

IFN- α exerts opposing effects on activation-induced and IL-7-induced proliferation of T cells that may impair homeostatic maintenance of CD4⁺ T cell numbers in treated HIV infection

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Running title: Opposing effects of IFN- α on T cell proliferation

Abstract

To determine if IFN- α is a cause of the T cell hyperactivation and IL-7 signaling pathway defects that are observed in some HIV patients receiving antiretroviral therapy (ART), we have investigated the effect of IFN- α on the proliferation of CD4⁺ and CD8⁺ T cells from healthy donors (n=30) and treated HIV⁺ donors (n=20). PBMC were cultured for 7 days with Staphylococcal enterotoxin B (SEB) or IL-7 in the absence or presence of 100U/mL IFN- α 8. Total and naïve CD4⁺ and CD8⁺ T-cells were assessed for proliferation (via Ki67 expression), CD127 expression and phosphorylated (p)-STAT5 levels using flow cytometry. IFN- α significantly enhanced activation induced proliferation (via SEB stimulation) but inhibited homeostatic proliferation (IL-7-induced) of CD4⁺ and CD8⁺ T-cells. Both of these effects may adversely affect CD4⁺ T cell homeostasis in HIV patients. CD127 expression was increased in both healthy and HIV⁺ donors following culture with IFN- α 8 and levels of IL-7-induced pSTAT5 were increased by IFN- α 8 in healthy donors only. Hence, the inhibitory effects of IFN- α on IL-7-induced proliferation of CD4⁺ T cells are unlikely to be mediated by down-regulation of CD127 expression or inhibition of STAT5 phosphorylation. These data suggest that increased IFN- α activity may promote the loss of T cells by accelerating cell turnover and activation-induced cell death while decreasing the renewal of T cells by inhibiting the proliferative effect of IL-7.

Introduction

Between 5 and 56% of HIV-infected individuals do not achieve a normal blood CD4⁺ T cell count (>500 μ L) after a mean time of 7.5 years on antiretroviral therapy (ART) (1). Persistent depletion of CD4⁺ T cells in lymphoid tissue, such as gut associated lymphoid tissue, is even more pronounced than in blood (2). CD4⁺ T cell deficiency on ART is associated with a higher rate of mortality and morbidity (3, 4), which includes atherosclerotic vascular disease (3, 5), cardiovascular disease (4), osteoporosis and fractures (6) and non-AIDS-defining cancer (4, 7, 8).

Recovery of CD4⁺ T cell numbers during ART is dependent on de novo production of naïve CD4⁺ T cells from the thymus as well as homeostatic proliferation of the naïve CD4⁺ T cell pool. The latter process is regulated by IL-7 and its receptor via the Jak/STAT signaling pathway. The IL-7 receptor complex is a heterodimer of an IL-7 receptor α -chain (CD127) and the γ -chain that is common to the receptor for other ' γ c' cytokines (CD132). Defects of T cell proliferation persist in HIV patients receiving ART and are associated with T cell hyperactivation and impaired IL-7-mediated homeostasis as a result of defects of the CD127 signaling pathway. Decreased signaling through CD127 is in part a consequence of reduced CD127 expression on both CD8⁺ and CD4⁺ T cells but post-receptor signaling pathways are also defective (9). Hyperactivation of CD4⁺ T cells in patients with treated HIV infection is associated with increased basal levels of the TCR signaling molecules phospho- ZAP70, -ERK and -JNK, which appears to result in a hyporesponsiveness to signaling through the TCR (10).

Persistent CD4⁺ T cell depletion in HIV patients receiving ART that suppresses HIV replication is closely associated with persistent immune activation (11). However, the underlying pathogenic mechanisms are still a topic of debate. Three potential causes of immune activation have been identified: a) increased translocation of microbial products across the gut wall (12), b) CMV co-infection (13, 14) and c) residual low-level HIV replication, identified in studies of ART

intensification therapy with HIV integrase inhibitors (15, 16). Irrespective of whether one or more of these factors cause persistent immune activation, we and others have demonstrated that elevated expression of interferon-stimulated genes (ISG) in separated CD4⁺ T cells (17), separated accessory cells (18) or PBMC (19) is the strongest correlate of persistent CD4⁺ T cell deficiency in treated HIV patients. Increased IFN- α activity may therefore be a central component of the pathogenic mechanisms that result in poor CD4⁺ T cell recovery in HIV patients receiving ART.

Increased IFN- α activity (20-22) and elevated production of IFN- α in PBMC and plasmacytoid dendritic cell cultures is a characteristic of HIV infection (23) and is associated with both CD4⁺ T cell depletion and disease progression (21). IFN- α is an essential mediator of the innate immune response and its expression is rapidly up-regulated in response to viral pathogens, reflecting its potent anti-viral activity. There are 12 functional subtypes of IFN- α , which form part of a large family of type I interferons. All of the IFN- α subtypes are structurally similar and bind to a common receptor. Activation of the IFN- α receptor triggers a signaling cascade that initially includes the tyrosine kinases, Tyk2 and Jak1 and eventually results in activation of transcription factor complexes, such as IFN stimulated gene factor 3, and phosphorylation and dimerisation of STAT molecules, which translocate to the cell nucleus and regulate the expression of numerous ISG. Untreated HIV infection is associated with increased expression of mRNA of several ISG in monocytes (24) and T cells (20).

IFN- α displays contrasting proliferation-inducing and pro-apoptotic properties (25). At low concentrations, IFN- α enhances cell proliferation. Conversely, at high concentrations it acts in an anti-proliferative fashion and may have detrimental effects on T cell development, maturation and homeostasis. These actions may in part be mediated by an effect of IFN- α on IL-7 and the IL-7 receptor as in studies assessing thymocyte differentiation and maturation, IFN- α impaired IL-7-mediated proliferation, down regulated CD127 expression and reduced overall cell numbers in the thymus (26, 27).

Interleukin-7 is produced by stromal cells in the bone marrow and lymphoid tissue and plays a critical role in T cell homeostasis (28). Serum levels of IL-7 are increased in HIV infection (29) and decrease after ART is commenced, which has been attributed to increased numbers of CD127⁺ T cells and receptor clearance of IL-7 (30). It is also possible that immune activation in HIV infection affects the production of IL-7 by stromal cells because cytokines potently affect the production of IL-7 from bone marrow stromal cells with IFN- γ increasing production and IL-1 β reducing production (31). The effect of IFN- α on IL-7 production by stromal cells is unknown.

We hypothesised that IFN- α plays a key role in the immune activation and impaired recovery of CD4⁺ T cells that occurs in a proportion of otherwise successfully treated HIV patients, by impairing homeostatic proliferation of CD4⁺ T cells and promoting acceleration of T cell turnover. We have explored this by examining the effect of IFN- α on key pathways involved in CD4⁺ T cell homeostasis in both HIV patients and individuals who are not infected by HIV. We have also examined the effect of IFN- α on IL-7 production by bone marrow stromal cells.

Methods

Study groups and sample collection

Thirty healthy donors and twenty HIV positive donors were studied. Healthy donors were categorized according to age into those under 30 years (younger donors; n = 15) and over 50 years (older donors; n = 15). HIV positive donors had undergone ART for at least 12 months, with stable suppression of viral replication (<50 copies/mL of plasma HIV RNA) for more than 8 months. Patients were selected on the basis of a history of extreme immunodeficiency (nadir CD4⁺ T cell counts of less than 40 cells/ μ L of blood) prior to ART. A wide range of current CD4⁺ T cell counts was represented. Demographic data are displayed in Table 1. This study was approved by the Human Research Ethics Committees of Royal Perth Hospital and the University of Western Australia. Informed consent was obtained from all subjects.

PBMC cultures and detection of proliferating cells and CD127 expression

Cryopreserved PBMC were thawed, washed and resuspended at 10⁶cells/mL in culture medium (RPMI 1640 supplemented with 10% FCS, 1% Penicillin/Streptomycin solution and 2mM L-glutamine). PBMC were seeded in 24-well plates at 10⁶ cells/well and stimulated with 1 μ g/mL SEB (Sigma-Aldrich, Sydney, Australia) or 5ng/mL IL-7 (R&D Systems, Minneapolis, MN) either alone or in combination with 100U/mL IFN- α 8 (PBL Interferon Source, Piscataway, NJ). PBMC that were cultured without stimuli or in the presence of 100U/mL IFN- α 8 only were included as negative controls. Plates were incubated at 37°C / 5% CO₂ for 7 days. PBMC were washed once in cold flow buffer (1% BSA in PBS) then stained for surface (15 min) and intracellular (30 min) markers using the Human FoxP3 Buffer Set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. This permeabilisation buffer set was selected as it is optimal for the detection of nuclear antigens such as Ki67. The following fluorochrome-conjugated monoclonal antibodies were used: CD3-V450 (clone UCHT1), CD4-APCH7 (SK3), CD8-V500 (RPA-T8), CD45RA-APC (HI100),

CCR7-PECy7 (3D12), CD127-PerCPCy5.5 (hIL-7R-M21) and Ki67-PE (B56) from BD Biosciences. After staining PBMC were washed and resuspended in cold flow buffer for acquisition.

In a subset of 10 healthy donors, the proliferation of T cells was also assessed via carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling. Briefly, cryopreserved PBMC were thawed, washed, resuspended at 10^7 cells/mL in warm PBS/0.1% BSA and incubated with 10 μ M CFSE (Molecular Probes) for 10 min. CFSE staining was quenched by the addition of 5 volumes of ice cold culture media and cells were pelleted by centrifugation. Cells were washed an additional 2 times with ice cold culture media before being resuspended at 10^6 cells/mL in warm culture medium and stimulated as described above. After 7 days of culture, cells were stained with the same fluorochrome-conjugated monoclonal antibodies described above.

Assessment of STAT5 phosphorylation

Cryopreserved PBMC were thawed, washed and resuspended in culture medium at 10^6 cells/mL and incubated alone or with 100U/mL IFN- α 8 for 2 hours at 37°C / 5% CO₂. PBMC were pelleted and resuspended in 100 μ L of sterile flow buffer and stained with the following fluorochrome-conjugated monoclonal antibodies; CD3-V450 (UCHT1), CD4-V500 (RPA-T4), CD8-PE (RPA-T8) and CD27-FITC (L128) for 15 min. PBMC were washed with flow buffer and resuspended in 1mL culture media containing 5ng/mL IL-7 for 15 min at 37°C/5% CO₂ before fixation and permeabilisation using the Cytotfix™ Fixation Buffer and Phosflow Perm Buffer III (BD Biosciences) according to the manufacturer's instructions. After washing, PBMC were stained for CD45RA-APCH7 (HI100) and phosphorylated (p)-STAT5-AF647 (47) for 15 min, washed twice and resuspended in cold flow buffer for acquisition.

Flow cytometry acquisition and analysis

Data were acquired on a FACSCanto™ II using Diva software (BD Biosciences). For the 7-day stimulation cultures, acquisition stopping gates were set at 50,000 CD4⁺ T cell events defined by co-expression of CD3 and CD4 markers. The acquisition settings for the STAT5 phosphorylation protocol were fixed at 250,000 lymphocyte events defined by forward and side scatter measurements. Data files were visualized using FlowJo software (v7.6, Treestar, USA).

Quantification of IL-7 production by HS-27A cells

The HS-27A stromal cell line was obtained from the American Type Culture Collection and propagated in RPMI 1640 media supplemented with 10% FCS. To determine whether IFN- α modified IL-7 production by the cell line, HS-27A cells were seeded in 96-well plates at 2×10^5 cells/well alone or in the presence of IFN- α 8 at 1, 10, 100 or 1000U/mL. All cultures were performed in triplicate. Supernatants were collected after 6, 24 and 48 hours of incubation and stored at -80°C. IL-7 levels in supernatants were measured using a high sensitivity commercial ELISA (Quantikine® HS Human IL-7 Immunoassay; R&D systems, USA) according to the manufacturer's instructions. Optical density measurements were determined immediately using a Bio-Rad microplate plate reader set to 490nm with wavelength correction set to 650nm. The assay had a lower detection threshold of 0.25pg/mL.

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). Non-parametric tests were conducted to determine statistical significances. Wilcoxon matched pairs tests were used to compare results within healthy donor and patient groups and Mann-Whitney tests were used to compare results between donor and patient groups. Correlation coefficients were calculated with Spearman's tests. P-values less than 0.05 were considered to be statistically significant.

Results

SEB- and IL-7- dependent proliferation of T cells is negatively influenced by older age and HIV⁺ status

To determine the effects of IFN- α on T cell proliferation induced by activation via the T cell receptor or on homeostatic proliferation induced by IL-7, we cultured PBMC with SEB or IL-7 and determined the effect of IFN- α on proliferation by adding IFN- α 8 to the cultures. IFN- α 8 was chosen following a series of experiments comparing the effects of IFN- α 2b, IFN- α 8 and IFN- α 10 which demonstrated that all three subtypes exhibited similar levels of anti-proliferative activity (Supplemental Figure 1).

SEB and IL-7 induced substantial proliferation of total and naïve CD4⁺ and CD8⁺ T cells in PBMC of younger, older and HIV positive donors (Figure 1A-D). Overall, naïve T cells were less responsive to stimulation than the total T cell population although naïve CD8⁺ T cells were highly responsive to IL-7. SEB was more effective at inducing proliferation than IL-7 within all T cell populations assessed, with the exception of naïve CD8⁺ T cells where the reverse was observed. Proliferation was not observed in unstimulated cell cultures or those stimulated with IFN- α 8 alone.

When assessing the proliferative potential of the total CD4⁺ T cell pool, both younger and older healthy donors were more responsive to SEB than HIV⁺ donors (Figure 1A; $p=0.02$ and $p=0.0002$, respectively). Younger healthy donors also had a greater proliferative response to IL-7 than HIV⁺ donors (Figure 1A; $p=0.02$). In the naïve CD4⁺ T cell subset, younger healthy donors had a significantly greater proliferative response to IL-7 than older healthy donors (Figure 1B; $p=0.004$) but no other differences between donor groups was observed.

After stimulation with SEB, a significantly greater proportion of proliferating cells was observed in the total CD8⁺ T cell pool in both younger and older healthy donors compared to HIV⁺ donors (Figure 1C; $p=0.001$ for both). IL-7-induced proliferation was also significantly higher in CD8⁺ T cells of

younger healthy donors than HIV⁺ donors (p=0.0007). Within the naïve CD8⁺ T cell pool, older healthy donors had a greater proliferative response to SEB than HIV⁺ donors (Figure 1D; p=0.02) and younger healthy donors had a greater proliferative response to IL-7 than HIV⁺ donors (p=0.03).

IFN- α 8 enhanced SEB-dependent proliferation but impaired IL-7-dependent proliferation of T cells in all donor groups

To compare the effects of IFN- α on T cell proliferation between donor groups, the proportion of proliferating cells observed following addition of IFN α -8 to either SEB- or IL-7-stimulated cells was subtracted from the proportion of proliferating cells observed after culture with either SEB or IL-7 alone and the difference was plotted (Figure 1E-H). The majority of the SEB-representative plots lie in the positive region (demonstrating an overall increase) whereas the majority of the IL-7-representative plots lie in the negative region (demonstrating an overall decrease).

IFN- α 8 significantly enhanced SEB-induced proliferation of total CD4⁺ T cells (Figure 1E) in the younger healthy donors (p=0.0001), older healthy donors (p=0.0007) and HIV⁺ donors (p=0.003). The degree to which SEB-induced proliferation was increased by IFN- α 8 was similar in all 3 donor groups. IFN- α 8 significantly inhibited IL-7-induced proliferation of total CD4⁺ T cells (Figure 1E) in each of the donor groups (p<0.0001, p=0.0003 and p=0.0002, respectively). The degree of inhibition was greater in younger healthy donors than HIV⁺ donors (median of inhibition -13.2% vs -4.2% respectively, p=0.004).

Within the naïve CD4⁺ T cell subset (Figure 1F), IFN- α 8 significantly enhanced SEB-induced proliferation in both healthy donor groups (p=0.01 and p=0.001, respectively) but not HIV⁺ donors (p=0.9). In fact, the effect of IFN- α upon SEB-induced proliferation of naïve CD4⁺ T cells was moderately inhibitory in HIV patients and was significantly lower than that observed in the younger and older healthy donors (p=0.007 and p=0.0005, respectively). Inhibition of IL-7-induced

proliferation of naïve CD4⁺ T cells by IFN- α 8 was observed in all donor groups ($p < 0.0001$, $p = 0.03$ and $p = 0.01$, respectively) and was significantly greater in the younger healthy donors compared to both the older healthy donors and HIV⁺ donors, which showed a much wider range of effects ($p = 0.002$ and $p = 0.02$, respectively).

IFN- α 8 enhanced SEB-induced proliferation of total CD8⁺ T cells in all donor groups (Figure 1G) but the effect was only significant in the younger healthy ($p < 0.0001$) and HIV⁺ donors ($p = 0.0004$). The enhancement was greater in younger healthy donors compared to both older healthy and HIV⁺ donors ($p = 0.02$ for both). In contrast, IFN- α 8 significantly inhibited IL-7-induced proliferation of total CD8⁺ T cells in all donor groups ($p = 0.0002$, $p = 0.0001$ and $p = 0.003$, respectively) and the degree of inhibition did not differ between groups.

Within the naïve CD8⁺ T cell subset (Figure 1H), IFN- α 8 significantly enhanced SEB-induced proliferation ($p = 0.008$, $p = 0.008$ and $p = 0.0006$, respectively) and inhibited IL-7-induced proliferation ($p = 0.008$, $p = 0.2$, $p = 0.008$). The degree of enhancement or inhibition of proliferation was equivalent in all donor groups.

In summary, the addition of IFN- α 8 to PBMC cultures stimulated with SEB enhanced the observed proliferation in all T cell subsets across all donor groups. In contrast, the addition of IFN- α 8 to PBMC cultures stimulated with IL-7 inhibited the observed proliferation in all T cell subsets across all donor groups.

In a subset of 10 healthy donors, the effect of IFN- α 8 on SEB- and IL-7 induced proliferation was assessed using both Ki67 and CFSE staining as indicators of proliferation. The results observed with CFSE were equivalent to those observed with Ki67 (Figure 2). Representative flow cytometry scatter

plots from one donor comparing the proliferation observed with Ki67 or CFSE under all conditions of stimulation are shown in Figure 3.

Culture of PBMC with IFN- α 8 up-regulated CD127 expression by T cells

Because IFN- α 8 generally reduced IL-7-induced proliferation of T cells, we examined the effect of IFN- α 8 on the expression of CD127, the α chain of the IL-7 receptor. The mean fluorescence intensity (MFI) of CD127 expression on CD4⁺ T cells was significantly up-regulated in the younger healthy (p=0.02), older healthy (p<0.0001) and HIV⁺ (p=0.005) donor groups after 7 days of culture with IFN- α 8 (Figure 4A). A similar up-regulation of CD127 expression was observed after culture with IL-7 alone for 7 days in the younger and older healthy donors but not the HIV⁺ donors (p=0.04 and p=0.004, respectively).

When naïve CD4⁺ T cells were assessed (Figure 4B), CD127 expression was not altered by culture with IFN- α 8 in either of the healthy donor groups but an upregulation of CD127 was observed in the HIV⁺ donors (p=0.002). Culture with IL-7 alone did not influence CD127 expression in any donor group.

The MFI of CD127 expression on CD8⁺ T cells was significantly up-regulated in the younger healthy (p=0.01), older healthy (p=0.05) and HIV⁺ (p=0.0002) donor groups after 7 days of culture with IFN- α 8 (Figure 4C). A similar up-regulation of CD127 expression was observed after culture with IL-7 alone for 7 days in the younger and older healthy donors only (p=0.008 and p=0.007, respectively).

The MFI of CD127 expression on naïve CD8⁺ T cells was significantly up-regulated in the younger healthy (p=0.007), older healthy (p=0.0002) and HIV⁺ (p=0.0001) donor groups after 7 days of culture with IFN- α 8 (Figure 4D). Up-regulation of CD127 expression was also observed after culture with IL-7 in the younger and older healthy donors (p=0.02 and p=0.01, respectively).

In summary, culture of PBMC in the presence of IFN- α 8 for 7 days led to either up-regulated or static CD127 expression in comparison to PBMC that were cultured alone for 7 days.

IFN- α 8 enhanced IL-7-induced pSTAT5 in all T cell populations of healthy donors

In order to determine the effect of IFN- α on phosphorylation of STAT5, PBMC were pre-exposed to IFN- α 8 for 2 hours prior to assessment of IL-7-induced pSTAT5 expression. Overall, the frequency of pSTAT5⁺ CD4⁺, naïve CD4⁺ and naïve CD8⁺ T cells after stimulation with IL-7 was significantly higher in HIV patients (Figure 5). This was also observed in cells pre-treated with IFN- α 8. In healthy donors pre-treatment of PBMC for 2 hours with IFN- α 8 resulted in a significantly higher frequency of CD4⁺ T cells (p=0.006), naïve CD4⁺ T cells (p=0.004), CD8⁺ T cells (p=0.002) and naïve CD8⁺ T cells (p=0.002) expressing pSTAT5 (Figure 5). Pre-treatment of PBMC with IFN- α 8 did not significantly affect the frequency of pSTAT5⁺ T cells in HIV patients (Figure 5). Similar findings were observed when PBMC were pre-treated with IFN- α 8 for 24 hours (data not shown).

IFN- α increased IL-7 production by bone marrow stromal cells

We used HS27A cells (spontaneous producers of IL-7) to determine whether IFN- α modulates IL-7 production by bone marrow stromal cells. We first confirmed expression of the IFN- α receptor 1 and 2 subunits on the HS27A cells (data not shown). Overall, IL-7 production was low after 6 hours but considerably increased after 24 and 48 hours of culture. At all 3 time points, the highest concentrations of IFN- α 8 tested (100 and 1000U/mL) induced the HS27A cell line to produce significantly more IL-7 (p<0.0001; Figure 6). To exclude IFN- α induced proliferation of the HS27A cells as a possible cause of the observed increase in IL-7 production, cell counts were performed on HS27A cells following culture with increasing concentrations of IFN- α 8. No effect of IFN- α 8 upon cell growth was observed (data not shown).

Discussion

Here, we compared the proliferative potential of CD4⁺ and CD8⁺ T cells in response to activation-induced stimulation via the TCR or homeostatic proliferation via the IL-7 signaling pathway in healthy and HIV⁺ donor groups and evaluated the effect of IFN- α on these processes. We also examined the effect of IFN- α on production of IL-7 by bone marrow stromal cells.

We first assessed HIV- and age-associated differences in the proliferative potential of CD4⁺ and CD8⁺ T cells in the three donor groups. HIV patients were less responsive to SEB stimulation than both younger and older healthy donors. These data are in agreement with that of Downey and colleagues who have shown that CD4⁺ and CD8⁺ T cells from HIV patients are poorly responsive to activation through the TCR. Their data were obtained using IL-2 and CD3/CD28 as a TCR stimulus rather than SEB (10). Interestingly, these trends were less apparent when the naïve CD4⁺ and naïve CD8⁺ T cell pools were assessed separately, suggesting that the memory T cell pool in treated HIV patients may be particularly unresponsive to stimulation via the TCR. This may reflect the replicative exhaustion of the memory T cell pool that is characteristic of chronic, treated HIV disease as a consequence of constant cell turnover and activation-induced senescence (32, 33).

IL-7-induced proliferation was also impaired in the HIV⁺ donors, but significant differences were only noted in relation to the younger healthy donors, which may reflect age rather than HIV-associated differences. The median ages of the younger and older healthy donors studied here were 34 and 15 years, younger and older than the HIV donors, respectively. Therefore, T cells from HIV patients on ART may have the potential to undergo “normal” (relative to age) proliferation in response to a homeostatic signal such as IL-7 – in the absence of external negative factors.

The effect of age was evaluated further by directly comparing proliferative responses in the younger and older healthy donor groups. Age significantly influenced proliferative responses with older

healthy donors demonstrating less proliferation of total CD8⁺ T cells in response to SEB and total and naïve CD4⁺ T cells in response to IL-7. It is well-established that the function of the immune system declines with age (34, 35). Diminished responses to mitotic stimuli, such as IL-2, have been observed in aging people and associated with reduced receptor signaling (36). A similar mechanism involving age-related defects in CD127 (IL-7 receptor) signaling may affect IL-7 responsiveness.

Across all donor groups, IFN- α enhanced SEB-induced proliferation and inhibited IL-7-induced proliferation (assessed by Ki67 and CFSE staining) of both the total and naïve CD4⁺ and CD8⁺ T cell populations. It would be important to confirm these effects on proliferation with live cell counting in future studies, an option that was not available to us here due to small cell numbers. Nevertheless, these findings suggest that IFN- α has potent but contrasting effects on these two different activation pathways.

SEB binds to MHC class II which stimulates the TCR to activate the MAPK pathway (37). The resulting production of various pro-inflammatory cytokines, such as IL-2, facilitates T cell proliferation and clonal expansion as well as promotes the production of other pro-inflammatory cytokines (38). Therefore, in an environment where IFN- α levels are elevated, such as during a viral infection, IFN- α may induce CD4⁺ and CD8⁺ clonal expansion via these mechanisms. IFN- α could also enhance TCR-binding and/or transduction of the signaling pathway, thereby increasing production of IL-2 and promoting further expansion of the T cell pool. Interestingly, the pro-proliferative effect of IFN- α on SEB-induced proliferation was least evident in the naïve CD4⁺ T cell population from HIV⁺ patients – where in fact, the effect of IFN- α was moderately inhibitory (Figure 1F). Naïve CD4⁺ T cell cycle entry is delayed by IFN- α exposure, perhaps as a defence mechanism to prevent cell death (25). In an environment with persistently high levels of IFN- α , as is the case in HIV disease, this delay could become permanent.

In contrast, stimulation of the IL-7 signaling pathway involves the catalytic activation of Jak1 and Jak3. This leads to the phosphorylation of STAT5, which in turn dimerizes and translocates to the nucleus, inducing gene transcription (39). Several studies have documented that IFN- α affects IL-7-mediated proliferation in foetal and post-natal thymic progenitor cells thus impairing thymopoiesis and T cell maturation (26, 27). The effect of IFN- α on homeostatic maintenance of the naïve CD4⁺ T cell pool (that is also governed by the IL-7 signaling pathway) has not been previously reported. Our data demonstrates that IFN- α inhibits IL-7-induced proliferation in total and naïve T cells. Therefore, in contrast to its effects on activation-induced proliferation, IFN- α may exhibit anti-proliferative activity during homeostatic proliferation. At physiological levels, IFN- α may play a pivotal role in regulating T cell homeostasis by establishing an equilibrium between its proliferative and anti-proliferative activities. However at high concentrations, such as in the context of HIV disease, IFN- α may also have a deleterious effect. For example, IFN- α may play an important role in inducing apoptosis of CD4⁺ T cells via upregulation of TRAIL (40, 41), and has been linked to increased expression of the pro-apoptotic molecules Bak and CD95 (Fas) on CD4⁺ T cells and subsequent CD95-mediated apoptosis of T-cells in chronically infected HIV patients (42). Indeed, the induction of these pro-apoptotic signals may be sufficient to overcome IL-7-mediated cell survival signals that would promote proliferation.

We hypothesized that the inhibition of IL-7-induced proliferation by IFN- α may be due to down-regulation of the IL-7R α chain, CD127. However, CD127 expression in all T cell populations, except naïve CD4⁺ T cells from healthy donors, was up-regulated by IFN- α . This result is in contrast to that of Baron et al, who demonstrated a down-regulation of CD127 expression following culture of thymic progenitor cells with recombinant IFN- α (26). However, cells at such an early stage of primary thymic development would likely be more sensitive to IFN- α activity than mature, circulating peripheral T cells. It is also worth noting that our data reports the level of CD127 expression following a period of 7 days of culture either alone or in the presence of IFN- α 8 – which may be too long to observe IFN-

$\alpha 8$ induced changes in CD127. To exclude this as a factor, we performed a small study using five healthy donors to assess CD127 expression after 6, 24, 48 and 72 hours of culture with IFN- $\alpha 8$ (data not shown). Our results confirmed that IFN- $\alpha 8$ was not mediating its effects on IL-7 induced proliferation by down-regulating expression of the IL-7R α chain.

IFN- α also enhanced IL-7-induced STAT5 phosphorylation in all T cell populations. This is in contrast to a study by Schmidlin and colleagues that demonstrated no significant effect of IFN- α on IL-7-mediated STAT5 phosphorylation in thymic progenitor cells (27). Interestingly, Erickson et al have shown that IFN- α inhibited STAT5 binding to DNA in T cells stimulated by IL-2, a cytokine very closely related to IL-7 (43). With this in mind, a recent study by Landires and colleagues found that the translocation of IL-7-induced phosphorylated STAT5 to the nucleus of CD4⁺ T cells is perturbed in patients with HIV infection (44). Therefore, although there was no suppressive activity of IFN- α observed upon CD127 expression and STAT5 phosphorylation, IFN- α may impair the IL-7 signaling pathway by impairing the migration of phosphorylated STAT5 to the nucleus and therefore prevent gene transcription.

It is important to note that in addition to the STAT5 signalling pathway, the PI3K/Akt pathway is also essential in IL-7/IL-7R α -mediated T cell homeostasis. Both pathways are primarily induced by activation of IL-7R α and they can act either independently or synergistically. For example, both pathways are required to stimulate T cell proliferation while STAT5 signalling is more important in T cell survival (regulation of Bcl-2). In addition, STAT5 and Akt have been shown to interconnect in regulating T cell metabolism (glucose trafficking and uptake) through STAT5-dependent activation of Akt. For this study, we focused on the JAK/STAT pathway as previous work from our group has shown a significant decrease in pSTAT5 in the total and naïve CD4⁺ T cell compartments in HIV patients with low CD4⁺ T cell recovery (45). Given that the inhibition of IL-7-induced proliferation

by IFN- α we observed could not be attributed to a decrease in pSTAT5, it would be pertinent to assess the effects of IFN- α on the PI3K pathway in future studies.

The findings of our study provide supportive evidence for our hypothesis that increased IFN- α activity contributes to persistent CD4⁺ T cell deficiency in HIV patients receiving ART. We demonstrated that IFN α decreased homeostatic proliferation of CD4⁺ T cells, which had undergone approximately 5 rounds of proliferation over 7 days, by 10- 20%. The life span of human T cells in the absence of T cell receptor signalling is 2-3 years (46-48), therefore the decrease in proliferation that we have observed could have a significant cumulative effect on CD4⁺ T cell numbers over time. Given our findings that IFN- α also had similar effects on the proliferation of CD8⁺ T cells, increased IFN- α activity may contribute to the persistent deficiency of naïve CD8⁺ T cells that is observed in some patients receiving ART (11). However, total CD8⁺ T cell counts are not decreased and may be increased in patients with treated HIV infection. Studies have shown that type I IFNs have an important role in inducing CD8⁺ T cell expansion either directly (49) or indirectly by stimulating the synthesis of IL-15, which induces the expansion of memory CD8⁺ T cells (50, 51). In addition, other factors may contribute to persistent CD4⁺ T cell depletion, including an increased propensity of CD4⁺ T cells to undergo apoptosis mediated by molecules of the TNF family, such as TRAIL (23, 40) and TNF- α , which has been linked with CD4⁺ T cell depletion in a study of patients with a defined resumption of HIV replication after cessation of ART (52). Indeed, there is evidence that IFN- α contributes to the increased sensitivity of CD4⁺ T cells to apoptosis in HIV patients (23, 53).

It is worth noting that the HIV patients studied here, all experienced severe immunodeficiency (nadir CD4⁺ T cell counts of less than 40 cells/ μ L of blood) prior to commencing ART. Under current treatment protocols in developed nations, it would be rare for a HIV infected individual to commence treatment at such a late stage. Therefore, it would be valuable to confirm the present data in a cohort of HIV patients that better reflects the current treatment scenario. However, given that the pro- and

anti-proliferative effects of IFN- α upon TCR activation-induced proliferation and homeostatic proliferation of T cells, respectively, were also evident in the 2 healthy donor populations studied here, it is likely that these results would be consistent regardless of how late ART was commenced.

Our final consideration was the effect of IFN- α on IL-7 production by bone marrow stromal cells. We demonstrated that IFN- α exhibited a similar effect to IFN- γ (31) and promoted IL-7 production from HS27A bone marrow stromal cells. This finding raises the possibility that increased serum levels of IL-7 in HIV patients reflects the effects of immune activation, specifically IFN- α production, on stromal cells in bone marrow and lymphoid tissue and that serum IL-7 levels decline on ART because of a reduction in immune activation.

In summary, IFN- α displays contrasting effects on TCR activation-induced proliferation and homeostatic proliferation of T cells, which together, and in association with an increased propensity of CD4⁺ T cells to apoptosis, would impair the recovery of CD4⁺ T cell numbers in some HIV patients receiving ART. Our findings provide an explanation for increased ISGs in CD4⁺ T cells (17) or PBMC (18, 19) of HIV patients with persistent CD4⁺ T cell deficiency on ART. Increased IFN- α activity may promote the loss of T cells by accelerating cell turnover and activation-induced cell death while decreasing the renewal of T cells by inhibiting the proliferative effect of IL-7. Adding therapies that inhibit IFN- α activity to ART may therefore be a strategy for increasing CD4⁺ T cell counts in HIV patients with persistent CD4⁺ T cell deficiency on ART.

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Footnotes

1. The work was supported by a program grant (510448) from the National Health and Medical Research Council of Australia.

2. Abbreviations used in this article: ART, antiretroviral therapy; ISG, interferon stimulated gene; MFI, mean fluorescent intensity; pSTAT5, phosphorylated STAT5; SEB, staphylococcal enterotoxin B

Figure Legends

Figure 1. IFN- α 8 enhanced SEB-induced proliferation but inhibited IL-7-induced proliferation of both CD4⁺ and CD8⁺ T cells in healthy donors and HIV⁺ donors. PBMC were cultured alone or with 100U/mL IFN α 8, 1 μ g/mL SEB or 5ng/mL IL-7 and the proportions of proliferating CD4⁺ (A), naïve CD4⁺ (B), CD8⁺ (C) or naïve CD8⁺ (D) cells were plotted. To evaluate the effects of IFN- α on SEB- and IL-7-induced proliferation, the proportion of proliferating CD4⁺ (E), naïve CD4⁺ (F), CD8⁺ (G) or naïve CD8⁺ (H) T-cells following addition of IFN- α 8 to either SEB- (open boxes) or IL-7- (shaded boxes) stimulated cells was subtracted from the proportion of proliferating cells after stimulation with SEB or IL-7 alone. A positive value indicates an increase in proliferation while a negative value indicates a decrease in proliferation. Similar results were observed for healthy donors and HIV+ patients. (***=p<0.0005, **=p<0.005, *=p<0.05).

Figure 2. The change in SEB- or IL-7-induced proliferation of CD4⁺, naïve CD4⁺, CD8⁺ or naïve CD8⁺ T cells with IFN- α 8 was assessed using both Ki67 and CFSE as indicators of proliferation in 10 healthy donors. IFN- α induced an upregulation of SEB-induced proliferation (A) but inhibited IL-7-induced proliferation (B), regardless of whether Ki67 (results shown in grey) or CFSE (results shown in white) staining was utilised. A positive value indicates an increase in proliferation while a negative value indicates a decrease in proliferation.

Figure 3. Representative flow cytometry scatter plots demonstrating the proliferation observed using Ki67 (top row) or CFSE (bottom row) staining in one donor. From left to right, plots for unstimulated cells, SEB-stimulated cells, SEB-stimulated cells + IFN- α 8, IL-7-stimulated cells and IL-7-stimulated cells + IFN- α 8 are shown. The proportion of proliferating CD4⁺ T cells in indicated in each plot.

Figure 4. IFN- α 8 caused an upregulation of CD127 expression in healthy and HIV⁺ donors. PBMC were cultured alone (unstim) or with 100U/mL IFN α 8 or 5ng/mL IL-7 and the MFI of CD127 expression was plotted. IFN- α 8 induced an upregulation of CD127 expression in CD4⁺ (A), naïve CD4⁺ (B), CD8⁺ (C) and naïve CD8⁺ (D) T cells.

Figure 5. IL-7-induced pSTAT5 was increased by IFN- α 8 in healthy donors. IL-7-induced pSTAT5 levels were examined in T cells from 10 healthy donors (circles) and 10 treated HIV patients (triangles). PBMC were pre-treated with IFN- α 8 for 2 hours and then pSTAT5 was induced by IL-7 and detected by flow cytometry. HIV patients had a significantly greater frequency of pSTAT5⁺ CD4⁺ (A), naïve CD4⁺ (B) and naïve CD8⁺ (D) T cells than healthy donors. In healthy donors only, pre-treatment with IFN- α 8 resulted in enhancement of IL-7-induced pSTAT5 levels in CD4⁺ T cells (A; p=0.006), naïve CD4⁺ T cells (B; p=0.004), CD8⁺ T cells (C; p=0.002) and naïve CD8⁺ T cells (D; p=0.002). IFN- α 8 alone did not induce pSTAT5.

Figure 6. IL-7 protein production by HS27A cells was increased by high concentrations of IFN- α 8. IL-7 production was significantly increased after 6, 24 and 48 hours in the presence of 100 or 1000U/mL of IFN- α 8. The results represent the mean values of three different experiments, each performed in triplicate. *** = p<0.0001.

Table 1. Demographic characteristics of HIV patients and healthy donors

	Younger	Older	HIV
N	15	15	20
Age (years)	24 (20-28) ^A	58 (50-73)	43 (33-67)
Male/Female	7/8	13/2	19/1
Current CD4 count (cells/μL)	N/A	N/A	470 (72-1334)
Nadir CD4 count (cells/μL)	N/A	N/A	11 (0-56)
Viral load (copies/mL)	N/A	N/A	<50
Months on ART	N/A	N/A	66 (19-178)

^Adata presented as median (range)

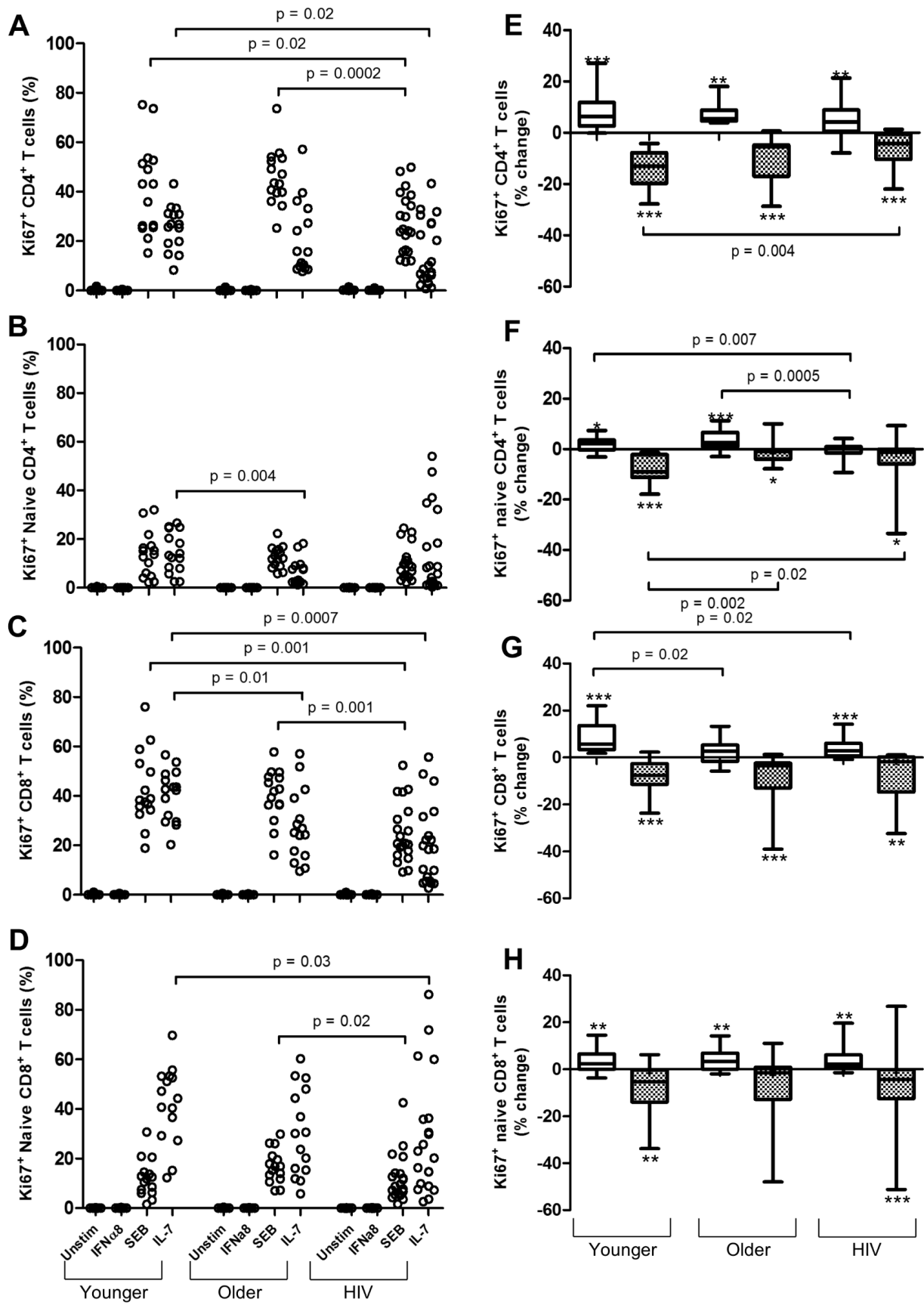


Figure 1.

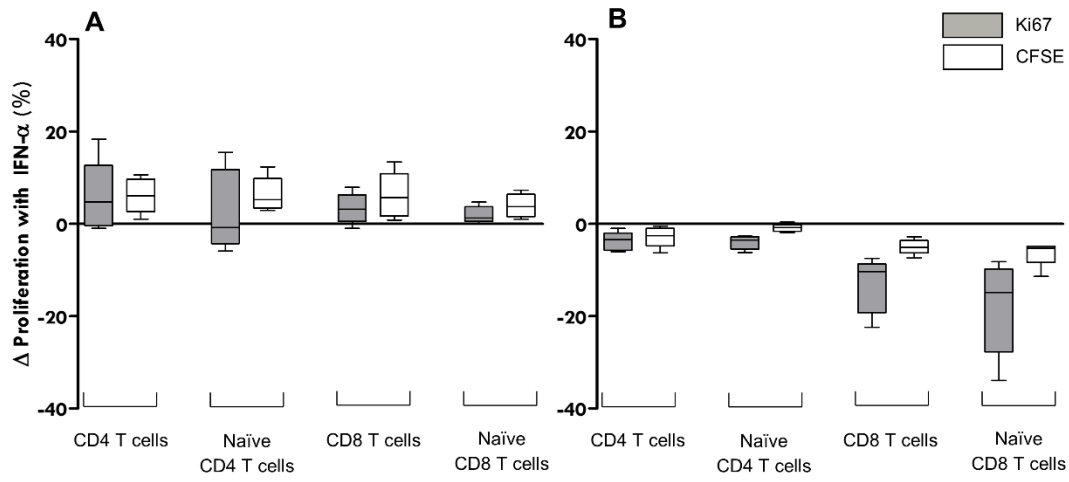
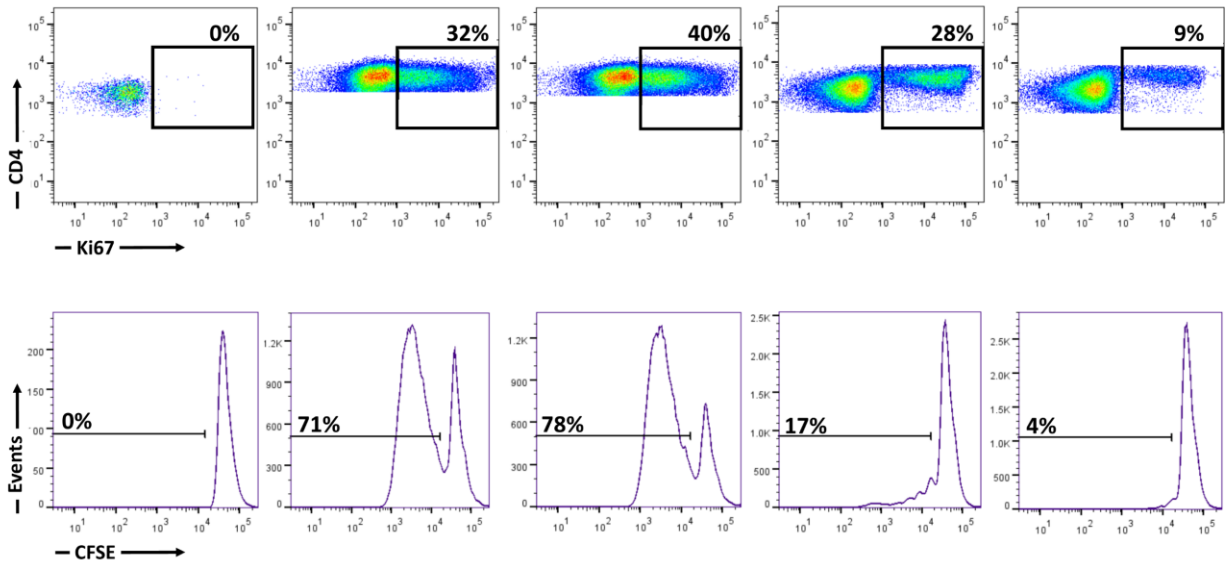


Figure 2.



SEB	-	+	+	-	-
IL-7	-	-	-	+	+
IFN- α 8	-	-	+	-	+

Figure 3.

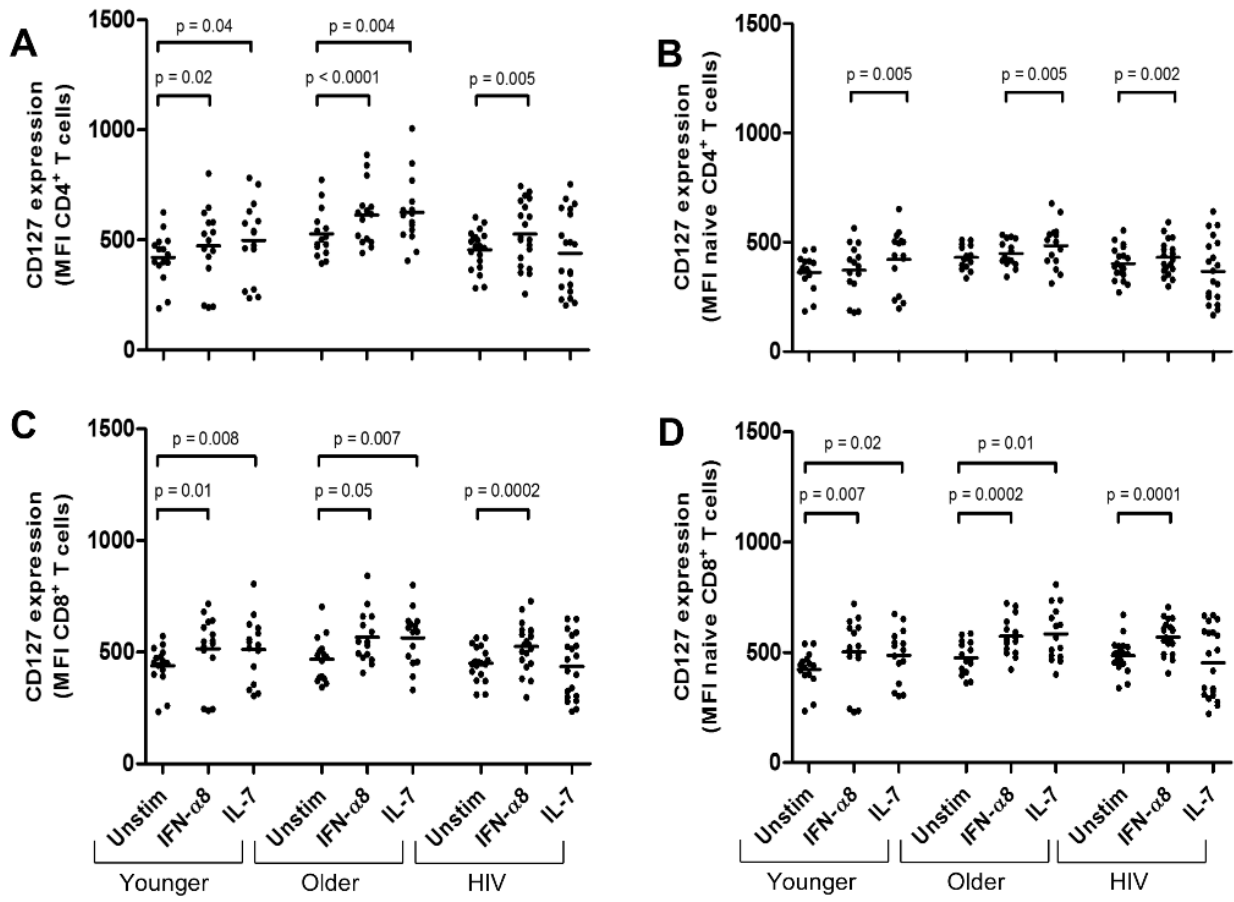


Figure 4.

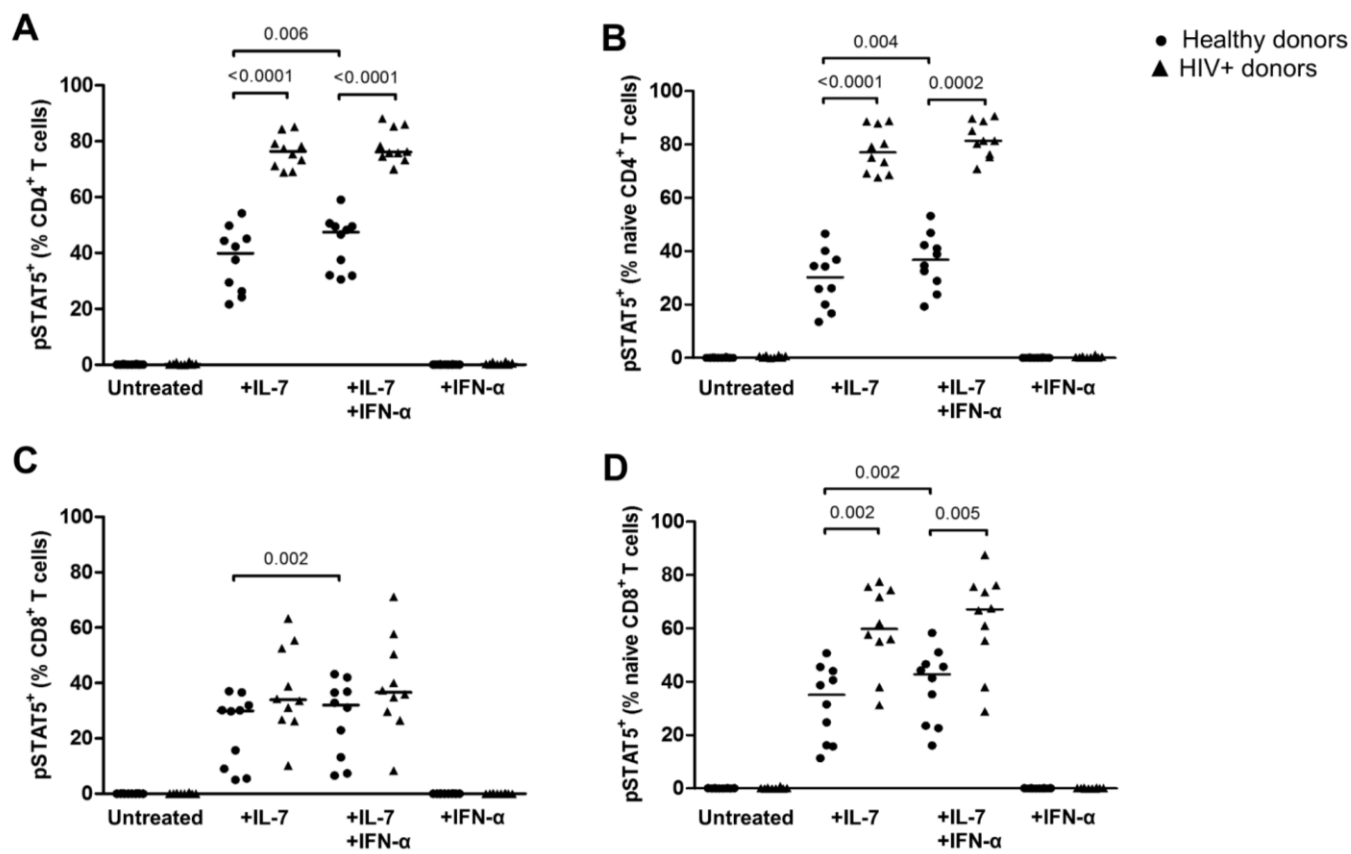


Figure 5.

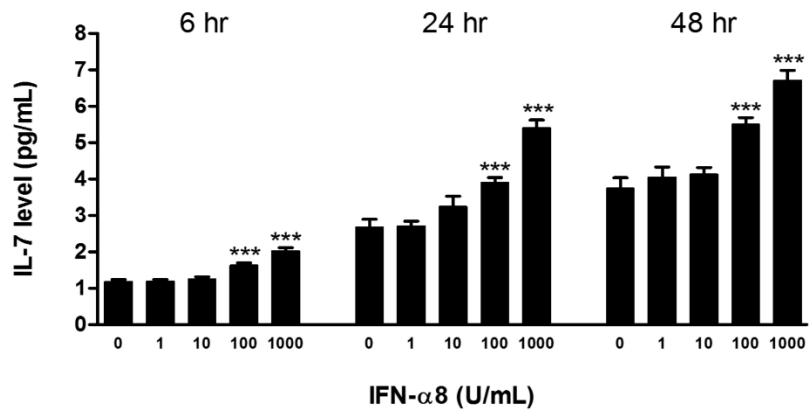
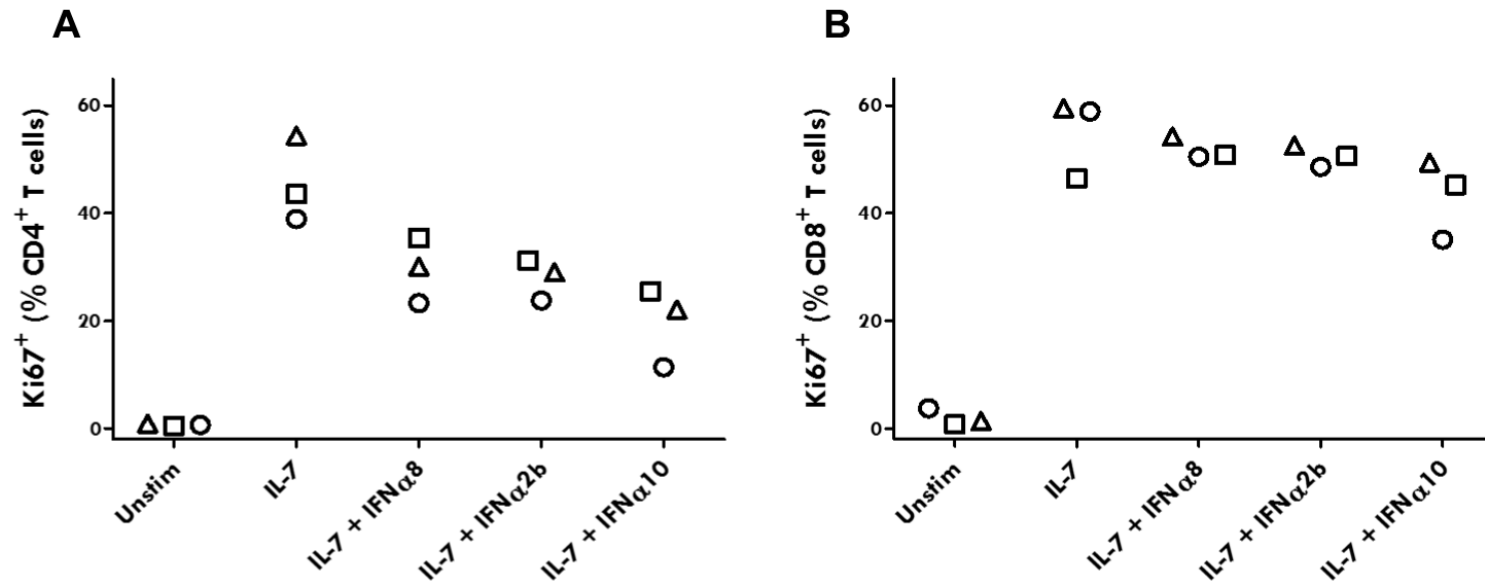


Figure 6.



Supplemental Figure 1. The anti-proliferative effects of three IFN- α subtypes (IFN- α 2b, IFN- α 8 and IFN- α 10) were compared in three non-HIV donors (depicted as a triangle, square and circle). PBMC were cultured with 5ng/mL IL-7 either alone or in the presence of 100U/mL of IFN- α 2b, IFN- α 8 or IFN- α 10. After 7 days of culture, proliferation of CD4⁺ T cells (A) and CD8⁺ T cells (B) was assessed using flow cytometry. Proliferating T cells are shown as the proportion of total CD4⁺ or CD8⁺ T cells expressing the proliferation marker Ki67. All three subtypes of IFN- α inhibited IL-7 induced proliferation of T cells to a similar degree.