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In: AIDS, Vol. 28, No. 6, 27.03.2014, p. 821-830.

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This is a non-final version of an article published in final form in AIDS (see citation above).

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IL-7 signalling defects in naïve CD4⁺ T cells of HIV patients with CD4⁺ T cell deficiency on ART associate with T cell activation and senescence

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Submitted as an Original Paper

Word counts: Abstract 247, Text 3584

Running Head: Defects of the IL-7 signalling pathway in HIV

Conflicts of Interest and Source of Funding: The authors certify that they do not have a commercial or other association that might pose a conflict of interest. The work was supported by a program grant (510448) from the National Health and Medical Research Council of Australia.

ABSTRACT

Objective: To examine the relationship of defects in IL-7-induced naïve CD4⁺ T-cell homeostasis with residual immune activation and CD4⁺ T-cell senescence in HIV patients receiving ART who exhibit persistent CD4⁺ T cell deficiency.

Design: IL-7-induced proliferation of, and IL-7R signalling in, total and naïve CD4⁺ T-cells of HIV patients who had low (<350/μL) or normal (>500/μL) CD4⁺ T-cell counts on ART were examined and related to markers of CD4⁺ T-cell activation and senescence and innate immune activation.

Methods: Total, naïve (CD45RA⁺ CD27⁺) and CD31⁺ naïve CD4⁺ T-cells from aviremic HIV patients (n=39) with nadir CD4⁺ T-cell counts <100 cells/μL, who had received ART for a median time of 7 (range 1-11) years, were assessed for CD127 expression, proliferation (Ki67), STAT5 phosphorylation (pSTAT5), and CD127 modulation following IL-7 stimulation. Changes were related to proportions of CD4⁺ T-cells expressing HLA-DR or CD57 and plasma levels of sCD14, CXCL9 and CXCL10.

Results: Patients with CD4⁺ T-cell deficiency exhibited lower expression of CD127 on total, naïve and CD31⁺ naïve CD4⁺ T-cells. Downregulation of CD127 after culture with IL-7 correlated inversely with CD4⁺ T cell counts and directly with Ki67 expression. Induction of pSTAT5 in CD4⁺ T-cell subsets was greater in patients with normal CD4⁺ T-cell counts. CD127 expression correlated inversely with proportions of CD4⁺CD57⁺ T-cells, and pSTAT5 induction correlated inversely with CD4⁺ T-cell expression of HLA-DR and CD57.

Conclusions: Defects of IL-7 signalling in HIV patients with persistent CD4⁺ T-cell deficiency receiving ART are associated with CD4⁺ T-cell activation and senescence.

Keywords: CD127; CD4⁺ T-cell; human immunodeficiency virus; immune activation; interleukin-7; senescence; STAT5 phosphorylation

INTRODUCTION

A proportion of HIV patients receiving antiretroviral therapy (ART) retain low CD4⁺ T-cell counts, despite optimal suppression of HIV replication. The frequency of persistent CD4⁺ T-cell deficiency varies from 8% in patients observed over 2 years on ART [1] to 35.8% over 5 years on ART [2]. A low nadir CD4⁺ T-cell count increases the risk of persistent CD4⁺ T-cell deficiency, so that 40% of patients with a nadir CD4⁺ T-cell count of <200/ μ L have this problem after 10 years on ART [3]. CD4⁺ T-cell deficiency on ART is associated with a higher rate of mortality and morbidity [4], which includes atherosclerotic vascular disease [5, 6], osteoporosis and fractures [7] and non-AIDS-defining cancers [8-10].

CD4⁺ T-cell deficiency on ART is associated with older age, ongoing immune activation [11, 12], fibrosis of lymphoid tissue [13, 14] and a failure to regenerate naïve T-cells through thymopoiesis [15-17] and/or peripheral homeostasis. The latter may reflect a reduced capacity of naïve T-cells to proliferate following stimulation with interleukin-7 (IL-7) and/or T-cell receptor (TCR) ligation [18]. Furthermore, we have shown that the naïve T-cell subset is particularly affected by immune activation [19]. CD4⁺ T cell deficiency on ART is also associated with activation of the innate immune system, particularly monocytes [20].

IL-7 is produced by stromal cells of the bone marrow and thymus, and by dendritic cells in secondary lymphoid organs. It has an essential role in thymopoiesis, homeostatic proliferation and survival of naïve and memory T-cells [21-23] with CD31⁺ naïve T-cells being more responsive than long-term resident peripheral T-cells [24]. T-cell responses to IL-7 are initiated through the IL-7 receptor (IL-7R) [25]. The IL-7R is expressed by cells of the lymphoid lineage and consists of a unique α chain (IL-7R α ; CD127) and a common cytokine receptor γ chain. IL-7 binding to IL-7R activates Janus kinases, JAK1 and JAK3, and

stimulates the intracellular domain of CD127. This leads to phosphorylation of the transcription factor STAT5 (pSTAT5), which dimerizes and translocates to the nucleus to bind to its target DNA and activate transcription [26, 27].

Untreated HIV infection is associated with increased circulating levels of IL-7 and low expression of CD127 on peripheral T-cells [28-31]. Elevated levels of IL-7 have been detected in other lymphopenic conditions, such as severe combined immunodeficiency, chemotherapy-induced lymphopenia [28] and idiopathic CD4⁺ lymphopenia [32], so increased IL-7 production may be a compensatory mechanism invoked as a homeostatic response to T-cell depletion.

Serum IL-7 levels decline with increased CD4⁺ T-cell numbers in HIV patients receiving ART, but CD127 expression on naïve, effector and terminally differentiated CD4⁺ T-cells may remain low [31, 33, 34]. IL-7 binding and IL-7R signalling in naïve and memory CD4⁺ T-cells from viremic and aviremic patients appears to be normal, despite elevated levels of pSTAT5 and reduced expression of the survival protein Bcl-2 in naïve T-cells [35]. However, T-cells from HIV patients display impaired access of STAT5 to the nuclear compartment, which may prevent the induction of downstream pro-survival signals such as Bcl-2 [26].

Recombinant IL-7 therapy can increase CD4⁺ T-cell counts in HIV patients with CD4⁺ T-cell deficiency on ART [36] but it is unclear whether this reflects increased thymopoiesis or homeostatic proliferation of CD4⁺ T-cells. Here, we examine IL-7-induced homeostatic mechanisms in naïve CD4⁺ T-cells of HIV patients with good or poor CD4⁺ T-cell recovery on ART to determine if they are affected by immune activation and CD4⁺ T-cell senescence.

METHODS

Study population

Thirty-nine adult HIV-1-infected patients (37 males) receiving combination ART were recruited in 2007-2008. Criteria for inclusion were nadir CD4⁺ T-cell counts <100/ μ L, ART for >12 months and undetectable plasma HIV RNA (<50 copies/mL) for >6 months. Patients were categorized as having normal (n=20) or low (n=13) CD4⁺ T-cell counts based on values of >500/ μ L or <350/ μ L, respectively. For correlations between the parameters measured, an additional six patients with intermediate CD4⁺ T-cell counts (350-500/ μ L) were included. Informed consent was obtained from all subjects, and the study was approved by an institutional ethics committee.

HIV-1 RNA viral load

Plasma HIV RNA levels were assayed by quantitative reverse transcription polymerase chain reaction (AmplicorTM Version 1.5, Ultrasensitive Protocol, 50-75,000 copies/mL) (Roche Diagnostic Systems, USA).

T-cell immunophenotyping

Total and naïve 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, USA) by the Flow Cytometry Unit at Royal Perth Hospital. Peripheral blood mononuclear cells (PBMC) were separated from lithium heparin-treated whole blood by Ficoll-PaqueTM density centrifugation and washed twice in RPMI 1640. Viable cells were counted by trypan blue exclusion and resuspended in 10% dimethylsulphoxide/90% heat inactivated fetal calf serum at >10⁷ cells/mL for storage in liquid nitrogen. Thawed PBMC were stained for

surface markers for 15 minutes. The following fluorescently conjugated mAbs were used for assessment of CD127 expression: CD3-V450, CD4-V500, CD45RA-APC-H7, CCR7-PeCy7, CD27-PerCP-Cy5.5, CD28-PE, CD31-FITC, and CD127-AF674 (BD Biosciences). For assessment of immune activation molecules, PBMC were incubated with: CD3-V450, CD4-V500, CD45RA-APC-H7, CCR7-PeCy7, CD27-PerCP-Cy5.5, HLA-DR-PE, CD38-APC, and CD31-FITC (BD Biosciences). 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 for 20 minutes, followed by 1 mL FACSlyse (BD Biosciences) for a further 15 minutes. A minimum of 100,000 events per sample were analysed and gates were set using appropriate controls. Lymphocytes were identified by their forward and side light scatter, and subsequently total CD4⁺ T-cells were identified as CD3⁺CD4⁺ while naïve CD4⁺ T-cells were identified as CD3⁺CD4⁺CD45RA⁺CD27⁺. The CD31⁺ subpopulation of naïve CD4⁺ T-cells was also assessed as this phenotype is thought to mark recent thymic emigrants. All analyses were performed using a FACS Canto II cytometer (BD Biosciences). Files were exported in FCS 3.0 format and visualised using FlowJo software, version 7.6.3 (Tree Star, USA).

Assessment of STAT5 phosphorylation

Cryopreserved PBMC were thawed, washed and incubated for 15 minutes at room temperature with the following fluorescently conjugated mAbs: CD3-V450, CD4-V500, CD27-FITC and CD31-PE (BD Biosciences). Following surface staining, cells were cultured in the presence or absence of IL-7 (5ng/mL) at 37°C for 15 minutes and then fixed using Cytotfix Buffer (BD Biosciences). Cells were then permeabilised using Phosflow Perm Buffer III and stained with fluorescently conjugated mAbs to STAT5-AF647 and CD45RA-APC-H7 (BD Biosciences).

Proliferation assay and Ki67 expression

Thawed PBMC were cultured (10^6 cells/mL) in the presence or absence of IL-7 (5ng/mL) at 37°C for 5 days. Cells were then surface stained with the following fluorescently conjugated mAbs: CD3-V450, CD4-V500, CD45RA-APC-H7, CCR7-PeCy7, CD27-PerCP-Cy5.5, CD28-APC and CD31-PE (BD Biosciences). Intracellular staining using Ki67-FITC was then performed using the Human FoxP3 Buffer Set (BD Biosciences) according to the manufacture's protocol.

Measurement of plasma levels of sCD14, CXCL9 and CXCL10

Plasma levels of soluble (s) CD14 were assayed by enzyme-linked immunosorbent assay (R&D Systems) as described previously [37]. Plasma levels of CXCL9 and CXCL10 were assayed by cytometric bead arrays [38].

Statistical analysis

Statistical analyses were performed with Graph Pad Prism software, version 5.01 (USA). The Mann-Whitney test was performed for continuous variables and correlation coefficients were determined by the Spearman's rank correlation test. Fisher's exact test was performed for categorical analyses. For all tests, $p < 0.05$ was considered to represent a significant difference.

RESULTS

Patients

Demographic and clinical data on patients are presented in Table 1. All patients had received ART for >12 months but the duration of therapy was longer in patients with normal CD4⁺ T-cell counts. However, time on ART did not correlate with proportions of total or naive CD4⁺

T-cells in an analysis that combined both patient groups ($r=0.23-0.24$, $p=0.14-0.17$, respectively). Furthermore, 5 years after study entry, all participants originally classified as having CD4⁺ T-cell deficiency had CD4⁺ T-cell counts $<340/\mu\text{L}$ after a median (range) time of 12 (4-17) years on ART. In addition, CD4⁺ T-cell counts obtained from the start of ART until the time of study entry were previously modelled for each patient and best-fit curves used to estimate a 10-year CD4⁺ T-cell count [37]. Using these data, all patients classified as having CD4⁺ T-cell deficiency here had estimated CD4⁺ T-cell counts of $<350/\mu\text{L}$ 10 years after commencing ART.

IL-7-induced 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 IL-7 (5ng/mL) in 5 day cultures was assessed by subtracting the percentages of CD4⁺ T-cells expressing Ki67 in unstimulated cultures from the percentages expressing Ki67 after stimulation (ΔKi67). Low lymphocyte viability (absent lymphocyte population on flow cytometry plots; Supplemental Figure 1) precluded analysis of data from cultures in some patients. This was more common in patients with CD4⁺ T-cell deficiency than patients with normal CD4⁺ T-cell counts (38% vs 5%; Fisher's exact test, $p=0.02$). Analysis of data from patients with CD4⁺ T-cell deficiency demonstrated no differences in any demographic characteristics described in Table 1 between those with and without viable cells in culture. When analyses were restricted to samples with good cell viability ($n=26$), ΔKi67 was similar in patients with low or normal CD4⁺ T-cell counts ($p=0.93$) (Supplemental Figure 2). Similarly, ΔKi67 in 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).

HIV patients with low CD4⁺ T-cell counts exhibited diminished CD127 expression on all CD4⁺ T-cell populations

To assess whether CD127 expression is perturbed on CD4⁺ T-cells, we measured mean fluorescence intensity (MFI) of CD127 expression at day 0 and following 5 day cultures with and without IL-7. At day 0, CD127 expression on total, naïve and CD31⁺ naïve CD4⁺ T-cells was higher in patients with normal CD4⁺ T-cell counts ($p=0.03-0.04$; Fig. 1A). Stimulation with IL-7 for 5 days downregulated CD127 in all CD4⁺ T-cell populations ($p=0.008-0.0001$), but no differences in CD127 expression were evident when patients with CD4⁺ T-cell deficiency were compared to those with normal CD4⁺ T-cell counts ($p=0.15-0.70$; Fig. 1B).

To evaluate the relative degree of downregulation of CD127, we subtracted the CD127-MFI of cells incubated in medium alone from that of cells incubated with IL-7 (Δ CD127-MFI). The downregulation of CD127 was reported as an absolute value. The Δ CD127-MFI on total CD4⁺ T-cells was lower in patients with normal CD4⁺ T-cell counts compared to patients with CD4⁺ T-cell deficiency, although statistical significance was not reached (medians of 245 and 398, respectively; Fig. 1C). Furthermore, the Δ CD127-MFI correlated inversely with total CD4⁺ T-cell counts ($r=-0.37$, $p=0.04$) but not naïve CD4⁺ T-cell counts ($r=-0.03$, $p=0.89$) in all patients ($n=39$) (Fig. 1D). Levels of CD127 at day 0 did not correlate with the Δ CD127-MFI ($r=0.27$, $p=0.16$).

CD4⁺ T-cell proliferation correlated inversely with downregulation of CD127 expression

In 5 day cultures stimulated with IL-7, the Δ CD127-MFI on total, naïve and CD31⁺ naïve CD4⁺ T-cell subsets correlated with Δ Ki67 in total ($r=0.39$, $p=0.03$; Fig. 2A), naïve ($r=0.60$,

$p=0.002$; Fig. 2B) and $CD31^+$ naive ($r=0.59$, $p=0.003$; Fig. 2C) $CD4^+$ T-cells. Levels of CD127 expression at day 0 in total, naive and $CD31^+$ naive $CD4^+$ T-cell subsets did not correlate with IL-7-induced $\Delta Ki67$ following 5 day cultures in total, naive or $CD31^+$ naive $CD4^+$ T-cell subsets. Thus, greater downregulation of CD127 was associated with increased proliferation.

IL-7-induced STAT5 phosphorylation in $CD4^+$ T-cells correlated with circulating $CD4^+$ T-cell counts

To assess the IL-7R signalling pathway, PBMCs were incubated for 15 minutes with or without IL-7, and the increase in the proportion of cells positive for pSTAT5 above the background levels ($\Delta pSTAT5$) was determined. $\Delta pSTAT5$ was higher in HIV patients with normal $CD4^+$ T-cell counts than patients with $CD4^+$ T-cell deficiency when assessed in total $CD4^+$ T-cells ($p=0.003$), naive $CD4^+$ T-cells ($p=0.003$) and $CD31^+$ naive $CD4^+$ T-cells ($p=0.008$; Fig. 3A). Accordingly, $\Delta pSTAT5$ in each population correlated directly with numbers of total ($r=0.38$, $p=0.03$), naive ($r=0.43$, $p=0.01$) and $CD31^+$ naive ($r=0.35$, $p=0.04$) $CD4^+$ T-cells.

Induction of STAT5 phosphorylation by IL-7 correlated with CD127 expression on $CD4^+$ T-cells but not with proliferation

IL-7-induced $\Delta pSTAT5$ in total, naive and $CD31^+$ naive $CD4^+$ T-cells correlated with levels of CD127 expression at day 0 in total ($r=0.62$, $p=0.0002$; Fig. 3B), naive ($r=0.54$, $p=0.001$; Fig. 3C) and $CD31^+$ naive ($r=0.37$, $p=0.03$; Fig. 3D) $CD4^+$ T-cells. However, $\Delta pSTAT5$ did not correlate with $\Delta Ki67$ [$r=-0.08$ -(-0.16), $p=0.46$ -0.68] or $\Delta CD127$ -MFI [$r=-0.002$ -(-0.18), $p=0.36$ -0.9] in any $CD4^+$ T-cell subset.

IL-7-induced STAT5 phosphorylation in CD4⁺ T-cells correlated inversely with T-cell activation and senescence

Finally, we examined the relationship between Δ pSTAT5 in CD4⁺ T-cells and markers of CD4⁺ T-cell activation and senescence and innate immune system activation. As previously demonstrated [19], patients with low CD4⁺ T-cell counts had higher expression of both HLA-DR ($p=0.0007$) and CD57 ($p=0.01$; Fig. 4A) on total CD4⁺ T-cells than patients with normal CD4⁺ T-cell counts.

Expression of CD57 on total CD4⁺ T-cells correlated inversely with Δ pSTAT5 in total ($r=-0.62$, $p<0.001$; Fig. 4B), naïve ($r=-0.61$, $p=0.002$; Fig. 4C) and CD31⁺ naïve CD4⁺ T-cells ($r=-0.65$, $p<0.001$; Fig. 4D). Furthermore, inverse correlations were observed between CD57 expression on total CD4⁺ T-cells and expression of CD127 (at day 0) on total ($r=-0.54$, $p=0.008$; Fig. 4E), naïve ($r=-0.40$, $p=0.02$; Fig. 4F) and CD31⁺ naïve CD4⁺ T-cells ($r=-0.64$, $p=0.03$; Fig. 4G). We also observed inverse correlations between the expression of HLA-DR on CD4⁺ T-cells and Δ pSTAT5 in total ($r=-0.37$, $p=0.03$) and naïve CD4⁺ T-cells ($r=-0.36$, $p=0.03$), but not CD31⁺ naïve CD4⁺ T-cells ($r=-0.17$, $p=0.32$). However, no correlations were evident between HLA-DR expression on total CD4⁺ T-cells and expression of CD127 (at day 0) on total ($r=-0.11$, $p=0.54$), naïve ($r=-0.19$, $p=0.27$) and CD31⁺ naïve CD4⁺ T-cells ($r=-0.21$, $p=0.22$).

In contrast to the findings for T cell activation and senescence, Δ pSTAT5 in CD4⁺ T-cells was not clearly associated with plasma markers of innate immune system activation. While Δ pSTAT5 in total CD4⁺ T cells showed a weak inverse correlation with plasma levels of

CXCL9 ($r=-0.35$, $p=0.04$), there was no association with sCD14 ($r=-0.08$, $p=0.63$) or CXCL10 ($r=0.11$, $p=0.50$) (data not shown).

DISCUSSION

Substantial disruption of the IL-7/IL-7R pathway has been described in patients with HIV infection receiving effective ART, contributing to impaired naïve T-cell homeostasis and persistent CD4⁺ T-cell deficiency. A greater understanding of these defects may assist in identifying patients likely to benefit from recombinant IL-7 therapy.

While low cell viability precluded analysis of data from cell cultures in 38% of patients with persistent CD4⁺ T-cell deficiency, we demonstrated that IL-7-induced proliferation of CD4⁺ T-cells (Ki67 expression) was not associated with numbers of circulating total, naïve or CD31⁺ naïve CD4⁺ T-cells. Similarly, Bazdar *et al.* showed that TCR responsiveness is diminished in naïve CD4⁺ T-cells from viremic HIV infected patients, whereas responsiveness to IL-7-induced stimulation is relatively preserved and IL-7 enhances responses after TCR stimulation [18]. Hence, diminished TCR responsiveness may be more important than impaired responsiveness to IL-7 in causing decreased CD4⁺ T-cell proliferation in patients with persistent CD4⁺ T-cell deficiency.

Our findings of lower CD127 expression at day 0 on total, naïve and CD31⁺ naïve CD4⁺ T-cells in patients with persistent CD4⁺ T-cell deficiency are in accord with those of previous studies [31, 33, 34]. Bai *et al.* examined immunological non-responders, and found that low CD127 expression on CD4⁺ T-cells was the only marker associated with incomplete CD4⁺ T-cell recovery [39]. Furthermore, CD4⁺ T-cell recovery during ART is associated with genetic polymorphism of CD127 [34]. Hence, low CD4⁺ T-cell counts may be attributed to the lack

of IL-7-induced signalling caused by decreased CD127 availability, resulting in reduced survival of these cells [23]. Our study furthers the work of Bai *et al.* [39] by examining the effects of IL-7 stimulation on IL-7R expression on, and induction of pSTAT5 in, naïve CD4⁺ T cells, as well as the relationship with immune activation.

We found that total, naïve and CD31⁺ naïve CD4⁺ T-cells from all patients were responsive to IL-7 and able to modulate CD127 expression (Fig. 1B), which occurred to a greater degree in total CD4⁺ T-cells of patients with CD4⁺ T-cell deficiency and negatively correlated, albeit weakly, with CD4⁺ T-cell counts (Fig. 1C-D). It is established that transcription and expression of IL-7R α is suppressed by IL-7 and other pro-survival cytokines [24, 25, 40, 41]. The transient downregulation of IL-7R α on T-cells that have recently received an IL-7 signal, ensures that they will not compete with unstimulated T-cells for any remaining IL-7, thus increasing T-cell survival.

STAT5 activation is crucial in signalling pathways controlling CD4⁺ T-cell survival and proliferation, through the induction of anti-apoptotic molecules such as Bcl-2, and through the phosphatidylinositol 3-kinase/Akt pathway [42, 43]. We therefore examined IL-7-induced pSTAT5 in CD4⁺ T-cell subsets. This was greater in total, naïve and CD31⁺ naïve CD4⁺ T-cells from patients with normal CD4⁺ T-cell counts. In these patients, levels of pSTAT5 were relatively uniform (50-95%) and never fell below 50% in any CD4⁺ T-cell subset. In contrast, levels of pSTAT5 were more variable (8-87%) in patients with CD4⁺ T-cell deficiency (Fig. 3A). Our findings further those of Camargo *et al.* who showed that levels of pSTAT5 in total T-cells were higher in patients with CD4⁺ T-cell counts >500/ μ L compared to patients with counts <500/ μ L [44].

Lower IL-7-induced pSTAT5 expression in CD4⁺ T-cell of patients with CD4⁺ T-cell deficiency on ART may have a negative impact on cell survival through reduced induction of anti-apoptotic factors such as Bcl-2 [35, 45, 46]. We demonstrated that IL-7-induced pSTAT5 expression in total, naïve and CD31⁺ naïve CD4⁺ T-cells correlated with CD127 expression on the corresponding CD4⁺ T-cell subset, as has previously been observed for total T-cells [44]. However, IL-7-induced pSTAT5 was not correlated with proliferation in any CD4⁺ T-cell subsets. Furthermore, when analysis was divided into CD31⁺ and CD31⁻ naïve CD4⁺ T-cell subsets, the same associations between CD127, pSTAT5 and Ki67 expression were observed (data not shown). The discordance between pSTAT5 and proliferation may have arisen because pSTAT5 was measured following a 15 minute incubation with IL-7, whilst Ki67 expression was assessed 5 days post-stimulation with IL-7. In addition, CD4⁺ T-cells from HIV patients receiving ART may be capable of phosphorylating STAT5, but have a reduced ability to translocate STAT5 to the nucleus [26]. Therefore, the link between IL-7-induced pSTAT5 and proliferation may not be linear.

Interestingly, HIV patients with the lowest levels of pSTAT5 (less than 20%) had non-viable cells after stimulation with IL-7 for 5 days (data not shown). The level of CD127 expression has been shown to correlate with IL-7-induced Bcl-2 and CD25 in healthy donors whilst IL-7-induced Bcl-2 and CD25 expression was reduced in untreated viremic HIV patients, who exhibited low CD127 expression [45]. Colle *et al.* examined CD4⁺ T-cells following 3-6 days of culture with and without IL-7, and found lower induction of Bcl-2 in treated patients with CD4⁺ T-cell counts of <250/ μ L than in patients with counts of >400/ μ L [46]. These results suggest that successful ART can partially correct IL-7R signalling defects, but this is least effective in patients with lower CD4⁺ T-cell counts.

We and others have associated CD4⁺ T-cell deficiency on ART with immune activation [12, 19]. T-cell activation (assessed by HLA-DR or CD38 expression) correlates with reduced CD127 expression on CD4⁺ T-cells in HIV patients [33, 47, 48], and loss of CD127 expression in HIV infection may be driven by immune activation [49, 50]. A notable finding of our study was that CD4⁺ T-cell activation (HLA-DR⁺) and senescence (CD57⁺) correlated inversely with IL-7-induced pSTAT5 in CD4⁺ T-cells and that increased CD4⁺ T-cell senescence was associated with lower expression of CD127 at day 0 on total, naïve and CD31⁺ naïve CD4⁺ T-cells. Hence, HIV patients who have a greater accumulation of senescent CD4⁺ T-cells may be less responsive to IL-7. These findings raise the possibility that IL-7-induced CD4⁺ T-cell homeostasis in HIV patients receiving ART might be improved by therapies that reduce T-cell activation and senescence. Furthermore, the proportion of CD57⁺CD4⁺ T-cells should be investigated as a simple predictor of response to recombinant IL-7 therapy.

The association of immunosenescence (CD57⁺) in total CD4⁺ T-cells with defects of IL-7/IL-7R signalling in naïve CD4⁺ T-cells is notable as naïve T-cells express very low amounts of CD57. We propose that CD4⁺ T-cell senescence on the one hand, and low pSTAT5 expression within, and CD127 expression upon, naïve CD4⁺ T-cells on the other hand, result from a common cause of T-cell activation. One possible cause is persistent inflammation in lymphoid tissue. (13, 14)

Although activation of the innate immune system, particularly monocytes, persists in HIV patients receiving ART [20], we did not demonstrate a clear relationship between plasma markers of innate immune system activation and defects in the IL-7 signalling pathway of naïve CD4⁺ T cells.

Limitations to our study include the low number of patients with CD4⁺ T-cell deficiency on ART, particularly for studies after PBMC cultures. Also, patients with CD4⁺ T-cell deficiency had received ART for a shorter period of time. However, we undertook very rigorous analyses to demonstrate the stability of CD4⁺ T-cell deficiency over time. Particular strengths of our study were the inclusion criterion of a nadir CD4⁺ T-cell count of <100/μL, as low nadir CD4⁺ T-cell counts are a strong predictor of poor immune reconstitution on ART [51-53], and our definition of CD4⁺ T cell deficiency (<350 cells/μL) and normal CD4⁺ T-cell counts (>500 cells/μL), as these values predict mortality [54] and, arguably, are more relevant than those used in other studies [39, 44, 55].

In summary, our findings provide evidence that impaired IL-7/IL-7R signalling, which leads to poor immune reconstitution in HIV patients receiving ART, is associated with activation and senescence of CD4⁺ T-cells. Enhancement of IL-7-induced CD4⁺ T-cell homeostasis might therefore be achieved by resolution of CD4⁺ T cell activation and senescence. Furthermore, assessment of CD4⁺ T-cell senescence may identify patients who are most likely to benefit from IL-7 therapy for persistent CD4⁺ T-cell deficiency.

ACKNOWLEDGEMENTS

The work was supported by a program grant (510448) from the National Health and Medical Research Council of Australia. This is manuscript number 2013-06 for the Department of Clinical Immunology, Royal Perth Hospital. The authors certify that they do not have a commercial or other association that might pose a conflict of interest.

Author contributions: M.F. conceived and developed the study, and recruited patients for the study. S.F. and S.T. designed the study. S.T. performed the experiments, conducted data analysis and interpretation, and wrote the manuscript. S.F., P.P. and M.F. were involved in the interpretation of data along with review of the manuscript. All authors have read and approved the text as submitted to AIDS.

REFERENCES

1. Renaud M, Katlama C, Mallet A, Calvez V, Carcelain G, Tubiana R, *et al.* Determinants of paradoxical CD4 cell reconstitution after protease inhibitor-containing antiretroviral regimen. *AIDS* 1999,**13**:669-676.
2. Kaufmann GR, Furrer H, Ledergerber B, Perrin L, Opravil M, Vernazza P, *et al.* Characteristics, determinants, and clinical relevance of CD4 T cell recovery to <500 cells/microL in HIV type 1-infected individuals receiving potent antiretroviral therapy. *Clin Infect Dis* 2005,**41**:361-372.
3. Kelley CF, Kitchen CM, Hunt PW, Rodriguez B, Hecht FM, Kitahata M, *et al.* Incomplete peripheral CD4+ cell count restoration in HIV-infected patients receiving long-term antiretroviral treatment. *Clin Infect Dis* 2009,**48**:787-794.
4. Helleberg M, Kronborg G, Larsen CS, Pedersen G, Pedersen C, Obel N, *et al.* CD4 decline is associated with increased risk of cardiovascular disease, cancer, and death in virally suppressed patients with HIV. *Clin Infect Dis* 2013,**57**:314-321.
5. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, *et al.* Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 2007,**8**:359-368.
6. van Lelyveld SF, Gras L, Kesselring A, Zhang S, De Wolf F, Wensing AM, *et al.* Long-term complications in patients with poor immunological recovery despite virological successful HAART in Dutch ATHENA cohort. *AIDS* 2012,**26**:465-474.
7. Yong MK, Elliott JH, Woolley IJ, Hoy JF. Low CD4 count is associated with an increased risk of fragility fracture in HIV-infected patients. *J Acquir Immune Defic Syndr* 2011,**57**:205-210.
8. Calabresi A, Ferraresi A, Festa A, Scarcella C, Donato F, Vassallo F, *et al.* Incidence of AIDS-defining cancers and virus-related and non-virus-related non-AIDS-defining cancers among HIV-infected patients compared with the general population in a large health district of northern Italy, 1999-2009. *HIV Med* 2013.
9. Helleberg M, Kronborg G, Larsen CS, Pedersen G, Pedersen C, Obel N, *et al.* CD4 Decline Is Associated With Increased Risk of Cardiovascular Disease, Cancer, and Death in Virally Suppressed Patients With HIV. *Clin Infect Dis* 2013.
10. Monforte A, Abrams D, Pradier C, Weber R, Reiss P, Bonnet F, *et al.* HIV-induced immunodeficiency and mortality from AIDS-defining and non-AIDS-defining malignancies. *AIDS* 2008,**22**:2143-2153.
11. Benito JM, Lopez M, Lozano S, Ballesteros C, Martinez P, Gonzalez-Lahoz J, *et al.* Differential upregulation of CD38 on different T-cell subsets may influence the ability to reconstitute CD4+ T cells under successful highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* 2005,**38**:373-381.

12. Lederman MM, Calabrese L, Funderburg NT, Clagett B, Medvik K, Bonilla H, *et al.* Immunologic failure despite suppressive antiretroviral therapy is related to activation and turnover of memory CD4 cells. *J Infect Dis* 2011,**204**:1217-1226.
13. Zeng M, Paiardini M, Engram JC, Beilman GJ, Chipman JG, Schacker TW, *et al.* Critical role of CD4 T cells in maintaining lymphoid tissue structure for immune cell homeostasis and reconstitution. *Blood* 2012,**120**:1856-1867.
14. Zeng M, Southern PJ, Reilly CS, Beilman GJ, Chipman JG, Schacker TW, *et al.* Lymphoid tissue damage in HIV-1 infection depletes naive T cells and limits T cell reconstitution after antiretroviral therapy. *PLoS Pathog* 2012,**8**:e1002437.
15. Fernandez S, Nolan RC, Price P, Krueger R, Wood C, Cameron D, *et al.* Thymic function in severely immunodeficient HIV type 1-infected patients receiving stable and effective antiretroviral therapy. *AIDS Res Hum Retroviruses* 2006,**22**:163-170.
16. Li T, Wu N, Dai Y, Qiu Z, Han Y, Xie J, *et al.* Reduced thymic output is a major mechanism of immune reconstitution failure in HIV-infected patients after long-term antiretroviral therapy. *Clin Infect Dis* 2011,**53**:944-951.
17. Teixeira L, Valdez H, McCune JM, Koup RA, Badley AD, Hellerstein MK, *et al.* Poor CD4 T cell restoration after suppression of HIV-1 replication may reflect lower thymic function. *AIDS* 2001,**15**:1749-1756.
18. Bazdar DA, Sieg SF. Interleukin-7 enhances proliferation responses to T-cell receptor stimulation in naive CD4+ T cells from human immunodeficiency virus-infected persons. *J Virol* 2007,**81**:12670-12674.
19. Fernandez S, Price P, McKinnon EJ, Nolan RC, French MA. Low CD4+ T-cell counts in HIV patients receiving effective antiretroviral therapy are associated with CD4+ T-cell activation and senescence but not with lower effector memory T-cell function. *Clin Immunol* 2006,**120**:163-170.
20. Lederman MM, Funderburg NT, Sekaly RP, Klatt NR, Hunt PW. Residual immune dysregulation syndrome in treated HIV infection. *Adv Immunol* 2013,**119**:51-83.
21. Bradley LM, Haynes L, Swain SL. IL-7: maintaining T-cell memory and achieving homeostasis. *Trends Immunol* 2005,**26**:172-176.
22. Rathmell JC, Farkash EA, Gao W, Thompson CB. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol* 2001,**167**:6869-6876.
23. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, *et al.* IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci U S A* 2001,**98**:8732-8737.
24. Swainson L, Kinet S, Mongellaz C, Sourisseau M, Henriques T, Taylor N. IL-7-induced proliferation of recent thymic emigrants requires activation of the PI3K pathway. *Blood* 2007,**109**:1034-1042.

25. Park JH, Yu Q, Erman B, Appelbaum JS, Montoya-Durango D, Grimes HL, *et al.* Suppression of IL7R α transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* 2004,**21**:289-302.
26. Landires I, Bugault F, Lambotte O, de Truchis P, Slama L, Danckaert A, *et al.* HIV infection perturbs interleukin-7 signaling at the step of STAT5 nuclear relocalization. *AIDS* 2011,**25**:1843-1853.
27. Leonard WJ. Cytokines and immunodeficiency diseases. *Nat Rev Immunol* 2001,**1**:200-208.
28. Fry TJ, Connick E, Falloon J, Lederman MM, Liewehr DJ, Spritzler J, *et al.* A potential role for interleukin-7 in T-cell homeostasis. *Blood* 2001,**97**:2983-2990.
29. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, *et al.* Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* 2001,**7**:73-79.
30. Rethi B, Fluor C, Atlas A, Krzyzowska M, Mowafi F, Grutzmeier S, *et al.* Loss of IL-7R α is associated with CD4 T-cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients. *AIDS* 2005,**19**:2077-2086.
31. Bazdar DA, Kalinowska M, Sieg SF. Interleukin-7 receptor signaling is deficient in CD4⁺ T cells from HIV-infected persons and is inversely associated with aging. *J Infect Dis* 2009,**199**:1019-1028.
32. Puronen CE, Thompson WL, Imamichi H, Beq S, Hodge JN, Rehm C, *et al.* Decreased interleukin 7 responsiveness of T lymphocytes in patients with idiopathic CD4 lymphopenia. *J Infect Dis* 2012,**205**:1382-1390.
33. Benito JM, Lopez M, Lozano S, Gonzalez-Lahoz J, Soriano V. Down-regulation of interleukin-7 receptor (CD127) in HIV infection is associated with T cell activation and is a main factor influencing restoration of CD4(+) cells after antiretroviral therapy. *J Infect Dis* 2008,**198**:1466-1473.
34. Rajasuriar R, Booth D, Solomon A, Chua K, Spelman T, Gouillou M, *et al.* Biological determinants of immune reconstitution in HIV-infected patients receiving antiretroviral therapy: the role of interleukin 7 and interleukin 7 receptor alpha and microbial translocation. *J Infect Dis* 2010,**202**:1254-1264.
35. Juffroy O, Bugault F, Lambotte O, Landires I, Viard JP, Niel L, *et al.* Dual mechanism of impairment of interleukin-7 (IL-7) responses in human immunodeficiency virus infection: decreased IL-7 binding and abnormal activation of the JAK/STAT5 pathway. *J Virol* 2010,**84**:96-108.
36. Levy Y, Sereti I, Tambussi G, Routy JP, Lelievre JD, Delfraissy JF, *et al.* Effects of recombinant human interleukin 7 on T-cell recovery and thymic output in HIV-infected patients receiving antiretroviral therapy: results of a phase I/IIa randomized, placebo-controlled, multicenter study. *Clin Infect Dis* 2012,**55**:291-300.

37. Fernandez S, Tanaskovic S, Helbig K, Rajasuriar R, Kramski M, Murray JM, *et al.* CD4⁺ T-cell deficiency in HIV patients responding to antiretroviral therapy is associated with increased expression of interferon-stimulated genes in CD4⁺ T cells. *J Infect Dis* 2011,**204**:1927-1935.
38. Oliver BG, Elliott JH, Price P, Phillips M, Cooper DA, French MA. Tuberculosis after commencing antiretroviral therapy for HIV infection is associated with elevated CXCL9 and CXCL10 responses to Mycobacterium tuberculosis antigens. *J Acquir Immune Defic Syndr* 2012,**61**:287-292.
39. Bai F, Bellistri GM, Tincati C, Savoldi A, Pandolfo A, Bini T, *et al.* Reduced CD127 expression on peripheral CD4⁺ T cells impairs immunological recovery in course of suppressive highly active antiretroviral therapy. *AIDS* 2010,**24**:2590-2593.
40. Sasson SC, Zaunders JJ, Zanetti G, King EM, Merlin KM, Smith DE, *et al.* Increased plasma interleukin-7 level correlates with decreased CD127 and increased CD132 extracellular expression on T cell subsets in patients with HIV-1 infection. *J Infect Dis* 2006,**193**:505-514.
41. Vranjkovic A, Crawley AM, Gee K, Kumar A, Angel JB. IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human CD8⁺ T cells. *Int Immunol* 2007,**19**:1329-1339.
42. Jiang Q, Li WQ, Hofmeister RR, Young HA, Hodge DR, Keller JR, *et al.* Distinct regions of the interleukin-7 receptor regulate different Bcl2 family members. *Mol Cell Biol* 2004,**24**:6501-6513.
43. Yao Z, Cui Y, Watford WT, Bream JH, Yamaoka K, Hissong BD, *et al.* Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* 2006,**103**:1000-1005.
44. Camargo JF, Kulkarni H, Agan BK, Gaitan AA, Beachy LA, Srinivas S, *et al.* Responsiveness of T cells to interleukin-7 is associated with higher CD4⁺ T cell counts in HIV-1-positive individuals with highly active antiretroviral therapy-induced viral load suppression. *J Infect Dis* 2009,**199**:1872-1882.
45. Colle JH, Moreau JL, Fontanet A, Lambotte O, Delfraissy JF, Theze J. The correlation between levels of IL-7Ralpha expression and responsiveness to IL-7 is lost in CD4 lymphocytes from HIV-infected patients. *AIDS* 2007,**21**:101-103.
46. Colle JH, Moreau JL, Fontanet A, Lambotte O, Joussemet M, Jacod S, *et al.* Regulatory dysfunction of the interleukin-7 receptor in CD4 and CD8 lymphocytes from HIV-infected patients--effects of antiretroviral therapy. *J Acquir Immune Defic Syndr* 2006,**42**:277-285.
47. Dunham RM, Cervasi B, Brenchley JM, Albrecht H, Weintrob A, Sumpter B, *et al.* CD127 and CD25 expression defines CD4⁺ T cell subsets that are differentially depleted during HIV infection. *J Immunol* 2008,**180**:5582-5592.

48. Koesters SA, Alimonti JB, Wachihi C, Matu L, Anzala O, Kimani J, *et al.* IL-7 α expression on CD4⁺ T lymphocytes decreases with HIV disease progression and inversely correlates with immune activation. *Eur J Immunol* 2006,**36**:336-344.
49. Crawley AM, Angel JB. The influence of HIV on CD127 expression and its potential implications for IL-7 therapy. *Semin Immunol* 2012,**24**:231-240.
50. Kiazzyk SA, Fowke KR. Loss of CD127 expression links immune activation and CD4(+) T cell loss in HIV infection. *Trends Microbiol* 2008,**16**:567-573.
51. Florence E, Lundgren J, Dreezen C, Fisher M, Kirk O, Blaxhult A, *et al.* Factors associated with a reduced CD4 lymphocyte count response to HAART despite full viral suppression in the EuroSIDA study. *HIV Med* 2003,**4**:255-262.
52. Kaufmann GR, Perrin L, Pantaleo G, Opravil M, Furrer H, Telenti A, *et al.* CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study. *Arch Intern Med* 2003,**163**:2187-2195.
53. Moore RD, Keruly JC. CD4⁺ cell count 6 years after commencement of highly active antiretroviral therapy in persons with sustained virologic suppression. *Clin Infect Dis* 2007,**44**:441-446.
54. Rodger AJ, Lodwick R, Schechter M, Deeks S, Amin J, Gilson R, *et al.* Mortality in well controlled HIV in the continuous antiretroviral therapy arms of the SMART and ESPRIT trials compared with the general population. *AIDS* 2013,**27**:973-979.
55. Marziali M, De Santis W, Carello R, Leti W, Esposito A, Isgro A, *et al.* T-cell homeostasis alteration in HIV-1 infected subjects with low CD4 T-cell count despite undetectable virus load during HAART. *AIDS* 2006,**20**:2033-2041.

LEGENDS TO FIGURES

Fig. 1. Mean fluorescence intensity (MFI) of CD127 expression at day 0, on total, naïve and CD31⁺ naïve CD4⁺ T-cells was lower in patients with CD4⁺ T-cell deficiency (●) compared to patients with normal CD4⁺ T-cell counts (▲) (p=0.04, p=0.04, p=0.03, respectively) (A). Following 5 day cultures, CD127 expression (MFI) in unstimulated (open symbol) and stimulated (closed symbol) PBMC did not differ between patients with low or normal CD4⁺ T-cell counts in total, naïve or CD31⁺ naïve CD4⁺ subsets. CD127-MFI was lower after stimulation when compared to unstimulated cells in total, naïve and CD31⁺ naïve CD4⁺ subsets in patients with low or normal CD4⁺ T-cell counts (B). PBMC were incubated with or without IL-7 for 5 days. The CD127-ΔMFI represents the relative decrease of CD127-MFI in cells following stimulation with IL-7 for 5 days (reported as an absolute value). The CD127-ΔMFI did not differ between patients with low or normal CD4⁺ T-cell counts in CD4⁺ total (p=0.13), naïve (p=0.66) or CD31⁺ naïve subsets (p=0.44) (C). Following 5 day cultures stimulated with IL-7, the CD4⁺ CD127-ΔMFI correlated inversely with CD4⁺ T-cell count (D; r=-0.37, p=0.04). (*p≤0.05, **p≤0.005, ***p≤0.0005)

Fig. 2. The change in proportion of CD4⁺ T-cells expressing CD127 correlated with IL7-induced proliferation (measured by Ki67 expression). ΔCD127-MFI represents the decrease of CD127 MFI in cells following stimulation with IL-7 for 5 days (reported as an absolute value). The ΔKi67 represents the increase in the percentages of cells that expressed Ki67 as compared with cells incubated without IL-7. Induction of Ki67 in total, naïve and CD31⁺ naïve CD4⁺ T-cells correlated with CD127-ΔMFI (A; r=0.39, p=0.03), (B; r=0.60, p=0.002) and (C; r=0.59, p=0.0003).

Fig. 3. Levels of pSTAT5 following IL-7 stimulation for 15 minutes, were higher in patients with normal (\blacktriangle) CD4⁺ T-cell counts than patients with CD4⁺ T-cell deficiency (\bullet) in total (p=0.003), naïve (p=0.003) and CD31⁺ naïve (p=0.008) CD4⁺ T-cell subsets. The Δ pSTAT5 represents the proportion of T-cells that phosphorylated STAT5 following stimulation with IL-7 minus the percentage of T-cells that phosphorylated STAT5 without stimulation (**A**). Levels of pSTAT5 following IL-7 stimulation for 15 minutes in total, naïve and CD31⁺ naïve CD4⁺ subsets correlated with levels of CD127 expression (at day 0) in total CD4⁺ (**B**; r=0.62, p=0.0002), naïve CD4⁺ (**C**; r=0.54, p=0.001) and CD31⁺ naïve CD4⁺ subsets (**D**; r=0.37, p=0.03). (*p \leq 0.05, **p \leq 0.005, ***p \leq 0.0005)

Fig. 4. Expression of CD57 on CD4⁺ T-cells was higher in patients with persistent CD4⁺ T-cell deficiency (\bullet) than in patients with normal (\blacktriangle) CD4⁺ T-cell counts (p=0.01) (**A**). The proportion of CD4⁺ T-cells expressing CD57 at day 0 correlated inversely with levels of pSTAT5 following IL-7 stimulation for 15 minutes in CD4⁺ total (**B**; r=-0.62, p<0.0001), naïve (**C**; r=-0.61, p=0.002), and CD31⁺ naïve subsets (**D**; r=-0.65, p<0.0001). The proportion of CD4⁺ T-cells expressing CD57 at day 0 correlated inversely with CD127 expression at day 0 in CD4⁺ total (**E**; r=-0.54, p=0.008), naïve (**F**; r=-0.40, p=0.02) and CD31⁺ naïve subsets (**G**; r=-0.64, p=0.03). (*p \leq 0.05, **p \leq 0.005, ***p \leq 0.0005)

Supplemental Fig. 1. Forward and side light scatter profiles of cryopreserved PBMC. Cells were stained immediately or were incubated for 5 days with or without 5ng/mL of IL-7 are shown. 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 (38% vs 5%; p=0.02).

Supplemental Fig. 2. Induction of Ki67 expression in CD4⁺ T-cells following stimulation with IL-7 was similar in samples from patients with low (●) or normal (▲) CD4⁺ T-cell counts (p=0.93). Δ Ki67 represents the increase in the frequency of CD4⁺ T-cells that expressed Ki67 5 days post-stimulation with IL-7, over cells incubated in medium alone.

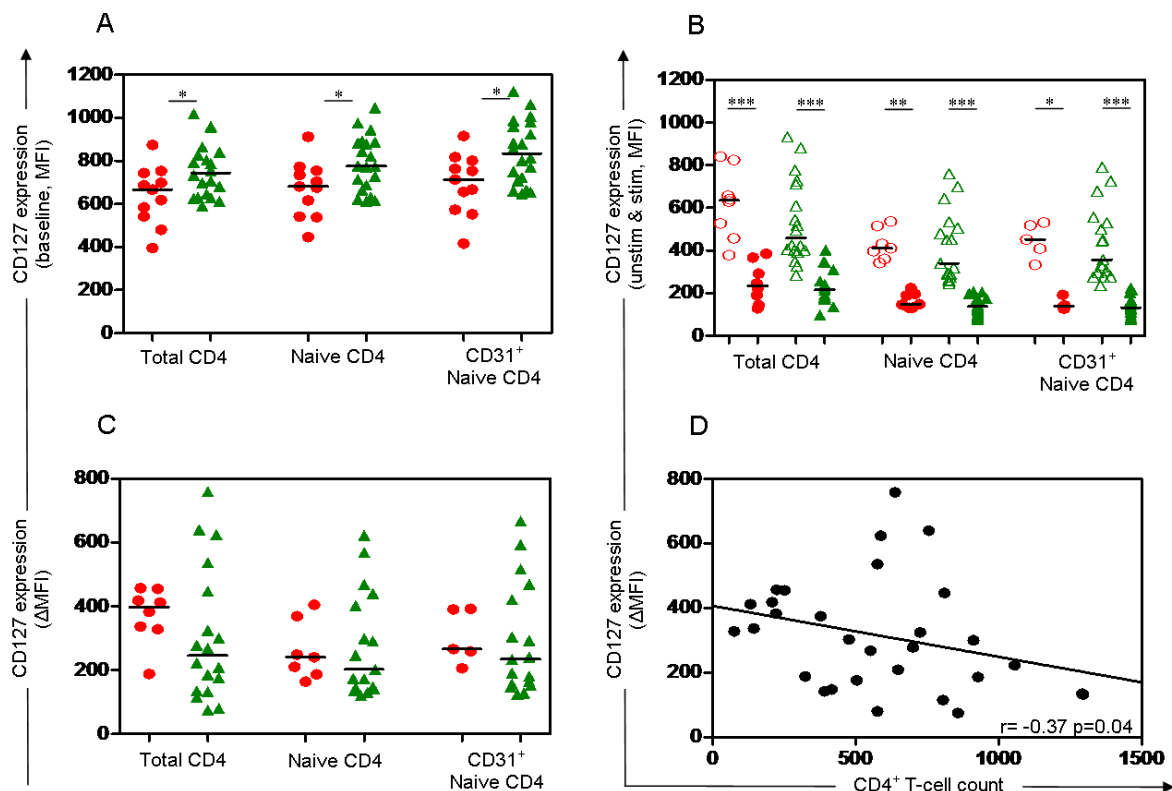


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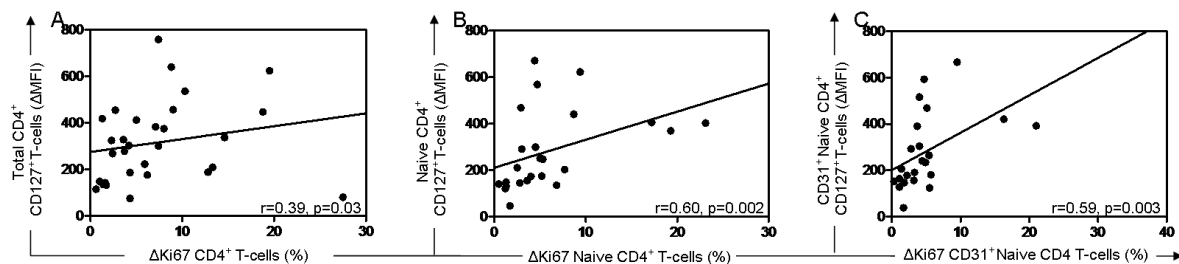


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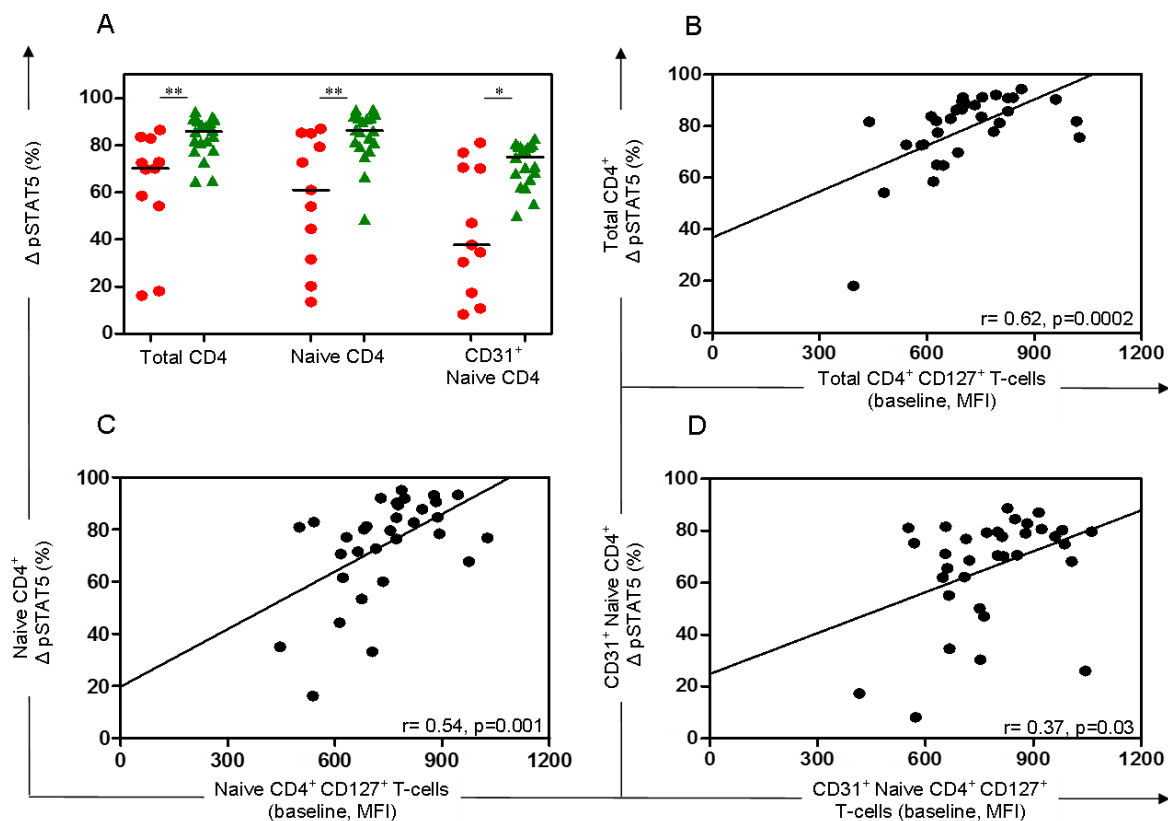


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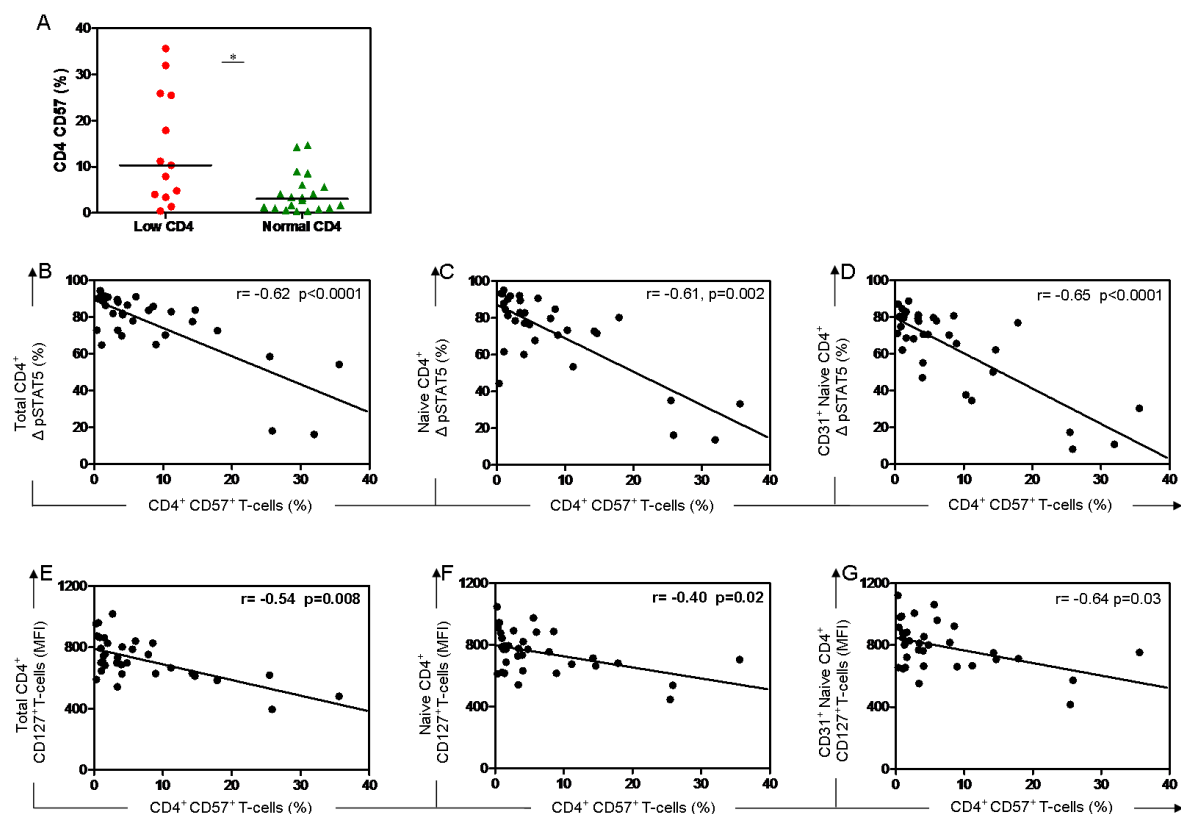
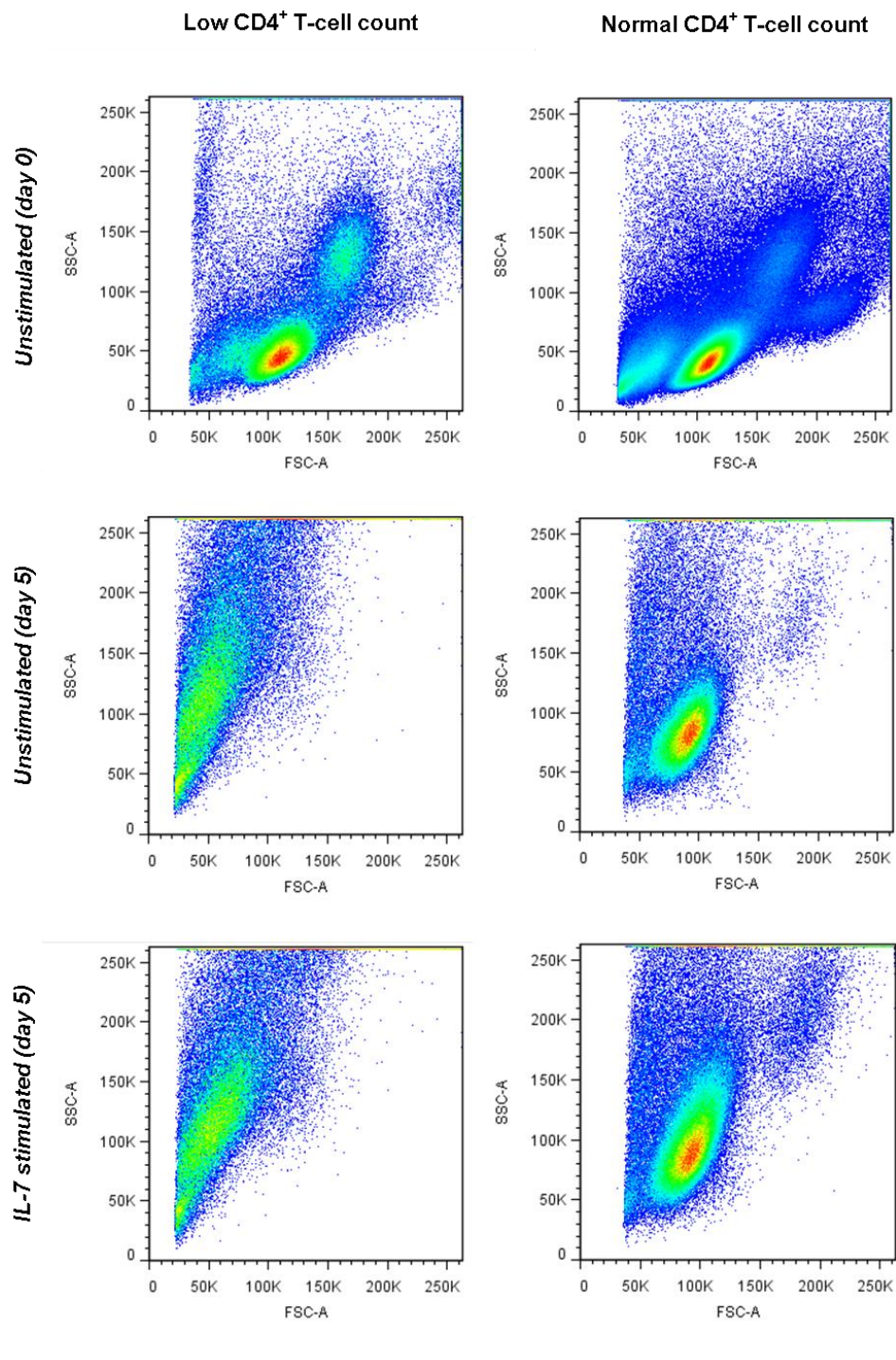
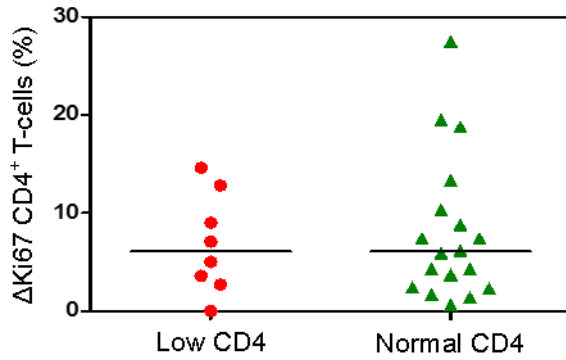


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Table 1. Patient characteristics and clinical data

	Low CD4 ⁺ T-cells (n=13)	Normal CD4 ⁺ T-cells (n=20)	Intermediate CD4 ⁺ T-cells (n=6)	<i>p</i> ^b
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 (17-125)	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 (75-384)	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 (5-84)	145 (25-364)	44 (26-85)	<0.0001
Naïve CD8 ⁺ T-cells/ μ L blood	139 (10-210)	282 (25-448)	108 (32-476)	0.008

^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