IL-7 downregulates IL-7Rα expression in human CD8 T cells by two independent mechanisms

Feras M Ghazawi1,2,5, Elliott M Faller1,2,5, Scott M Sugden1,2, Juzer A Kakal1,2 and Paul A MacPherson1,2,3,4

Interleukin (IL)-7 is an essential nonredundant cytokine, and throughout the lifespan of a T-cell signaling via the IL-7 receptor influences cell survival, proliferation and differentiation. It is therefore no surprise that expression of the IL-7 receptor alpha-chain (CD127) is tightly regulated. We have previously shown that IL-7 downregulates expression of CD127 at the cell surface and now elucidate the kinetics of that suppression and demonstrate that IL-7 downregulates CD127 transcripts and surface protein in primary human CD8 T cells by two separate pathways. We show that IL-7 induces the initial reduction in cell-surface CD127 protein independent of transcriptional suppression, which is delayed by 40–60 min. Although IL-7-mediated downregulation of CD127 transcripts is dependent on Janus kinase (JAK)/STAT5, the early downregulation of surface CD127 protein is independent of JAK activity. The data further illustrate that low levels of IL-7 induce smaller and transient decreases in CD127 transcripts and surface protein, whereas higher concentrations induce more profound and sustained suppression. Such flexibility in receptor expression likely allows for fine-tuned immune responses in human CD8 T cells in different microenvironments and in response to different immunological challenges.

**Keywords:** interleukin-7; interleukin-7 receptor; CD127; cytotoxic T lymphocytes

Interleukin (IL)-7 has essential, nonredundant roles in the development, maintenance and function of T cells. It is a master regulator of human and murine T-cell homeostasis and is required for survival of naive and mature peripheral T cells and for the establishment and maintenance of T-cell memory. IL-7 also has a pivotal role in the regulation of the expression and activity of a number of downstream transcription (STAT) proteins and the p85 subunit of phosphoinositide 3 (PI3) kinase, both of which subsequently regulate the expression and activity of a number of downstream genes and gene products. Despite the central importance of IL-7 and its receptor in the development, maintenance and function of T cells, the mechanisms regulating expression of the IL-7 receptor in response to IL-7 signaling have been only partially characterized.

We and others have shown that IL-7 downregulates expression of the CD127 receptor subunit on the surface of T cells. Although some groups have shown transcriptional suppression of the CD127 gene in response to IL-7, others have found that IL-7 does not affect CD127 mRNA levels in human T cells. Further, where IL-7 has been shown to suppress CD127 gene transcription, it has not been established whether the loss of CD127 protein from the cell surface is the result of transcriptional downregulation and reduced protein synthesis or the result of a second mechanism acting at the cell membrane. Indeed, it was recently reported IL-7 downregulates surface CD127 protein in the T-leukemia cell lines HPB-ALL and TAIL7 through receptor internalization. Whether this applies to primary human CD8 T cells has yet to be determined. In addition, the detailed kinetics and roles that PI3K and JAK and downstream phosphorylation of STAT proteins have in regulating the amount of CD127 on the cell membrane or CD127 gene transcription or both have never been delineated.

In view of this, we set out to clearly and systematically define the mechanisms by which IL-7 downregulates CD127 expression on the surface of resting human CD8 T cells and to determine to what extent the early downregulation of surface CD127 protein is dependent on
transcriptional suppression. We demonstrate for the first time that IL-7 suppresses expression of its own receptor in primary human CD8 T cells by two independent mechanisms, one transcriptional and one at the level of surface protein. Through these pathways, IL-7 provides negative feedback on its own signaling cascades, allowing for fine-tuned immune responses in human CD8 T cells in different microenvironments and in response to different immunological challenges.

RESULTS
IL-7 downregulates CD127 mRNA transcripts and surface protein in a time- and dose-dependent manner
We first set out to elucidate the kinetics of IL-7-mediated suppression of CD127 surface protein and mRNA transcripts in primary human CD8 T cells. To do this, purified CD8 T cells were incubated with increasing concentrations of IL-7 (100–10 000 pg ml\(^{-1}\)), and levels of CD127 mRNA transcripts were measured by quantitative PCR (qPCR) and compared with untreated cells, normalizing to RPS18 expression. Surface CD127 protein expression was monitored by flow cytometry. As shown in Figure 1a, IL-7 suppresses the level of CD127 mRNA transcripts in CD8 T cells in a dose-dependent manner. At least 100 pg ml\(^{-1}\) IL-7 was required to see a decrease in the level of CD127 mRNA (34 ± 4% reduction), with 10 000 pg ml\(^{-1}\) inducing a 78 ± 8% decline in transcripts. Maximal suppression at all concentrations of IL-7 occurred at 3 h. At lower concentrations of IL-7 (100–1000 pg ml\(^{-1}\)), the reduction in CD127 transcripts was transient with a dose response in time to recovery, whereas 10 000 pg ml\(^{-1}\) IL-7 maintain transcriptional suppression for over 72 h. Similarly, the

![Figure 1](https://example.com/figure1.png)

Figure 1 IL-7 downregulates CD127 mRNA transcripts and surface protein in CD8 T cells in a time- and dose-dependent manner. Purified CD8 T cells were incubated in media alone or in the presence of increasing concentrations of IL-7 (100–10 000 pg ml\(^{-1}\)) for up to 72 h. (a) CD127 mRNA transcript levels were measured by qPCR normalizing to RPS18. (b) Surface CD127 protein expression was analyzed by flow cytometry. Values represent relative CD127 mRNA or surface protein expression (percent positive cells) compared with media controls. Error bars represent s.e. of the mean (s.e.m.) of four independent experiments. (c) Representative flow-cytometry histograms from one individual in (b) showing CD127 expression on CD8 T cells incubated in media alone or with IL-7 (250 and 10 000 pg ml\(^{-1}\)) for 3–12 h.
effects of IL-7 on CD127 surface protein are also dose dependent both in the extent of the downregulation and in the duration of suppression (Figure 1b). A transient (17 ± 3%) decrease in surface CD127 protein could be detected on CD8 T cells with as little as 100 pg ml⁻¹ of IL-7. This effect became more marked with increasing concentrations of IL-7, where 5000 pg ml⁻¹ induced a 41 ± 2% reduction in CD127 surface protein within 3 h, with a maximal suppression of 90 ± 2% at 12 h. Recovery of CD127 on the cell surface took progressively longer with increasing doses of IL-7, but concentrations of 5000–10 000 pg ml⁻¹ maintained suppression for over 72 h. Thus, we show that the effects of IL-7 on suppressing CD127 mRNA transcripts and surface protein are both dose- and time-dependent, with transient reductions in expression at lower IL-7 concentrations (100–1000 pg ml⁻¹) and sustained suppression at higher concentrations (10 000 pg ml⁻¹). The maximal reductions in CD127 mRNA and surface protein occur at 3 and 12 h, respectively, at all concentrations of IL-7.

**IL-7 downregulates CD127 protein at the cell surface independent of transcriptional suppression**

As shown in Figure 1, the maximal effect of IL-7 on surface CD127 protein expression was observed several hours following maximal transcriptional suppression. This could suggest that loss of CD127 protein from the cell membrane is entirely dependent on transcriptional suppression of the CD127 gene and natural turnover of surface protein. We felt this was unlikely, however, because IL-7 induced a maximal reduction in surface CD127 protein within 12 h, whereas the natural half-life of CD127 at the cell surface is approximately 55 h.26 To determine to what extent the downregulation of surface CD127 protein is dependent on transcriptional suppression, we treated CD8 T cells with IL-7 (10 ng ml⁻¹) and measured CD127 mRNA transcripts and surface protein at 20 min intervals by qPCR and flow cytometry, respectively. Interestingly, the downregulation of CD127 protein at the cell surface occurred within 20 min, following addition of IL-7, and leveled off at 54 ± 4% pretreatment levels over the next 2 h (Figure 2a). In contrast, IL-7-induced suppression of CD127 mRNA transcripts was delayed by 40–60 min post treatment (Figure 2b). These data then demonstrate that the initial downregulation of CD127 protein at the cell surface actually precedes and is therefore likely independent of transcriptional suppression.

To further distinguish downregulation of surface CD127 protein from transcriptional suppression, we treated CD8 T cells simultaneously with IL-7 and dexamethasone (Dex), a glucocorticoid previously shown to upregulate CD127 transcripts in murine B and T cells.27,28 We reasoned if IL-7 is able to downregulate CD127 surface protein independent of transcriptional suppression, we would see this reduction at the cell surface, even when levels of CD127 transcripts are augmented by the simultaneous addition of Dex. As expected (Figure 3a), treatment with Dex alone (20 μM) increased CD127 mRNA transcript levels in human CD8 T cells some 2.6-fold within 1 h, whereas IL-7 alone (10 ng ml⁻¹) reduced CD127 transcripts by 64 ± 5% at 4 h, reaching 80 ± 4% suppression at 24 h. In contrast, the combination of IL-7 plus Dex together maintained CD127 mRNA transcripts at levels comparable to that in untreated cells. Interestingly, when added in combination with IL-7, Dex appeared to induce an initial increase in CD127 transcripts within 1 h presumably due to the rapid effect of Dex binding to the glucocorticoid receptor in the cytoplasm and its translocation to the cell nucleus. This was followed by an apparent decline in CD127 transcripts by 4 h likely due to the delayed suppressive effects of IL-7. Although these variations were consistent, the changes were not statistically significant compared with cells maintained in medium alone, and, ultimately, CD127 mRNA leveled off to pretreatment levels at 24 h. Despite the initial increase and overall lack of change in the level of CD127 transcripts, IL-7 still downregulated CD127 protein at the cell surface by 55 ± 8% in the presence of Dex (Figure 3b). These data then confirm that IL-7 downregulates surface CD127 protein independent of transcription. Notably, at 24 h, the level of surface CD127 protein was significantly higher in the presence of Dex plus IL-7 over IL-7 alone. This is most likely due to synthesis and replenishment of new CD127 protein in the presence of normal transcript levels and highlights the contribution of IL-7-induced transcriptional suppression in further limiting CD127 surface expression at later time points.

Finally, to fully separate IL-7-induced downregulation of CD127 protein at the cell surface from transcriptional suppression, we utilized a Jurkat-CD127 cell line engineered to express the CD127 cDNA from the cytomegalovirus promoter.22 Because IL-7 is not known to attenuate transcription from the cytomegalovirus promoter, we expected CD127 transcripts would remain unchanged in the presence of IL-7, whereas surface CD127 protein would be downregulated. As shown in Figure 4, this is exactly what we found. Although treatment with IL-7 did not suppress CD127 mRNA transcripts in the Jurkat-CD127 cell line (Figure 4a) over 6 h, IL-7 did significantly downregulate CD127 protein at the cell surface.
This downregulation of surface CD127 protein in the absence of changes in CD127 mRNA levels confirms that IL-7 induces the loss of CD127 protein from the cell surface independent of mRNA suppression.

**IL-7-mediated downregulation of CD127 mRNA transcripts is dependent on JAK/pSTAT5**

JAK/STAT and PI3K are considered the major IL-7-mediated signaling pathways in T cells, and we therefore set out to determine which, if either, of these pathways is involved in IL-7-induced downregulation of CD127 transcripts and surface protein. The role of IL-7 in activating JAK3/STAT5 signaling is well established, and we confirm here that IL-7 induces phosphorylation of STAT5 in primary human CD8 T cells (Figure 5a). Phosphorylation of STAT3 in response to other γ chain cytokines, such as IL-2, IL-4, IL-9, IL-15, and IL-21, has been previously reported, but to our knowledge, induction of STAT3 phosphorylation by IL-7 in human CD8 T cells has not been investigated. To examine this, we treated CD8 T cells with IL-7 (10 ng ml⁻¹) or IL-10 (10 ng ml⁻¹) as a positive control and measured intracellular phospho-STAT3 by flow cytometry. As shown in Figure 5b, IL-10 induces STAT3 phosphorylation, whereas IL-7 does not. Finally, we used a synthetic STAT5 inhibitor that specifically targets the SH2 domain of STAT5 to block its phosphorylation in the presence of IL-7. Figure 5c shows a dose titration and complete block of STAT5 phosphorylation in the presence of IL-7 plus 500 µM inhibitor.

To examine the role of the JAK/STAT5 and PI3K signaling pathways in IL-7-induced downregulation of CD127 transcripts, we pretreated CD8 T cells with inhibitors of either JAK or PI3K for 1 h followed by IL-7 (10 ng ml⁻¹) for 80 min. The PI3K inhibitor (LY294002; 10 µM) successfully blocked phosphorylation of Akt following IL-7 stimulation in primary human CD8 T cells, whereas the JAK inhibitor (1.5 µM) blocked IL-7-induced STAT5 phosphorylation (data not shown). As shown in Figure 6a, preincubating cells with JAK inhibitor completely blocked IL-7-mediated downregulation of CD127 transcripts, whereas inhibition of PI3K signaling had no effect. These results clearly indicate a requirement for JAK activity but not PI3K in the downregulation of CD127 mRNA by IL-7. We next questioned whether STAT5 phosphorylation had a role in the suppression of CD127 transcripts by IL-7. To investigate this, CD8 T cells were preincubated with 500 µM STAT5 inhibitor followed by IL-7 (10 ng ml⁻¹) for 60 min, and levels of CD127 mRNA transcripts were measured by qPCR. As shown in Figure 6b, inhibition of STAT5 phosphorylation blocked the effect of IL-7, confirming that transcriptional suppression of CD127 by IL-7 is dependent on the JAK/STAT5 signaling pathway.
Although IL-7-mediated downregulation of CD127 protein at the cell surface occurs independently of JAK/STAT5 and PI3K, IL-7-induced degradation of CD127 is JAK dependent.

To examine the requirement of the JAK/STAT5 and PI3K signaling pathways in IL-7-stimulated downregulation of CD127 protein at the cell surface, CD8 T cells were pretreated with inhibitors of either JAK, PI3K or STAT5 for 1 h followed by IL-7 (10 ng ml$^{-1}$) for 80 min in the case of JAK and PI3K inhibitors or for 60 min in the case of the STAT5 inhibitor. Levels of surface CD127 protein were then measured by flow cytometry. As shown in Figures 7a and b, inhibition of JAK, PI3K and STAT5 phosphorylation had no effect on IL-7-mediated downregulation of CD127 protein at the cell surface.

**Figure 6** IL-7-mediated suppression of CD127 transcripts is dependent on JAK activity and STAT5 phosphorylation but not on PI3K. Purified CD8 T cells were maintained in media alone or preincubated with inhibitors of JAK (1.5 µM), PI3K (10 µM) or STAT5 phosphorylation (500 µM) for 1 h followed by treatment with IL-7 (10 ng ml$^{-1}$) for 80 min in the case of the JAK and PI3K inhibitors (a) and for 60 min in the case of the STAT5 inhibitor (b). CD127 transcript levels were measured by qPCR normalizing to RPS18. Data are represented as relative changes compared with media control. (a) *P < 0.005 relative to media. (b) P = 0.02 IL-7 compared with media, *P = 0.37 STAT5i+IL-7 compared with STAT5i and **P = 0.01 STAT5i+IL-7 compared with IL-7. Error bars represent s.e.m of four independent experiments.

**Figure 5** IL-7 induces the phosphorylation of STAT5 but not STAT3 in human CD8 T cells. Purified CD8 T cells were maintained in media alone or treated with IL-7 (10 ng ml$^{-1}$) or IL-10 (10 ng ml$^{-1}$) for 45 min and then fixed, permeabilized and stained with fluorescein isothiocyanate (FITC)-labeled anti-phospho-STAT3 or anti-phospho-STAT5 antibodies. (a) Representative flow-cytometry histogram showing phospho-STAT5 in untreated cells (gray fill) and cells stimulated with IL-7 (black line). (b) Representative flow-cytometry histogram showing phospho-STAT3 in untreated cells (gray fill) and cells stimulated with IL-7 (black line) or IL-10 (dashed line). (c) Purified CD8 T cells were maintained in media alone, treated with IL-7 (10 ng ml$^{-1}$) for 8 h or preincubated with increasing concentrations of STAT5 inhibitor (100–500 µM) for 1 h and then treated with IL-7 (10 ng ml$^{-1}$) for an additional 8 h. Cells were then fixed, permeabilized and stained with FITC-labeled anti-phospho-STAT5 antibodies and analyzed by flow cytometry. Data show percent positive cells for each condition (*P < 0.03 and #P = 0.49 compared with media). Error bars represent s.e.m of four independent experiments.
removal of CD127 from the cell surface. In all cases, IL-7 stimulated a reduction in surface CD127 protein equally in the presence and absence of inhibitors. As expected, IL-7 induced the phosphorylation of tyrosine 449 (Y449) in the cytoplasmic tail of CD127 within 15 min, and inhibition of JAK prevented this from occurring (Figure 7c). Taken together, these data indicate that phosphorylation of Y449 by JAK is not required to mark CD127 for removal from the cell membrane. Interestingly, inhibition of JAK did prevent subsequent degradation of CD127 protein. When examined by western blot of whole-cell extracts, CD127 was markedly reduced in CD8 T cells treated with IL-7 for 6 h, but in the presence of JAK inhibitor, CD127 remained at levels equivalent to media control following IL-7 stimulation. Thus, although IL-7-mediated removal of CD127 from

Figure 7 Although IL-7-induced downregulation of CD127 protein at the cell surface is independent of JAK activity and phosphorylation of tyrosine 449, subsequent degradation of CD127 is dependent on JAK. Purified CD8 T cells were preincubated with inhibitors of JAK and PI3K (a) or STAT5 phosphorylation (b) followed by IL-7 (10 ng ml⁻¹) as described in Figure 6, and surface CD127 expression was analyzed by flow cytometry. Data are represented as relative changes compared with media control (*P<0.05 compared with media). Error bars represent s.e.m of at least four independent experiments. (c) Western blot showing phosphorylation of tyrosine 449 on the cytoplasmic tail of CD127 is induced by IL-7 and requires JAK. Purified CD8 T cells were maintained in media alone, treated with IL-7 (10 ng ml⁻¹) for 15 min, JAK inhibitor (1.5 μM) for 1 h and 15 min or JAK inhibitor for 1 h followed by IL-7 (10 ng ml⁻¹) for 15 min. Whole-cell lysates were then analyzed by western blot probing for phospho-Y449 using a polyclonal rabbit anti-human CD127 phospho-Tyr449 antibody. Blots were then stripped and reprobed for total CD127 as control using a polyclonal goat anti-human CD127 antibody. (d) Western blot showing IL-7-induced degradation of CD127 is JAK dependent. Purified CD8 T cells were maintained in media, treated with IL-7 (10 ng ml⁻¹) for 6 h, JAK inhibitor (1.5 μM) for 6 h or JAK inhibitor for 1 h followed by the addition of IL-7 (10 ng ml⁻¹) for 6 h. Whole-cell lysates were then analyzed by western blot probing for total CD127 with a polyclonal goat anti-human CD127 antibody. Blots were then stripped and reprobed for β-actin as loading control. (e) CD127 reaccumulates on the cell surface in the presence of IL-7 and JAK inhibitor. Purified CD8 T cells were maintained in media, pretreated with JAK inhibitor (1.5 μM) for 1 h followed by the addition of IL-7 (10 ng ml⁻¹) or treated with IL-7 alone, and CD127 surface expression was monitored by flow cytometry at the times indicated. Data are represented as relative changes compared with media controls. Error bars represent s.e.m. of at least four independent experiments.
Once in the early endosome, CD127 may then be sorted, such that CD127 phosphorylated at Y449 by JAK is targeted for degradation, whereas unphosphorylated CD127 is recycled back to the cell surface.

DISCUSSION

Although IL-7 signaling has pivotal roles in the development and function of T cells, the mechanisms regulating expression of the IL-7 receptor have only been partially characterized. Although several studies have already reported that IL-7 downregulates the expression of CD127 mRNA transcripts and surface protein in human and murine T cells,19,22 conflicting data have been provided suggesting that IL-7 does not affect CD127 gene transcription.21 Whether these discordant results are due to the source of T cells used in each study (for example, umbilical cord versus adult peripheral blood), culture conditions or the single time point of analysis is unclear. Further, it has not been determined whether IL-7-mediated downregulation of CD127 mRNA and surface protein at 3 and 12 h, respectively. Low concentrations of IL-7 (100–1000 pg ml⁻¹) induced transient declines in CD127 mRNA and protein, whereas higher concentrations (10000 pg ml⁻¹) sustained suppression for over 72 h. Although this may be due to either a bona fide concentration effect or sustained signaling at higher IL-7 concentrations, either way, this allows flexibility in regulating CD127 expression in different microenvironments with different levels of IL-7. Although decreased transcription of the CD127 gene was viewed as the primary mechanism by which IL-7 downregulates surface CD127 protein,19,20,22,26 we now show that transcriptional suppression contributes only at later time points and serves to maintain low CD127 levels after the receptor has been removed from the cell membrane by IL-7. We demonstrate by several methods that IL-7 reduces CD127 transcripts and surface protein through separate pathways. In both the Jurkat-CD127 cell line and when added to primary human CD8 T cells in combination with Dex, IL-7 removed CD127 protein from the cell surface in the absence of changes in CD127 mRNA levels. We also show that IL-7-mediated downregulation of CD127 protein at the cell surface is rapid and occurs within 20 min before there is any reduction in CD127 transcripts. This results in an initial new equilibrium in protein turnover, maintaining lower but relatively constant CD127 expression on the cell surface over the next 2 h until CD127 transcriptional suppression kicks in to prevent protein replenishment and further downregulate CD127 expression. The contribution of transcriptional suppression in reducing CD127 expression at later time points is evident in CD8 T cells treated with IL-7 and Dex, where CD127 mRNA and surface protein are higher at 24 h compared with cells treated with IL-7 alone. This effect is less evident in the Jurkat-CD127 cells because protein turnover on the surface of this cell line is much more rapid compared with primary cells (data not shown). These data together suggest that brief exposure to IL-7 may allow rapid transient decreases in surface CD127 protein, whereas sustained exposure to IL-7 results in transcriptional suppression and further reductions in receptor expression over time. This would provide a finely tuned mechanism by which CD8 T cells could respond to small changes in IL-7 concentrations in the microenvironment and to different immunological challenges.

Suppression of CD127 mRNA transcripts by IL-7 is dependent on the JAK/STAT5 signaling pathway. It is already established that IL-7 stimulation leads to phosphorylation of CD127 at Y449 by JAK3,1 allowing docking via their SH2 domains and subsequent phosphorylation of STAT5 proteins.1,18,37 Phospho-STAT5 then dimerizes and translocates to the nucleus where it may either directly or indirectly suppress CD127 transcription. Given the delay in transcriptional suppression by 40–60 min following stimulation with IL-7, we hypothesize that IL-7 signaling via STAT5 results in the upregulation of a transcriptional repressor or an epigenetic modifier protein that in turn suppresses CD127 gene expression. Consistent with this hypothesis, the translational inhibitor cycloheximide blocks the ability of IL-7 to downregulate CD127 transcripts in human CD8 T cells (Ghazawi et al., manuscript in preparation), confirming that new protein synthesis is in fact required for the transcriptional suppression. Park et al.19 reported a similar effect with cycloheximide and implicated the transcriptional repressor Gr-1 in the downregulation of CD127 gene expression in CD8 T cells in mice. However, to date, the repressor induced by IL-7 and responsible for suppressing CD127 gene transcription in human CD8 T cells has yet to be identified.

The IL-7-mediated downregulation of CD127 protein on the surface of human CD8 T cells is rapid and occurs within 20 min. Similarly, Swainson et al.20 reported that surface CD127 protein is reduced on human CD4 T cells within 30 min of IL-7 stimulation (10 ng ml⁻¹), and Henriques et al.24 documented the rapid internalization of CD127 protein from the surface of the leukemic cell line HPB-ALL following addition of IL-7 (50 ng ml⁻¹). We show here that, as expected, IL-7 induces the phosphorylation of tyrosine 449 within the cytoplasmic tail of CD127 by JAK and that this phosphorylation is not required for the initial removal of CD127 protein from the cell membrane. Indeed, IL-7 still downregulates surface CD127 protein in the presence of JAK inhibitor. However, inhibition of JAK does prevent subsequent degradation of CD127 and allows the reaccumulation of receptor on the cell surface over 12–24 h. Taken together, the data suggest that IL-7 binding to CD127 directly triggers receptor internalization, perhaps, through dimerization with CD132 or by inducing a conformational shift in the protein. Once in the early endosome, CD127 may then be sorted, such that CD127 phosphorylated at Y449 by JAK is targeted for degradation, whereas unphosphorylated CD127 is recycled back to the cell surface. These pathways are currently being further delineated in our laboratory.

The rapid downregulation of surface CD127 protein by IL-7 before CD127 transcriptional suppression and the dose response in extent and duration of suppression highlights the importance of a fine-tuned response to IL-7 in CD8 T cells. IL-7 has several pivotal roles in CD8 T-cell survival and function including but not limited to the upregulation of Bcl-2,23 induction of perforin expression,15 enhanced glucose metabolism38 as well as the establishment and maintenance of memory.6,39,40 The fine-tuned modulation of surface CD127 protein may allow CD8 T cells to regulate these functions in response to varying IL-7 concentrations in different microenvironments and in response to different immunological challenges. A disruption in this dynamicity could also lead to poor or inappropriate CD8 T-cell responses. Indeed, dysregulation of IL-7 signaling has been implicated in a number of diseases, such as

Immunology and Cell Biology
multiple sclerosis,4,5 rheumatoid arthritis and juvenile idiopathic arthritis, as well as in breast cancer.4,5 The expression of CD127 is also dysregulated in Epstein–Barr virus,4–7 cytomegalovirus4,7 and hepatitis C virus48 infections. HIV infection in particular is associated with decreased expression of CD127 on CD4 and CD8 T cells.25,29–55 In fact, we have previously shown that soluble HIV Tat protein downregulates CD127 on the surface of CD8 T cells isolated from healthy donors and targets the receptor for degradation by the proteasome.12,26 Understanding the detailed mechanisms by which IL-7 regulates expression of its own receptor may well lead to novel therapeutic strategies aimed at improving T-cell number and function in lymphopenic conditions, as well as in chronic viral infections and several autoimmune diseases.

MATERIALS AND METHODS

Jurkat-CD127 cell line and primary human CD8 T cells

The Jurkat-CD127 cell line was a kind gift from Dr René van Lier (Academic Medical Centre, Amsterdam, The Netherlands). These cells contain the retroviro-transduced CD127-TRES-GFP sequence and express the human CD127 cDNA from the cytomegalovirus promoter.22 Primary human CD8 T cells were isolated as previously described.15,23,26 Peripheral blood mononuclear cells were isolated from the blood of healthy volunteers by Ficoll-Paque density centrifugation followed by CD8 T-cell purification using magnetic-activated cell sorting using human CD8 microbeads and the AutoMACS Isolation System (Miltenyi Biotech, Auburn, CA, USA). Purified CD8 T cells and Jurkat-CD127 cells were cultured at a density of 1 × 10^6 cells per ml at 37 °C in media comprised of RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 0.2 mM L-glutamine (Gibco, Grand Island, NY, USA) and either 20% fetal calf serum (Cansaer, Rexdale, Ontario, Canada) for primary CD8 T cells or 10% fetal calf serum for the Jurkat-CD127 cells. All research conducted using human blood was reviewed and approved by the Ottawa Hospital Research Ethics Board.

Reagents

IL-7 and IL-10, obtained from Invitrogen Biosource (Carlsbad, CA, USA), were resuspended in phosphate-buffered saline plus 0.1% bovine serum albumin at a stock concentration of 10 ng ml⁻¹ and stored at −80 °C. Dexamethasone was obtained from BioVision (San Francisco, CA, USA) and was resuspended in dimethyl sulfoxide at a stock concentration of 10 mM and stored at −20 °C. STAT5 inhibitor (573108; 0.1 M), JAK inhibitor 1 (420097; 10 mM) and PI3K inhibitor (LY294002; 10 mM) were purchased from EMD Biosciences (San Diego, CA, USA). All fluorochrome-labeled antibodies used for flow cytometry were purchased from Invitrogen (Carlsbad, CA, USA). Anti-CD8-phycoerythrin-Cy5 (B9.11) and anti-CD127-phycoerythrin (R34.34) fluorochrome-labeled monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). All fluorochrome-labeled antibodies used for flow cytometry were titrated and used at saturating concentrations.

Flow cytometry

Anti-CD8-phycocerythrin-Cy5 (PC5) (B9.11) and anti-CD127-phycocerythrin (PE) (R34.34) fluorochrome-labeled monoclonal antibodies were purchased from Immunotech Beckman Coulter (Marseille, France). Monoclonal anti-phospho-STAT3-fluorescein isothiocyanate (FITC) (Y694) and anti-phospho-STAT5-fluorescein isothiocyanate (Y705) antibodies were purchased from BD Biosciences (San Jose, CA, USA). All fluorochrome-labeled antibodies used for flow cytometry were purified and used at saturating concentrations.

Western blot analysis

CD8 T cells (1 × 10^6 cells per condition) were collected by centrifugation at 6000 g for 10 min, and whole-cell extracts were prepared by resuspending the cell pellets in 50 µl RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS with 1 × Halt Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA)) and incubating at 4 °C for 45 min. Protein concentration was determined by the Bradford protein assay. Equal amounts of total cell protein were then resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus (Bio-Rad) for 30 min at 15 V. Following transfer, membranes were blocked overnight in Tris-buffered saline with Tween-20 (TBST; 50 mM Tris–HCl pH 7.6, 150 mM NaCl and 0.1% Tween-20), containing 5% nonfat milk. Total CD127 protein was detected using a polyclonal goat anti-human CD127 antibody (R&D Systems, Minneapolis, MN, USA), whereas CD127 phosphorylated at Tyr449 was detected using a polyclonal rabbit anti-human CD127 phospho-Tyr449 antibody (Rockland, Biomol, Hamburg, Germany) at a concentration of 1 µg ml⁻¹ in TBST blocking solution. Horseradish peroxidase-conjugated Donkey anti-goat (1:5000) and Donkey anti-rabbit (1:5000) from R&D Systems were used as secondary antibodies, and proteins were visualized by chemiluminescence using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Chalfont St Giles, UK) according to the manufacturer’s instructions. For reprobing for loading controls, membranes were stripped for 30 min at 50 °C in stripping buffer (2% SDS, 62.5 mM Tris–HCl pH 6.8 and 100 mM β-mercaptoethanol), washed five times with TBST; blocked with 5% nonfat milk in TBST for 1 h and then reprobed with a monoclonal mouse anti-human β-actin antibody (C4; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200, 1 µg ml⁻¹ in TBST blocking solution followed by an horseradish peroxidase-conjugated Donkey anti-mouse secondary antibody (R&D Systems) at 1:5000 dilution.

Flow cytometry

Anti-CD8-phycocerythrin-Cy5 (PC5) (B9.11) and anti-CD127-phycocerythrin (PE) (R34.34) fluorochrome-labeled monoclonal antibodies were purchased from Immunotech Beckman Coulter (Marseille, France). Monoclonal anti-phospho-STAT3-fluorescein isothiocyanate (FITC) (Y694) and anti-phospho-STAT5-fluorescein isothiocyanate (Y705) antibodies were purchased from BD Biosciences (San Jose, CA, USA). All fluorochrome-labeled antibodies used for flow cytometry were purified and used at saturating concentrations.

Surface CD127 protein expression was analyzed by incubating cells with anti-CD127-phycocerythrin antibodies for 30 min in the dark at room temperature followed by analysis on a Coulter Epics ALTRA flow cytometer (Fullerton, CA, USA). The protocol was modified slightly in Figure 2a. To prevent further internalization of CD127 while staining with the antibody, CD8 T cells were treated with IL-7 for the times indicated and then incubated with anti-CD127-phycocerythrin antibodies for 30 min on ice. Intracellular phospho-STAT3 and phospho-STAT5 staining was performed as follows: isolated CD8 T cells were fixed in 2% paraformaldehyde 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 phosphate-buffered saline, cells were incubated with anti-phospho-STAT3-fluorescein isothiocyanate or anti-phospho-STAT5-fluorescein isothiocyanate antibodies in the dark for 30 min and then analyzed by flow cytometry. Live cells were gated on the basis of side and forward scatter, and 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), and statistical analysis was done using a two-tailed, paired Student’s t-test with 95% confidence intervals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
ACKNOWLEDGEMENTS

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR). PAM is the principal recipient of the research grant. FMG and SMS are recipients of CIHR doctoral scholarships and EMF is a recipient of a postdoctoral fellowship from the Ontario HIV Treatment Network. PAM is supported in part by a mid-career salary award from the Department of Medicine, University of Ottawa. We thank the volunteers who donated blood samples for this study.

Author contributions: FMG carried out the qPCR in CDB T cells and Jurkat-CD127 cells and flow cytometry in CDB T cells (Figures 1a, 2b, 3a, 4a, 6, and 7c and e), wrote and revised the manuscript. EMF carried out flow cytometry in CD8 T cells (Figures 1b and c, 2a, 3b, and 5). SMS performed flow cytometry in Jurkat-CD127 cells and CDB T cells and carried out western blot analysis (Figures 1a, 2a, 3a, 4a, 7a, c,e). JAK participated in the ground work of the project. PAM designed the research, analyzed and interpreted data, and co-wrote the manuscript.

7 Denoue JB, Viaud SD, McVey MJ, Kakal JA, MacPherson PA. Soluble HIV Tat protein removes the IL-7 receptor alpha chain from the surface of resting CDB T cells and targets it for degradation. J Immunol 2010; 185: 2854–2866.
25 Vudattu NK, Magalhaes I, Schmidt M, Seyfert-Margolis V, Maeurer MJ. Reduced expression of the IL-7 receptor (CD127) expressing immune cells and IL-7-signalizing defects in peripheral blood from patients with breast cancer. Int J Cancer 2007; 121: 1512–1519.


52 MacPherson PA, Fex C, Sanchez-Dardon J, Hawley-Foss N, Angel JB. Interleukin-7 receptor expression on CD8(+) T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy. J Acquir Immune Defic Syndr 2001; 28: 454–457.

