. A paired t test was used to compare paired means. Data are means ± sem and significant differences (P < 0.05) are depicted in the figure: #, differences between CD4+CD28null and CD8+CD28null under the same treatments; †, differences with respect to cells in medium; ‡, differences with respect to cells treated with both IL-15 and IL-21; §, differences with respect to anti-CD3-stimulated cells; ¶, differences with respect to anti-CD3-stimulated cells treated with both IL-15 and IL-21. (C and D) Histogram plots showing representative experiments for proliferative capacity. A paired t test was used to compare paired means, and P values are depicted in the panels.
In contrast, IL-21 counteracted IL-15 inhibition of CD28 expression, and even increased CD28 levels in resting and anti-CD3-stimulated CD4+ and CD8+ T lymphocytes. In a similar manner, IL-21 sustains CD28 expression in naive CD8+ T lymphocytes [58]. We previously failed to generate CD28null cells in vitro from elderly CD4+CD28+ T lymphocytes treated with IL-15, but we found a similar reduction in CD28 expression levels in both CD4+ and CD8+ T lymphocytes from the studied HIV-infected patients. CD28 is a key costimulatory molecule required for proper T lymphocyte activation, and CD28null cells present a low activation threshold in response to TCR stimulation, which could be implicated in their predisposition to the breakdown of self-tolerance [59]. Increases in CD28 expression induced by IL-21 may also affect the activation threshold, which would explain the reduced responses to anti-CD3 in the presence of IL-21.

Other possible explanation would be the induction of Bcl-6 expression by IL-21. T-bet, the lineage-defining transcription factor for Th1 cells, recruits Bcl-6 to the Ifng locus in late stages of Th1 differentiation and represses its activity, possibly to prevent the overproduction of IFN-γ [60].

In contrast, IL-21 significantly increased perforin and granzyme B expression in effector and effector memory CD4+ and CD8+, and NK cells [61]. Accordingly, CD28null T lymphocytes from HIV-infected patients showed greater perforin and granzyme B stores and a superior ability to degranulate upon TCR stimulation in response to in vitro IL-21 treatment, at levels similar to those induced by IL-15.

T lymphocytes could loss CD28 expression after a recent, common priming event, and this fact could increase their susceptibility to IL-15 and IL-21 cytokines. However, it is unlikely,
given that some individuals have more than one-half of their CD8 T cells with no expression of CD28 and it has been demonstrated that loss of CD28 expression is a stable process and cells need repeated division to become CD28null [4, 5].

In summary, our data show that IL-15 and, to lesser extent, IL-21 have similar effects on CD4+CD28null and CD8+CD28null T lymphocytes in HIV-infected patients, not only on the resting cells but also on their TCR-specific responses. The effects of IL-15 and IL-21 may be interesting because the accumulation of CD28null T lymphocytes, which show high frequencies of anti-HIV- and anti-CMV-specific cells, is associated with a reduced overall immune response to pathogens and vaccines in the aging immune system, as occurs in HIV-infected individuals. Therefore, enhancing the functional properties of CD28null T cells may be important because they provide antigen-specific defense against chronic infections.

**AUTHORSHIP**

The authors' responsibilities were as follows. R.A.-A. and C.L.-L. designed the study. M.A.M.-G. and A.E. prepared protocols, collected, and processed all samples; performed or oversaw the

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**Figure 7.** IL-15 and IL-21 up-regulate granzyme B and perforin expression and degranulation induced by anti-CD3 in CD4+CD28null and CD8+CD28null T lymphocytes. (A–D) Expression of granzyme B and perforin was studied by intracellular staining in CD4+CD28null and CD8+CD28null T lymphocytes from HIV-infected patients (n = 12 and n = 9, respectively) after 5 h of culture in medium alone or medium containing IL-15 (50 ng/ml), IL-21 (50 ng/ml), or both. (A and B) Histogram plots showing representative experiments. (C and D) Histogram plots showing the percentage of, and the MFI of, positive cells (means ± SEM) in cultured in CD4+CD28null (white bars) and in CD8+CD28null T lymphocytes (black bars). (E) PBMCs from HIV-infected patients (n = 8) were stimulated with anti-CD3 (1 μg/ml), IL-15 (50 ng/ml), IL-21 (50 ng/ml), or with all 3 over 5 h. Surface CD107a expression was analyzed in CD4+CD28null and CD8+CD28null T lymphocytes by flow cytometry. Paired t test was used to compare paired means. Data are means ± SEM, and significant differences (P < 0.05) are depicted in the figure: # indicates differences between CD4+CD28null and CD8+CD28null under the same treatments; †, differences with respect to cells in medium; ‡, differences with respect to cells treated with both IL-15 and IL-21; §, differences with respect to anti-CD3-stimulated cells; and ¶, differences with respect to anti-CD3-stimulated cells treated with both IL-15 and IL-21.
experimental protocols; and analyzed data. A.E. and M.A.M.-G. wrote the manuscript. V.A. and J.A.C. selected, recruited, and followed up with volunteers. C.I.-L. and R.A-A. reviewed the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

REFERENCES


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CD4⁺CD28null T lymphocytes resemble CD8⁺CD28null T lymphocytes in their responses to IL-15 and IL-21 in HIV-infected patients

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