Journal of NeuroVirology, 11: 319–328, 2005

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280500187005

Taylor & Francis
Taylor & Francis Group

Exogenous IL-7 induces Fas-mediated human neuronal apoptosis: potential effects during human immunodeficiency virus type 1 infection

Giuseppe Nunnari, Yan Xu, Edward A Acheampong, Jianhua Fang, Rene Daniel, Chune Zhang, Hui Zhang, Muhammad Mukhtar, and Roger J. Pomerantz

Center for Human Virology and Biodefense, Division of Infectious Diseases and Environmental Medicine, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA

The use of exogenous cytokines is part of translational immune-antiretroviral approaches to induce immune reconstitution and possibly eliminate the persistence of human immunodeficiency virus type 1 (HIV-1) in virally suppressed infected individuals on highly active antiretroviral therapy (HAART). Recently, our laboratories demonstrated that interleukin-7 (IL-7) has significant efficiency in stimulating HIV-1 replication from proviral latency in CD4+ T lymphocytes of infected patients. The authors now investigated the possible role of IL-7 in HIV-1–associated dementia (HAD). The authors demonstrated that the IL-7 receptor is expressed on both human neurons (i.e., differentiated NT2 cells) and human astrocytes, with relatively higher mRNA levels in neurons. The translational protein levels of IL-7 receptor α were not proportional to those of the mRNA levels in these central nervous system (CNS)-based cell types. Exogenous IL-7 was observed to only slightly down-regulate IL-7 receptor α expression on both neurons and astrocytes, as assayed by Western blotting. Instead of promoting survival, surprisingly, exogenous IL-7 induced neuronal apoptosis, as detected by TUNEL assays. Furthermore, IL-7 augmented neuronal apoptosis induced by HIV-1 gp120. Human apoptosis genomic microarray analyses of IL-7–treated human neurons showed up-regulated expression of proapoptotic genes: protein kinases, caspase-10, FAST kinase, tumor necrosis factor (TNF) receptor, and BCL2-antagonist of cell death. These data suggest that IL-7 leads to neuronal apoptosis by a molecular mechanism(s) that occurs via Fas-mediated activation-induced cell death. These studies may therefore not only be key in evaluating the potential use of IL-7 *in vivo* as a therapeutic modality, but also suggest that IL-7, which is increased endogenously in HIV-1–infected individuals late in disease, may be involved in the neuronal apoptosis demonstrated during HAD. *Journal of NeuroVirology* (2005) **11,** 319–328.

Keywords: apoptosis; CNS; HIV-1; IL-7; neurons

Introduction

Despite the use of highly active antiretroviral therapy (HAART), eliminating latent human immunodeficiency virus (HIV)-1 reservoirs remains a significant challenge. The use of exogenous cytokine treatment to purge latently infected cells and improve immune function is a potential immune-adjunctive therapy to HAART. Interleukin-6 (IL-6), IL-2, and tumor necrosis factor alpha (TNF-α) treatment have been shown to activate latently infected CD4+ T cells, increase the turnover rate of latent virus *in vitro* and also

Address correspondence to Giuseppe Nunnari or Roger J. Pomerantz, Center for Human Virology and Biodefense, Division of Infectious Diseases and Environmental Medicine, Department of Medicine, Thomas Jefferson University, Jefferson Alumni Hall, 1020 Locust Street, Suite 329, Philadelphia, PA 19107, USA. E-mail: Giuseppe.Nunnari@jefferson.edu or RogerJ.Pomerantz@jefferson.edu

The authors wish to thank Dr. Avindra Nath of Johns Hopkins University for producing the human primary astrocytes, and Brenda O. Gordon and Rita M. Victor for excellent secretarial assistance. This work was supported in part by USPHS grants NS41864, NS44513, AA13849, and AI43289 to R. J. P.

Received 20 February 2005; revised 25 April 2005; accepted 7 May 2005.

elevate the CD4+ T-cell count in a well-established $SCID_{hu}$ mouse model (Brooks *et al*, 2001; Chun *et al*, 1998). Recently, IL-7 was reported to induce the expression of latent HIV-1 with minimal effects on T-cell phenotype (Llano *et al*, 2001; Scripture-Adams *et al*, 2002). Moreover, we recently demonstrated that IL-7 is a potent and proviral strain-specific inducer of latent, *in vivo* HIV-1 cellular reservoirs of infected individuals on virally suppressive HAART (Wang *et al*, 2005).

Immunologically, IL-7 is a nonredundant cytokine produced by stromal cells of the thymus and the bone marrow and by keratinocytes (Sudo *et al*, 1989; Wolf *et al*, 1992; Heufler *et al*, 1993). It plays an important role in B-cell lymphopoiesis and differentiation of thymocytes into mature T cells (Fry *et al*, 2001; Grabstein *et al*, 1990). The high-affinity IL-7 receptor (IL-7R) is responsible for mediating the effects of IL-7 (Goodwin *et al*, 1990). The IL-7 receptor complex is composed of α and γc subunits, which are shared by IL-2, IL-4, IL-9, and IL-15 (Leonard *et al*, 2001). γc serves primarily to activate signal transduction through the complex, whereas IL-7Rα determines specific signaling events through its association with cytoplasmic signaling molecules (Lai *et al*, 1997). IL-7Rα has three cytoplasmic domains, A (acidic), S (serine-containing), and T (tyrosine-containing), serving as potential docking regions for these kinases (Lin *et al*, 1995). The IL-7 receptor complex signals through phosphorylation of tyrosine, serine, threonine protein kinases, activates signal transducer and activator of transcription (STAT), plus it also increases transcription of early response genes (Miyazaki *et al*, 1994; Lin *et al*, 1995; Wilks *et al*, 1994; Cosenza *et al*, 2002). Recent evidence suggests that the actions of survival/death factors are affected by modulation of the phosphorylation status of important elements of the apoptotic process (Cross *et al*, 2000). Signal transduction pathways also could activate the death receptors, such as Fas, TNF, and TNF-related ligand (TRAIL), leading to cell death (Cross *et al*, 2000; Dudley *et al*, 2004).

Of note, there is an inverse correlation between CD4+ T-cell depletion and the serum levels of IL-7 in patients with HIV-1 infection (Llano *et al*, 2001; Napolitano *et al*, 2001). These data suggest that IL-7 is not only an indicator of CD4+ T-cell depletion, but also consequently a marker of disease progression towards acquired immunodeficiency syndrome (AIDS). Taken together, these studies suggest that IL-7 could possibly be a candidate for future study as an adjunctive therapy to HAART.

Jaleco *et al* (2003) demonstrated that IL-7 regulates T-cell homeostasis by modulating the balance between proliferation and apoptosis in recent thymic emigrants (RTEs), and mature naïve and memory T cells. They found that RTEs and mature naïve and memory CD4+ T cells were sensitive to Fas-mediated cell death following exposure to either IL-2 or IL-7 alone. Furthermore, Fas engagement in the presence of both IL-2 and IL-7 resulted in high levels of caspase-dependent apoptosis.

Cytokines could originate from the brain or the periphery (Raber *et al*, 1994), and penetrate the blood-brain barrier (BBB) (Ellison *et al*, 1987). During HIV-1 infection, elevated cytokines from infected periphery cells and brain cells could induce neuronal apoptosis (Kaul *et al*, 2001), leading to HIV-1–associated dimentia (HAD). Interestingly, HAD occurs during the latest stages of AIDS, in which the highest levels of IL-7 are reached. It is reasonable to speculate the IL-7 might play either a direct or indirect role in the pathogenesis of HAD.

IL-7R has been reported to be widely present in different human organs (Cosenza *et al*, 2002; Dus *et al*, 2003). Nonetheless, little is known about the role of IL-7 in the central nervous system (CNS) in general, and during HAD in particular.

In this study, we demonstrated that IL-7R mRNA and protein are expressed in human mature neurons from differentiated NT2 cells and human astrocytes. We found that IL-7 only slightly down-regulated IL-7Rα expression in these CNS cell types. Finally, we observed that IL-7 induced neuronal apoptosis, but did not induce programmed cell death in early passage primary astrocytes. Furthermore, IL-7 augmented neuronal apoptosis induced by the HIV-1 envelope protein gp120. Gene microarray analyses showed that up-modulated proapoptotic genes were related to Fas-mediated activation-induced cell death in human neurons.

Results

Expression of IL7R mRNA and protein on human neurons and astrocytes

In the present study, we investigated whether the IL-7 receptor α (IL-7Rα) is expressed on human neurons and astrocytes. First, we determined the expression of IL-7 mRNA by these cells *in vitro.* Nalm-6 B-cells were used as positive controls for the detection of IL7Rα mRNA. As shown in Figure 1, IL7Rα mRNA was detected on both human differentiated neurons and astrocytes, but with repeatedly higher levels demonstrated in neurons.

The expression of human IL-7 receptor protein, an integral strongly glycosylated membrane protein of 76 kDa, designated as CD127 (Cosenza *et al*, 2002), was then measured. The p76 IL-7Rα protein was detected by immunoblotting on both human neurons and astrocytes, as illustrated in Figure 2.

IL-7 and IL-7Rα expression in both human neurons and astrocytes

The biological effects of IL-7 are mediated through a receptor complex containing the IL-7Rα and the γc subunits (Lai *et al*, 1997). As such, we compared the baseline levels of IL-7Rα expression with those

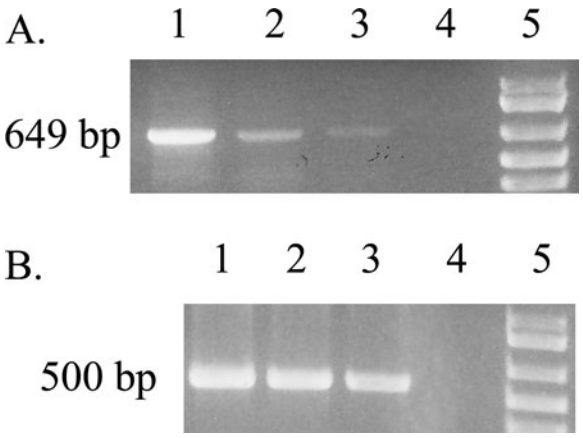

Figure 1 RT-PCR analyses of human mature neurons and astrocytes for IL-7R. Total mRNA extracted from Nalm-6 cells (positive control), human mature neurons, and primary human astrocytes were analyzed for IL-7Rα and GAPDH mRNA expression by RT-PCR analyses. **(A)** IL-7Rα RT-PCR, 649-bp fragment. **(B)** GAPDH RT-PCR, 500-bp fragment. Lane 1: Nalm-6; lane 2: human mature neurons; lane 3: primary human astrocytes; lane 4: negative control; lane 5: 1Kb plus DNA ladder (Gibco, BRL). These results are representative of independent experiments performed at least three times.

after IL-7 treatment. These immunoblotting studies demonstrated that, in response to IL-7 treatment, the levels of IL-7Rα expression were only slightly down-regulated in both neurons and astrocytes (Figure 3). These results suggest that IL-7Rα might be actively involved in signal transduction in these key CNS cell types, but that other signaling pathways could certainly not be ruled out.

IL-7 induces human neuronal apoptosis

In these next studies, it was demonstrated that IL-7 induced neuronal apoptosis, as shown by positive TUNEL staining (Figure 4, left panel, and Table 1). IL-7 induced apoptosis occurred in a dose-dependent pattern (0.01, 0.1, 1, 10, and 100 ng/ml of IL-7 were utilized, with P values $< .05$, by Student's t tests, for all comparisons except IL-7 10 ng/ml versus IL-7 100 ng/ml). On the other hand, IL-7 did not yield any demonstrable programmed cell death in astrocytes (data not shown). Of note, the addition of anti-IL-7

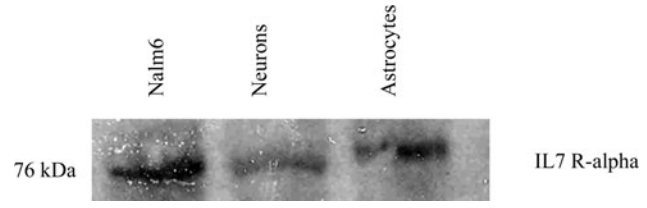

Figure 2 IL-7Rα immunoblot analysis of mature human neurons and astrocytes. IL-7Rα immune precipitates were analyzed for the expression of IL-7Rα protein. Immune precipitates were obtained with polyclonal antibody, blotted, and then detected with the same antibody. These results are representative of independent experiments performed at least three times.

Table 1 Semiquantitative determination of exogenous IL-7–induced apoptosis in human neurons

Treatment	*RGB values*
No addition	35 ± 5
IL-7 (ng/ml)	
0.01	77 ± 2
0.1	95 ± 5
1	113 ± 1
10	124 ± 4
100	134 ± 8
gp120 (50 ng/ml)	161 ± 1
gp120 (50 ng/ml) + IL-7 (ng/ml)	
0.01	164 ± 1
0.1	179 ± 9
1	178 ± 1
10	190
100	224 ± 1

antibodies was able to neutralize the IL-7–induced proapoptotic effects in neurons (Figure 5). Preincubation with anti-IL-7Rα antibodies was not able to inhibit apoptosis (Figure 5). Thus, these data suggest that IL-7 might play a specific direct role in inducing human neuronal apoptosis, at least *ex vivo*, but perhaps not via direct IL-7 receptor binding.

IL-7 synergistically augments gp120-induced neuronal apoptosis

Because the HIV-1 envelope protein gp120 is considered to have a crucial role in the pathogenesis of HAD due to its major neuronal apoptotic effects (Xu *et al*, 2004), we thus sought to determine whether IL-7 might act synergistically with HIV-1 gp120 in inducing neuronal apoptosis. When neurons were treated with both gp120 and different doses of IL-7, higher levels of apoptosis were detected as compared to gp120 alone, reaching statistical significance at the concentration of 1 ng of IL-7 per ml ($P < .05$) (Figure 4, right panel). Apoptosis values are reported in Table 1. Thus, IL-7 may interact with HIV-1 gp120 to up-regulate neuronal programmed cell-death during late stage HIV-1 infection.

Cell apoptosis genomic arrays

To assess the molecular mechanisms involved in the induction of neuronal apoptosis by exogenous IL-7 targeted, programmed cell death–targeted gene microarray analyses were used. Up-regulated expression of several pro-apoptotic genes was detected (Table 2). V-raf murine sarcoma viral oncogene and v-jun sarcoma virus 17 oncogene, encoding a serine/threonine kinase (Pollock *et al*, 2003), were dramatically up-regulated by nearly 12-fold. Of note, caspase-10, an initiator caspase in death receptor signaling, was up-regulated 10-fold. This molecule can function independently of caspase-8 in initiating Fas- and TNF-related apoptosis by inducing ligand-receptor–mediated apoptosis (Wang *et al*, 2001). Caspase-10 cleaves and activates caspases-3, -4, -6, -7, -8, and -9, which cause the proteolytic cleavage of

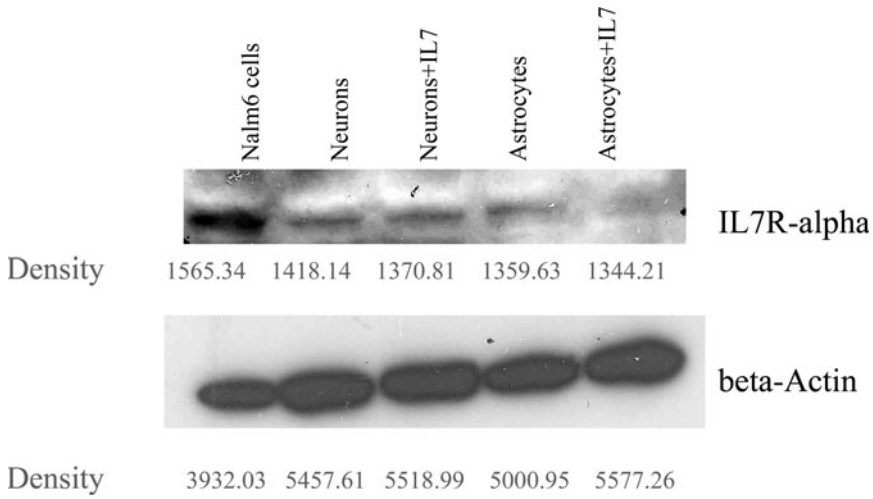

Figure 3 IL-7Rα immunoblot analysis of human mature neurons and astrocytes, after treatment with exogenous IL-7. Whole-cell lysates were analyzed for the expression of IL-7Rα protein, after treatment with exogenous IL-7 (10 ng/ml) for 48 h. A total of 25 μg of protein from each sample was loaded. Nalm-6 cells were the positive control. Autoradiographs of the blots were scanned to determine the density of the protein positions by Molecular Dynamics Densitometer (Molecular Dynamics) and IMAGE QUANT 5.0 software. These results are representative of independent experiments performed at least three times.

many key proteins, such as PARP poly (ADP-ribose) polymer. PCTAIRE protein kinase 3, a member of the PCTAIRE subfamily of cdc2-related serine/threonine protein kinases (Herskovits *et al*, 2004), was up-modulated sevenfold. FAST kinase, designated Fas-activated serine/threonine kinase, was up-regulated fivefold. These two genes encode proteins that are members of the serine/threonine protein kinase family. The genes moderately up-regulated were growth arrest and DNA damage inducible, fourfold; TNF receptor–associated factor 3, threefold; glutathione *S*-transferase A2, cyclin-dependent kinase inhibitor 2D, and lipopoly saccharide (LPS)-induced TNF-alpha factor, twofold; retinoblastoma-binding protein 4, ubiquitin C, 1.6-fold. Tubulin and Bcl2-antagonist of cell death were up-regulated only very modestly.

Table 2 Apoptosis genomics array of mature human neurons treated with IL-7

Human apoptosis genes	*Fold increases*
v-jun sarcoma virus 17 oncogene homology (avian)	12
v-raf murine sarcoma viral oncogene homolog B1	11
Caspase-10	11
PCTAIRE protein kinase 3	7
FAST kinase	5
DNA-damage-inducible, alpha	4
Growth arrest and DNA-damage-inducible, alpha	4
TNF receptor-associated factor 3	3
Glutathione *S*-transferase A2	2.6
Cyclin-dependent kinase inhibitor 2D	2
LPS-induced TNF-alpha factor	2
Retinoblastoma-binding protein 4	1.6
Ubiquitin C	1.6
Tubulin, alpha, ubiquitous	0.4
BCL2-antagonist of cell death	0.35

These results suggest that IL-7 may induce neuronal apoptosis via activating TNF and Fas death receptors, involving phosphoregulation of protein kinases.

IL-7 up-regulates the expression of STAT5 in astrocytes but not neurons

Because the IL-7:IL-7R interaction is known to act through the JAK-STAT pathway (Porter *et al*, 2001), adherent human mature neurons and astrocytes were left untreated or treated with IL-7, and the expression of JAK3 and STAT5 were analyzed by Western blotting.

As shown in Figure 6, IL-7 induced the down-regulation of JAK3 and the up-regulation of STAT5 in astrocytes, while having minimal effect on neurons. The up-regulation of STAT5 can be induced by either JAK 1, through the IL-7Rα, or through JAK3 via the IL-7 receptor gamma, the common receptor for IL-7, IL-2, IL-4, IL-9, and IL-15 (Schindler and Darnell, 1995). Thus, we speculate that, because JAK3 was unchanged in astrocytes, the up-regulation of STAT5 could be induced by the IL-7Rα. As for the neurons, the JAK/STAT pathway did not show any specific up- or down-regulation, further confirming that other signal transduction pathways or mechanisms might be involved in the signaling pathways for IL-7 in neurons.

Discussion

In this study, we provide the initial demonstration that IL-7R is expressed in mature human neurons (i.e., fully differentiated NT2 cells) and astrocytes at the mRNA and protein levels. IL-7Rα expression in human neurons and astrocytes was only slightly

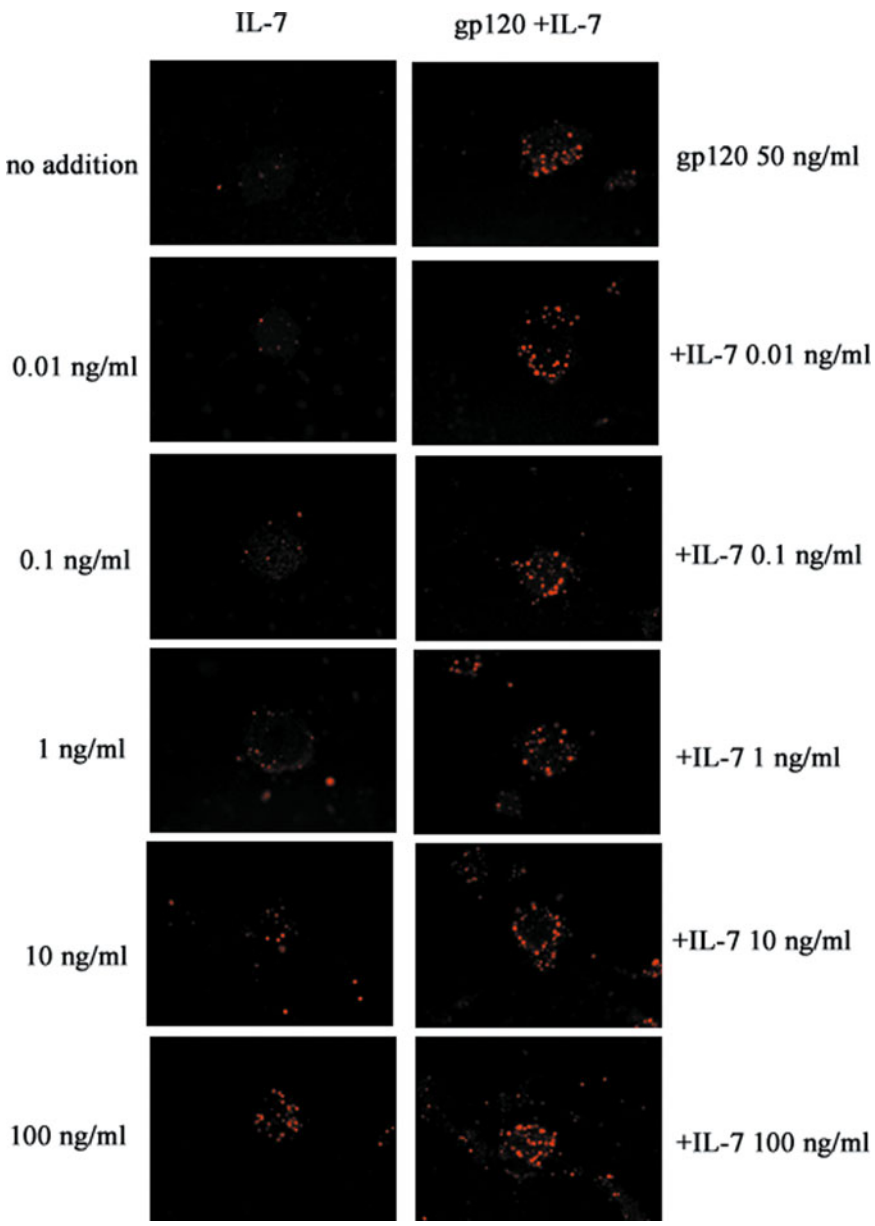

Figure 4 IL-7–induced human neuronal apoptosis and augmented gp120-induced neuronal apoptosis. (*Left*) Representative TUNEL staining of human neurons treated with 0.01, 0.1, 1, 10, and 100 ng/ml of IL-7 for 3 days. (*Right*) Representative TUNEL staining of human neurons treated with 50 ng/ml of gp120 for 48 h, then treated with 0.01, 0.1, 1, 10, 100 ng/ml of IL-7 for 3 days. The neurons were analyzed with fluorescence microscopy (Olympus, model BX60, with fluorescence attachment BX-FLA). Magnification: 20×. These studies were repeated at least three times.

down-modulated by the addition of exogenous IL-7. Interestingly, IL-7 induced human neuronal apoptosis and augmented HIV-1 gp120-induced human neuronal apoptosis. Of note, we found that the IL-7 neuronal proapoptotic effects could be induced via TNF-α/TNF-R1 receptor and FasL/Fas receptor programmed cell death pathways.

The effect of IL-7 in the CNS has not at all been fully elucidated. In Sprague-Dawley rats (Michaelson *et al*, 1996), IL-7 demonstrated neuropoietic properties and IL-7R was expressed in the developing brain. On the other hand, although the presence of IL-7R in nonhematopoietic cells has been reported, a functional IL-7R was not found in different CNS tumors, mostly gliobastoma cell lines, despite detectable levels of IL-7 mRNA, but was never evaluated in either primary astrocytes or neurons cell-lines. (Michaelson *et al*, 1996, Cosenza *et al*, 2002).

In this study, we demonstrated that IL-7R is expressed on both human neurons and astrocytes, although a quantitative discordance between the expression of IL-7Rα mRNA and protein in astrocytes was found, in accordance with previous studies suggesting that IL-7R transcripts might not be stable

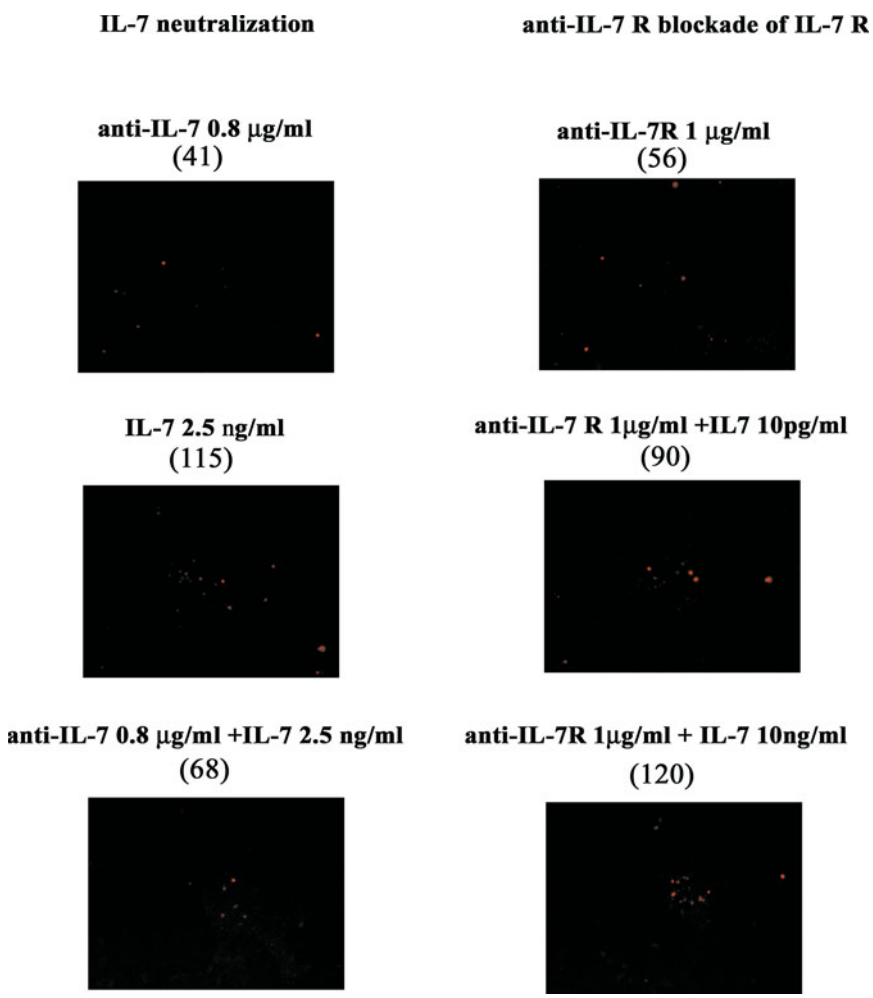

Figure 5 Anti-IL-7 antibodies neutralized the IL-7–induced apoptotic effect. Neurons were incubated overnight with anti-IL-7 antibodies at a concentration of 0.8 μg/ml, as per the manufacturer's instructions. IL-7 at 2.5 ng/ml was added once and TUNEL staining was performed, as illustrated. Anti-IL-7 receptor antibodies were added and left overnight at a concentration of 1 μg/ml, as per the manufacturer's instructions, to block IL-7Rα. Levels of apoptosis are shown in parenthesis.

in certain human cells (Smiers *et al*, 1995; Cosenza *et al*, 2002).

Plasma levels of IL-7 in HIV-1–infected individuals were found previously to be significantly higher than those in the healthy donors, correlating directly with plasma HIV-1 RNA levels and indirectly with CD4+ T-cell counts (Llano *et al*, 2001). Because plasma IL-7 levels increase along with HIV-1 disease progression, and HAD usually occurs in the late stages of HIV-1 disease, we speculated that IL-7-may play a role in the pathogenesis of HAD. We demonstrated that IL-7–induced neuronal apoptosis in a dose-dependent manner, and that it was inhibited by the addition of anti-IL-7 antibodies. Importantly, the well-described proapoptotic effect of the HIV-1 envelope protein, gp120 (Xu *et al*, 2004) was enhanced by the addition of IL-7, showing a neuronal apoptotic synergistic effect.

Previous studies demonstrated that IL-7 induced IL-7Rα down-regulation and Fas up-regulation in peripheral human T cells isolated from healthy donors (Rethi *et al*, 2004; Lelievre *et al*, 2004), also reported that IL-7 down-modulated IL-7Rα expression and increased HIV-1 mediated Fas-induced T-cell death. We showed in the present study that IL-7 down-modulated IL-7Rα expression in human neurons and astrocytes, and induced the up-regulation of STAT5 in astrocytes, but not in neurons. IL-7 leads to the activation of STAT5 either through JAK1 or JAK3 (Porter *et al*, 2001). We found that IL-7 did not induce significant changes in the expression of JAK3, neither in neurons nor in astrocytes. Because JAK3 is associated with the common IL-7 receptor gamma (shared by IL-7, IL-2, IL-4, IL-9, and IL-15) and JAK1 with IL-7Rα we suggest that IL-7–induced neuronal apoptosis might be related to other specific pathways, such as phosphoinositol (PI) 3-kinase or the activation of the *src* family tyrosine Kinase $p56^{lck}$ or $p59^{fyn}$, which associate with the A region of IL-7Rα (Porter *et al*, 2001). In addition, by a targeted genomics microarray analysis, we showed that IL-7–induced apoptosis was related to the up-regulation of several genes involved in

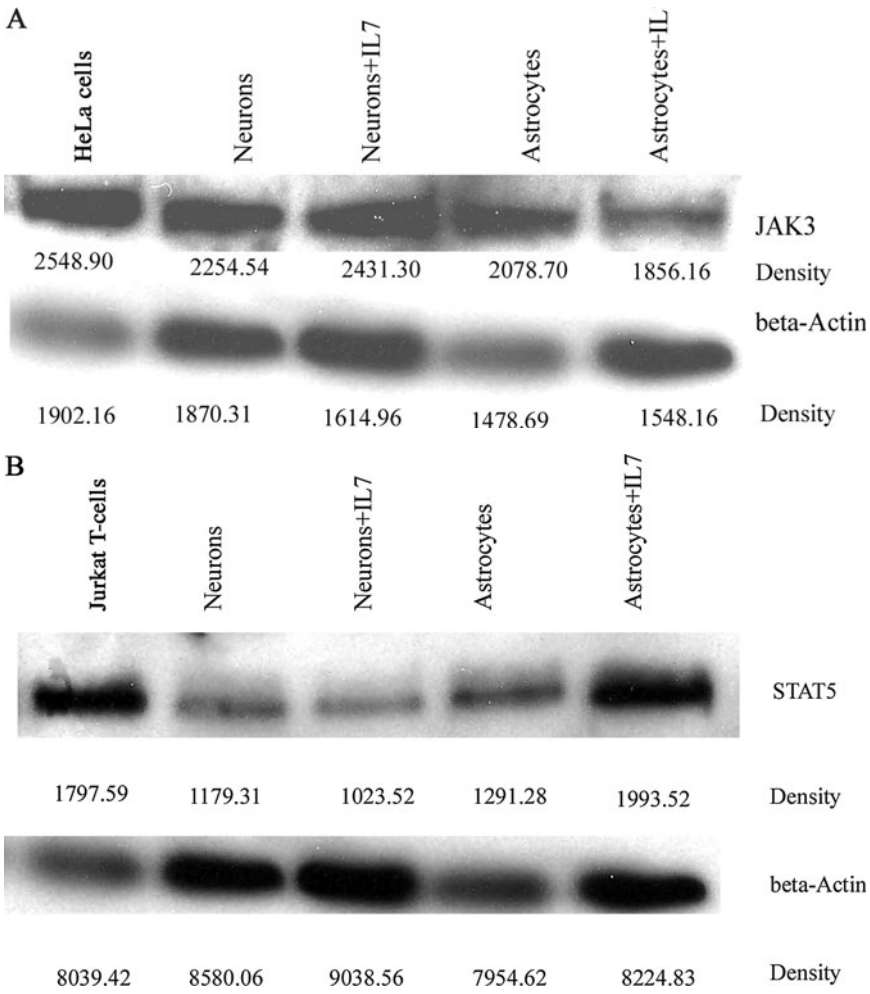

Figure 6 Immunoblot analysis of human neurons and astrocytes for expression of JAK3 and STAT5. HeLa cells (**A**) and Jurkat cells (**B**) were utilized as controls for JAK3 and STAT5 analyses, respectively. A total of 25 μg of protein from each sample was loaded in each lane. Autoradiographs of the blots were scanned to determine the density of the protein positions by Molecular Dynamics Densitometer (Molecular Dynamics) and IMAGE QUANT 5.0 software. These results are representative of independent experiments performed at least three times.

different cell death pathways, such as caspase-10, PCTAIRE protein kinase 3, TNF receptor–associated factor 3, FAST kinase, V-raf murine sarcoma viral oncogene, and v-jun sarcoma virus 17 oncogene. At this time, it is not clear whether phosphoregulation of signal transduction pathways are possibly involved in IL-7–induced neuronal apoptosis. Further studies investigating the major signaling cascades induced by IL-7 in the human CNS are necessary.

The present study revealed not only the presence of IL-7R in human neurons and astrocytes, but also a potential neuronal proapoptotic effect. Because IL-7 has been proposed as an adjunctive therapy for HIV-1 infection, not only as an immune-based adjuvant but also for its properties of inducing HIV-1 from latency in HIV-1–infected individuals on HAART (Wang *et al*, 2005), future *in vitro* and *in vivo* investigations will be critical to determine whether IL-7 plays a role in HAD, which usually occurs in late stages of HIV-1 infection. These clinical stages are characterized by high plasma viral loads and low CD4+ T-cell counts. Reduced CD4+ T cells are believed to induce stromal cells to produce high levels of IL-7, to maintain T-cell homeostasis by stimulating the production of T-cells in the bone marrow (Napolitano *et al*, 2001). If the increase of IL-7 circulating levels is associated with the appearance of HAD, it will be crucial to attempt to modulate its effects by either attempting to maintain CD4+ T-cell counts above a threshold under which IL-7 production will be significantly increased, or by blocking certain of its downstream effects.

Determining the optimal IL-7 immune-therapeutic dosage, as previously established for other cytokines and growth factors, such as IL-2, interferon gamma, or granulocyte-macrophage colony stimulating factor, will be fundamental in attempting to optimize its pleiotropic effects for potential therapy in HIV-1–infected patients. In addition, its possible interactions with HIV-1 virions and lentiviral proteins

in inducing neuronal programmed cell death may also represent a rational target towards interdicting in HAD.

Materials and methods

Cell cultures and IL-7 treatment

hNT-2 neuronal precursor cells were purchased from Stratagene Cloning System (Stratagene, La Jolla, CA). A total of 2.6×10^6 cells were seeded in T75 flasks in the complete Dulbecco's modified Eagle's medium DMEM/F12 medium (containing 0.05 U penicillin–0.05 U streptomycin and 10% fetal bovine serum) and treated with 10 μm retinoic acid (Sigma, St Louis, MO) three times per week for 6 weeks. The differentiated cells were detached by 0.05% trypsin-EDTA and replated in T175 flask in complete DMEM/F12 medium containing 5% fetal bovine serum, and further treated with mitotic inhibitors (1 μm cytosine arabinoside, 10 μm fluorodeoxyuridine, and 10 μm uridine; Sigma) three times per week for 10 days (Xu *et al*, 2004). Mature human neurons were replated on poly-D-lysine and laminin coated (both were 10 μg/ml, Sigma) four-well glass chamber slides in the conditioned medium from the first replating for TUNEL assays, and T25 flasks for human apoptosis gene arrays.

Primary human astrocytes (passage 2), obtained from Dr. Avindra Nath of Johns Hopkins University, were cultured in DMEM medium containing 0.05 U penicillin–0.05 U streptomycin and 10% fetal bovine. Nalm-6 B-cells were cultured in RPMI-1640 medium containing 0.05 U penicillin–0.05 U streptomycin, 10% fetal bovine serum, and 2-mercaptoethanol (1000,000×). All cultures were maintained at 37°C in an incubator with 5% CO_2. Recombinant human IL-7 (R&D Systems) at 0.01, 0.1, 1, 10, and 100 ng/ml was added once at the initiation of the experiments to human neurons and human astrocytes.

To attempt to inhibit IL-7–induced apoptotic effects, anti-IL-7 antibodies (R&D Systems) were used in parallel experiments. Anti-IL-7R antibodies were utilized in competition assays to further determine the role played by the IL-7R (R&D Systems). Human neurons were treated overnight with anti-IL-7R prior to IL-7 exposure.

IL-7 receptor alpha mRNA determinations

Human neurons and astrocytes untreated and treated with IL-7 were lysed in 1 ml of Trizol reagent (Gibco BRL, Baltimore, MD) and 0.2 ml of chloroform was added afterwards. The samples were centrifuged at 13,000 rpm for 30 min at 4°C. Total RNA was precipitated from the aqueous phase by adding 0.5 ml of isopropanol. After incubation at room temperature for 10 min, the precipitates were pelleted by centrifugation at 13,000 rpm for 10 min at 4°C. The RNA pellets were washed with 75% ethanol, air dried, and then dissolved in Diethyl polycarbonate (DEPC)-treated water.

The SuperScript First-Strand Synthesis kit was purchased from Gibco BRL (Baltimore, MD). Total RNA (1 to 5 μg) was hybridized with 1 μl Oligo (dT) in a total volume of 10 μl at 65°C for 5 min, then on ice for 1 min. The RNA/primer mixture was first incubated with reverse transcriptase (RT) reaction mixture (2 μl 10× RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M Dithiothreithol (MDTT), and 1 μl RNase Out recombinant RAase inhibitor) at 42°C for 2 min, and then incubated with 1 μl of SuperScript II RT for 50 min. The reaction was terminated by incubation at 70°C for 15 min. RNase H, 1 μl, was added to the reaction to remove any remaining RNA. Oligonucleotides used for the polymerase chain, reactions, (PCR) amplification were (IL7R1) 5′ TGGAGACTTGGAAGATGCA 3′; (IL7R2) 5′ TTAGTAAGATAGGATCCAT 3′ for IL7Rα and (G1) 5′ CAAAGTTGTCATGGATGAC 3′; (G2) 5′ CAAAGTTGTCATGGACC 3′ for GAPDH. PCR was performed using a 9700 PCR thermal cycler (Perkin-Elmer), under the following conditions: denature at 95°C for 45 s, primer annealing 57°C for 45 s, and DNA extension at 72°C for 1 min for 40 cycles.

The gel photographs were scanned and further analyzed for density comparison by using Molecular Dynamics Densitometer and IMAGE QUANT 5.0 software. The density ratio of IL-7Rα RNA bands versus the GAPDH bands served as a standard, according to known fold dilution of cDNA from Nalm-6 cells. The linearity between the density ratio and fold dilution was confirmed.

Immunoprecipitation

Adherent human mature neurons and astrocytes were cultured in T75 flasks until confluent, prior to the experiments. A total of 1×10^7 nonadherent Nalm-6 B cells, neurons, and astrocytes, were lysed in 1 ml cold lysis buffer (Novagen, Madison, WI) containing 1× protease inhibitor cocktail (Pierce, Rockford, IL) on ice for 30 min. The cell lysates were spun at 10,000 *g* for 15 min at 4°C. The supernatants were collected, 10 μg of polyclonal anti-IL-7Rα (R&D Systems, Minneapolis, MN) were added to each sample, and incubated at 4°C overnight. Fifty microliters of a protein G slurry (Calbiochem, Darmstadt, Germany) was added to each sample and incubated at 4°C for 1 h on a rocking platform. Samples were spun at 10,000 *g* for 30 s at 4°C. After supernatants were completely removed, the beads were washed three times with 500 μl of lyses buffer. After the last washing, 50 μl of 1× Laemmli sample buffer was added to each sample bead pellet. Samples were heated at 95°C for 10 min and centrifuged again. The supernatant of each sample was collected and loaded on to gels.

Determination of IL-7Rα by Western blotting

Human neurons and astrocytes untreated and treated with IL-7 were lysed with Cytobuster Protein Extraction Reagent (Novagen, Madison, WI). Protein concentrations were determined with the BCA Protein assay kit (BioRad, Herculas, CA). Approximately

25 μg of each protein preparation was resolved on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (BioRad, Herculas, CA) and transferred to PVDF membranes (Amersham Biosciences, Piscathaway, NJ) by electroblotting. The membranes were incubated in blocking buffer (10 mM phosphate-buffered saline (PBS), pH 7.2, 150 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk) at 4°C for overnight. The membranes were then incubated with primary antibody (polyclonal goat anti-h IL-7Rα antibody, 1:1000, R&D Systems, Minneapolis, MN), rabbit polyclonal anti-JAK3 and anti-STAT5 antibodies at concentrations of 1:500 (Santa Cruz Biotechnology, CA), for 1 h at room temperature in PBST (10 mM Phosphate-Buffered Difluoride PBS, pH 7.2, 150 mM NaCl, 0.1% Tween 20). The secondary antibody, horseradish peroxidase labeled anti-goat immunoglobulin G (IgG) antibody (1:500, for anti-IL-7R and 1:5000 for JAK3 and STAT5; Santa Cruz Biotechnology) was applied at room temperature for 1 h in PBST. The membranes were rinsed in PBST, and proteins were visualized using an enhanced chemoluminscent (ECL) Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ), and exposure to Kodak BioMax MS autoradiographic film (Kodak, Rochester, NY).

Immunoblot analyses of immune precipitation complexes prepared from human neurons and astrocytes were performed for the expression of IL-7Rα protein, JAK3, and STAT5. The Nalm-6 B cells were used as positive controls for the expression of IL-7Rα protein, plus HeLa cells and Jurkat cells were utilized for JAK3 and STAT5 analyses, respectively. We utilized Restore Western blot stripping buffer (Pierce, Rockford, IL) to strip the blots, using normal goat IgG (R&D Systems) as an isotype control.

HIV-1 viral envelope protein gp120 and IL-7 treatment

Recombinant viral Env protein of the HIV-1 strain gp120 from BaL (R5-tropic) (endotoxin levels less than 10 EU/ml) was purchased from Intracel Corporation (Rockville, MD). The protein was added at aconcentration of 50 ng/ml to the neuronal cell culture medium, as a relatively low concentration which we previously demonstrated would induce apoptosis in human neurons (Xu *et al*, 2004). After 48 h, the medium was removed and cells were washed twice with complete DMEM/F12 medium. The gp120-treated neurons were then treated once with IL-7 at concentrations of 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml, and then cultured for 3 days before DNA fragmentation and terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end-labeling (TUNEL) assays.

TUNEL assays and semiquantitative apoptosis determinations

The *in situ* cell death detection kit, TMR red, was purchased from Roche Diagnostics (Indianapolis, IN). Human neurons and astrocytes seeded in four chamber slides were washed twice with PBS, pH 7.4, fixed with acetone for 10 min at room temperature, and assayed for apoptosis according to the manufacture's instructions. The cells were analyzed by fluorescence microscopy (Olympus System Microscope Model BX60 with fluorescence attachment BX-FLA).

For semiquantitative measurements of apoptosis, images generated with a CCD array camera (RT Color, Diagnostic Instruments, Sterling Heights, MI) were subjected to fluorescence brightness value determinations on a monochromatic scale in light of red, blue, green (RGB) values (0–255 ASCII numbers). Apoptosis was defined based on a red monochromatic scale in the range of 0 to 255. Blank values were subtracted from the average of seven random values from different cells (Xu *et al*, 2004).

Human apoptosis gene microarray

The CLONTECH's Atlas TM cDNA expression human apoptosis array was purchased from BD Biosciences Clontech (Palo Alto, CA), and used according to the instructions. The total RNA was isolated from untreated or treated neurons using the RNAgents kit from Promega (Madison, WI). cDNA probe mixtures were synthesized by reverse transcribing respective RNA using cDNA synthesis primer mix provided in a kit and α-^{32}P dATP (Perkin Elmer Sciences Inc, Boston, MA). Each radioactively labeled probe mix was then hybridized to separate Atlas arrays overnight at 68°C. After washing, the hybridization patterns were analyzed by autoradiography and quantified with a phosphor imager (Molecular Dynamics). Differences in gene expression levels were quantitated by the BD AtlasImage 2.7 Software.

References

Alper O, Hacker NF, *et al* (1999). Protein kinase A-Ialpha subunit-directed antisense inhibition of ovarian cancer cell growth: crosstalk with tyrosine kinase signaling pathway. *Oncogene* **18:** 4999–5004.

Becker EB, Howell J, *et al* (2004). Characterization of the c-Jun N-terminal kinase-BimEL signaling pathway in neuronal apoptosis. *J Neurosci* **24:** 8762–8770.

Bellacosa A, Testa JR, *et al* (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254:** 274–277.

Brooks DG, Kitchen SG, *et al* (2001). Generation of HIV latency during thymopoiesis. *Nat Med* **7:** 459–464.

Chun TW, Engel D, *et al* (1998). Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med* **188:** 83–91.

Cosenza L, Gorgun G, *et al* (2002). Interleukin-7 receptor expression and activation in nonhaematopoietic neoplastic cell lines. *Cell Signal* **14:** 317–325.

Cross TG, Scheel-Toellner D, *et al* (2000). Serine/threonine protein kinases and apoptosis. Exp *Cell Res* **256:** 34–41.

Deacon EM, Pongracz J, *et al* (1997). PKC isoenzymes: differential involvement in apoptosis and pathogenesis. *J Clin Pathol Mol Pathol* **50:** 124–131.

Dudley AC, Thomas D, *et al* (2004). The STATs in cell stress-type responses. *Cell Communi Signal* **2:** 1478–1481.

Dus D, Krawczenko A, *et al* (2003). IL-7 receptor is present on human microvascular endothelial cells. *Immunol Lett* **86:** 163–168.

Ellison M, Povlishock JN, *et al* (1987). Blood-brain barrier dysfunction in cats following recombinant interleukin-2 infusion. *Cancer Res* **47:** 5765–5770.

Fry AS, Connick E, *et al* (2001). A potential role for interleukin-7 in T-cell homeostasis. *Blood* **97:** 2983–2990.

Goodwin RG, Friend DJ, *et al* (1990). Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell* **60:** 941–951.

Grabstein KH, Namen AE, *et al* (1990). Regulation of T cell proliferation by IL-7. *J Immunol* **144:** 3015–3020.

Herskovits AZ, Davies P, *et al* (2004). Cloning and expression analysis of two novel PCTAIRE 3 transcripts from human brain. *Gene* **328:** 59–67.

Heufler C, Tppar G, *et al* (1993). Interleukin 7 is produced by murine and human keratinocytes. *J Exp Med* **178:** 1109–1114.

Ichijo H (1999). From receptor to stress-activated MAP kinases. *Oncogene* **18:** 6087–6093.

Jaleco S, Swainson L, *et al* (2003). Homeostasis of naïve and memory CD4+ T-cell: IL-2 and IL-7 differentially regulate the balance between proliferation and Fas-mediated apoptosis. *J Immunol* **171:** 61–68.

Kaul M, Garden GA, *et al* (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410:** 988–994.

Lai SY, Molden J, *et al* (1997). Shared ϒc subunit within the human interleukin-7 receptor complex. *J Clin Invest* **99:** 169–177.

Lelievre JD, Petit F (2005). Interleukin 7 increases human immunodeficiency HIV-1 LAI-mediated Fas-induced T cell death. *J Virol* In press.

Le-Niculescu H, Bonfoco E, *et al* (1999). Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol. Cell Biol* **19:** 751–763.

Leonard WJ (2001). Cytokines and immunodeficiency disease. *Nat Rev Immunol* **1:** 200–208.

Lin JX, Migone TS, *et al* (1995). The role of shared receptor motifs and common stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* **2:** 331–339.

Llano A, Barretina J, *et al* (2001). Interleukin-7 in plasma correlates with CD4 T-cell depletion and may be associated with emergence of syncytium-inducing variants human immunodeficiency virus type 1-positive individuals. *J Virol* **75:** 10319–10325.

Marte BM, Downward J (1997). PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem Sci* **22:** 355–358.

Macaluso M, Russo G, *et al* (2002). Ras family genes: an interesting link between cell cycle and cancer. *J Cell Physiol* **192:** 125–130.

Michaelson MD, Mehler MF, *et al* (1996). Interleukin-7 is trophic for embryonic neurons and is expressed in developing brain. *Dev Biol* **179:** 251–263.

Miyazaki T, Kawahara A, *et al* (1994). Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* **266:** 1045–1047.

Napolitano LA, Grant RM, *et al* (2001). Increased production of IL-7 accompanies HIV-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* **7:** 73–79.

Pimentel-Muinos FX, Seed B (1999). Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity* **11:** 783–793.

Pollock PM, Harper UL, *et al* (2003). High frequency of BRAF mutations in nevi. *Nat Genet* **33:** 19–20.

Porter BO, Scibelli P, *et al* (2001). Control of T cell development *in vivo* by subdomains within the IL-7 receptor alpha-chain cytoplasmic tail. *J Immunol* **166:** 262–269.

Raber J, Merlo PE, *et al* (1994). IL-1β potentiates the acetycholine-induced release of vasopressin from the hypothalamus in vitro, but not from the amygdala. *Neuroendocrinology* **59:** 208–217.

Rethi B, Fluur C, *et al* (2004). Decreased IL-7Rα expression of T cell is associated with CD4+ T cell depletion and high levels of serum IL-7 during HIV infection. *Immuno 2004* online abstracts 840.

Sasson S, Zanetti G, *et al* IL-7 Down-regulates Surface Expression of IL-7Ralpha in vitro and in vivo. The 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, USA. Abstract N 249.

Schmitz I, Krueger A, *et al* (2003). An IL-2-dependent switch between CD95 signaling pathways sensitize primary human T cells toward CD95-mediated activation-induced cell death. *J Immunol* **171:** 2930–2936.

Scripture-Adams DD, Brooks DG, *et al* (2002). Interleukin-7 induces expression of latent human immunodeficiency virus type 1 with minimal effects on T-cell phenotype. *J Virol* **76:** 13077–13082.

Smiers FJ, van Paassen M, *et al* (1995). Heterogeneity of proliferative responses of human B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells to interleukin 7 (IL-7): no correlation with immunoglobulin gene status and expression of IL-7 receptor or IL-2/IL-4/IL-7 receptor common gamma chain genes. *Leukemia* **9:** 1039–1045.

Sudo T, Ito M, *et al* (1989). Interleukin 7 production and function in stromal cell-dependent B cell development. *J Exp Med* **170:** 333–338.

Wang J, Chun HJ, *et al* (2001). Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A* **98:** 13884–13888.

Wang FX, Xu Y, *et al* (2005). IL-7 is a potent and proviral strain-specific inducer of latent, in vivo HIV-1 cellular reservoirs of infected individuals on virally-suppressive HAART. *J Clin Inves* **115:** 128–137.

Wilks AF, Harpur AG, *et al* (1994). Cytokine signal transduction and the JAK family of protein tyrosine kinase. *Bioassays* **16:** 313–320.

Wolf SS, Cohen A, *et al* (1992). Expression of cytokines and their receptors by human thymocytes and thymic stromal cells. *Immunology* **77:** 362–368.

Xia Z, Dickens M, *et al* (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270:** 1326–1331.

Xu Y, Kulkosky J, *et al* (2004). HIV-1 mediated apoptosis of neuronal cells: proximal molecular mechanisms of HIV-1-induced encephalopathy. *Proc Natl Acad Sci U S A* **101:** 7070–7075.