IL-7 and the HIV Tat protein act synergistically to down-regulate CD127 expression on CD8 T cells

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Abstract

IL-7 signaling is essential for optimal CD8 T cell function, homeostasis and establishment of memory. We have previously shown decreased expression of the IL-7 receptor α-chain (CD127) on CD8 T cells from HIV-infected patients with active viral replication. We have also shown that soluble HIV Tat protein specifically down-regulates CD127 on the surface of CD8 T cells and impairs cell proliferation and cytolytic potential following stimulation with IL-7 *in vitro*. We now show that soluble HIV Tat protein and IL-7 at near physiologic concentrations act synergistically to suppress CD127 expression. While soluble HIV Tat protein and IL-7 both independently reduce CD127 expression on the surface of CD8 T cells, Tat concentrations of 10 μg ml^{-1} and IL-7 concentrations of 500 pg ml^{-1} are required *in vitro* to have an appreciable effect. However, where 0.5 μg ml^{-1} of Tat has no effect on CD127 expression and 200 pg ml^{-1} of IL-7 decreases CD127 by only 14%, these two together at these same concentrations induce a 35% reduction in CD127 expression after 24 h. Inhibition of Janus kinase (JAK) completely blocks IL-7's ability to down-regulate CD127 on the surface of CD8 T cells and also abolishes synergy with Tat. Interestingly, while Tat acts synergistically with IL-7 to reduce CD127 expression, it antagonizes IL-7-induced cell proliferation and Ki-67 expression and has no effect on IL-7-mediated signal transducer and activator of transcription 5 (STAT5) phosphorylation or expression of the anti-apoptotic gene Bcl-2. Thus, by affecting different IL-7 signal transduction pathways, HIV Tat protein is able to impair both CD8 T cell activation and proliferation without inducing apoptosis.

Introduction

IL-7 is essential for the normal development and function of lymphocytes. Indeed, IL-7 plays a pivotal role at a number of T cell developmental stages (1–3) and is critical for peripheral immune homeostasis and maintenance of naive (4–8) and memory T cells in mice (9–11). IL-7 is also essential for the activation and proliferation of CD8 T cells. By both up-regulating telomerase in naive T cells (12) and increasing proliferation (12–15), IL-7 enhances clonal expansion following T cell stimulation. IL-7 further potentiates CD8 T cell cytolytic activity (16–22) by up-regulating perforin (23), a protein used by CD8 T cells to kill their targets.

The IL-7 receptor (IL-7R) is a heterodimer composed of a unique α-chain (CD127) (24) and a common γ-chain (CD132) (25) that is shared with the receptors for IL-2, -4, -9, -15 and -21 (26). We and others have shown decreased expression of the IL-7R α-chain on CD8 T cells in HIV+ patients with uncontrolled viral replication (27–33). This down-regulation occurs on both naive and memory cells

(28, 31) and correlates with an impaired response to antigen stimulation and IL-7 (34, 35). Given the important role that IL-7 plays in CD8 T cell development and function, decreased IL-7 signaling may contribute to the impaired cell-mediated immunity and inefficient immunologic control of viral replication evident in HIV+ patients with progressive disease.

Several authors have suggested that the generalized decrease in CD127 expression on CD8 T cells in HIV-infected patients is the result of chronic antigen stimulation and immune activation. This model is unsatisfying as it does not explain down-regulation of CD127 on non-HIV-specific CD8 T cells (28, 31) in HIV-infected patients. Further, the reduced CD127 expression documented on T cells with a naive phenotype (27–29, 31, 32) is difficult to reconcile with chronic activation. IL-7 down-regulates the expression of its own receptor (36) and since HIV-infected individuals have increased plasma concentrations of IL-7 compared with

uninfected controls (37, 38), it is possible that IL-7 itself is responsible for reduced expression of CD127 on CD8 T cells in HIV+ patients. While this hypothesis is attractive, it is notable that IL-7 concentrations in the range of 500–10 000 pg ml^{-1} are required *in vitro* to down-regulate CD127 on purified CD8 T cells isolated from healthy volunteers (33, 36). Since plasma IL-7 concentrations average 2.2 pg ml^{-1} in HIV-negative individuals and 12–55 pg ml^{-1} (5, 32, 38, 39) in HIV-infected patients, it is questionable whether the concentrations of IL-7 required to down-regulate CD127 *in vitro* can be achieved *in vivo*. This is reflected in Simian immunodeficiency virus-infected macaques where administration of supra-physiologic concentrations of IL-7 exceeding 1000 pg ml^{-1} in plasma were required to down-regulate CD127 on circulating CD8 T cells (8). Perhaps more revealing are three other studies (29, 31, 40) demonstrating no correlation between plasma IL-7 levels and expression of CD127 on CD8 T cells in HIV-infected individuals. It therefore appears that elevated serum IL-7 concentrations alone are not solely responsible for the low CD127 expression on CD8 T cells in HIV-infected patients.

The HIV Tat protein is a small 14 kdal polypeptide known to increase viral gene transcription by binding to a secondary stem-loop structure termed transactivation response element located at the 5′ end of HIV transcripts and enhancing the processivity of RNA polymerase II (41, 42). Interestingly, Tat is secreted by HIV-infected cells and can be found in the media during *in vitro* infection (43, 44) as well as in the serum of HIV-infected patients (45). Acting in a paracrine fashion, Tat is rapidly taken up by neighboring cells including lymphocytes (44, 46, 47) by binding via its arginine-rich basic domain to heparin sulfate proteoglycans on the cell surface (47–49) followed by internalization in T cells through clathrin-coated pits (50).

We have recently shown that soluble HIV Tat protein down-regulates CD127 on the surface of CD8 T cells isolated from healthy HIV-negative volunteers (51). Tat's effect on CD127 is both dose and time dependent and can be blocked by either anti-Tat antibodies or pre-incubation with heparin. Tat down-regulates CD127 expression on the bulk CD8 T cell population including both naive and memory CD8 T cells mirroring decreased CD127 expression *in vivo*. Importantly, Tat has no effect on CD8 T cell viability in culture which remains >90% over 72 h. Notably, not only does Tat down-regulate CD127 on CD8 T cells but also this down-regulation results in deficits in CD8 T cell activity. Pre-incubating CD8 T cells with Tat inhibits proliferation and also prevents accumulation of intracellular perforin in response to IL-7. As with IL-7, we found that Tat is required *in vitro* at higher than physiologic concentrations to down-regulate CD127 on CD8 T cells. This may reflect a lower amount of biologically active protein in the total preparation or the need for post-translation modification to enhance Tat function. Indeed, acetylation of Tat at Lys28 and Lys50 both independently increase Tat activity at least in transcription assays (52–54). Alternatively, host factors may work in concert with Tat to down-regulate CD127 *in vivo*. With that in mind, we questioned whether Tat and IL-7 together affect CD127 expression to a greater extent than either one alone.

We show here that soluble HIV Tat protein and IL-7 in fact act synergistically to down-regulate CD127 on CD8 T cells

isolated from healthy volunteers. This synergism appears to be mediated at least in part by the Janus kinases (JAKs) as addition of JAK inhibitor 1 completely blocked IL-7's ability to down-regulate CD127 and abolished synergy with Tat. Interestingly, while Tat and IL-7 work synergistically to decrease CD127 expression, Tat opposes IL-7-induced cell proliferation and Ki-67 expression. In addition, Tat had no effect on IL-7-mediated signal transducer and activator of transcription 5 (STAT5) phosphorylation or expression of CD25 and Bcl-2.

Materials and methods

Reagents

Purified HIV-1 Tat protein (86 amino acids) was purchased from Advanced Bioscience Laboratories Inc. (Kensington, MD, USA). Protein was received lyophilized and was re-suspended to 1 mg ml^{-1} in PBS containing 1 mg ml^{-1} BSA and 0.1 mM dithiothreitol. Tat protein is reportedly >95% pure by heparin-affinity chromatography and reverse-phase HPLC. Anti-CD8–PE–Cy5 (PC5) (B9.11), anti-CD127–PE (R34.34), anti-CD25–FITC (B1.49.9) and anti-CD62L–PE–Texas Red (ECD) (DREG56) fluorochrome-labeled mAbs were purchased from Immunotech Beckman Coulter (Marseille, France). All fluorochrome-labeled antibodies were titrated and used at saturating concentrations. Anti-CD3 (HIT3a) and anti-CD28 (CD28.2) mAbs were purchased from BD Biosciences: PharMingen (Mississauga, Ontario, Canada). IL-7 was obtained from Invitrogen Biosource (Burlington, Ontario, Canada), re-suspended in PBS plus 0.1% BSA and stored at −80°C. Anti-phospho-STAT5 (Y694) mAb, Bcl-2 (Blc-2/100) and Ki-67 (B56) staining kits were purchased from BD Biosciences: PharMingen. JAK inhibitor 1 was purchased from Calbiochem (San Diego, CA, USA). Reagents for real-time PCR including the iScript cDNA Synthesis Kit and IQ SYBR Green Supermix® were purchased from Bio-Rad (Mississauga, Ontario, Canada). [^{3}H]-thymidine ([^{3}H]-TdR) was obtained from Amersham Biosciences (Baie d'Urfe, Quebec, Canada).

Cell purification and culture

Blood from healthy HIV-negative donors was drawn into tubes containing sodium heparin, and PBMC were isolated by Ficoll-Paque density centrifugation. CD8 T cells were then purified from PBMC using the magnetic cell sorting Microbead CD8+ Cell AutoMACS Isolation System (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's directions. Cell purity was consistently >95% CD8+ by flow cytometric analysis.

Following isolation, purified CD8 T cells were allowed to recover overnight at 1×10^6 cells per milliliter in media comprised of RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 20% FCS (Cansera, Rexdale, Ontario, Canada) plus penicillin and streptomycin (RPMI-20). The following day, CD8 T cells were incubated in media alone (RPMI-20) or in media supplemented with purified HIV Tat protein, IL-7 or other reagents as indicated. All cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO_2. For inhibitor studies, cells were pre-incubated for 1 h with inhibitors as indicated prior to treatment with Tat protein and/or IL-7.
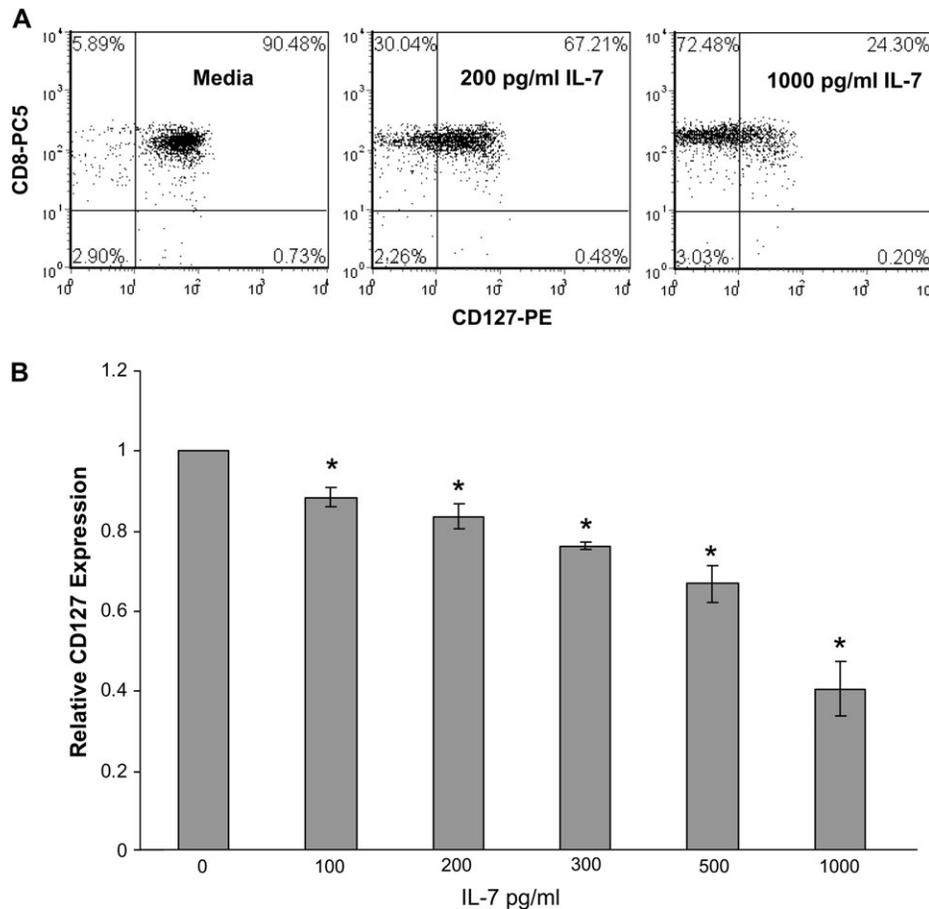
Fig. 1. IL-7 down-regulates CD127 on the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative volunteers and incubated in media alone or with IL-7 at the concentrations indicated for 24 h and then analyzed for CD127 expression by flow cytometry. (A) Representative dot plots of CD8 T cells from one individual incubated in media alone or with IL-7 at 200 or 1000 pg ml^{-1}. (B) Composite data for $n = 8$. Values represent percent positive CD8 T cells expressing CD127 relative to media control $\pm$ standard error of the mean. Bars marked with an * indicate values that are statistically different ($P < 0.05$) from media control.

Flow cytometry

At times indicated, cells were incubated with the appropriate fluorochrome-labeled antibodies for 30 min in the dark at room temperature and then analyzed by flow cytometry using a Coulter Epics ALTRA flow cytometer (Fullerton, CA, USA). Live cells were gated on the basis of side and forward scatter. At least 10 000 events were recorded for each sample. Isotype controls were performed for each fluorochrome-conjugated antibody. Resulting profiles were analyzed with FCS Express 2 software (De Novo, Los Angeles, CA, USA). Intracellular Ki-67 and Bcl-2 staining was performed as specified by the manufacturer (BD Biosciences: PharMingen).

Intracellular phospho-STAT5 staining was performed as follows. Isolated CD8 T cells were fixed in 2% PFA for 10 min at room temperature and then permeabilized by incubating in cold 100% methanol for an additional 10 min at 4°C. After washing in PBS, cells were incubated with anti-phospho-STAT5 antibodies in the dark for 30 min and analyzed using a BD FACS Calibur flow cytometer (San Jose, CA, USA). Profiles were analyzed with FCS Express 2 software.

Reverse transcription–PCR

Total RNA was harvested from isolated CD8 T cells using the RNAqueous®-4PCR for isolation of DNA-free RNA (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. RNA was quantified on a Genequant Pro instrument (GE Healthcare, Piscataway, NJ, USA) and reverse transcribed using the iScript cDNA Synthesis Kit. The resulting cDNA was used to quantify CD127 transcripts relative to expression of the RPS18 reference gene using the IQ SYBR Green Supermix® from Bio-Rad. Both reverse transcription and quantitative PCR were carried out in an iCycler thermal cycler (Bio-Rad). Real-time PCR was carried out in triplicate against a standard curve in order to account for amplification efficiency. CD127 transcripts were quantified using a forward primer spanning the boundary of exons 4 and 5 (CD127fwd 5′-ATGGACGCATGTGAATTTATC-3′) and a reverse primer within exon 5 (CD127rev 5′-GGGAGATG-GATCCTATC-3′). The forward and reverse primers used for RPS18 were as follows: RPS18fwd 5′-CTGCCAT-TAAGGGTGTGG-3′ and RPS18rev 5′-TCCATCCTTTA-CATCCTTCTG-3′. Data analysis was carried out according to the $2^{-\Delta\Delta Ct}$ principle using the Bio-Rad Gene Expression

Analysis Macro. The resulting expression values were plotted using GraphPad Prism software (San Diego, CA, USA) and statistical analysis was done using a one-tailed, unpaired Student's *t*-test with 95% confidence intervals.

Proliferation assays

Purified CD8 T cells were transferred to a 96-well tissue culture plate at 5×10^5 cells per milliliter and stimulated in triplicate with IL-7 (0.1–10 ng ml^{-1}) or with anti-CD3 plus anti-CD28 mAbs (3 and 2 µg ml^{-1}, respectively). After 48 h of incubation, cultures were pulsed with 1 µCi [^{3}H]-TdR for 18 h. Cells were then harvested onto Filtermat paper (Perkin Elmer, Wellesley, MA, USA) and β-radioactivity was measured using a 96-well liquid scintillation counter.

This work was reviewed and approved by the Ottawa Health Research Institute Research Ethics Board.

Results

*IL-7 and HIV Tat protein independently down-regulate CD127 on the surface of CD8 T cells*

IL-7 has previously been shown to down-regulate surface expression of CD127 on T cells isolated from both humans

(33) and mice (36). In agreement with this, we found that IL-7 decreased CD127 on the surface of purified CD8 T cells isolated from healthy volunteers in a dose-dependent manner. As shown in Fig. 1, cells incubated in RPMI-20 with increasing concentrations of IL-7 (100–1000 pg ml^{-1}) for 24 h demonstrated an incremental decrease in CD127 expression. The effect was linear with 200 pg ml^{-1} inducing a 16.4 ± 3.2% decline in receptor expression and 500 pg ml^{-1} causing a 33.2 ± 6.9% decline compared with cells maintained in media alone ($P = 0.002$).

We have previously demonstrated that soluble HIV Tat protein also down-regulates CD127 on the surface of CD8 T cells (51). The effect was specific to CD127 and we found no change in the expression of a number of other cell surface proteins including CD25, CD38 and HLA-DR suggesting a stable CD8 T cell phenotype in the presence of purified Tat protein. Notably, Tat did not down-regulate CD132, the common γ-chain that associates with CD127 forming the heterodimeric IL-7R (51). The effect on CD127 was specifically mediated by Tat and could be blocked with either anti-Tat antibodies or heparin and was not due to LPS or induction of apoptosis (51). As shown in Fig. 2 and consistent with our previous findings, Tat-induced down-regulation of CD127 on the surface of CD8 T cells is dose
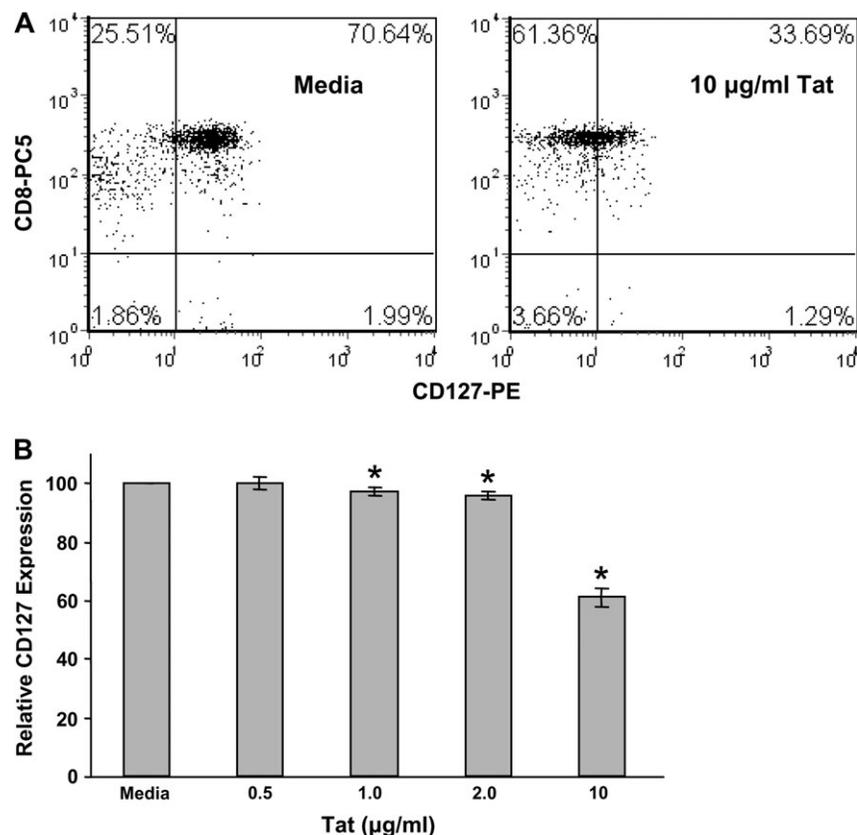
Fig. 2. Soluble HIV Tat protein down-regulates CD127 on the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative volunteers and incubated in media alone or with purified Tat protein at the concentrations indicated for 24 h and then analyzed for CD127 expression by flow cytometry. (A) Representative dot plots of CD8 T cells from one individual incubated in media alone or with purified soluble Tat protein at 10 µg ml^{-1}. (B) Composite data for $n = 4$. Values represent percent positive CD8 T cells expressing CD127 relative to media control ± standard error of the mean. Bars marked with an * indicate values that are statistically different ($P < 0.05$) from media control.
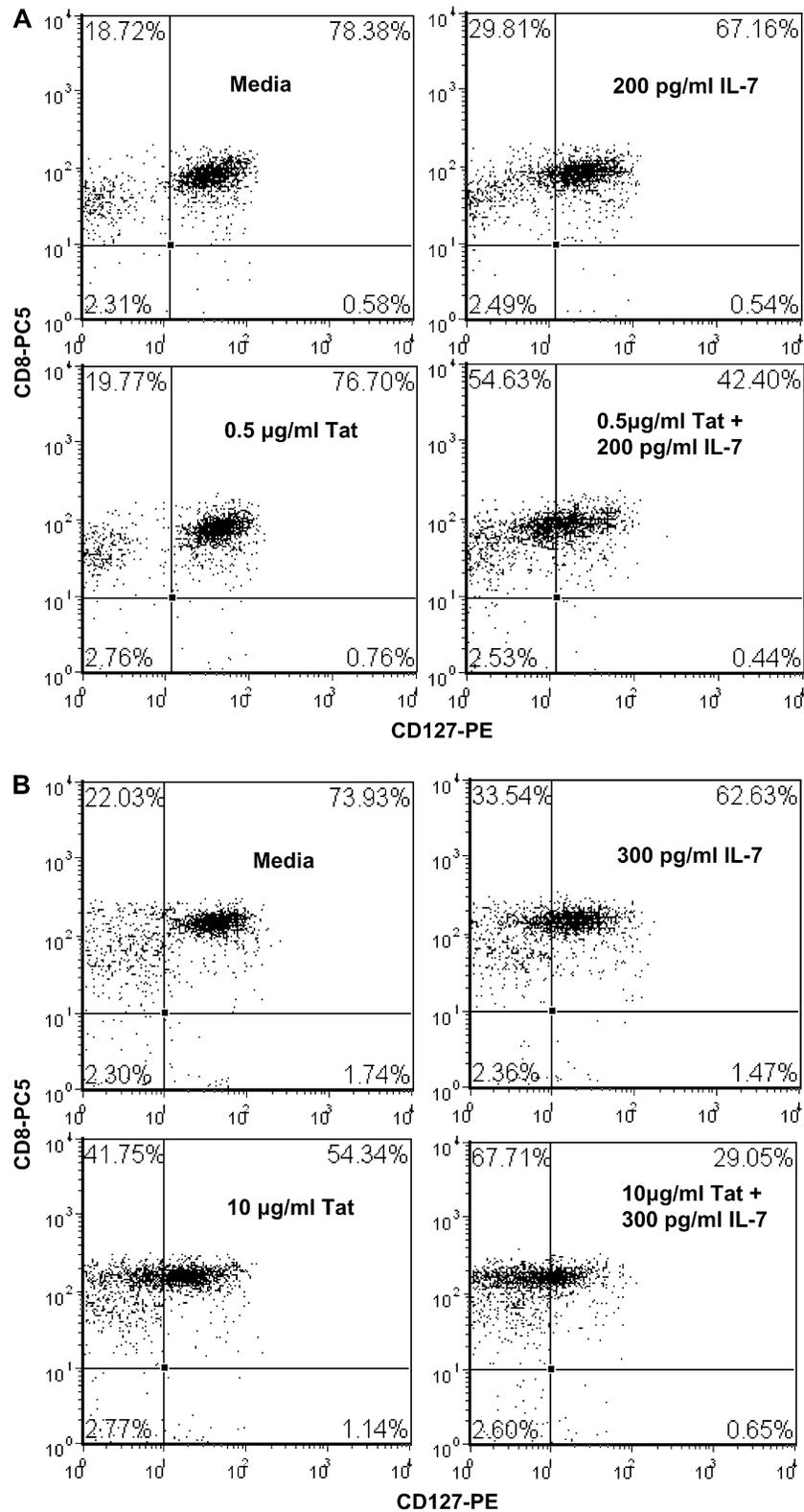
Fig. 3. HIV Tat and IL-7 act synergistically to down-regulate CD127 on the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative volunteers and incubated in media alone or with purified Tat protein and IL-7 either alone or in combination at the indicated concentrations for 24 h. Cells were then analyzed for CD127 expression by flow cytometry. (A) Representative dot plots of CD8 T cells from one individual incubated in media alone, 0.5 μg ml^{-1} Tat, 200 pg ml^{-1} IL-7 or both. (B) Representative dot plots of CD8 T cells from a second individual incubated in media alone, 10 μg ml^{-1} Tat, 300 pg ml^{-1} IL-7 or both.

dependent where 2 μg ml^{-1} soluble Tat protein induces a 4 ± 1% decrease in CD127 expression and 10 μg ml^{-1} Tat induces a 39 ± 3% decline compared with cells maintained in media alone (P = 0.003).

HIV Tat and IL-7 act synergistically to down-regulate CD127 on CD8 T cells

The concentrations of IL-7 required in our experiments (200–1000 pg ml^{-1}) to down-regulate CD127 exceed the concentrations of IL-7 reported in the sera of both healthy controls (average 2.2 pg ml^{-1}) and in HIV-infected individuals (12–55 pg ml^{-1}) (5, 32, 38, 39). Likewise, Tat is required at nanomolar concentrations (5–10 μg ml^{-1}) *in vitro* to down-regulate CD127, some 10- to 20-fold higher than estimates of Tat concentrations in patient sera (300–500 ng ml^{-1}) (45). In view of the fact that Tat and IL-7 each independently down-regulate CD127 expression, we questioned whether these viral and host proteins could act together at more physiologic concentrations to reduce CD127 on CD8 T cells. Indeed, when Tat and IL-7 were added at low concentrations where each alone has only a small effect on CD127 expression, they demonstrated synergy when added in combination. While 0.5 μg ml^{-1} of Tat has no effect on CD127 expression and 200 pg ml^{-1} of IL-7 induces only a 14 ± 1% decline, these two together at these same concentrations cause a 35 ± 9% drop in surface CD127 expression at 24 h (Fig. 3A, Table 1). Synergy was evident over a range of concentrations and was observed whether Tat concentration was held constant relative to IL-7 or vice versa (Fig. 3B, Table 1).

IL-7-induced decrease in CD127gene transcripts is not affected by Tat

In mice, IL-7 has been shown to decrease CD127 gene expression (36). In view of this, we asked whether the synergy between Tat and IL-7 occurs at the level of transcription. As shown in Fig. 4(A), IL-7 (10 ng ml^{-1}) induced a 60 ± 10% decrease in the level of CD127 mRNA in CD8 T cells within 24 h. In contrast, Tat (10 μg ml^{-1}) alone had no effect on the level of CD127 transcripts and did not induce a further decrease in mRNA when added in combination with IL-7

beyond that seen for IL-7 alone. We wondered if in these experiments the effects of Tat could have been overshadowed by the relatively high concentrations of IL-7. To address this, lower concentrations were used. As seen in Fig. 4(B), neither IL-7 at 200 pg ml^{-1} nor Tat at 2 μg ml^{-1} had any effect on the level of CD127 transcripts. When Tat and IL-7 were added together at these same concentrations, the level of CD127 mRNA remained unchanged. These data indicate that IL-7 is in fact able to decrease the level of CD127 transcripts in CD8 T cells but is required at relatively high concentrations to have an effect. In contrast, Tat appears to have no effect on CD127 gene transcription. Further, the synergy observed between Tat and IL-7 in down-regulating CD127 surface expression does not occur at the level of gene expression.

Down-regulation of CD127 by IL-7 requires activation of JAK

Binding of IL-7 to its receptor results in heterodimerization of CD127 and the common γ-chain (CD132) bringing together
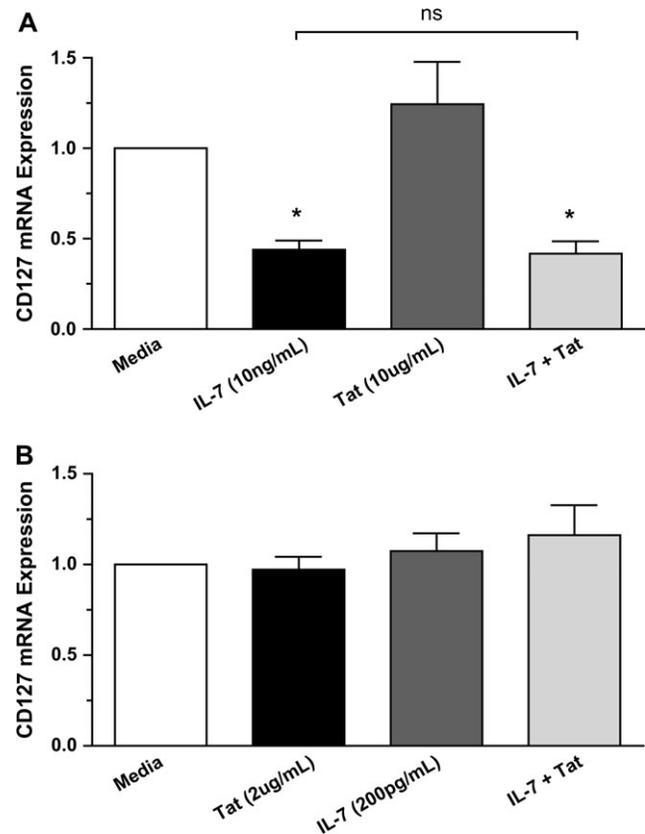
Fig. 4. Down-regulation of CD127 mRNA transcripts by IL-7 is unaltered by Tat. Purified CD8 T cells from healthy HIV-negative volunteers were incubated with purified Tat protein, IL-7 or both for 24 h after which total RNA was isolated and CD127 transcripts quantified by real-time PCR normalizing to RPS18. Levels of transcripts are shown in each case relative to cells in media alone ± standard error of the mean. (A) Concentrations were IL-7: 10 ng ml^{-1}; Tat: 10 μg ml^{-1} (n = 4). (B) Concentrations were IL-7: 200 pg ml^{-1}; Tat: 2 μg ml^{-1} (n = 8). Bars marked with an * are statistically different (P < 0.05) from media control. Values that are not statistically different are indicated by 'NS', not significant.

Table 1. HIV Tat and IL-7 act synergistically to down-regulate CD127 on CD8 T cells

% CD127 ± SEM	IL-7 concentration (pg ml^{-1})			
	0	50	100	200
Media	100	93 ± 2	93 ± 1*	86 ± 1*
0.5 μg ml^{-1} Tat	100 ± 2	92 ± 2*	89 ± 1*	65 ± 9*
1 μg ml^{-1} Tat	97 ± 1	88 ± 2*	84 ± 2*	64 ± 3*
2 μg ml^{-1} Tat	96 ± 1*	90 ± 1*	78 ± 3*	59 ± 2*

Purified CD8 T cells from healthy HIV-negative volunteers were incubated with purified Tat protein, IL-7 or both at concentrations indicated for 24 h and then analyzed for CD127 expression by flow cytometry. Data were accumulated from n = 4. Values represent percent positive CD8 T cells expressing CD127 relative to media control ± standard error of the mean (SEM). Values marked with an * indicate values that are statistically different (P < 0.05) from media control.

JAK1 and JAK3 and allowing their trans-phosphorylation. Subsequent phosphorylation of CD127 and CD132 allows STAT5 to bind to the receptor via its SH2 domain permitting in turn its phosphorylation and signal transduction (55, 56). In view of this, we questioned whether JAK activity was required for CD127 down-regulation by IL-7 and if so if this contributed to the synergy with Tat. To investigate this, CD8 T cells were incubated in increasing concentrations of IL-7 in the presence or absence of the general JAK inhibitor, JAK inhibitor 1. As expected, surface CD127 expression decreased with increasing concentrations of IL-7 ranging from 200 to 500 pg ml^{-1} (Fig. 5A). Addition of the JAK inhibitor, however, completely abolished IL-7's ability to down-regulate CD127 (Fig. 5B and C). At concentrations of IL-7 up to 500 pg ml^{-1}, CD127 expression on CD8 T cells remained unchanged in the presence of JAK inhibitor 1 compared with cells maintained in media alone while IL-7 on its own at 200 and 500 pg ml^{-1} induced a significant decline in CD127 compared with media controls ($P = 0.04$ and $P = 0.02$, respectively). As expected, inhibition of JAK activity also blocked IL-7-induced STAT5 phosphorylation (Fig. 5D).

We next asked whether CD127 down-regulation by HIV Tat was also dependent on the JAKs. To address this, CD8 T cells were incubated with Tat (10 μg ml^{-1}) in the presence or absence of JAK inhibitor 1. As seen in Fig. 6, inhibition of JAK activity had no effect on Tat's ability to down-regulate CD127. Taken together, these data indicate that IL-7 and Tat down-regulate CD127 surface expression through different mechanisms, one dependent on JAKs and the other not.

Inhibition of JAK activity abolishes synergy between Tat and IL-7

We next asked if JAK activity plays a role in the synergy between IL-7 and Tat in down-regulating CD127 surface
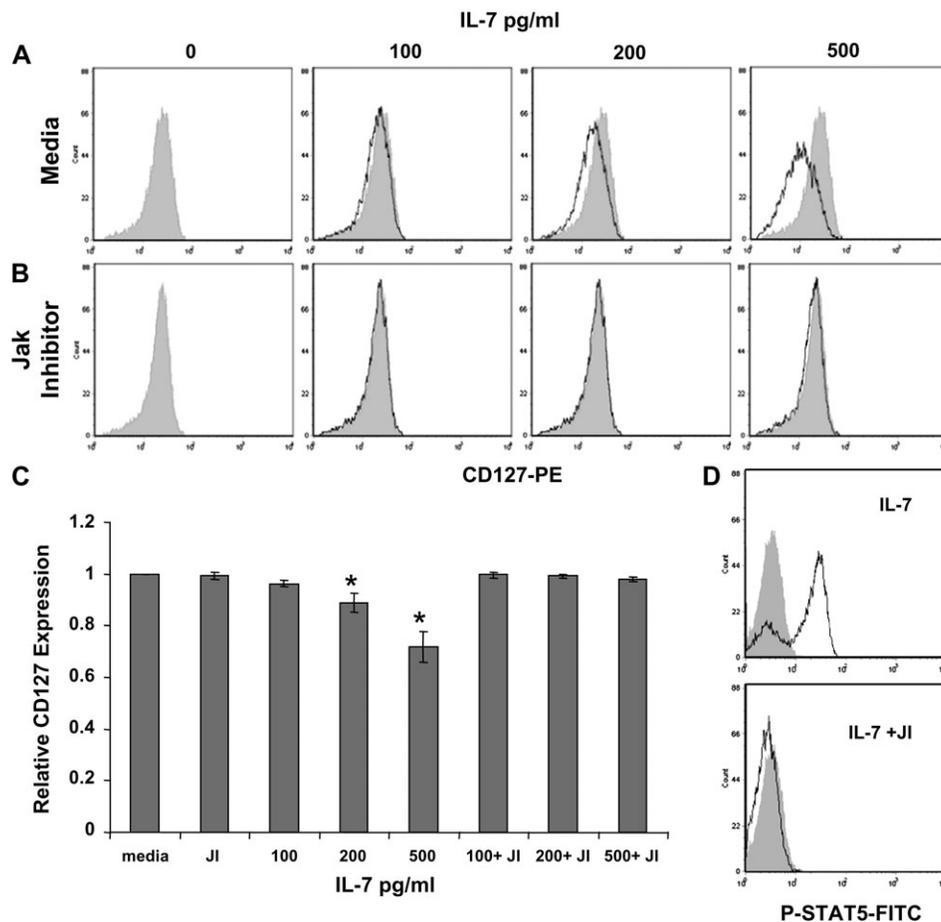
Fig. 5. JAK inhibitor 1 abolishes IL-7-induced down-regulation of CD127 and STAT5 phosphorylation. Purified CD8 T cells from healthy HIV-negative volunteers were incubated in media alone or media containing IL-7 at the concentrations indicated plus or minus JAK inhibitor 1 (30 nM). After 24 h, cells were stained for CD127 and analyzed by flow cytometry. Shown are representative histograms of CD127 expression on CD8 T cells from one individual incubated with (A) IL-7 or (B) IL-7 plus JAK inhibitor 1. (C) Composite data of CD127 expression from $n = 4$. Values represent percent positive CD8 T cells expressing CD127 relative to media control ± standard error of the mean. (D) Purified CD8 T cells were incubated in media alone or media containing IL-7 (1 ng ml^{-1}) or IL-7 plus JAK inhibitor 1 (30 nM) for 15 min. Cells were immediately fixed and permeabilized, stained with anti-phospho-STAT5 antibodies and analyzed by flow cytometry. Shown are representative histograms from one individual comparing phospho-STAT5 expression in cells maintained in media alone (gray fill) to cells treated with IL-7 or IL-7 plus JAK inhibitor 1 (black lines). Bars marked with an * indicate values that are statistically different ($P < 0.05$) from media control.
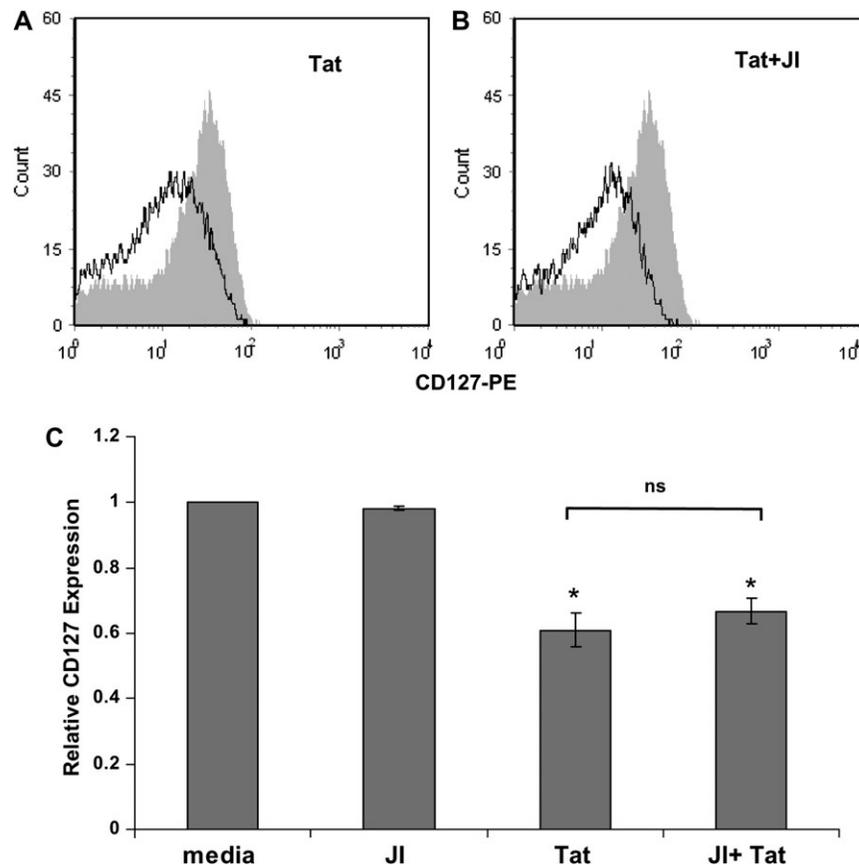
Fig. 6. JAK inhibitor 1 does not block CD127 down-regulation by soluble HIV Tat protein. Purified CD8 T cells from healthy HIV-negative volunteers were incubated in media alone or media containing Tat (10 µg ml^{-1}), JAK inhibitor 1 (30 nM) or both. After 24 h, cells were stained for CD127 and analyzed by flow cytometry. Shown are representative histograms from one individual comparing CD127 expression on CD8 T cells maintained in media alone (gray fill), with (A) Tat (black line) or (B) Tat plus JAK inhibitor 1 (black line). (C) Composite data of CD127 expression from *n* = 4. Values represent percent positive CD8 T cells expressing CD127 relative to media control ± standard error of the mean. Bars marked with an * indicate values that are statistically different (*P* < 0.05) from media control.

expression. To address this, CD8 T cells were incubated with soluble Tat and IL-7 in the presence of JAK inhibitor 1. As shown in Fig. 7, inhibition of JAK activity had no effect on Tat's ability to down-regulate CD127 expression. By completely blocking IL-7 activity, however, JAK inhibitor 1 was able to abolish synergy with Tat. As before, 2 µg ml^{-1} Tat plus 200 pg ml^{-1} IL-7 induced a 41 ± 2% decrease in surface CD127 expression, more than an additive effect and much greater than either one alone (*P* = 0.002 Tat + IL-7 versus Tat only at 2 µg ml^{-1}; *P* = 0.0002 Tat + IL-7 versus IL-7 only at 200 pg ml^{-1}). Addition of JAK inhibitor 1 reduced the effect of Tat (2 µg ml^{-1}) plus IL-7 (200 pg ml^{-1}) to only 8 ± 1%, equivalent to that seen with 2 µg ml^{-1} Tat alone. This indicates that the synergy between Tat and IL-7 in down-regulating CD127 requires the activity of JAKs.

Tat does not affect IL-7-induced STAT5 phosphorylation

As described, IL-7 binding to its receptor leads to activation of JAK1 and JAK3 and subsequent signal transduction via STAT5 phosphorylation. In view of the fact that IL-7-induced down-regulation of CD127 and synergy with Tat could be blocked by inhibition of the JAKs, we questioned whether

the synergistic effect with Tat was mediated through STAT5 signaling. Specifically, we asked if Tat could amplify STAT5 phosphorylation. While IL-7 did not induce STAT3 phosphorylation in primary CD8 T cells (data not shown), it did induce phosphorylation of STAT5 within 15 min (Fig. 8A). In contrast, Tat alone had no effect on STAT5 (Fig. 8B) and did not enhance STAT5 phosphorylation in the presence of IL-7 (Fig. 8C). Consistent with this and in agreement with our previous data, Tat alone had no effect on the expression of Bcl-2, CD25 or CD62L nor did it enhance induction of Bcl-2 (Fig. 9A) or CD25 (Fig. 9B) or down-regulation of CD62L (Fig. 9C) by IL-7. Thus, while JAK activity plays an essential role in the synergistic down-regulation of CD127 by IL-7 and Tat, we found no evidence to suggest that this synergy was mediated by increased STAT5 phosphorylation.

Tat opposes IL-7-induced CD8 T cell proliferation

IL-7 stimulates proliferation of both human and mouse CD8 T cells in culture, (12–15) while T cells from IL-7R−/− mice proliferate poorly with about half undergoing apoptosis (57, 58). While the pathway regulating cell proliferation by IL-7 has not been fully elucidated, stimulation with IL-7 appears
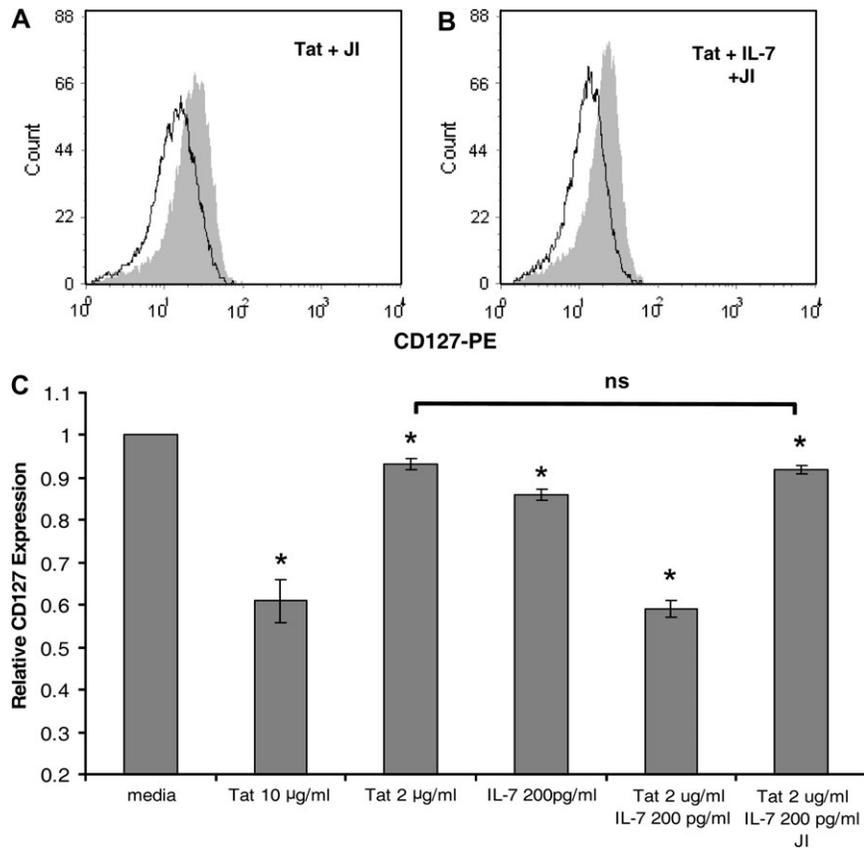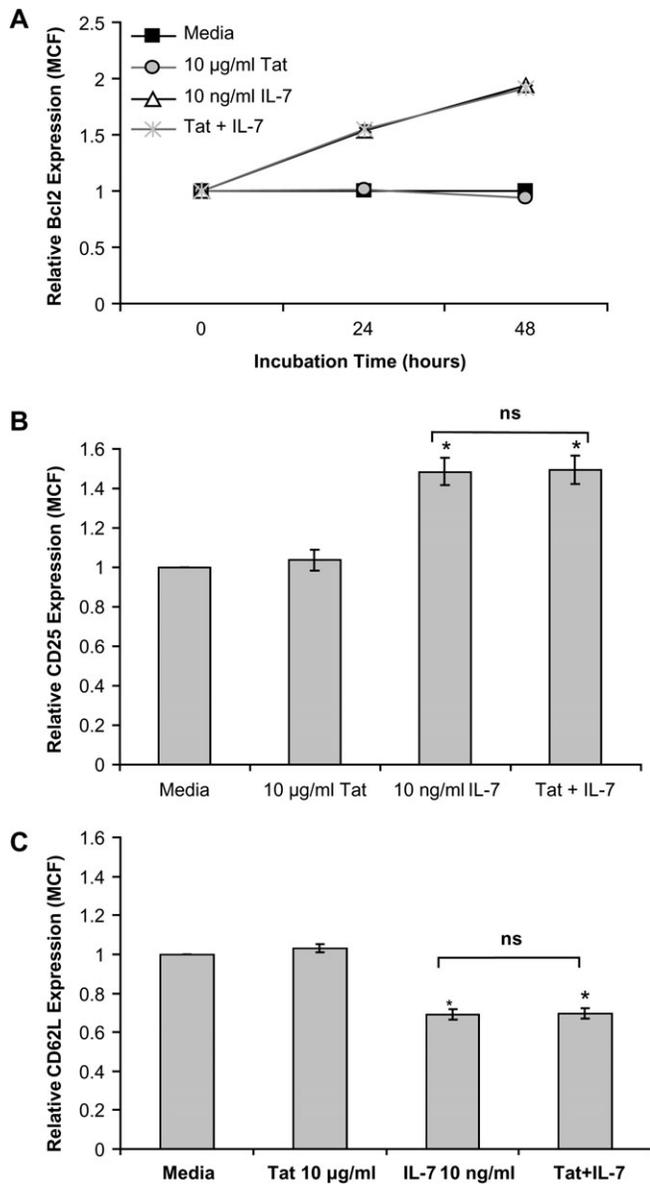
Fig. 7. JAK inhibitor 1 abolishes synergy between Tat and IL-7. Purified CD8 T cells from healthy HIV-negative volunteers were incubated in media alone or media containing Tat and/or IL-7 at the concentrations indicated in the presence or absence of JAK inhibitor 1 (30 nM). After 24 h, cells were stained for CD127 and analyzed by flow cytometry. Shown are representative histograms from one individual comparing CD127 expression on CD8 T cells incubated in media alone (gray fill), with (A) 2 µg ml^{-1} Tat plus JAK inhibitor 1 (black line) or (B) 2 µg ml^{-1} Tat with 200 pg ml^{-1} IL-7 and JAK inhibitor 1 (black line). (C) Composite data of CD127 expression from $n = 4$. Purified CD8 T cells were incubated with Tat (2 or 10 µg ml^{-1}), IL-7 (200 pg ml^{-1}) or Tat (2 µg ml^{-1}) plus IL-7 (200 pg ml^{-1}) in the presence or absence of JAK inhibitor 1 (JI; 30 nM). Values represent percent positive CD8 T cells expressing CD127 relative to media control ± standard error of the mean. Bars marked with an * are statistically different ($P < 0.05$) from media control. Values that are not statistically different are indicated by 'NS', not significant.
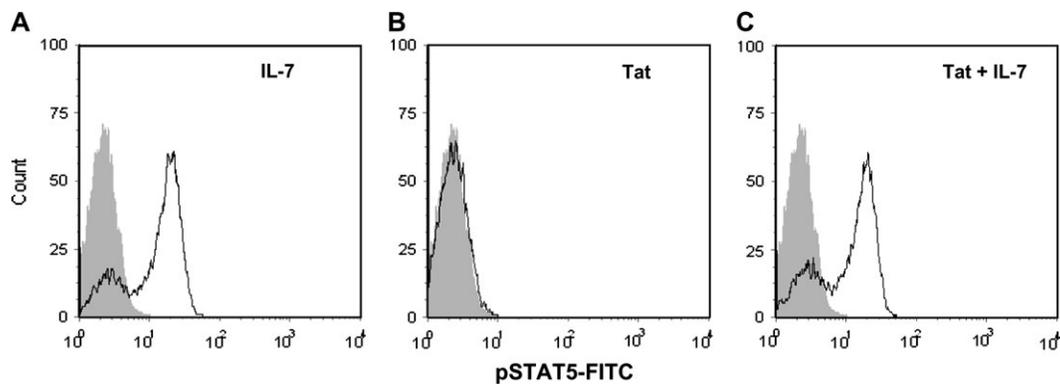
Fig. 8. Tat does not affect IL-7-induced STAT5 phosphorylation. Purified CD8 T cells from healthy HIV-negative volunteers ($n = 4$) were incubated in media alone (gray fill) or media containing (A) 10 ng ml^{-1} IL-7 (black line), (B) 10 µg ml^{-1} Tat (black line) or (C) Tat plus IL-7 (black line) for 15 min. Cells were immediately fixed and permeabilized, stained with anti-phospho-STAT5 antibodies and analyzed by flow cytometry. Shown are representative histograms from one of four individuals.

to result in phosphorylation of the retinoblastoma protein, thereby inducing progression from G1 to S phase of the cell cycle (55). Previously, we showed that when CD8 T cells are

pre-incubated with Tat, CD127 is down-regulated and IL-7 is no longer able to stimulate cell proliferation (51). Since Tat and IL-7 together down-regulate CD127 on the surface of

Fig. 9. Tat does not affect IL-7-induced changes in Bcl-2, CD25 or CD62L expression. Purified CD8 T cells from healthy HIV-negative volunteers were incubated in media alone or media containing 10 μg ml^{-1} Tat, 10 ng ml^{-1} IL-7 or both. After 24 h, cells were stained for Bcl-2 (A), CD25 (B) or CD62L (C) expression and analyzed by flow cytometry. For each experiment, $n = 4$. Values represent percent positive CD8 T cells relative to media control $\pm$ standard error of the mean (SEM). SEM for (A) range 0.02–0.03. Bars marked with an * indicate values that are statistically different ($P < 0.05$) from media control.

CD8 T cells, we questioned what the simultaneous addition of these factors might have on cell division. We confirmed IL-7's ability to stimulate proliferation of primary CD8 T cells in culture by both [^{3}H]-TdR incorporation and by induction of Ki-67. IL-7 had a dose-dependent effect on proliferation reaching a maximum at 5 ng ml^{-1} (Fig. 10A; $P = 0.006$) and increased Ki-67 expression within 48 h (Fig. 10B). Consistent with our previous report demonstrating that Tat did not stim-

ulate [^{3}H]-TdR incorporation in CD8 T cells (51), Tat also did not induce Ki-67 expression (Fig. 10C). Perhaps somewhat surprisingly, we found Tat inhibited the induction of Ki-67 by IL-7 (Fig. 10D). This suggests that rather than simply having no effect on CD8 T cell proliferation, Tat may actively suppress IL-7-induced cell division. Whether Tat achieves this by reducing the number of accessible CD127 molecules on the cell surface or by directly inhibiting signal transduction downstream of the IL-7R requires further investigation.

Discussion

In a previous report, we demonstrated that soluble HIV Tat protein down-regulates surface CD127 expression on CD8 T cells (51). The effect was both dose and time dependent and required the continuous presence of Tat. Importantly, Tat did not induce apoptosis or otherwise affect CD8 T cell viability as measured by propidium iodide exclusion and annexin V staining. To have an appreciable effect on CD127 expression, however, Tat was required *in vitro* at concentrations some 20-fold higher than those found in patient sera. Here, in agreement with other reports (33, 36), we show that IL-7 also down-regulates CD127 on the surface of CD8 T cells but like Tat is required *in vitro* at supra-physiologic concentrations. A number of explanations may be put forward as to why high concentrations of protein are required in *in vitro* assays. First, the level of biologically active protein is rarely 100% in any purified preparation. Second, recombinant proteins produced in bacterial expression systems are unlikely to be post-translationally modified and as a result may lack full activity. Third, it is possible that while extracellular protein concentrations are low in the serum, high concentrations similar to those used *in vitro* may be achieved in the micro-environments *in vivo*. With respect to the IL-7R, this latter explanation is perhaps less satisfying as CD127 expression has generally been measured on T cells taken from the peripheral circulation (27–33). Finally, *in vitro* assays may overlook important interactions that occur *in vivo* where two different proteins at lower concentrations may act in concert to generate a response. Here we have in fact shown that soluble HIV Tat protein and IL-7 act synergistically to down-regulate CD127 on CD8 T cells. While 10 μg ml^{-1} of Tat or 500 pg ml^{-1} of IL-7 were each required alone to induce a 40–50% decrease in CD127 surface expression, together only 2 μg ml^{-1} of Tat plus 200 pg ml^{-1} of IL-7 were able to achieve the same effect.

While we have yet to fully elucidate the mechanism by which Tat and IL-7 together down-regulate CD127, several possibilities may be considered. We have shown here that while Tat does not enhance STAT5 phosphorylation, the synergistic effect between Tat and IL-7 on CD127 expression is dependent on JAK activity. Morelon *et al.* (59) have suggested that phosphorylation of the common γ-chain (CD132) marks this receptor subunit for endocytosis and degradation. While phosphorylation of neither CD127 nor CD132 has yet been demonstrated in IL-7R endocytosis, we have found that inhibition of JAK activity completely blocks IL-7-induced down-regulation of CD127 on the surface of CD8 T cells. Whether JAK directly phosphorylates CD127 marking it for removal from the cell membrane or
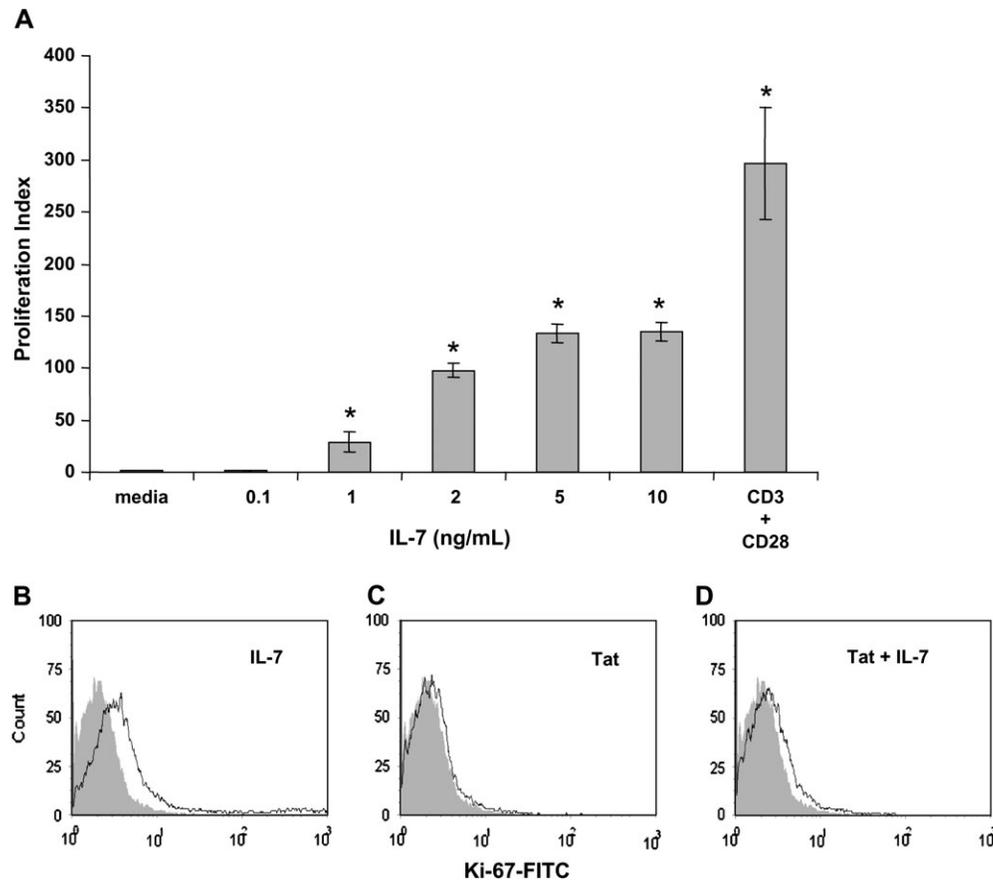
Fig. 10. Tat inhibits IL-7-induced CD8 T cell proliferation. (A) Purified CD8 T cells from healthy HIV-negative volunteers ($n = 4$) were incubated in media alone, with IL-7 at the concentrations indicated or with anti-CD3 plus anti-CD28 mAbs (3 µg ml^{-1} and 2 µg ml^{-1}, respectively) for 48 h after which [^{3}H]-TdR incorporation was measured. Values represent proliferation indices relative to unstimulated media controls (mean ± standard error of the mean). (B–D) Purified CD8 T cells from healthy HIV-negative volunteers ($n = 4$) were incubated in media alone (gray fill) or media containing 10 ng ml^{-1} IL-7 (B), 10 µg ml^{-1} Tat (C) or both (D). After 24 h, cells were stained for Ki-67 and analyzed by flow cytometry. Flow histograms from one representative individual are shown. Bars marked with an * indicate values that are statistically different ($P < 0.05$) from media control.

phosphorylates other proteins or adaptors that mediate CD127 down-regulation awaits further investigation. It may be, consistent with the suggestion of Morelon *et al.* (59), that on binding IL-7 and dimerization of CD127 and CD132, JAK3 phosphorylates CD132 and targets it for internalization. CD127 may as a result be removed from the cell membrane by its association with the common γ-chain. In this case, CD127 and CD132 would be removed from the cell surface in a 1:1 ratio. Preliminary evidence from our laboratory indicates that Tat induces clustering of CD127 molecules on the cell surface and subsequent internalization (E. Faller, M. McVey, S. Sugden, J. Kakal and P. MacPherson, in preparation). One hypothesis is that IL-7 induces binding of a single CD132 molecule to a cluster of CD127 molecules in the presence of Tat and via JAK3-mediated phosphorylation of CD132 activates endocytosis of the entire complex. In this way, Tat and IL-7 together may remove CD127 from the cell membrane more efficiently than either alone. This hypothesis requires further investigation.

Alternatively, soluble Tat protein and IL-7 may interact directly at the cell surface. Tat secreted into the external environment binds via its arginine-rich basic domain to heparin sulfate proteoglycans on the cell surface (47–49) and is subsequently internalized in T cells through clathrin-coated pits (50). Similarly, IL-7 like other cytokines has also been shown to interact with heparin sulfate proteoglycans on the cell membrane (60–62). This interaction may ensure that IL-7 remains localized to surrounding cells and protected from proteolytic degradation. It is possible then that the synergy noted between Tat and IL-7 occurs at the cell surface where Tat binding to heparin sulfate proteoglycans may enhance the localization of IL-7 at the cell surface and thus promotes IL-7 signaling.

We have found here that Tat has a number of effects on IL-7 signal transduction in CD8 T cells. First, Tat acts synergistically with IL-7 to down-regulate CD127 on the cell surface. Our data suggest that Tat amplifies a direct effect of JAK on the receptor. Tat has no effect, however, on STAT5 phosphorylation. In the presence of Tat, IL-7 induces STAT5 phosphorylation to the same degree and up-regulates Bcl-2 and CD25 and down-regulates CD62L. We have also found that Tat directly inhibits IL-7-induced up-regulation of Ki-67 and cell proliferation. These different responses likely reflect the cascade of intracellular signaling events influenced by

both Tat and IL-7. For example, IL-7-induced STAT5 phosphorylation results in increased histone acetylation presumably through recruitment of the histone acetyltransferases CBP/p300 and pCAF (63, 64). Similarly, Tat has also been shown to alter the activity of several acetyltransferases including Tip60 (65, 66), CBP/p300 (67, 68) and TAF$_{II}$250 (69). By influencing the acetylation state of histones and other cellular proteins, Tat and IL-7 may act together or in opposition to affect target gene expression. In a similar vein, extracellular Tat and IL-7 have both been shown to activate phosphoinositide 3-kinase (55, 70–72). However, while extracellular Tat induces c-fos expression and AP-1 DNA-binding activity (73), IL-7 has in contrast been shown to suppress c-fos and c-jun gene transcription (74). Thus, by acting in concert with IL-7 to up-regulate some signaling pathways while opposing IL-7's effects on other pathways, Tat may significantly disrupt IL-7 signal transduction.

Consistent with previous reports, we have shown here that at supra-physiologic concentrations, soluble HIV Tat protein and IL-7 each independently down-regulate the IL-7R α-chain on the surface of CD8 T cells. Here we demonstrate for the first time the synergy between HIV Tat and IL-7 in the regulation of a key cytokine signaling pathway. IL-7 signaling plays a pivotal role in CD8 T cell function. We have shown here that IL-7 stimulates Ki-67 expression and cell proliferation and also induces a number of phenotypic changes on CD8 T cells including down-regulation of CD127 and CD62L and up-regulation of Bcl-2 and CD25 as well as perforin synthesis (51). The evidence we present sheds light on how HIV may effectively limit CD8 T cell activity. Acting in a paracrine manner, secreted Tat protein is taken up by CD8 T cells and down-regulates CD127 on the cell surface. In the presence of IL-7 and activation of JAK, the effects of Tat on CD127 are amplified. With decreased CD127 expression, CD8 T cells no longer accumulate perforin (51). While IL-7 may induce phenotypic changes on the surface of CD8 T cells, Tat effectively inhibits Ki-67 induction and cell proliferation. Thus, by targeting the IL-7R and potentially downstream signaling pathways, soluble HIV Tat protein may be able to severely impair CD8 T cell responses to foreign antigen. In light of this, Tat may be an important target for therapeutic intervention.

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Abbreviations

[^{3}H]-TdR	[^{3}H]-thymidine
IL-7R	IL-7 receptor
JAK	Janus kinase
STAT5	signal transducer and activator of transcription 5

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