

Impaired upregulation of the costimulatory molecules CD27 and CD28 on CD4⁺ T-cells from HIV patients receiving ART is associated with poor proliferative responses

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ABSTRACT

HIV patients beginning antiretroviral therapy (ART) with advanced immunodeficiency often retain low CD4⁺ T-cell counts despite virological control. We examined proliferative responses and upregulation of costimulatory molecules following anti-CD3 stimulation, in HIV patients with persistent CD4⁺ T-cell deficiency on ART. Aviraemic HIV patients with nadir CD4⁺ T-cell counts <100 cells/ μ l who had received ART for a median time of 7 (range 1-11) years were categorized into those achieving low (<350 cells/ μ L; n=13) or normal (>500 cells/ μ L; n=20) CD4⁺ T-cell counts. Ten healthy controls were also recruited. CD4⁺ T-cell proliferation (Ki67) and upregulation of costimulatory molecules (CD27 and CD28) after anti-CD3 stimulation were assessed by flow cytometry. Results were related to proportions of CD4⁺ T-cells expressing markers of T-cell senescence (CD57), activation (HLA-DR) and apoptotic potential (Fas). Expression of CD27 and/or CD28 on uncultured CD4⁺ T-cells was similar in patients with normal CD4⁺ T-cell counts and healthy controls, but lower in patients with low CD4⁺ T-cell counts. Proportions of CD4⁺ T-cells expressing CD27 and/or CD28 correlated inversely with CD4⁺ T-cell expression of CD57, HLA-DR and Fas. After anti-CD3 stimulation, induction of CD27^{hi}CD28^{hi} expression was independent of CD4⁺ T-cell counts, but lower in HIV patients than in healthy controls. Induction of CD27^{hi}CD28^{hi} expression correlated with induction of Ki67 expression in total, naïve and CD31⁺ naïve CD4⁺ T-cells from patients. In HIV patients responding to ART, impaired induction of CD27 and CD28 on CD4⁺ T-cells after stimulation with anti-CD3 is associated with poor proliferative responses, as well as greater CD4⁺ T-cell activation and immunosenescence.

Keywords: CD4⁺ T-cell; HIV; immune activation; anti-CD3 stimulation; CD27/CD28 costimulatory molecules; proliferation

INTRODUCTION

Despite control of HIV replication following treatment with effective antiretroviral therapy (ART), many patients fail to normalize CD4⁺ T-cell numbers. Approximately 40% of patients commencing therapy with fewer than 200 CD4⁺ T-cells/ μ L have less than 500 CD4⁺ T cells/ μ l after 10 years on ART ¹. This scenario is associated with a higher rate of mortality and morbidity ², which includes atherosclerotic vascular disease ^{3,4}, osteoporosis and fractures ⁵ and non-AIDS-defining cancers ⁶⁻⁸.

Persistent CD4⁺ T cell deficiency may reflect damage to the thymus that impairs its regeneration ⁹⁻¹¹, persistent immune activation ^{12, 13}, fibrosis of lymphoid tissue ^{14, 15} and/or failure to regenerate T-cells via peripheral homeostatic mechanisms. The latter of these may result from a reduced capacity of naïve T-cells to proliferate following stimulation ¹⁶. Homeostatic maintenance of naïve T-cells is regulated by interleukin (IL)-7 and/or T-cell receptor (TCR) ligation ^{17, 18}. We have shown that defects of IL-7 signaling in HIV patients with persistent CD4⁺ T-cell deficiency despite effective ART are associated with CD4⁺ T-cell activation and senescence ¹⁹. Similarly, the capacity for naïve T-cells to proliferate following TCR engagement may also be impaired in treated HIV disease ²⁰⁻²³. This may reflect poor signaling downstream of the TCR and its associated costimulatory molecules, CD27 and CD28. Indeed, HIV infection (regardless of treatment status) is characterized by aberrant expression of CD27 and CD28 on T-cells ²⁰.

CD27 and CD28 act in concert with the TCR to support T-cell expansion ²⁴. CD27 provides costimulatory signals for naïve CD4⁺ T-cells, and expression is transiently increased on the cell surface following TCR stimulation ²⁵. This is important in antigen-specific memory T-cell formation ²⁶ and T-cell survival after activation ²⁴. CD28 promotes assembly of the T-cell signaling complex and amplifies

signals received by the TCR, thereby decreasing the threshold required for T-cell expansion ²⁷. CD28 signaling also promotes IL-2 production, enhancing cell cycle activity and the survival of activated T-cells ²⁸.

Here, we investigate whether defects in anti-CD3 mediated stimulation of proliferation and regulation of CD27 and CD28 expression associate with poor CD4⁺ T-cell recovery in HIV patients responding to ART. This is assessed here in total, naïve and CD31⁺ naïve CD4⁺ T-cells, with expression of CD31 on naïve CD4⁺ T-cells marking recent thymic emigrants ²⁹. Our findings associate a failure to upregulate CD27 and CD28 after anti-CD3 stimulation with poor proliferative responses following anti-CD3 stimulation in total, naïve and CD31⁺ naïve CD4⁺ T-cells, and show that this is not related to the recovery of CD4⁺ T-cells after long-term ART.

MATERIALS AND METHODS

Study population

Thirty-nine adult HIV-1-infected patients (2 females, 37 males), attending clinics at Royal Perth Hospital, Western Australia, participated in the study. All patients were receiving ART, defined as treatment with at least three antiretroviral drugs. Criteria for inclusion were nadir CD4⁺ T-cell counts <100/μL, ART for >12 months and undetectable plasma HIV RNA for >6 months. Patients were categorized as having normal (n=20) or low (n=13) CD4⁺ T-cell counts based on values >500 or <350 CD4⁺ T-cells/μL, respectively. For correlations between the parameters measured, additional patients (6 males) with intermediate CD4⁺ T-cell counts (350-500 CD4⁺ T-cells/μL) were included for all correlations. The healthy control cohort comprised 10 adults (4 females, 6 males) aged 22 to 54 years.

Informed consent was obtained from all participants and the study was approved by the Human Research Ethics Committees of Royal Perth Hospital and the University of Western Australia.

HIV-1 RNA viral load

Plasma HIV RNA levels were assayed by quantitative reverse transcription polymerase chain reaction (Amplicor™ Version 1.5, Ultrasensitive Protocol, 50-75000 copies/mL) (Roche Diagnostic Systems, Branchburg, New Jersey, USA).

T-cell phenotyping

Total, naïve and memory CD4⁺ and CD8⁺ T-cells were enumerated in EDTA-treated whole blood using the following fluorescently conjugated monoclonal antibodies (mAbs); CD45-APC-H7, CD3-PerCP, CD4-APC, CD8-PeCy7, CD45RA-FITC and CD62L-PE (BD Biosciences, San-Jose, California, USA). Peripheral blood mononuclear cells (PBMC) were separated from lithium heparin-treated whole blood by Ficoll-Paque density centrifugation and cryopreserved in 10% dimethylsulphoxide/90% heat inactivated fetal calf serum. To assess CD27 and CD28 expression, thawed PBMC were incubated with CD3-V450, CD4-V500, CD45RA-APC-H7, CD27-PerCP-Cy5.5, CD28-PE and CD31-FITC for 15 minutes at room temperature. For assessment of immunosenescence molecules, EDTA-treated whole blood was incubated with CD4-PerCP-Cy5.5, CD8-APC-Cy7, CD57-FITC, HLA-DR-APC and Fas-PE (BD Biosciences) for 20 minutes, followed by 1mL FACSlyse (BD Biosciences) for a further 15 minutes. Analyses were performed using a FACS Canto II cytometer (BD Biosciences) at Royal Perth Hospital. Lymphocytes were distinguished by their forward and side light scatter and in subsequent gates, total CD4⁺ T-cells were identified as CD3⁺CD4⁺, naïve CD4⁺ T-cells as CD3⁺CD4⁺CD45RA⁺CD27⁺ and recent thymic emigrants as CD3⁺CD4⁺CD45RA⁺CD27⁺CD31⁺. A

minimum of 100 000 lymphocyte events per sample were analyzed using FlowJo software (Tree Star, Ashland, Oregon, USA).

Proliferation assay (Ki67 expression)

Thawed PBMC were cultured at 10^6 cells/mL in RPMI/10% FCS, with or without 10ng/mL anti-CD3 antibody (Mabtech, Nacka Strand, Stockholm SE, Sweden) at 37°C for 5 days. Cells were then surface stained with the following fluorescently conjugated mAbs from BD Biosciences: CD3-V450, CD4-V500, CD45RA-APC-H7, CD27-PerCP-Cy5.5, CD28-APC and CD31-PE. Intracellular staining using Ki67-FITC was then performed using the Human FoxP3 Buffer Set (BD Biosciences) according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, California, USA), using non-parametric statistics. Mann-Whitney tests were performed for continuous variables and correlation coefficients were determined by the Spearman's rank correlation test. Fisher's exact tests were performed for categorical analyses. For all tests, $p < 0.05$ was considered to represent a significant difference.

RESULTS

Patient groups were well matched

Demographic data are presented in Table 1. The duration of ART was longer in patients with normal CD4⁺ T-cell counts ($p=0.003$), however time on ART did not correlate with proportions of total or

naive CD4⁺ T-cells in an analysis that combined both patient groups ($r=0.23-0.24$, $p=0.14-0.17$). Furthermore, 5 years after study entry, all participants originally classified as having CD4⁺ T-cell deficiency had <340 CD4⁺ T-cells/ μ L after a median time of 12 (range 5-17) years on ART. Hence, our definition of poor recovery of CD4⁺ T-cell numbers was independent of time on ART in this group of patients.

Proliferation assessed in viable T-cells did not differentiate HIV patients with low or normal CD4⁺ T-cell counts

CD4⁺ T-cell proliferation induced by anti-CD3 was assessed by subtracting the percentages of CD4⁺ T-cells expressing Ki67 in unstimulated cultures (background proliferation) from the percentages seen after stimulation (Δ Ki67). Background proliferation was negligible, with Ki67 expression detected at rates of less than 5% in 90% of individuals. Low lymphocyte viability (absent lymphocyte population on flow cytometry plots; Figure 1) precluded analysis of data from some patients. This was more common in patients with low CD4⁺ T-cell counts than patients with normal CD4⁺ T-cell counts (54% vs 5%; Fisher's exact test, $p=0.003$), but could not be ascribed to any of the characteristics described in Table 1. When analyses were restricted to samples with good cell viability (distinct lymphocyte populations after 5 days of culture; $n=25$), Δ Ki67 values were similar in total CD4⁺ T-cells from patients with low or normal CD4⁺ T-cell counts ($p=0.45$) and healthy controls ($p=0.15$ and $p=0.44$, respectively; Figure 2). Moreover, Δ Ki67 values for total, naive and CD31⁺ naive CD4⁺ T-cells did not correlate with numbers of circulating total, naive or CD31⁺ naive CD4⁺ T-cells, respectively (data not shown).

As proliferative responses to IL-7 have also been investigated in this cohort of HIV patients ¹⁹, we compared Ki67 expression in response to anti-CD3 or IL-7. Δ Ki67 values generated by stimulation

with anti-CD3 or IL-7 were correlated in total, naïve and CD31⁺ naïve CD4⁺ T-cells [(r=0.35, p=0.08), (r=0.58, p=0.0004) and (r=0.62, p=0.0002), respectively]. Thus, patients with a diminished response to IL-7 also responded poorly to anti-CD3 stimulation.

Low CD4⁺ T-cell counts were associated with decreased proportions of CD4⁺ T-cells expressing CD27 and CD28

Proportions of CD4⁺ T-cells expressing CD27, CD28 or both markers at baseline (day 0, unstimulated) were lower in patients with low CD4⁺ T-cell counts than patients with normal CD4⁺ T-cell counts (p=0.007, p=0.03 and p=0.0009, respectively) or healthy controls (p=0.003, p=0.03 and p=0.003, respectively). Proportions were similar in patients with normal CD4⁺ T-cell counts and healthy controls (Table 2). The proportions of CD4⁺ T-cells expressing CD27 or coexpressing CD27/CD28 at day 0 correlated with CD4⁺ T-cell counts in HIV patients [(r=0.55, p=0.001) and (r=0.54, p=0.001), respectively], but not with anti-CD3 induced CD4⁺ T-cell proliferation [(r= -0.01, p=0.11) and (r=0.30, p=0.91), respectively].

Upregulation of CD27 and CD28 following anti-CD3 stimulation correlates with CD4⁺ T-cell proliferation

Surface expression of CD27 and CD28 on healthy T-cells increases following TCR activation^{20, 25, 30}. To investigate whether poor CD4⁺ T-cell recovery in HIV patients is associated with impaired upregulation of these costimulatory molecules following anti-CD3 stimulation, we assessed the proportion of CD27^{hi}CD28^{hi} CD4⁺ T-cells present after stimulation with anti-CD3 for 5 days (Figure 3A).

In samples with viable lymphocytes (low CD4⁺ T-cells, n=6; normal CD4⁺ T-cells, n=19), the proportion of CD4⁺ T-cells that upregulated expression of CD27 and CD28 to a CD27^{hi}CD28^{hi} phenotype after anti-CD3 stimulation did not differ between HIV patients with low or normal CD4⁺ T-cell counts (p=0.48), but were lower in both patient groups compared to healthy controls (p=0.008 and p=0.007, respectively) (Figure 4A). In a combined analysis of all patients following stimulation with anti-CD3, induction of CD27^{hi}CD28^{hi} expression on total, naïve and CD31⁺ naïve CD4⁺ T-cells correlated with Δ Ki67 in total (r=0.56, p=0.002; Figure 4B), naïve (r=0.46, p=0.02; Figure 4C) and CD31⁺ naïve (r=0.41, p=0.03; Figure 4D) CD4⁺ T-cells, respectively. In healthy controls, induction of CD27^{hi}CD28^{hi} expression on total, naïve and CD31⁺ naïve CD4⁺ T-cells also correlated with Δ Ki67 in total (r=0.57, p=0.09; Figure 4E), naïve (r=0.67, p=0.04; Figure 4F) and CD31⁺ naïve (r=0.65, p=0.04; Figure 4G) CD4⁺ T-cells, respectively.

Ki67 expression induced by anti-CD3 was greater in CD27^{hi}CD28^{hi} cells than CD27^{lo}CD28^{lo} cells from HIV patients and healthy controls

We next compared Ki67 expression (frequency distribution histograms) in CD27^{lo}CD28^{lo} and CD27^{hi}CD28^{hi} cells after culture with anti-CD3. The populations are illustrated in Figure 3B. In total, naïve and CD31⁺ naïve CD4⁺ T-cells, Ki67 expression was greater in cells expressing high levels of CD27 and CD28 (Figure 3C-E). This was observed in both HIV patients and healthy controls.

Baseline expression and upregulation of CD27 and/or CD28 expression after anti-CD3 stimulation correlate inversely with CD4⁺ T-cell senescence

Finally, we examined the relationship between CD27 and CD28 expression on CD4⁺ T-cells and markers of CD4⁺ T-cell activation, senescence and apoptotic potential. As previously demonstrated in

this cohort^{19, 31}, patients with low CD4⁺ T-cell counts had higher expression of HLA-DR (p=0.0002), CD57 (p=0.02) and Fas (p=0.01) on total CD4⁺ T-cells than patients with normal CD4⁺ T-cell counts.

The proportions of CD4⁺ T-cells expressing CD27, CD28 and CD27/CD28 at baseline correlated inversely with expression of CD57, HLA-DR and Fas on total CD4⁺ T-cells [r= -0.47-(-0.72), p<0.0001-0.01] (Table 3). Similarly, an inverse correlation was evident between the proportion of CD4⁺ T-cells that upregulated expression to CD27^{hi}CD28^{hi} after anti-CD3 stimulation and expression of CD57 on CD4⁺ T-cells (r= -0.39, p=0.04) (Table 3).

DISCUSSION

Here, we compared CD4⁺ T-cell proliferation and expression of CD27 and CD28 in response to anti-CD3 stimulation, in HIV patients receiving effective ART categorized as having either low or normal CD4⁺ T-cell counts, and healthy controls. The levels of proliferation induced by anti-CD3 were similar in all groups, suggesting that CD4⁺ T-cells from patients with low CD4⁺ T-cell counts are not defective in their capacity to proliferate. Our ability to detect differences in proliferation across groups may have been impeded by the small number of patients with viable cells, particularly in the low CD4⁺ T cell group. However, similar findings were observed in this same cohort of HIV patients when proliferative responses to IL-7 were assessed¹⁹ suggesting that what distinguishes this group of patients with low CD4⁺ T cell counts is that their CD4⁺ T cells are more prone to apoptosis in culture. In contrast, other studies found that Ki67 expression was induced less efficiently among naïve CD4⁺ T-cells in HIV patients compared to controls^{20, 21}. However, unlike our study, those studies were performed on combined cohorts of treated and untreated HIV patients, with varying viral loads, so the stage of HIV

disease (i.e. detectable viraemia) may be a critical factor in those findings. We did however observe a direct correlation between proliferative responses to anti-CD3 or IL-7. Thus, patients with a diminished response to IL-7 also responded poorly to anti-CD3.

CD27 and CD28 are important costimulatory molecules required for T-cell responses following TCR engagement. CD27⁺ T-cells have a higher proliferative capacity after HIV-specific stimulation compared to CD27⁻ T-cells, and signaling through CD27 results in increased IL-2 production and reduced apoptosis³². Moreover, the percentage of CD27 and CD28 expressing CD4⁺ T-cells correlated positively with CD4⁺ T-cell counts and negatively with viral load in a combined cohort of treated and untreated HIV patients³³. Similarly, we show that patients with low CD4⁺ T-cell counts had lower baseline proportions of CD4⁺ T-cells expressing CD27 and/or CD28 than patients with normal CD4⁺ T-cell counts and healthy controls. Accordingly, positive relationships were seen between CD4⁺ T-cell counts and both CD27 expression and CD27/CD28 coexpression. Although no correlations between baseline expression of CD27 and/or CD28 with anti-CD3 induced proliferation were observed, low cell viability after 5 days of culture precluded the analysis of proliferation in some individuals. This was more common amongst patients with persistent CD4⁺ T-cell deficiency, where the lowest levels of CD27 and CD28 expression on CD4 T-cells was also observed. As CD27 and CD28 signaling can promote the survival of T-cells following TCR activation^{26,28}, these two observations may be linked.

Following stimulation with anti-CD3, a subset of CD4⁺ T-cells upregulates expression of CD27 and CD28 to form a CD27^{hi}CD28^{hi} population. Therefore, we assessed the proportion of CD4⁺CD27⁺CD28⁺ T-cells that increased expression of CD27 and CD28 above background levels (CD27^{hi}CD28^{hi} CD4⁺ T-cells). Others have shown that naïve CD4⁺ T-cells from healthy donors upregulate expression of CD27 and CD28 after TCR activation, whereas in a combined cohort of both

treated and untreated HIV patients, an impairment in CD27^{hi}CD28^{hi} induction was evident, suggesting a possible mechanism for the naïve T-cell expansion failure seen in HIV disease ²⁰. Here, similar proportions of CD27^{hi}CD28^{hi} cells were induced in the total CD4⁺ T-cell population from patients with low or normal CD4⁺ T-cell counts. Low cell numbers precluded groupwise comparisons of CD27^{hi}CD28^{hi} expression on naïve and CD31⁺ naïve CD4⁺ T-cells. However the inclusion of patients with intermediate CD4⁺ T-cell counts established positive associations between proportions of cells with increased CD27^{hi}CD28^{hi} expression and Δ Ki67 in total, naïve and CD31⁺ naïve CD4⁺ T-cells.

As cell cycle progression may parallel the induction of the CD27^{hi}CD28^{hi} population, we analyzed Ki67 expression within the CD27^{lo}CD28^{lo} and CD27^{hi}CD28^{hi} subsets (Figure 3). Within total, naïve and CD31⁺ naïve CD4⁺ subsets, we found greater Ki67 expression in cells with the CD27^{hi}CD28^{hi} phenotype after anti-CD3 stimulation, when compared to CD27^{lo}CD28^{lo} cells from HIV patients or healthy controls. Hence, cells capable of upregulating CD27/CD28 have the greatest proliferative capacity. In a similar study combining treated and untreated HIV patients, after two days of anti-CD3 stimulation, cells that increased expression of CD27/CD28 were enriched for Ki67⁺, whereas Ki67 was rarely expressed in cells that did not increase CD27/CD28 expression ²⁰. Moreover, addition of soluble anti-CD28 to cultures (with anti-CD3) increased cell cycle entry, even in cells that were only able to weakly upregulate costimulatory molecule expression ²⁰. Hence, the authors suggest that agents capable of increasing CD27 and CD28 expression on T-cells, or the use of costimulatory agonists, may potentially be useful adjuvants in HIV disease ²⁰.

Persistent CD4⁺ T-cell deficiency in HIV patients receiving ART has been linked to chronic activation of the immune system ^{19, 31, 34}. We observed inverse correlations between CD27 and/or CD28 expression at baseline and markers of T-cell activation, senescence and pro-apoptotic potential.

Yonkers *et al.* examined hepatitis C virus (HCV)-infected patients with reduced naïve CD4⁺ T-cell numbers, and found associations between increased activation and impaired CD27 upregulation, or increased susceptibility to cell death after TCR stimulation ³⁰. Additionally in our cohort, impaired homeostatic IL-7/IL-7 receptor signaling was associated with activation and senescence of CD4⁺ T-cells ¹⁹. Accordingly, Bazdar and colleagues reported that IL-7 enhances Ki67 responses to TCR activation in naïve CD4⁺ T-cells from healthy controls and HIV patients ¹⁶. Therefore, an inability to respond to IL-7 may contribute to poor TCR mediated signaling

The clearest limitation to our study is the low number of patients with CD4⁺ T-cell deficiency on ART whose cells remained viable after culture. Furthermore as a viability dye was not included, we can not exclude the possibility that non-viable cells were included in the analysis. These results would also need to be validated using purified naïve T-cells, as stimulation is likely to modulate expression of CD27 and CD28 and induce differentiation of some naïve T cells into memory cells. Particular strengths of our study were the focus on patients with a nadir CD4⁺ T-cell count below 100 cells/ μ L, as low nadir CD4⁺ T-cell counts are a strong predictor of poor immune reconstitution on ART ³⁵⁻³⁷, and our definition of CD4⁺ T cell deficiency (<350 cells/ μ L) and normal CD4⁺ T-cell counts (>500 cells/ μ L), as these values predict mortality ³⁸.

In conclusion, our results show decreased expression of CD27 and CD28 on CD4⁺ T-cells in HIV patients with deficient CD4⁺ T-cell recovery on ART in association with increased markers of T cells activation and senescence. Furthermore, impaired upregulation of these costimulatory molecules in response to anti-CD3 stimulation was observed in all HIV patients, regardless of immune recovery status and this deficiency was associated with poor proliferative responses to anti-CD3 stimulation and increased markers of T-cell activation and senescence. Therefore, a failure to upregulate CD27 and

CD28 after TCR engagement may contribute towards the perturbations in naïve T-cell homeostasis that persist in some HIV patients on ART.

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Requests for reprints

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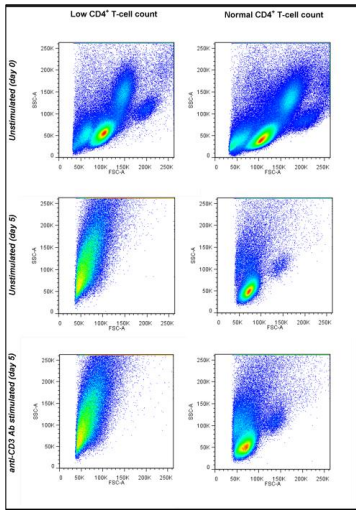


Figure 1

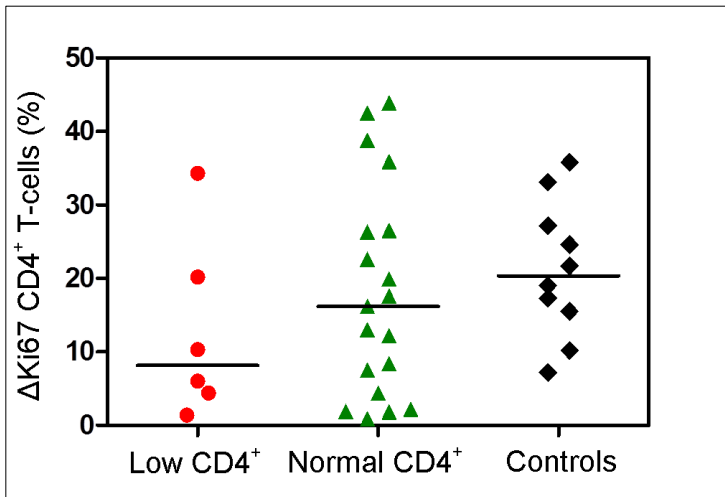


Figure 2

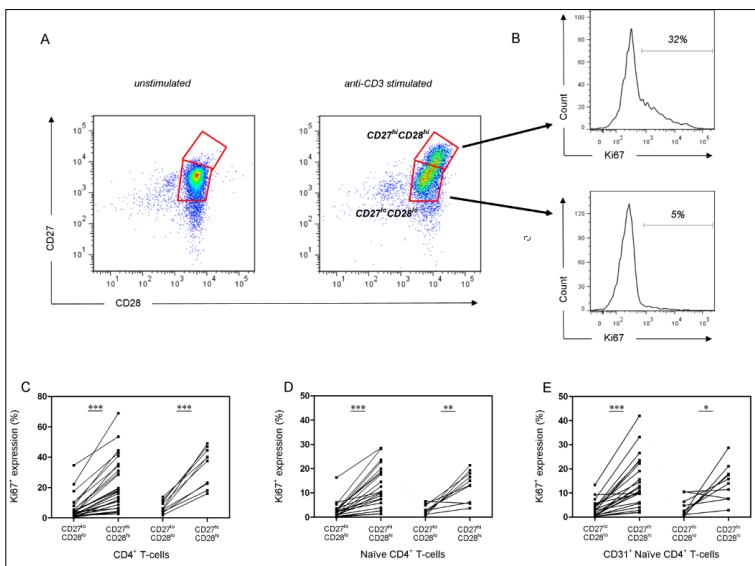


Figure 3

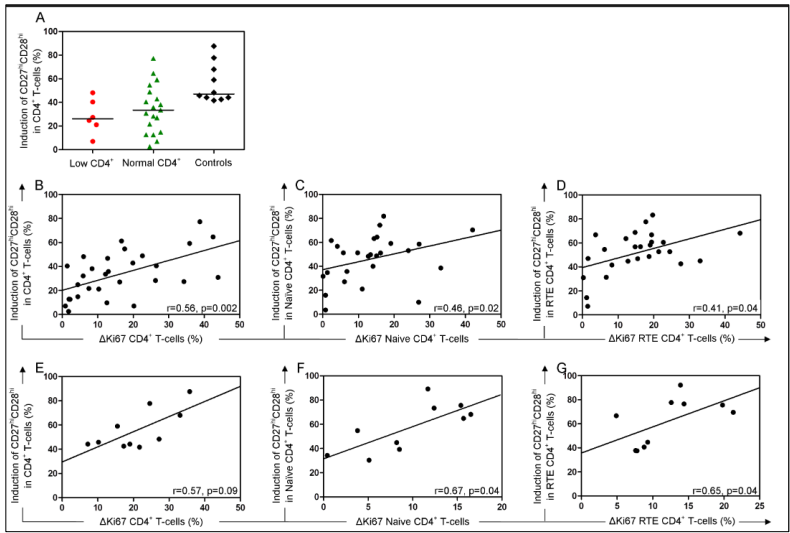


Figure 4

FIGURE CAPTIONS

Figure 1. Representative forward and side light scatter profiles of cryopreserved PBMC from two treated HIV patients with low or normal CD4⁺ T-cell counts. Cells were thawed and stained immediately or incubated for 5 days with or without 10ng/mL of anti-CD3. Low lymphocyte viability (evidenced by absence of a defined lymphocyte population on flow cytometry plots) precluded analysis of 5 day cultures from several patients. This was more common in patients with low rather than normal CD4⁺ T-cell counts (54% vs 5%; Fisher's exact test, p=0.003).

Figure 2. Anti-CD3 mediated proliferation of CD4⁺ T-cells (measured by Ki67 expression) in HIV patients and healthy controls. Induction of Ki67 expression in CD4⁺ T-cells following anti-CD3 stimulation for 5 days was similar in samples from patients with low (●) and normal (▲) CD4⁺ T-cell counts and healthy controls (◆). ΔKi67 represents the increase in the frequency of CD4⁺ T-cells that expressed Ki67 5 days post-stimulation with anti-CD3, over cells incubated in medium alone.

Figure 3. Upregulation of CD27 and CD28 expression on CD4⁺ T-cells following stimulation with anti-CD3 for 5 days. Cells incubated in medium alone were used to establish the background level of costimulatory molecule expression (CD27^{lo}CD28^{lo} double positives) so the proportion of cells that increased CD27/CD28 expression above this level could be calculated (A). Ki67 expression was analyzed (frequency distribution histograms) in the CD27^{lo}CD28^{lo} and CD27^{hi}CD28^{hi} subsets. Representative results are shown for a HIV patient (B). Induction of Ki67 5 days post stimulation with anti-CD3 was greater in CD27^{hi}CD28^{hi} cells than CD27^{lo}CD28^{lo} cells in HIV patients (⊙) and healthy controls (+) in total (C; p<0.0001 and p<0.0001), naïve (D; p<0.0001 and p=0.001) and CD31⁺ naïve CD4⁺ subsets (E; p<0.0001 and p=0.008). *p≤0.05, **p≤0.005, ***p≤0.0005

Figure 4. Upregulation of CD27 and CD28 expression after anti-CD3 stimulation correlated with proliferation (measured by Ki67 expression). The Δ Ki67 represents the increase in the percentages of cells that expressed Ki67 after stimulation with anti-CD3 for 5 days, over cells incubated in medium alone. The proportion of CD4⁺ T-cells that upregulated expression of CD27 and CD28 to a CD27^{hi}CD28^{hi} phenotype after anti-CD3 stimulation was similar in HIV patients with low or normal CD4⁺ T-cell counts (p=0.48), but lower in both patient groups compared to healthy controls (p=0.008 and p=0.007, respectively) (**A**). Following stimulation with anti-CD3, upregulation of CD27 and CD28 expression on total, naïve and CD31⁺ naïve CD4⁺ T-cells correlated with Δ Ki67 in total (**B**; r=0.56, p=0.002), naïve (**C**; r=0.46, p=0.02) and CD31⁺ naïve (**D**; r=0.41, p=0.03) CD4⁺ T-cells in HIV patients. After stimulation with anti-CD3, upregulation of CD27 and CD28 expression on total, naïve and CD31⁺ naïve CD4⁺ T-cells marginally correlated with Δ Ki67 in total CD4⁺ T-cells (**E**; r=0.57, p=0.09), and positively associated with naïve (**F**; r=0.67, p=0.04) and CD31⁺ naïve (**G**; r=0.65, p=0.04) CD4⁺ T-cells in healthy controls.

Table 1. Characteristics of the patient groups

	Low CD4⁺ T-cells	Normal CD4⁺ T-cells	Intermediate CD4⁺ T-cells	<i>p</i> ^b
	(n=13)	(n=20)	(n=6)	
Age (years)	55 (36-65) ^a	47 (34-66)	52 (33-69)	0.09 ^c
Male : Female	12 : 1	19 : 1	6 : 0	1.0 ^d
Nadir CD4 ⁺ T-cells/ μ L	22 (6-80)	17 (0-88)	5 (20-45)	0.15
Months on ART	52 (12-125)	112 (39-134)	93 (46-137)	0.003
Months with HIV RNA <50 copies/mL	33 (8-99)	73 (6-131)	65 (20-77)	0.02
Current CD4 ⁺ T-cells/ μ L blood	209 (75-324)	713 (504-1295)	404 (378-476)	<0.0001
Current CD8 ⁺ T-cells/ μ L blood	732 (205-1472)	958 (437-1628)	1390 (406-3234)	0.03
Naïve CD4 ⁺ T-cells/ μ L blood	26 (5-127)	145 (25-364)	44 (26-85)	<0.0001
Naïve CD8 ⁺ T-cells/ μ L blood	139 (10-413)	282 (25-448)	108 (32-476)	0.008
CD31 ⁺ naive CD4 ⁺ T cells (% CD4 ⁺ T cells)	13 (0.3-30)	27 (5-40)	22 (5-25)	0.003

^a median (range), ^b comparing patients with low and normal CD4⁺ T-cells, ^c *p* values obtained by Mann-Whitney test unless otherwise indicated, ^d *p* values obtained by Fisher's exact test

Table 2. Baseline expression of CD27 and/or CD28 on CD4⁺ T-cells is reduced in HIV patients with low CD4⁺ T-cell counts on ART

	A Low CD4⁺ T-cells <i>(n=13)</i>	B Normal CD4⁺ T-cells <i>(n=20)</i>	C Healthy controls <i>(n=10)</i>	D Intermediate CD4⁺ T-cells <i>(n=6)</i>	A vs B <i>p</i> value	A vs C <i>p</i> value	B vs C <i>p</i> value
CD4⁺CD27⁺ T-cells (%)	71 (20-92) ^a	89 (73-96)	91 (78-95)	74 (65–93) ^c	0.0007^b	0.003	0.26
CD4⁺CD28⁺ T-cells (%)	93 (30-99)	98 (86-100)	99 (87-100)	88 (78-99)	0.03	0.03	0.33
CD4⁺CD27⁺CD28⁺ T-cells (%)	71 (21-92)	87 (71-95)	91 (78-95)	74 (65-93)	0.0009	0.003	0.19

^a median (range), ^b *p* values obtained by Mann-Whitney test, ^c values presented for patients with intermediate CD4⁺ T-cells (column D) did not differ significantly from those of any other cohort (columns A, B and C),