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HIV-1 Tat Protein Down Regulates Interleukin-7 Receptor Alpha on CD8T Cells
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HIV-1 TAT PROTEIN DOWN REGULATES INTERLEUKIN-7 RECEPTOR ALPHA ON CD8 T CELLS

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies, University of Ottawa in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
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ABSTRACT

Expression of the IL-7 receptor α-chain (CD127) is decreased on CD8 T-cells in HIV infected patients and recovers in those receiving antiretroviral therapy with sustained viral suppression. Here I demonstrate that the HIV Tat protein, acting in a paracrine fashion, specifically down regulates cell surface expression of CD127 on human CD8 T-cells. The effect of Tat on CD127 is dose- and time-dependent, specific, and can be blocked with anti-Tat monoclonal antibodies or by pre-incubating Tat with heparin. Pre-incubation of purified CD8 T-cells with Tat protein inhibited CD8 T-cell proliferation and perforin synthesis following stimulation with IL-7.

I also show that low CD127 expression on CD8 T-cells from HIV+ patients recovers to normal levels when CD8 T-cells are isolated and cultured in vitro. Recovery of CD127 was completely inhibited by the addition of HIV Tat protein to the culture media. This suggests that soluble factor(s) are responsible for low CD127 expression on circulating CD8 T-cells in HIV+ individuals and further supports the role of Tat in this process.

I have elucidated the mechanism by which Tat removes CD127 from the cell surface. Soluble Tat protein is initially taken up by CD8 T-cells via endocytosis and exits the endosome through a process dependent on endosomal acidification. Once in the cytoplasm, Tat localizes to the inner leaflet of the cell membrane where it binds to the cytoplasmic tail of CD127 and induces receptor clustering and internalization. Through its association with Tat, CD127 is then directed to the proteasome for degradation.

While Tat and IL-7 both independently down regulate surface expression of CD127, I have demonstrated that at near physiologic concentrations these viral and host factors act synergistically to suppress CD127. Inhibition of JAK kinase 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.

Since IL-7 signalling is essential for optimal CD8 T-cell proliferation and function, the down regulation of CD127 and apparent inhibition of cytotoxic activity by Tat may play an important role in HIV induced immune dysregulation and impaired cell mediated immunity.

ACKNOWLEDGMENTS

I would like to thank Dr. Paul MacPherson for providing an environment that has allowed me to thrive in the field of HIV research. I am aware that a large part of my success as a student is due to the guidance and support of Dr. Paul MacPherson. As a member of his lab I have found ample opportunity to grow and develop through a variety of opportunities provided by Dr. MacPherson. I can say without hesitation that I am fortunate to have had the opportunity to benefit from his considerable experience.

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LIST OF ABBREVIATIONS

aa – amino Acid
AIDS - Acquired Immuodeficiency Syndrome
APC – Antigen Presenting Cell
BFA – Brefeldin A
BSA - Bovine Serum Albumin
CD - Cluster of differentiation
CMV - Cytomegalovirus
Cdk - cyclin dependent kinase
CHX - Cyclohexamide
CR – Complement Receptor
CTL- Cyttoxic Lymphocyte
CQ – Chloroquine
DAPI - 4',6-Diamidino-2-Phenylindole
DNA- Deoxyribonucleic Acid
ECD – PE texas red
ECL - Enhanced Chemiluminescence
EBV- Epstein-Barr Virus
ERK – Extracellular Siganlling Regulated Kinases
FITC – Fluorescein isothiocyanate
FCS – Fetal Calf Serum
HAART - Highly Active Antiretroviral Therapy
HIV - Human Immunodeficiency Virus
HLA – Human Leukocyte Antigen
HPLC – High Performance Liquid Chromatography
HRP - Horseradish Peroxidase
IFN – Interferon
Ig - Immunoglobulin
IL - Interleukin
Jak - Janus Kinase
Kda- Kilodalton
LPS – Lipopolysacharide
LNT – Lymph Node T-cells
LTR- Long Terminal Repeat
MAP – Microtubule Associated Protein
MCF – Mean Channel Flouresence
MHC - Major Histocompatibility Complex
mRNA- Messenger Ribonucleic Acid
NF-κB- nuclear factor-kappa B
PBMC - Peripheral Blood Mononuclear Cells
PBS – Phosphate Buiffered Saline
PE - Phycoerythrin
PI – Propidium Iodide
PCR - Polymerase Chain Reaction
PI3K - Phosphoinositide 3-kinase
PSD – Post Synaptic Dentity Protein

LIST OF ABBREVIATIONS (cont'd)

PVDF – polyvinylidene fluoride
RNA – Ribonucleic Acid
RON – Recepteur D'Origine Nantais
RPS18 - Ribosomal protein of 18 kDa
SEM – Standard Error of the Mean
SCID - Severe Combined Immunodeficiency
SDS – Sodium Doedecyl sulfate
SIV- Simian Immunodeficiency Virus
Src - kinase from the family of proto-oncogenic tyrosine kinases
STAT - Signal Transducer and Activator of Transcription
Tat – Transactivator of Transcription
TAR – Transactivation Response Region
TCR- Thymocyte Cell Receptor
TNF- Tumor Necrosis Factor

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CHAPTER 1 - INTRODUCTION

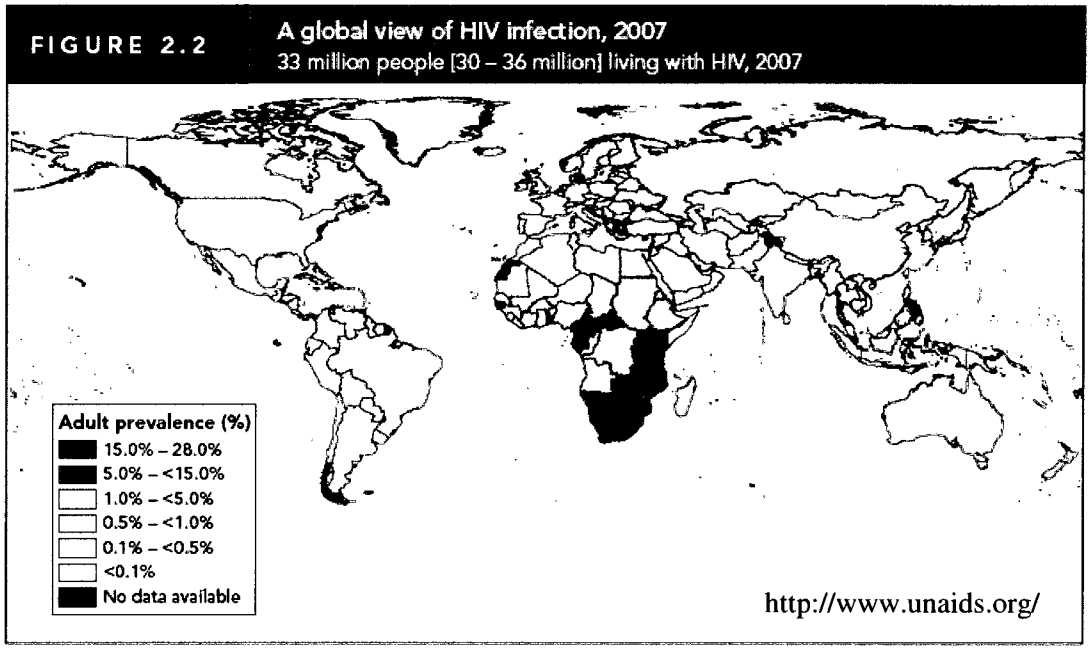
HIV-1

Since 1983, 60 million people had been infected with HIV, over one third of whom subsequently have died (1). It is estimated that approximately 40 million people world-wide are currently infected with HIV (figure 1(2)). The HIV pandemic has continued to expand with an estimated 3 million new infections in 2007 (3). The cost in human suffering and in health care dollars has been enormous. HIV infection results in severe immune deficiency leaving patients susceptible to opportunistic infections and malignancies. Understanding how HIV is able to suppress immune function is paramount to the development of new strategies for treating this devastating infection.

HIV-1 is a single stranded, positive sense RNA retrovirus, with each virus particle containing two different copies of the viral genome (4). Virus particles are 120 nm in diameter and spherical in appearance. The exterior viral envelope is covered with viral spike proteins (gp41/120) that are visible by scanning electron microscopy (figure 2A, B (5) C (6)). These viral spike proteins interact with host receptors (CD4) and co-receptors (CXCR4/CCR5) to promote viral attachment and fusion allowing the virus to gain entry to the cell (figure 3 (2)). Once inside the cytoplasm the virus reverse transcribes it's RNA genome into DNA, followed by the synthesis of a complementary DNA strand that is transported to the nucleus where the viral genome is integrated into the chromosome of the host cell. Once a part of the host chromosome, viral DNA may remain in a silent state in quiescent cells (7). Cellular activation induces rapid and efficient viral production that begins with transcription from the viral LTR followed by translation of viral proteins, virus assembly, packaging and budding from

Figure 1: (A) Global HIV infections in adults. Values represent percentage of infected individuals in a given region. Reproduced with kind permission from UNAIDS. **(B)** Global map of numbers of HIV infected individuals in specified regions. Reproduced with kind permission from The Gladstone Institutes.

A



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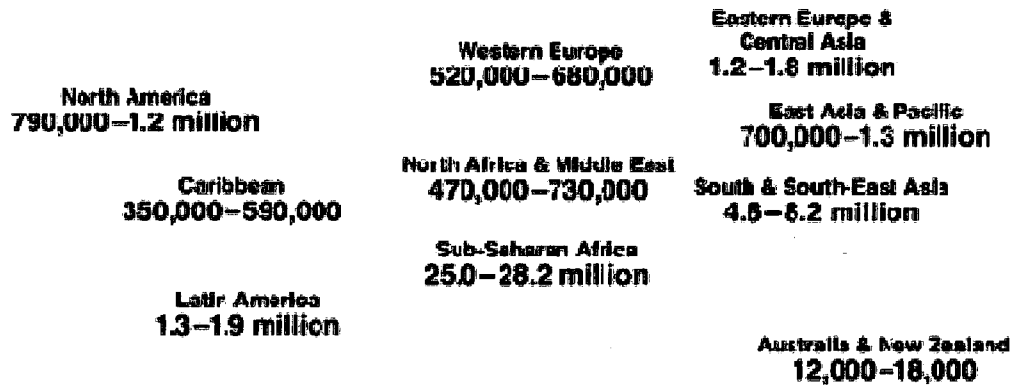
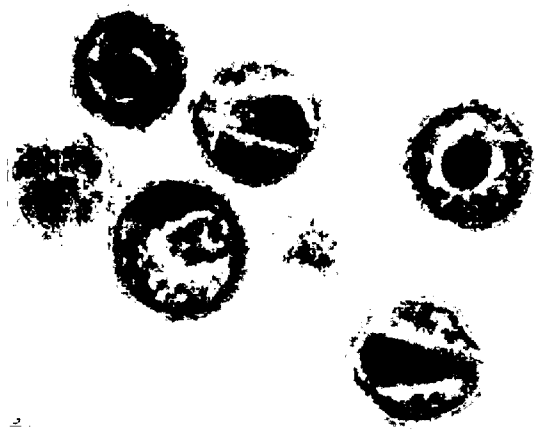


http://www.gladstone.ucsf.edu/gladstone/site/publicaffairs/section.php?id=1707

Figure 2: HIV structure. Transmission electron micrograph of **(A)** HIV-1 stained with ruthenium red to show surface glycoprotein knobs and **(B)** Cone-shaped cores, sectioned in various orientations. Viral genomic RNA is located in the electron-dense wider end of core. Reproduced with kind permission from the Centre for Disease Control, Public Health Library. Images captured by Dr. Edwin P. Ewing, Jr. **(C)** Cartoon depicting HIV structural components transcribed by pol: RT-Reverse Transcriptase (p55), PR- Protease, IN- Integrase (p11), gag: MA- Matrix (p17), CA-Capsid (p24), NC- Nucleocapsid (p6, p9), and env: SU –surface (gp120), TM-Transpmembrane (gp41). Reproduced with kind permission from the Journal of Chemical Education.

A



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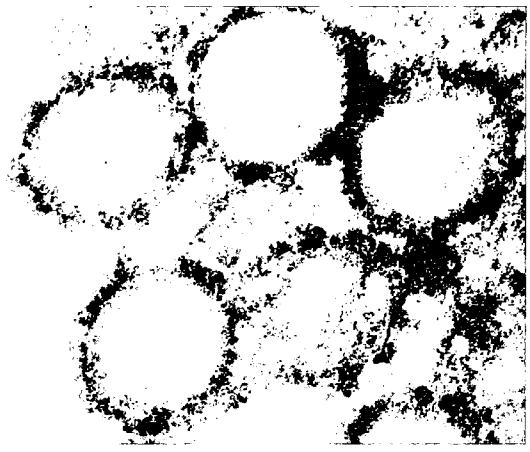

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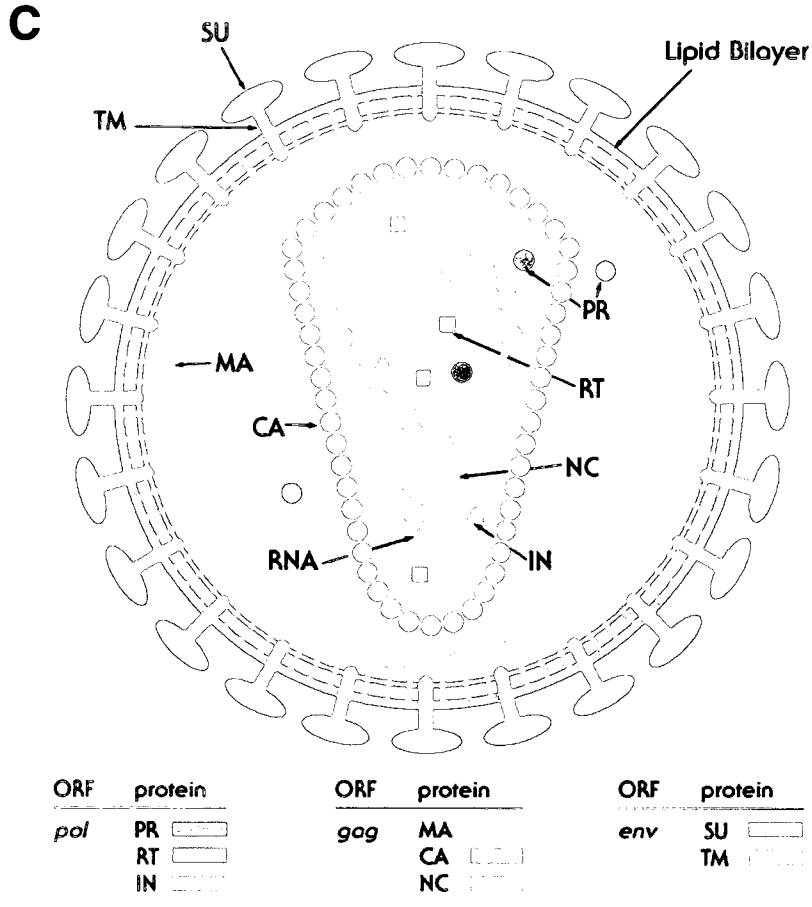
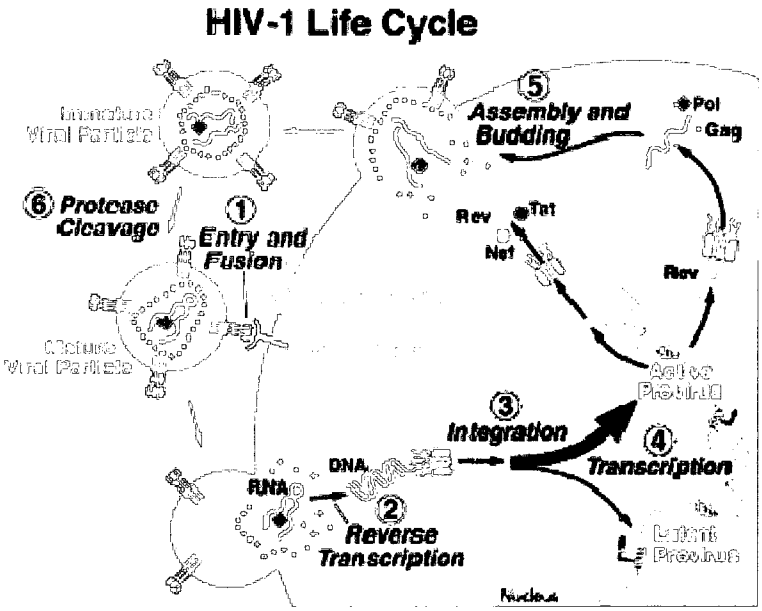


J. Chem. Educ. 1993, 70:3

Figure 3: HIV-1 Life cycle. Cartoon depicting the steps in the HIV-1 Life cycle. Reproduced with kind permission from The Gladstone Institutes.



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the cell membrane. Production of high titers of progeny virus may have cytopathic effects on the host cell. In addition to viral replication, HIV-1 infection can cause cell death though a variety of mechanisms including the activation of caspases, by compromising membrane integrity or by upregulation of FasL (7).

The 9.8kb genome of HIV-1 contains 9 genes that encode various structural (Env, Gag), enzymatic (Pol), regulatory (Tat, Rev). and accessory (Vif, Nef, Vpu, Vpr) proteins (7). In addition to direct effects on viral replication, HIV-1 accessory and regulatory proteins have various functions that contribute to HIV-1 pathogenicity and undermine host cell defenses (figure 4 (2)).

CD8 T-cells and HIV

In humans, CD8 T lymphocytes are the major effectors of cell-mediated immunity. These cells are first activated in lymph nodes when naïve CD8 T-cells come into contact with cells expressing peptide-loaded major histocompatibility complexes (MHC I). Peptides displayed by MHC I molecules are derived from intracellular pathogens (such as viruses) that are subject to degradation in the cytosol by the proteasome. Pathogen derived peptides (typically 8-10 aa) are then transported to the Endoplasmic reticulum, where they are loaded onto MHC I molecules. MHC I-peptide complexes are then transported though the golgi network to the cell surface. Once displayed on the cell surface, these complexes are recognized by the CD8 T cell's T cell receptor (TCR) (figure 5 (8)). Co-stimulation through the CD28 surface molecule, recognizing CD80 and CD86 on the surface of activated antigen presenting cells, is also required for CD8 T cell activation. In addition to receptor engagement CD8 T cell activity is further augmented by a variety of chemical signals, many of which are

Figure 4: HIV-1 genome. Cartoon depicting the HIV-1, 9kb genome and major functions of HIV-1 gene prodicts. Reproduced with kind permission from The Gladstone Institutes.

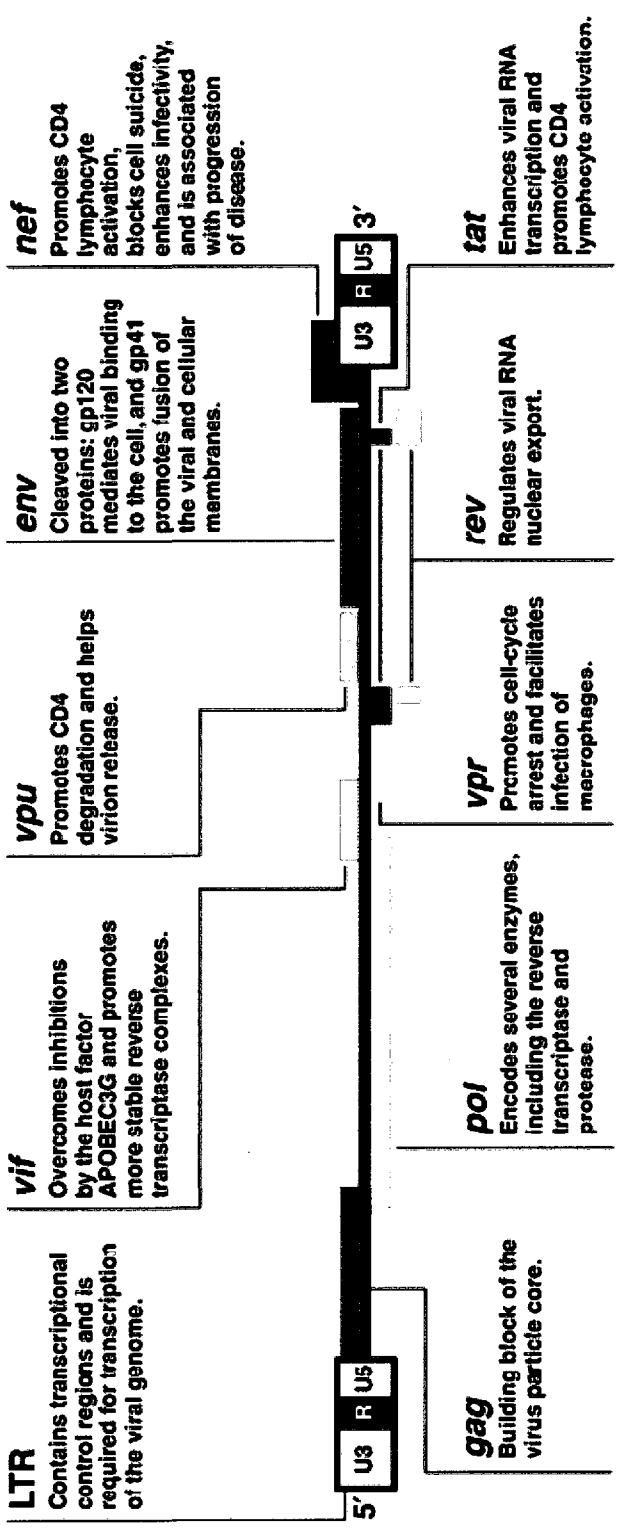

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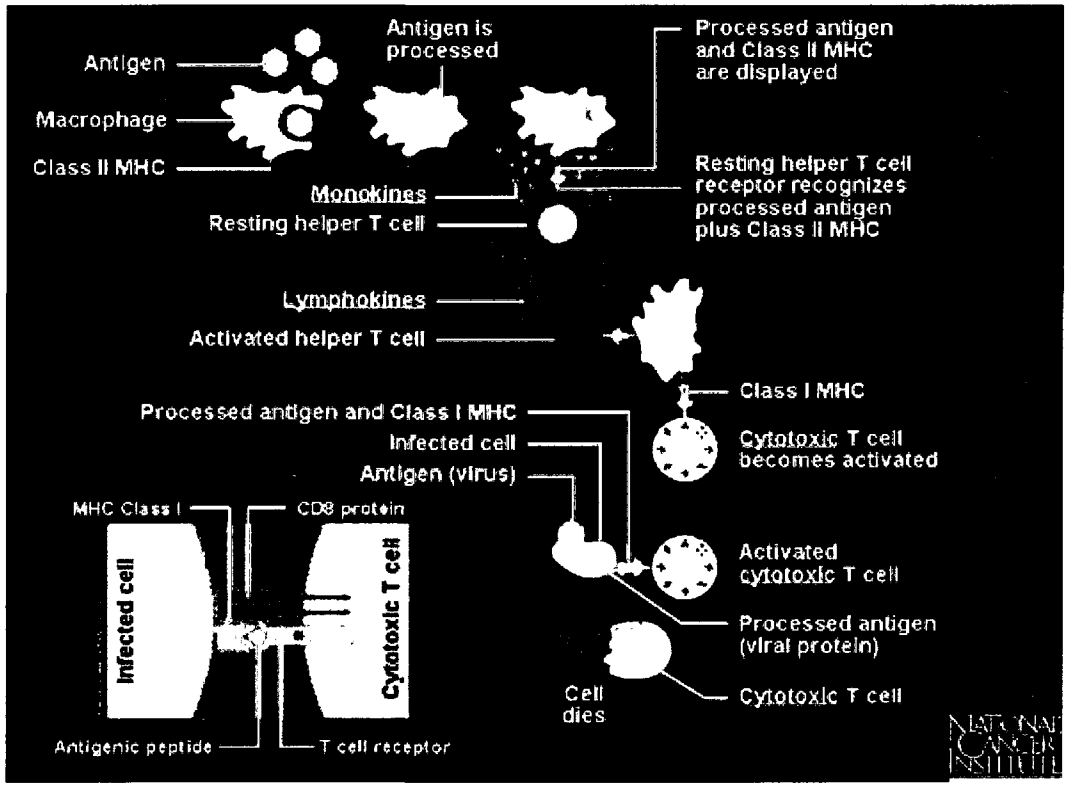
Figure 5: Activation of CD8 T cells. CD8 T cells only recognize antigen presented by Class I MHC markers. Resting cytotoxic CD8 T cells recognize virus fragments, which are displayed by a macrophage in combination with a Class I MHC marker. Receptors on a circulating, resting cytotoxic T cell in combination with CD8 surface proteins recognize the antigen-protein complex and bind to it. This binding process in combination with signals from activated helper T cells activates the cytotoxic T cell. Because the surfaces of other infected cells bear the same virus fragments in combination with Class I MHC markers, the activated cytotoxic T cells can then quickly recognize, attack, and destroy diseased cells. Figure reproduced with kind permission from the National Cancer Institute.

A



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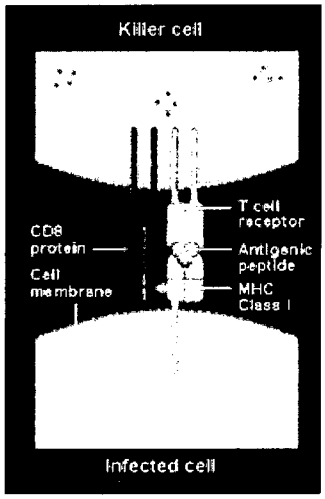

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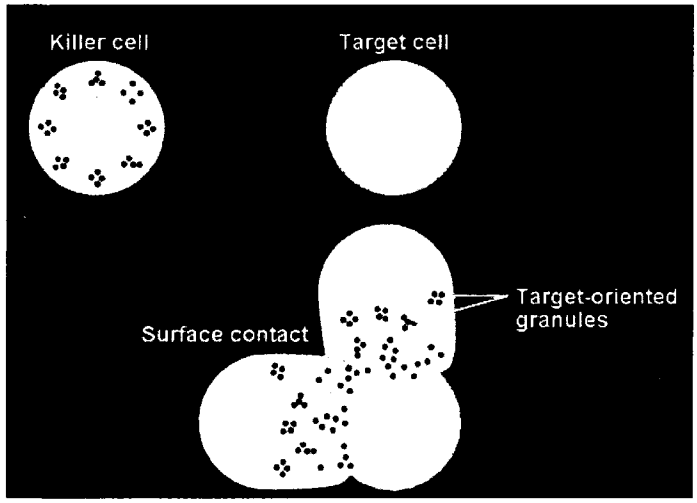
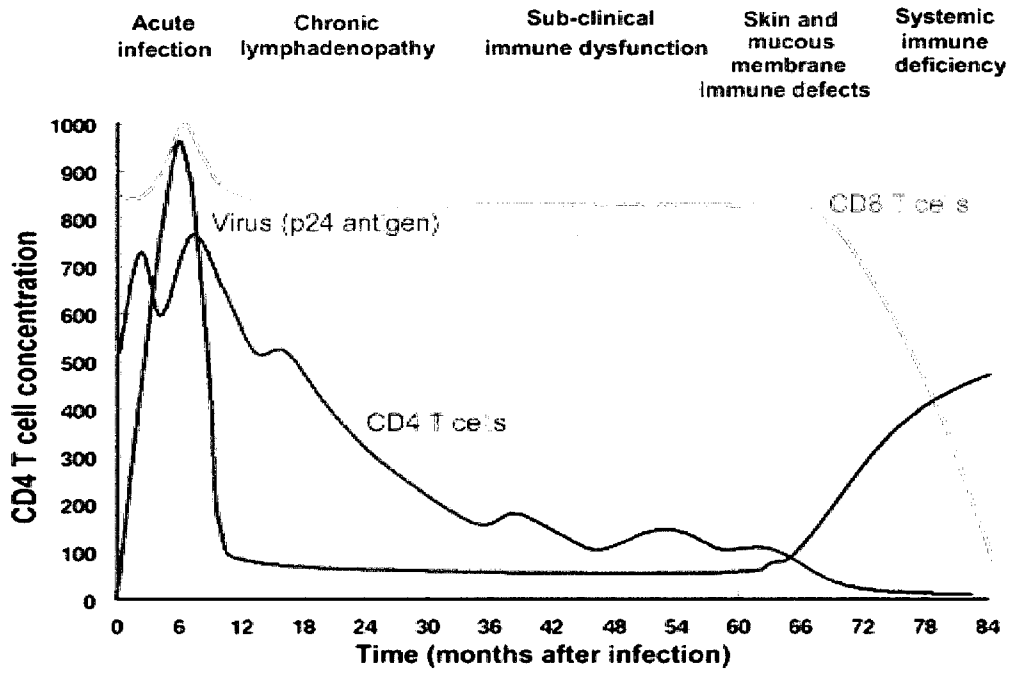


http://www.cancer.gov/cancertopics/understandingcancer/immunesystem

released by APCs and neighboring activated CD4 T cells (9, 10). A wide range of cytokines including IL-2, 4, 6, 7, 12, 15, 21, IFNα and IFNγ have been shown to promote CD8 T cell response to antigen (11). In particular, IL-12, IFNα and IL-21 provide co stimulatory signals that promote survival of activated CD8 T cells, which are distinct from signaling TCR and co-stimulatory receptors. Once activated, CD8 T cells undergo rapid proliferation and differentiation (12). These cytolytic effector CD8 T cells up regulate the expression of the cytolytic effector molecules perforin and granzymes and down regulate the expression of CD28. Clonally expanded effector CD8 T cells then traffic to areas of infection where they are able to target and destroy infected cells through the specific recognition of foreign antigens presented in the context of an MHC class I molecule on the infected cells' surfaces. Destruction of target cells is mediated by the directional release of cytotoxic vesicles, containing perforin and granzymes onto the target cell's surface. Perforin is able to form homomultimeric channels through the plasma membrane of target cells. Granzymes, entering the target cell through perforin-created pores, are able to enzymatically induce DNA damage and stimulate apoptotic cascades. As CD8 T cells are able to destroy cellular targets, they are the body's major adaptive defense against intracellular pathogens, especially viruses (13).

It is well established that HIV infection results in a loss of CD8 T-cell activity. Although CD8 T cells remain at high levels during the course of infection (figure 6 (14)), they become progressively anergic and unable to respond to foreign antigens. In vitro studies have demonstrated that cytotoxic T-cells isolated from HIV+ patients demonstrate lower proliferative capacity (in response to TCR stimulation) and decreased CTL activity (in response to alloantigen and mitogen) in chromium release assays compared to healthy controls (15-18). More recently it has been demonstrated

Figure 6: CD4/CD8 T cell counts in HIV Disease. Time course of CD4, CD8 T cell counts compared to viral titres during disease progression in HIV infected patients. Reproduced with kind permission from the University of South Carolina Faculty of Medicine.



http://pathmicro.med.sc.edu/lecture/HIV3.htm

that both HIV- and EBV-specific CD8 T-cells can be found in the circulation at relatively normal frequencies in HIV-infected patients with advanced disease (19-23), yet these cells respond poorly to their respective antigens, fail to express perforin and interferon (IFN)-γ, or demonstrate cytolytic activity in standard chromium release assays (20, 22-26). This is of obvious advantage to HIV, as by disarming cell mediated immunity the virus is able to avoid elimination and establish chronic infection.

Interleukin-7

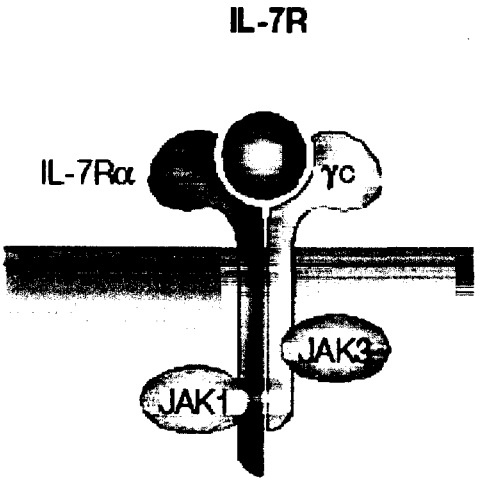
IL-7 was first discovered as a B- cell growth factor in mice and later as a non-redundant cytokine required for normal T-cell development and function (27, 28). IL-7 is a 25 kilodalton protein produced by a seeming ubiquity of cell types including thymic stromal epithelial cells, fetal hepatocytes, dendritic cells, megakarocytes, platelets, smooth muscle cells, fibroblasts and human microvascular endotheial cells of the lymph nodes (27, 29). Perhaps due in part to the fact that little is known about the regulation of IL-7 levels, it is generally accepted that IL-7 levels remain constant and IL-7 signaling events are controlled by altering IL-7 receptor expression on the cell surface (27, 30).

Expression of CD127 is regulated tightly during lymphocyte development, activation and differentiation. Naïve and memory CD8 T cells have high levels of CD127 compared to effector cells that have low CD127 expression. This is believed to benefit effectors by making them short lived and prone to apotosis, while high expression of CD127 on naive and memory cells make them long lived. CD127 expression is also down regulated on T cells in response to gamma chain cytokines (IL-2, 4, 6, 7, 15) and stimulation through the TCR, but up regulated in response to glucocorticoids (29, 31-37).

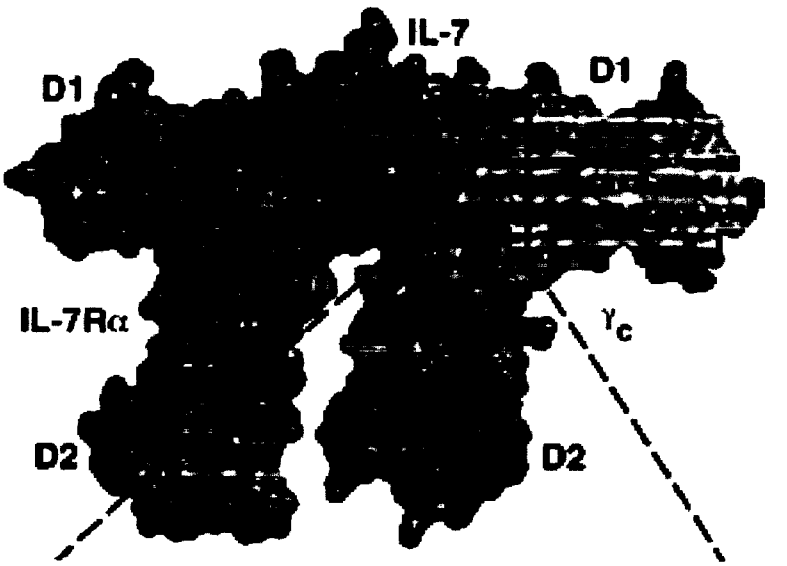
IL-7 signaling occurs via the IL-7 receptor, a heterodimer composed of a unique α-chain (CD127) (38), and a common γ-chain (CD132) (39) that is shared with the receptors for IL-2, -4, -9, -15 and -21 (29, 40) (figure 7A, (41) B (42)). CD127 is highly expressed on CD4 and CD8 T-cells and IL-7 mediated signal transduction, requires both receptor components. IL-7 first binds specifically to CD127, which promotes heterodimerization with CD132. Once these receptors are in close proximity, CD127 and CD132 associated Jak kinases (Jak 1 and 3 respectively) cross phosphorylate each other, activating a number of signaling cascades. Although IL-7 has been shown to stimulate a variety of signaling molecules including p56lck, p59fyn, JNK, p38 and STAT3, STAT5 (survival, cytolytic) and PI3K signaling (proliferation) are responsible for most relevant IL-7 functions in human CD8 T-cells (27, 29)

IL-7 promotes the survival and differentiation of immature T-cells within the thymus (43, 44) and is critical for immune homeostasis, both for the maintenance of naïve T-cells by upreglating the anti-apoptotic molecule Bcl-2 (45-49) and for the establishment of memory T-cells in mice (41, 50-52). IL-7 not only plays a pivotal role at a number of T-cell developmental stages (53-55) but also plays an important role in the activation and proliferation of cytotoxic CD8 T-cells. IL-7 has been shown to stimulate proliferation of CD8 T-cells in a time- and dose-dependent manner both in humans and in murine models (56-59). Consistent with this, CD8 T-cells from IL-7R-/- mice proliferate poorly with about half undergoing apoptosis (60, 61). IL-7 has also been shown to up regulate telomerase activity in CD45RA+ T-cells isolated from human cord blood (59), an activity necessary for clonal expansion of activated T-cells. In addition to its effects on proliferation, IL-7 also enhances CD8 T-cell anti-viral and anti-tumor

Figure 7: IL-7 receptor complex. **(A)** Cartoon representation of the IL-7 receptor complex. Reproduced with kind permission from the Nature Publishing group. **(B)** Crystal structure of the IL-7 receptor complex. Reproduced with kind permission from Elsevier.



Nat. Rev. Immunol. 2003, 3: 269-



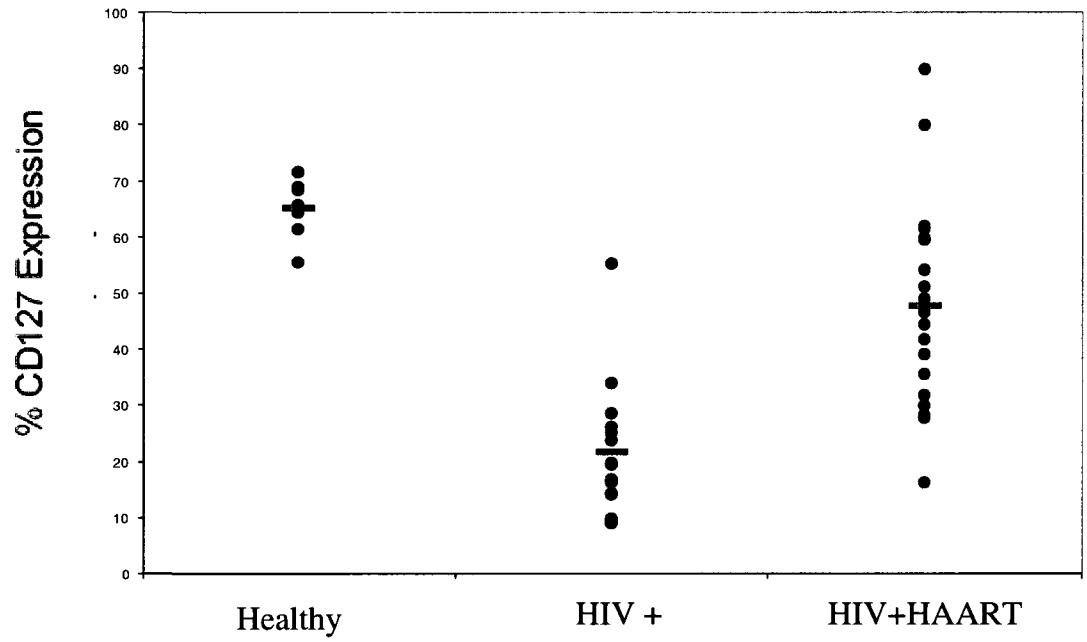
Structure. 2009, 17:54

cytolytic activity (62-68) and up regulates perforin expression, a protein used by CD8 T-cells to lyse their targets (69). Perforin regulation is thought to occur via activation of the transcription factor STAT5 (70). Thus, IL-7 plays an essential role in the activation of cell mediated immunity by enhancing the proliferation and cytolytic potential of CD8 T-cells.

Interleukin-7 and HIV

Given the importance of IL-7 in CD8 T-cell function, decreased IL-7 signaling could explain in part the impaired CD8 T-cell activity characteristic of progressive HIV disease. In a cross-sectional study comparing CD127, MacPherson et al. in fact reported a 67% reduction in the proportion of CD8 T-cells expressing the IL-7R α-chain in HIV+ patients with uncontrolled viral replication (mean CD4 cell count = 232) compared to healthy HIV seronegative controls (71) (figure 8). When HIV+ patients on antiretroviral therapy with sustained viral suppression were examined, CD127 expression approached normal levels to 73% of that seen in controls. The duration of viral suppression was the only parameter that correlated with the apparent recovery of CD127 expression in effectively treated patients. Several other groups have since reported similar findings.(71-77). Vingerhoets *et al* (78) also observed lower CD127 expression on CD8 T-cells from HIV infected individuals and found these cells were less able to form blasts and up regulate CD25 in response to IL-7 compared to controls. Consistent with this, Ferrari *et al* (79) demonstrated that anti-HIV cytotoxic T-lymphocytes (CTL) from patients with advanced disease could not be expanded *in vitro* following stimulation with HIV antigens

Figure 8: CD127 surface expression is reduced in HIV infected patients compared to healthy controls and patients on Highly Active Anti-Retroviral Treatment (HAART). Reproduced with kind permission from Wolters Kluwer Health.



JAIDS. 2001, 28(5): 454-457

and IL-7. Thus it appears that HIV infection is associated with down regulation of the IL-7 receptor which may in turn lead to impaired CD8 T-cell activity.

It has recently been demonstrated that IL-7 down regulates expression of its own receptor, both surface protein and mRNA transcripts (33, 34, 80). Since HIV infected individuals have increased plasma concentrations of IL-7 compared to uninfected controls (81, 82), it is possible that elevated plasma IL-7 causes reduced expression of CD127 on CD8 T-cells in HIV+ patients. I suspect, however, that the IL-7 concentrations found in HIV+ patients are alone insufficient to induce CD127 down regulation in vivo. First, IL-7 concentrations in the range of 500 to 10,000 pg/ml are required in vitro to down regulate CD127 on purified CD8 T-cells isolated from healthy volunteers (33, 77) . In HIV seronegative individuals, plasma IL-7 concentrations average 2.2 pg/ml and increase in HIV infected individuals to on average 18 pg/ml with a reported maximum of 55 pg/ml (45, 76, 82, 83). It is thus questionable whether the concentrations of IL-7 required to down regulate CD127 in vitro can be achieved in vivo. Similar results have been noted in SIV-infected rhesus macaques (48) where subcutaneous administration of IL-7 led to a significant decline in CD127 expression on CD8 T-cells. Here again supra-physiologic concentrations of IL-7 exceeding 1000 pg/ml in plasma were achieved, although lower concentrations were not tested. These findings are consistent with two recent studies (73, 75) demonstrating no correlation between plasma IL-7 levels (max: 40 and 60 pg/ml) and expression of CD127 on CD8 T-cells in HIV+ individuals. In addition, Mussini *et al* (84) noted that patients on antiretroviral therapy who experience a rapid increase in CD4 T-cells have plasma IL-7 levels some 10-fold higher than controls yet express CD127 on CD8 T-cells at near normal levels. Perhaps most convincing is that while T-cells isolated from HIV negative individuals down regulate CD127 in response

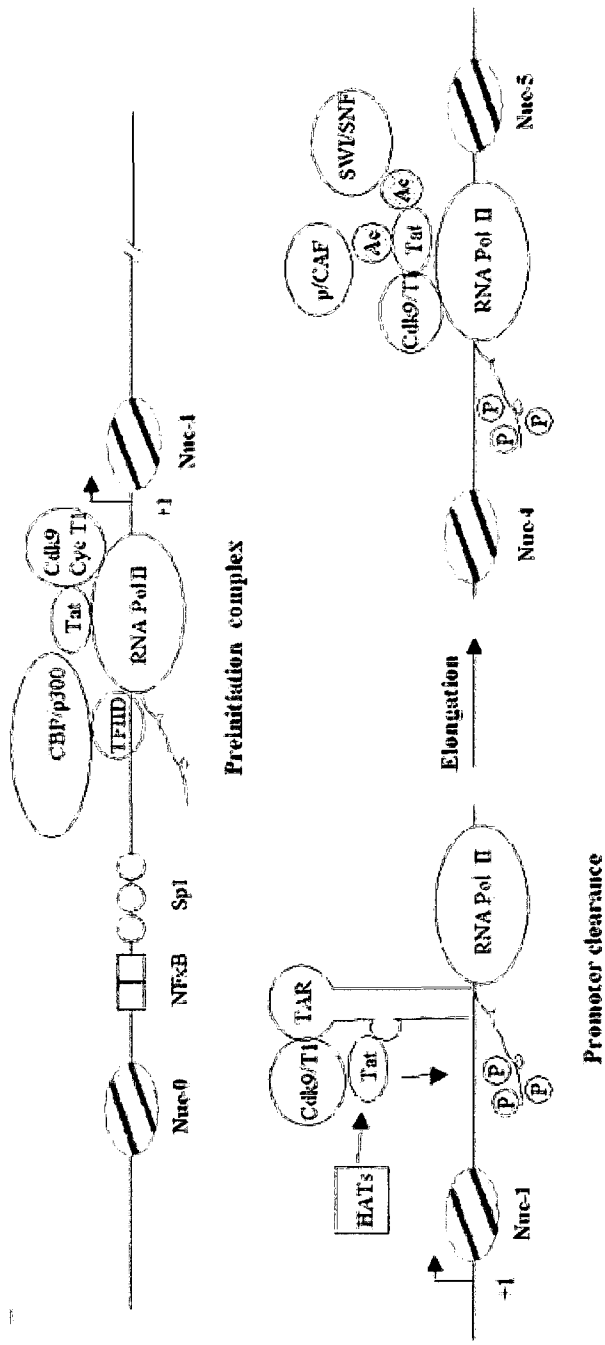
to high concentrations of IL-7 and recover the receptor once IL-7 is removed from the medium, T-cells isolated from HIV+ patients remain CD127 low/negative when cultured in the absence of IL-7 (76). While direct comparisons between in vitro studies using recombinant protein and in vivo observations can be difficult, it seems that IL-7 alone at concentrations typically found in HIV+ patients is unlikely to explain the down regulation of CD127 on CD8 T-cells in these individuals.

HIV-1 Tat

The HIV-1 Tat protein, a 14 kda polypeptide produced from multiply spliced viral transcripts, is a well known activator of viral gene transcription. Tat binds to a secondary stem-loop structure termed TAR (transactivation response region) located at the 5' end of all viral RNA transcripts and both alters histone acetylation around the viral LTR and directly enhances the processivity of RNA polymerase II (85, 86) (figure 9 (87)). In this way Tat causes an increased accumulation of full length viral transcripts.

The HIV-1 Tat protein has six functional domains (figure 10B (88)) that are responsible for the diverse effects Tat has on cellular functions. Depending on cell type, Tat has been shown to alter the expression of a number of cellular proteins. Tat up regulates the expression of IL-2, -8 and -10 in T-lymphoblastic cell lines (89-92), IL-6 in both HeLa and B-lymphoblastoid cells (93, 94) and TNF-α in monocytes (95). Tat also up regulates the IL-4 receptor on Raji cells, a B-lymphoblastoid cell line (96). While Tat has been shown to suppress IL-2 receptor α-chain (CD25) expression on the H9 T-lymphoid cell line (97), it appears to have no effect on the expression of this receptor on primary T-cells isolated from healthy donors (98). Finally, Tat also down regulates MHC

Figure 9: Transactivation of HIV by Tat. HIV-1 transcription is initiated by the recruitment of co-activators by the HIV Tat protein. These co-activators form a pre-initiation complex that assembles on the HIV-1 LTR Transcription is initiated by Tat mediated recruiting of CDK9 to the TAR structure, where it phosphorylates RNAPII. HATs present in the transcription complex then acetylate Tat proteins, resulting in Tat dissociating from the TAR complex, allowing for elongation of HIV transcripts. Reproduced with kind permission from Frontiers in Bioscience.



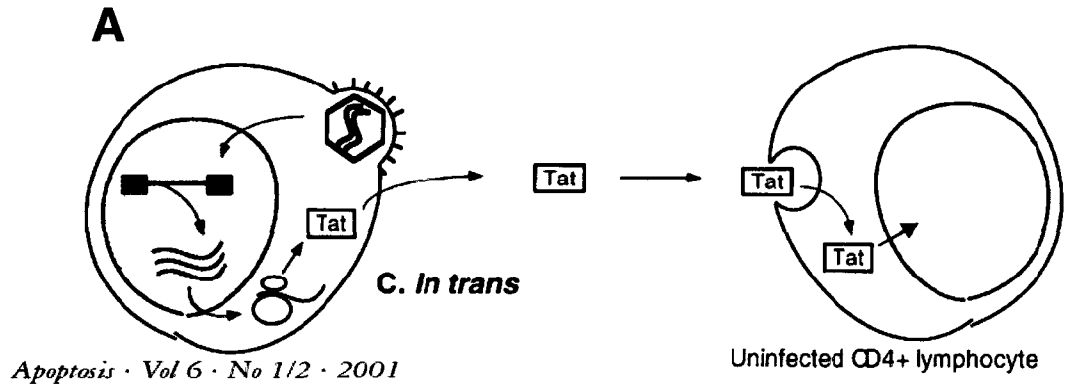
Frontiers in Bioscience. 2004, 9: 2388-2413

class I gene expression on Hela cells (99, 100). Tat appears to mediate these pleiotropic effects by interacting with cellular acetyltransferases. The reversible acetylation of lysine residues on both histones and transcription factors, regulated by a dynamic equilibrium between acetyltransferases and deacetylases, governs the assembly and activity of transcription complexes bound to cellular gene promoters (101-103).

Secreted Tat is rapidly internalized by a variety of cell types in culture including lymphocytes (88, 104, 105) (figure 10A (106)). The arginine-rich basic domain of Tat (amino acids 48-60) appears to be responsible for uptake across the cell membrane (107, 108). Tat binds via its basic domain to heparan sulfate glycosaminoglycans on the cell surface and is then internalized by endocytosis (88, 109-111) and specifically in lymphocytes (88, 104, 105) through clathrin-coated pits (112). Pre-incubating Tat protein with heparin blocks Tat binding to the cell membrane and entry into the cytoplasm (109-111). Several studies have demonstrated that Tat is taken up by cells within 90 minutes of being added to the medium and co-localizes with caveolin-1 containing cytoplasmic vesicles (111). Within 4 hours, Tat translocates to the nucleus where it up regulates transcription from the HIV LTR (105, 107-109, 111). Several authors have suggested that secreted Tat may act in a paracrine fashion to enhance HIV replication in unstimulated latently infected CD4+ cells (88).

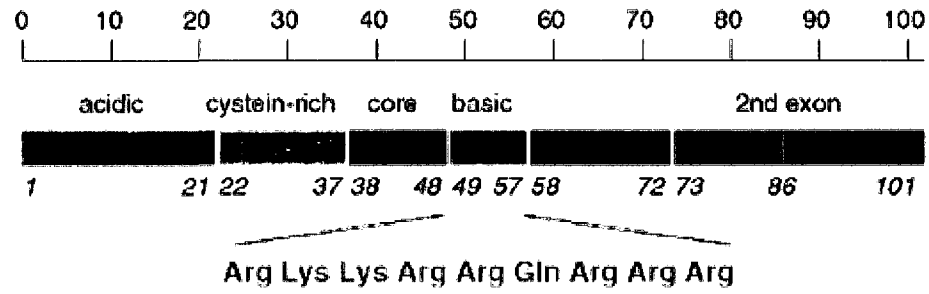
Tat appears to function in an autocrine and/or paracrine fashion. Full length Tat protein is secreted by HIV infected CD4+ T-cells and can be found in culture media during peak infection (105, 113). Soluble HIV Tat protein is found in the serum of HIV-infected patients (114) and plays a decisive role in immune dysregulation. Lymphocytes exposed to extracellular Tat in vitro no longer proliferate to tetanus toxiod (115, 116) Staphylococcal enterotoxin B (117) or anti-CD3 monoclonal antibodies (98, 118). In

Figure 10: The HIV-1 Tat protein. **(A)** Cartoon depicting trans effect of HIV-1 Tat protein. HIV-1 Tat is secreted by infected cells and taken up readily by various cell types. Reproduced with Kind permission from Springer **(B)** Domains of the HIV-1 Tat protein. Reproduced with kind permission from Elsevier.



Apoptosis. 2001, 6(1/2)

B



Adv. Drug Del. Rev. 2005, 57: 597-608

addition, neutralizing anti-Tat antibodies in the serum of HIV-infected individuals have been correlated with low viral loads and slower disease progression (116, 117, 119, 120). Indeed, Rezza *et al* (121) found patients with anti-Tat antibodies have a 60% lower risk of progressing to AIDS compared to those without such antibodies. Further, vaccination with HIV Tat protein and appearance of serum anti-Tat antibodies in cynomolgus monkeys was protective against infection with the highly pathogenic SHIV-89.6P virus (122). How soluble HIV Tat protein impairs lymphocyte function has not been previously elucidated.

CD8 T cells are vital in controlling viral infections. During the course of HIV infection, CD8 T cell function declines. IL-7 is an important cytokine that is required for optimal CD8 T -cell proliferation and cytolytic activity and expression of the IL-7Rα (CD127) is down regulated in HIV patients. In light of the fact that the HIV-1 Tat protein is present in patient serum, acts in a pararcrine fashion to alter protein expression and exhibits immunosuppressive activity, I questioned if soluble HIV-1 Tat protein plays a role in down regulating CD127 expression in CD8 T cells.

HYPOTHESIS

Soluble HIV-1 Tat protein is in part responsible for decreased CD127 expression on CD8 T cells in HIV infected patients.

Objectives:

1. Test the hypothesis that soluble Tat protein down regulates CD127 expression on CD8 T cells in vitro and characterize the effect of decreased CD127 expression on CD8 T cell function.

2. Determine if CD127 expression on CD8 T cells from HIV patients recovers ex vivo, and if so determine if CD127 suppression can be maintained by HIV-1 Tat soluble protein.

3. Determine if Tat mediated down regulation of CD127 is increased in the presence of IL-7.

4. Determine the mechanism of Tat mediated down regulation of CD127 on CD8 T cells.

CHAPTER 2-MATERIALS AND METHODS

Reagents

Purified HIV-1 Tat protein (86 amino acids) was obtained either from the NIH AIDS Research and Reference Reagent Program Division of AIDS, NIAID, NIH, or purchased from Advanced Bioscience Laboratories Inc. (Kensington, MD, USA). Protein was received lyophilized and was resuspended to 1 mg/ml in PBS containing 1 mg/ml BSA and 0.1 mM dithiotreitol. Tat protein is reportedly >95% pure by heparin-affinity chromatography and reverse phase high performance liquid chromatography (HPLC). Purified HIV-1 gp160 and Nef protein were obtained from Immunodiagnostics Inc (Woburn, MA). Whole killed Candida was from Greer Laboratories Inc (Lenoir, NC) The following fluorochrome-labeled monoclonal antibodies were purchased from Immunotech Beckman Coulter (Marseille, France): anti-CD2-PE (39C1.5), anti-CD3-FITC (UCHT1), anti-CD8-PC5 (B9.11), anti-CD16-PE (3G8), anti-CD25-FITC (B1.49.9), anti-CD28-ECD (CD28.2), anti-CD38-PE (T16), anti-CD45RA-ECD (2H4), anti-CD45RA-FITC (ALB11), anti-CD45RO-FITC (UCHL1), anti-CD56-PC5 (NKH-1), anti-CD62L-ECD (DREG56), anti-CD127-PE (R34.34), anti-HLA-DR-PE (Immu-357), and anti-perforin-FITC (δG9). Anti-CD132-PE (AG184), plus anti-CD3 (HIT3a) and anti-CD28 (CD28.2) monoclonal antibodies were purchased from BD Biosciences: Pharmingen (Mississauga, ON, Canada). All fluorochrome labeled antibodies were titrated and used at saturating concentrations. Anti-Tat monoclonal antibody (IgG1) and IgG1 isotype control (107.3) were purchased from Immunodiagnostics Inc and BD Biosciences: Pharmingen respectively. Interleukin-7 was obtained from Invitrogen Biosource (Burlington, ON, Canada), resuspended in PBS plus 0.1% BSA, and stored at

-80°C. The Annexin V staining kit and propidium iodide were purchased from BD Biosciences: Pharmingen (Mississauga, ON, Canada). Heparin was from Organon (Toronto, Canada), and lipopolysaccharide (LPS) from Sigma-Aldrich (St Louis, MO). Anti-phospho-STAT5 (Y694) monoclonal antibody, Bcl-2 (Blc-2/100) and Ki-67 (B56) staining kits were purchased from BD Biosciences: Pharmingen (Mississauga, ON, Canada). Unlabelled anti-CD127 monoclonal antibody (hIL-7R-M21) was purchased from BD Biosciences (Mississauga, Ontario, Canada) and polyclonal goat anti-human CD127 antibodies (AF-306-PB) were obtained from R&D Systems (Minneapolis, MN). Monoclonal anti-Tat (1002) and fluorescein isothiocyanate (FITC) conjugated anti-Tat (1002-f) antibodies were purchased from Immunodiagnostics Inc (Woburn, MA). All fluorochrome labeled antibodies used for flow cytometry were titrated and used at saturating concentrations. 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, ON, Canada). ^{3}H-thymidine was obtained from Amersham Biosciences (Baie d'Urfe, QC, Canada). Ammonium chloride, brefeldin A, chloroquine, colchicine, cyclohexamide, E64, leupeptin and MG132 were purchased from Sigma (St Louis, MO), and lactacystin was purchase from A.G. Scientific (San Diego, CA).

Patients

Patients were recruited from The Ottawa Hospital Immunodeficiency Clinic according to the following inclusion criteria: age over 18 years; naïve to antiretroviral therapy or off therapy for at least three months; a blood plasma HIV viral load above detection (>50 copies/ml); CD4 T-cell count >200 cells/μl; no co-morbidities; and able to

provide signed informed consent. Exclusion criteria included co-infection with hepatitis B and/or hepatitis C virus; and clinical or laboratory evidence of an active infection or malignancy. Patient blood was drawn into sodium heparin-containing tubes and processed within 2 hours.

Cell Purification and Culture

Blood from healthy donors was drawn with consent. Into tubes containing sodium heparin, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density centrifugation. Cells were washed with phosphate buffered saline (PBS) and resuspended at 1 x 10^6 cells/ml. CD8 T-cells were then purified from PBMC using the MACS Microbead CD8+ Cell AutoMACS Isolation System (Miltenyi Biotec) according to the manufacturer's directions. Cell purity was consistently >95% CD8+ by flow cytometric analysis with only 2.6 +/- 0.99% (mean +/- standard error of the mean (SEM)) CD8(+) CD3($^-$) CD16(+) CD56(+) NK cells. Our cultures therefore were comprised of >95% purified CD8 T-cells.

Following isolation, purified CD8 T-cells were generally allowed to recover overnight at 1 x 10^6 cells/ml in media comprised of RPMI 1640 (Hyclone) supplemented with 20% fetal calf serum (FCS; Cansera, Rexdale, Ont. Canada) plus 100U/mL penicillin and 100μg/mL streptomycin (RPMI-20). After overnight culture, CD8 T-cells were incubated in media alone (RPMI-20) or with purified HIV Tat (10 μg/ml, unless otherwise specified) or other proteins or 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 hour with inhibitors as indicated prior to treatment with Tat protein and/or IL-7.

Flow cytometry

At times indicated, cells were incubated with the appropriate fluorochrome-labeled antibodies for 30 minutes in the dark at room temperature, and then analyzed by flow cytometry using a Coulter Epics ALTRA flow cytometer. 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.

To detect intracellular Ki-67, 1 million cells were harvested and washed in cold PBS. Cell pellets were then resupended in 1 ml of cold 70% - 80% ethanol added dropwise, while vortexing and then incubated at -20°C for 2 hours. Cells were then washed 2 times in 15 ml wash buffer (PBS with 1% FBS, 0.09% NaN3 pH7.2) to the fixed cells. The cells were then resupended in 100 µl wash buffer and incubated with 20µl of FITC-conjugated anti Ki-67 (Clone B56, BD Biosciences: Pharmingen) at room temperature (RT) for 20-30 minutes in the dark. Cells were then washed a final time before analysis by flow cytometry.

To detect intracellular Perforin and Bcl-2, purified CD8 T-cells were washed at times and conditions indicated with PBS and then fixed and permeabilized using the Fix and Perm Cell permeablization kit from Caltag Laboratories (Burlingame, CA). Cells were then incubated with anti-Bcl2-FITC antibodies (clone Bcl2/10, BD Biosciences: Pharmingen) or anti-Perforin-FITC (clone δG9, BD Biosciences: Pharmingen) at room temperature for 60 minutes in the dark and analyzed by flow cytometry.

To detect intracellular Tat taken up by endocytosis, 10 μg of anti-Tat-FITC antibodies (1102-f) were incubated with 1 μg purified Tat protein (1:1 molar ratio) for 1 hour at 37°C. To reduce background staining, 1 x 10^5 CD8 T-cells in RPMI-20 were pre-incubated with 10 μg unlabelled anti-Tat antibodies (clone 1102) at 37°C for 60 minutes. The Tat bound anti-Tat-FITC complexes were then added to the CD8 T-cells and the cultures were returned to the 37°C incubator. At the time points indicated, cells were washed, resuspended in phosphate buffered saline (PBS) and subjected to flow cytometry.

To detect intracellular CD127, 5 x 10^5 purified CD8 T-cells were fixed in IC Fixation Buffer (eBioscience, San Diego, CA) at room temperature for 20 minutes. Cells were then washed in PBS and surface CD127 was blocked by the addition of 20 μl unlabelled anti-CD127 antibody (hIL-7R-M21) for 1 hour at 37°C. Cells were washed again to remove excess antibody and then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Cells were then incubated with anti-CD127-PE (R34.34) for 30 minutes in the dark at room temperature and analyzed by flow cytometry.

Intracellular phospho-STAT5 staining was performed as follows. Isolated CD8 T cells were fixed in 2% paraformaldehyde for 10 minutes at room temperature and then permeabilized by incubating in cold 100% methanol for an additional 10 minutes at 4°C. After washing in PBS, cells were incubated with anti-phospho-STAT5 antibodies in the dark for 30 minutes and analyzed using a BD FACS Calibur flow cytometer (San Jose, CA, USA). Resulting profiles were analyzed with the EXPO version 2.0 software package.

Immunofluorescence Microscopy

Anti-Tat-FITC (1102-f) antibodies (10 μg) were first complexed with 1 μg purified Tat protein for 1 hour at 37°C. To reduce background staining, 1 x 10^5 purified CD8 T-cells were pre-incubated in RPMI-20 with 10 μg unlabelled anti-Tat antibodies (1002) at 37°C for 60 minutes. Tat bound anti-Tat-FITC complexes were then added to the cells and the cultures were returned to the 37°C incubator. At the time points indicated, CD8 T-cells were fixed and permeabilized in ice cold ethanol:methanol (3:1) for 10 minutes at -20°C. Cells were then washed in PBS and incubated for 30 minutes with anti-CD127-PE (R34.34) antibodies at room temperature. Cells were counterstained with the addition of 0.5 μg/ml 4'-6-diamidino-2-phenylindole (DAPI) solution, fixed to microscope slides using a Shandon cytospin 4 (Thermo Scientific, Waltham, MA) and mounted with cover slips using Prolong gold antifade reagent (Invitrogen Biosource, Carlsbad, CA). Cells were analyzed using a Zeiss Axio Imager M1 upright microscope (Zeiss Canada, Toronto, ON) equipped with an Axiocam and Axiovision software version 4.6 (Zeiss Canada, Toronto, ON).

Cell Viability Assays

Purified CD8 T-cells were incubated in media alone or with Tat protein (10 μg/ml) in a humidified incubator at 37°C. At 24 and 72 hours cells were stained with Annexin V-FITC, and propidium iodide according to the manufacturer's directions using the Apoptosis Detection Kit I from BD Biosciences: Pharmingen. As a positive control for apoptosis, purified CD8 T-cells were incubated in parallel with camptothecin (Sigma-Aldrich) at a final concentration of 10 μM.

Proliferation Assays

Purified CD8 T-cells were pre-incubated in RPMI-20 at 1 x 10^6 cells/ml for 72 hours either in media alone or with purified Tat protein (10 μg/ml). The cells were then transferred to a 96-well tissue culture plate at 5 x 10^5 cells/ml and stimulated in triplicate with anti-CD3 plus anti-CD28 monoclonal antibodies (3 μg/ml and 2 μg/ml respectively) with or without IL-7 (5 ng/ml). After an additional 48 hours of incubation, cultures were pulsed with 1 μCi ^{3}H-thymidine for 18 hours. Cells were then harvested onto Filtermat paper (Perkin Elmer) and β-radioactivity measured using a 96-well liquid scintillation counter.

RT-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 (RT) and qPCR were carried out in an iCycler thermal cycler (Bio-Rad, Mississauga, ON, Canada). 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'- GGGAGATGGATCCTATC-3'). The forward and reverse primers used for RPS18 were as follows: RPS18fwd 5'-CTGCCATTAAGGGTGTGG-3', and RPS18rev 5'-TCCATCCTTTACATCCTTCTG-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 *t*-test with 95% confidence intervals.

Tat ELISA

To detect direct interactions with Tat, two forms of CD127 were used in an ELISA assay. The CD127-Fc chimera contains the extracellular and transmembrane domains of CD127 (amino acid residues 21 to 262) linked to the Fc portion of human IgG_1 (R&D Systems, Minneapolis, MN). Soluble CD127 lacking the transmembrane domain (38) was isolated from a CD127 secreting cell line, WI-26VA4 (American Type Culture Collection, Manassas, VA), and was a kind gift from Dr. Angela Crawley at the Ottawa Hospital Research Institute (35). Thrombospondin-1, an extracellular matrix protein previously shown to bind Tat with high affinity (123), was used as a positive control and was obtained from Protein Sciences Corporation (Meriden, CT). Bovine serum albumin (Sigma, St Louis, MO) was used a negative control. Equimolar amounts (200 nM) of each target protein were suspended in 1 mL of carbonate binding buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3.1 mM NaN_3, pH 9.0) and 100 µl of each protein suspension was added to individual wells of a 96 well plate and incubated overnight at 4°C. Plates were then blocked with 100µl/ well of 0.1% bovine serum albumin (BSA) in PBS containing

0.05% Tween-20. After washing the plates five times with PBS plus Tween-20, 40 ng of purified Tat protein was added to each well and incubated for 1 hour at 37^0C. Plates were washed again five times and bound Tat protein was detected by the addition of 100 µl of a 1:1000 dilution of biotinylated anti-Tat antibody (1002; Immunodiagnostics Inc., Woburn, MA) for 1 hour at 37^0C. After washing again, 100 µl of a 1:5 dilution of Streptavidin-peroxidase (Mandel Scientific, Guelph, Canada) was added to each well and incubated for 1 hour at 37^0C. Plates were washed again and developed using TMB Microwell Peroxidase Substrate and stop solutions (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's directions. Plates were read at 450nm using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA).

Co-Immunoprecipitation

Two million purified CD8 T-cells were lysed on ice for 1 hour in 200 µl WCL Buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% NP-40, 10% glycerol, 2mM dithiothreitol) with 1X HALT protease inhibitor cocktail (Thermo Scientific, Rockford, IL). Cell lysates were pre-cleared by adding 100µl Immunopure Immobilized Protein G beads (Thermo Scientific, Rockford, IL) and incubating at 4^oC for 30 minutes with constant mixing. Protein G beads were removed by centrifugation at 17000g and the supernatants were then incubated with 10 µg purified Tat protein for 1 hour at 37^oC. CD127 was immunoprecipitated by the addition of 1 µg of polyclonal goat anti-human CD127 antibodies (AF-306-PB) incubating overnight at 4^oC followed by the addition of 50µl Immunopure Immobilized Protein G beads for 2 hours. Antibody-Protein G bead complexes were pelleted and washed four times with IP buffer (50mM Tris-HCl pH 7.4,

150mM NaCl, 1mM EDTA, 0.1% NP-40, 10% Glycerol, 2mM dithiothreitol, and 0.2% Sodium Azide) containing 1X HALT protease inhibitor cocktail. Protein G bead complexes were then resuspended in SDS sample buffer (New England Biolabs, Ipswich, MA) and boiled for 10 minutes. Samples were analyzed by SDS polyacrylamide gel electrophoresis and Western blotting.

SDS-PAGE and Western Blotting

At the indicated time points, 1 x 10^6 CD8 T-cells were lysed in 100 µL RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS plus 1X Halt Protease Inhibitor Cocktail) at 4°C for 45 minutes. Protein was quantified using the BCA Assay (Thermo Scientific, Rockford, IL). Equal amounts of protein lysate was mixed with 10µL of Laemmli buffer (187.5 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol and 0.03% bromophenol blue supplemented with 5% β-mercaptoethanol), boiled for 10 minutes and then separated by SDS polyacrylamide gel electrophoresis through an 8% resolving gel. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) using a semi-dry transfer apparatus (Bio Rad, Hercules, CA) at 20 volts for 25 minutes. Membranes were blocked overnight at 4°C in 2% blocking solution (ECL Advance Chemiluminescence Kit: GE Healthcare, Buckinghamshire, UK) diluted in Tris-Buffered Saline with Tween-20 (TBST) (50mM Tris-HCl pH 7.6, 150mM NaCl, 0.1% Tween-20). The next day, membranes were incubated with a 1:500 dilution of polyclonal goat anti-human CD127 antibody (AF-306-PB) in 2% blocking solution diluted in TBST for 1 hour at room temperature with constant agitation. After washing three times for 10 minutes each with TBST,

membranes were incubated with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated-donkey anti-goat secondary antibody (R&D Systems, Minneapolis, MN) in 2% blocking solution in TBST for 1 hour again at room temperature with constant agitation. Membranes were again washed three times in TBST and CD127 protein was visualized by chemiluminescence.

Following co-immunoprecipitations, Tat protein was detected by incubating PVDF membranes with a 1:5000 dilution of anti-Tat monoclonal antibody (1002) in 2% blocking solution diluted in TBST for one hour at room temperature. Membranes were then washed in TBST and incubated for 1 hour with a 1:10000 dilution of an HRP-conjugated rabbit anti-mouse secondary antibody (R&D Systems, Minneapolis, MN).

Proteins were visualized by chemiluminescence using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's directions. Bands were photographed using the Alpha Innotech Imager and analyzed for densitometry using the Alpha Innotech AlphaEase software (Alpha Innotech, San Leandro, CA). Analysis was performed using an unsaturated image of the gel captured on a medium speed and high resolution setting normalizing CD127 band pixel intensity to β-actin in each lane.

Statistical analysis

Where indicated, error bars represent the standard error of the mean. Unless otherwise noted, statistical analysis was done using a one-tailed, paired student *t*-test with 95% confidence intervals.

CHAPTER 3 - INTERLEUKIN-7 RECEPTOR EXPRESSION ON CD8 T-CELLS IS DOWN REGULATED BY THE HIV TAT PROTEIN

RATIONALE

Several authors have suggested that the generalized decrease in CD127 expression on CD8 T-cells in HIV infected patients is the result of chronic immune activation. This model is unsatisfying in several aspects. Consistent with our previously published data, several groups have now reported a significant decrease in CD127 expression on CD8 T-cells isolated from untreated HIV-infected individuals (72, 73, 75, 76). Notably, all groups have documented reduced CD127 on the entire CD8 T-cell population. It remains unclear how persistent HIV antigens can cause chronic stimulation of non-HIV-specific CD8 T-cells. Indeed, Paiardini *et al.* (75) have shown that in untreated HIV+ patients CD127 is down regulated on not only HIV-specific CD8 T-cells but also on CMV- and EBV-specific CD8 T-cells compared to the respective cell populations in healthy HIV seronegative controls. It is significant that in this study the average CD4+ cell count within the untreated HIV+ patient group was 367 cells/μl (range 88-765 cells/μl). Since CMV reactivation rarely if ever occurs at CD4+ cell counts greater than 50 cells/μl, it cannot be argued that the down regulation of CD127 on CMV-specific CD8 T-cells was the result of chronic stimulation with CMV antigen. Further, Boutboul *et al.* (72) have reported that the majority of HIV-, CMV- and EBV-specific CD8 T-cells in untreated HIV+ patients are IL-7R$\alpha^{lo/neg}$Perforin^{lo} with very few IL-7R$\alpha^{neg/lo}$Perforin^{hi} cells. This is in contrast to the LCMV model in mice where chronic stimulation with persistent gp33 antigen caused a decrease in CD127 expression on LCMV gp33-specific CD8 T-cells but a concomitant increase in granzyme B (124).

Finally, consistent with our previous report (71) two groups have since demonstrated decreased CD127 expression on naïve (CD45RA+CCR7+) CD8 T-cells isolated from untreated HIV-infected patients compared to seronegative controls (73, 76). It is difficult to reconcile reduced expression of CD127 due to chronic stimulation with the naïve phenotype. Thus it seems that down regulation of CD127 on CD8 T-cells during active HIV replication may not be due to immune activation.

As mentioned previously, CD8 T cells are not infected with the HIV-1 virus. In light of the fact that immune activation cannot explain global down regulation of CD127, I questioned if a soluble factor was involved. Since Tat protein is secreted from infected cells during active HIV replication (105, 113) and in a paracrine fashion (88, 111) can alter protein expression in uninfected cells (90, 125-128), I questioned whether Tat protein exerted an effect on the expression of the IL-7 receptor α-chain on CD8 T-cells. I propose that Tat protein secreted by infected CD4+ cells acts in a paracrine fashion to down regulate CD127 on neighboring CD8 T-cells irrespective of their antigen specificity. In this case and consistent with the data, the majority of the CD8 T-cell population would be affected including naïve and memory cells. This may explain the generalized decrease in cell mediated immunity including both the ineffective control of HIV replication by HIV-specific CD8 T-cells and the increased susceptibility to opportunistic pathogens that is readily seen in HIV infected patients.

RESULTS

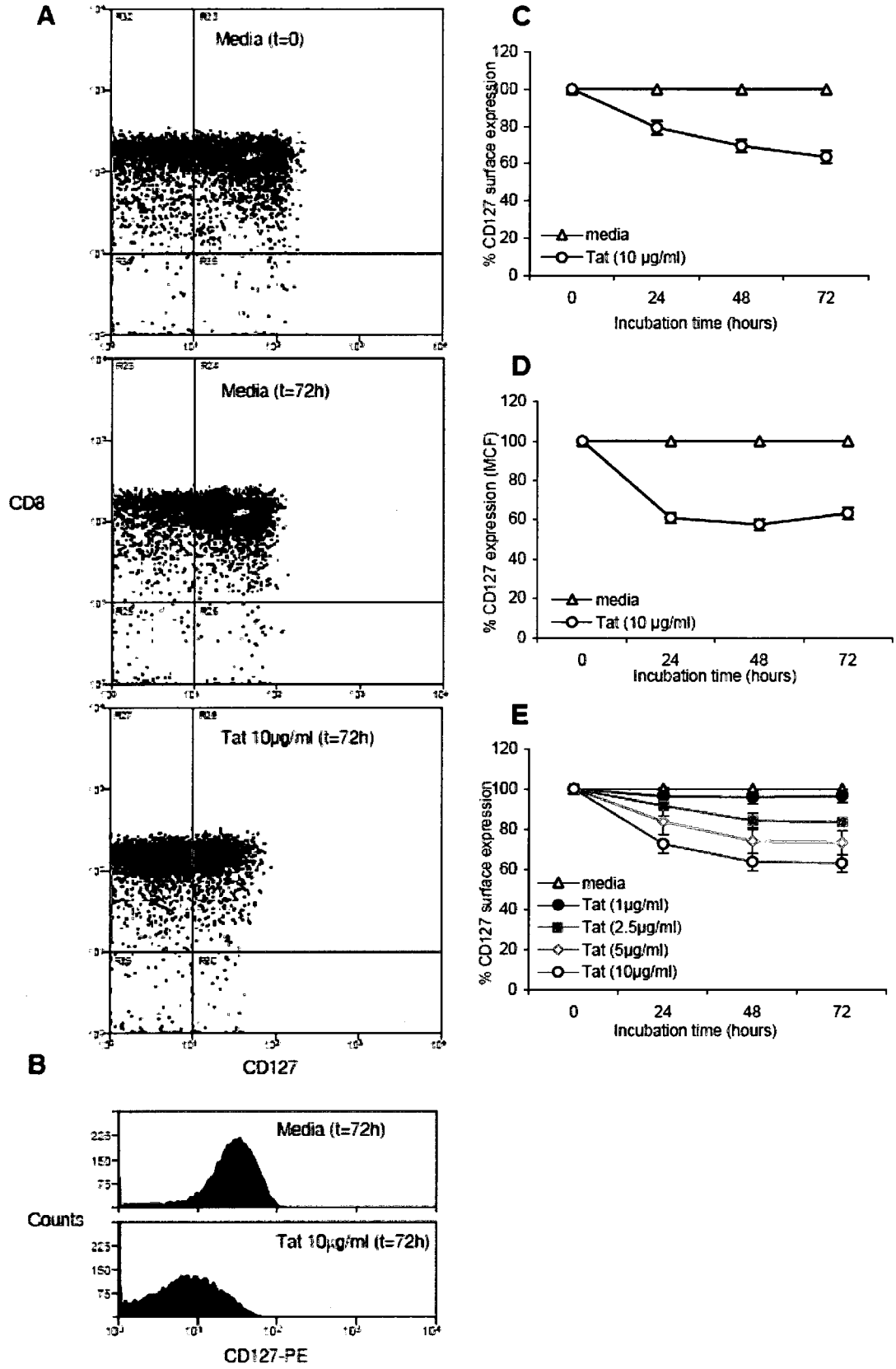
The IL-7 Receptor is down regulated on CD8 T-cells by soluble HIV Tat protein.

To determine if Tat plays a role in the down regulation of CD127, purified CD8 T-cells from healthy donors were incubated in RPMI-20 with or without purified Tat protein (10 μg/ml) and analyzed by flow cytometry at 24 hour intervals (n=15; figure 11, A-C). While the level of CD127 expression on CD8 T-cells cultured in media alone varied little over time, purified Tat protein induced a 35% +/- 3.3% reduction in the number of CD8 T-cells expressing CD127 over 72 hours compared to controls. Further, among those cells expressing CD127 at 72 hours, there was a 38% +/- 2.9% decline in the mean channel fluorescence indicating a reduction in the total number of receptors present on the cell surface (figure 11, B and D). These effects were time dependent, with the greatest decrease in CD127 expression occurring within the first 24 hours following exposure to Tat. The effects of Tat were also dose dependent. Increasing concentrations of purified Tat protein (1, 2.5, 5, 10 μg/ml) induced incremental reductions in CD127 expression (figure 11E).

Down regulation of CD127 on CD8 T-cells is mediated specifically by the HIV Tat protein.

To determine if the reduction in CD127 expression on CD8 T-cells was mediated by HIV Tat protein and not a contaminant in the preparation, 10 μg of purified Tat was pre-treated for 3 hours at 37°C with Proteinase K which was in turn inactivated at 95°C for one hour. Purified CD8 T-cells from healthy donors (n=4) were then incubated with

Figure 11: HIV-1 Tat down regulates CD127 on CD8 T-cells. **(A)**, representative dot plots and **(B)**, histograms from one individual are shown with purified CD8 T-cells at baseline, and after culture in either media alone or with Tat (10 μg/ml) for 72 hours. C, percent change in CD127 positive CD8 T-cells cultured in the presence of 10 μg/ml Tat compared to media controls (n=15). D, percent change in CD127 mean channel fluorescence relative to media controls for the same cultures as in C. E, dose response of increasing concentrations of Tat on CD127 positive CD8 T-cells compared to media controls (n=4). All graphs show mean values +/- standard error of the mean (SEM).


A
Media (t=0)
Media (t=72h)
Tat 10µg/ml (t=72h)
CD8
CD127
B
Media (t=72h)
Tat 10µg/ml (t=72h)
Counts
CD127-PE
C
% CD127 surface expression
media
Tat (10 µg/ml)
Incubation time (hours)
D
% CD127 expression (MCF)
media
Tat (10 µg/ml)
Incubation time (hours)
E
% CD127 surface expression
media
Tat (1µg/ml)
Tat (2.5µg/ml)
Tat (5µg/ml)
Tat (10µg/ml)
Incubation time (hours)

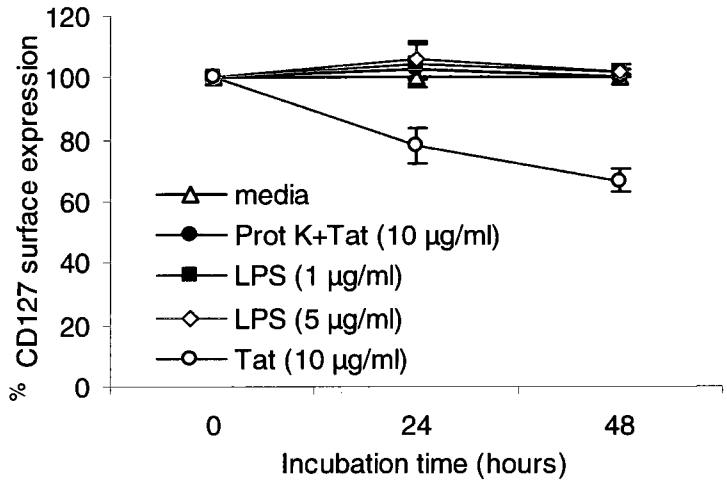
either native or Proteinase K treated Tat protein. As shown in figure 12A, prior treatment of Tat with Proteinase K abolished its ability to down regulate CD127.

While Tat is purified by HPLC and reported to be >95% pure, it is expressed in E. coli and as a result the preparation could contain LPS. Notably CD8 T-cells lack both CD14 and Toll-like receptor-4 and therefore should not respond to LPS. However, Kaya *et al.* (129) demonstrated LPS-induced activation of murine CD4 and CD8 T-cells via the complement receptors types 1 and 2 (CR1 and CR2), present on both T-cell subsets. To definitively rule out any effects of LPS, I incubated purified CD8 T-cells with 1 and 5 μg/ml LPS and followed CD127 expression by flow cytometry. As expected, LPS had no effect on CD127 expression (figure 12A).

To determine if the effects on CD127 expression were mediated specifically by HIV Tat, purified Tat protein was pre-incubated for 30 minutes with either an equi-molar concentration of anti-Tat IgG1 monoclonal antibody, or with heparin (3.5 μg/ml). Heparin binds directly to Tat protein and prevents its uptake by cells in culture (109, 111) and has been used in several studies to block the effects of extracellular Tat including Tat-induced trans-activation of the HIV LTR (110). Neither heparin alone nor isotype control IgG1 antibody had any effect on CD127 expression when incubated with CD8 T-cells over 72 hours (101% and 102% CD127 expression respectively) compared to media alone. As expected, purified Tat protein and Tat pre-incubated with nonspecific isotype control IgG1 antibody both induced a typical 36% and 35% reduction in CD127 expression respectively on CD8 T-cells after 72 hours. In contrast, pre-incubation of Tat with either anti-Tat monoclonal IgG1 or heparin completely abrogated the effects of Tat on CD127 expression. After 72 hours incubation, CD127 expression on CD8 T-cells was

Figure 12: (A) Down regulation of CD127 is induced by HIV-1 Tat. Purified CD8 T-cells (n=2) were incubated for 72 hours with purified LPS (1, 5 μg/ml), HIV-1 Tat protein (10 μg/ml) or HIV-1 Tat (10 μg/ml) pre-treated with Proteinase K. **(B)** CD127 is not down regulated by other HIV-1 proteins or Candida. Purified CD8 T-cells (n=3) were incubated for 72 hours with 10μg/ml of HIV Tat, HIV gp160, HIV Nef, or whole killed Candida. Percent changes in CD127 positive CD8 T-cells compared to media controls are shown (mean +/- SEM).

A


120
100
80
60
40
20
0
% CD127 surface expression
media
Prot K+Tat (10 µg/ml)
LPS (1 µg/ml)
LPS (5 µg/ml)
Tat (10 µg/ml)
0
24
48
Incubation time (hours)

B

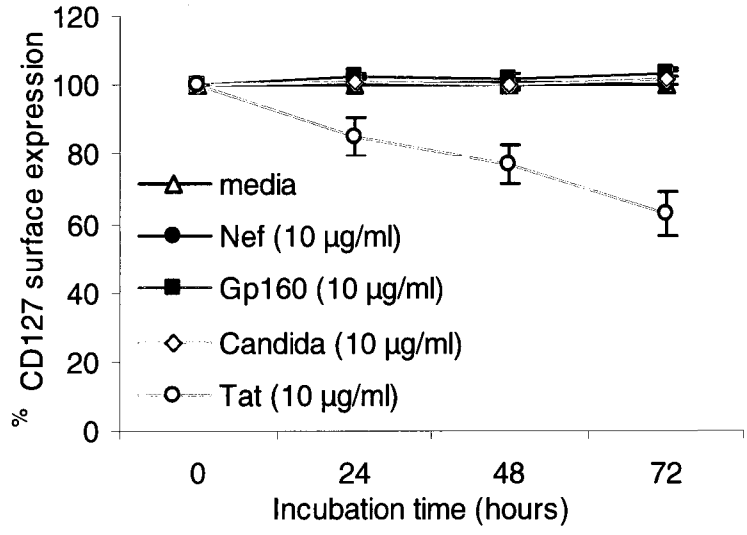

120
100
80
60
40
20
0
% CD127 surface expression
media
Nef (10 µg/ml)
Gp160 (10 µg/ml)
Candida (10 µg/ml)
Tat (10 µg/ml)
0
24
48
72
Incubation time (hours)

Table 1: Tat-induced down regulation of CD127 is blocked by an anti-Tat monoclonal antibodies and heparin. Anti-Tat monoclonal antibody and isotype control were used at equi-molar concentrations compared to Tat. Antibodies and heparin were pre-incubated with Tat for 30 minutes prior to addition to CD8 T-cells. CD127 expression is shown relative to media control.

Tat (10 µg/ml)	IgG1 (100 µg/ml)	IgG1 α-Tat (100 µg/ml)	Heparin (3.5 µg/ml)	% CD127 expression
				100
	+			101
			+	102
+				64
+	+			65
+		+		98
+			+	98

98% in the presence of Tat plus anti-Tat monoclonal and 102% in the presence of Tat plus heparin relative to media control (table I). The ability of both the anti-Tat monoclonal antibody and heparin to block the down regulation of CD127 on CD8 T-cells clearly indicates the effect is mediated by Tat protein.

Down regulation of CD127 on CD8 T-cells is not mediated by other HIV or nonviral proteins.

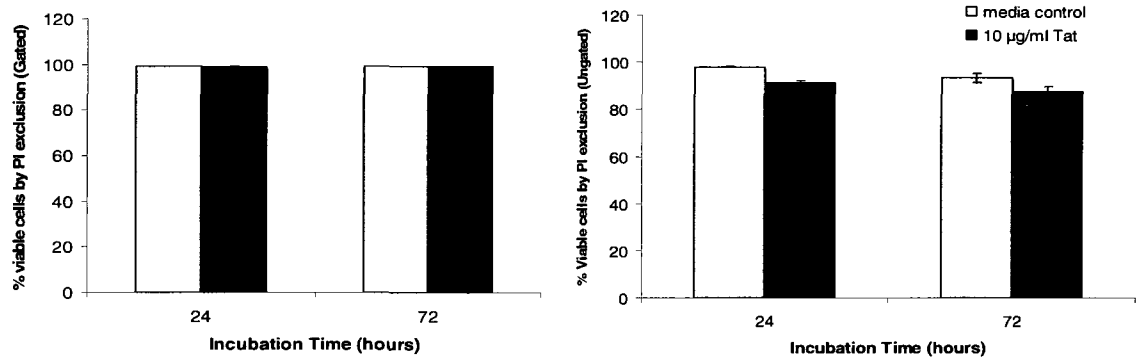
While CD127 is clearly down regulated by the HIV Tat protein, I considered the possibility that this is a nonspecific effect mediated by exposure of purified CD8 T-cells to foreign protein. To address this possibility, CD8 T-cells were isolated from healthy donors (n=3) and incubated in RPMI-20 with 10 μg/ml of either purified HIV gp160, HIV Nef protein or whole killed Candida. Among these proteins, Tat alone induced a down regulation of CD127 on CD8 T-cells (figure 12B).

Down regulation of CD127 on CD8 T-cells by Tat is not the result of cell death or apoptosis.

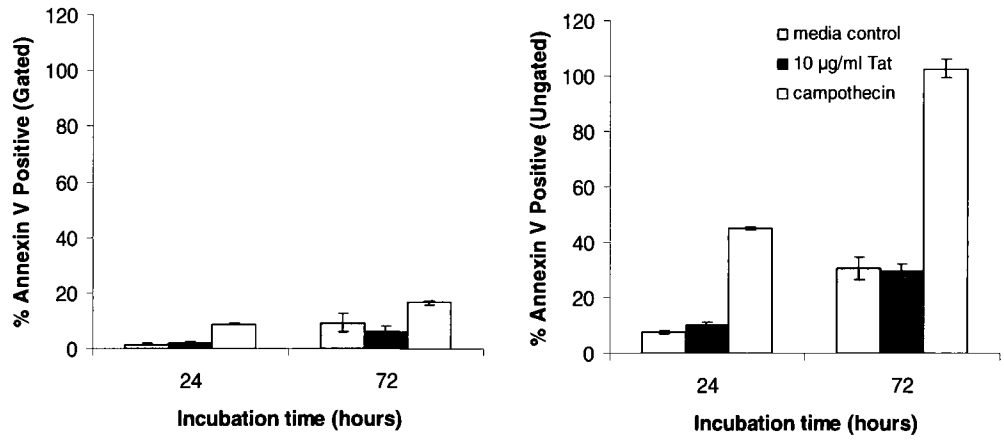
There are conflicting reports of exogenous Tat protein inducing apoptosis in cultured T-cells (130-133). To ensure that the down regulation of CD127 was in fact occurring on viable cells, purified CD8 T-cells isolated from healthy donors (n=4) were incubated with or without Tat (10 μg/ml) for up to 72 hours. Both in the presence and absence of Tat, viability of gated cells was 100% as indicated by propidium iodide exclusion (figure 13A). Forward and side scatter profiles of the entire cell population did not change significantly over 72 hours of incubation and viability of the entire population

Figure 13: Tat protein does not affect viability of isolated CD8 T-cells. Purified CD8 T-cells incubated in media alone or with Tat protein were stained with propidium iodide or annexin V-FITC at 24 or 72 hours and analyzed by flow cytometry. Analysis was carried out on cells within the lymphocyte gate based on forward and side scatter as well as on the ungated population. (**A**) Cell viability as indicated by propidium iodide exclusion was 100% in the gated cells and remained >90% in the ungated population, and was not different between cells maintained in media or treated with Tat. **(B)** Apoptosis as indicated by annexin V staining averaged 10% in gated cells and was not different between cells maintained in media alone or exposed to Tat protein. Campothecin which induces apoptosis was used as positive control. Graphs show mean values +/- standard error of the mean (SEM).

A


% viable cells by PI exclusion (Gated)
120
100
80
60
40
20
0
24
72
Incubation Time (hours)
% Viable cells by PI exclusion (Ungated)
media control
10 µg/ml Tat
120
100
80
60
40
20
0
24
72
Incubation Time (hours)

B


% Annexin V Positive (Gated)
120
100
80
60
40
20
0
24
72
Incubation time (hours)
% Annexin V Positive (Ungated)
media control
10 µg/ml Tat
campothecin
120
100
80
60
40
20
0
24
72
Incubation time (hours)

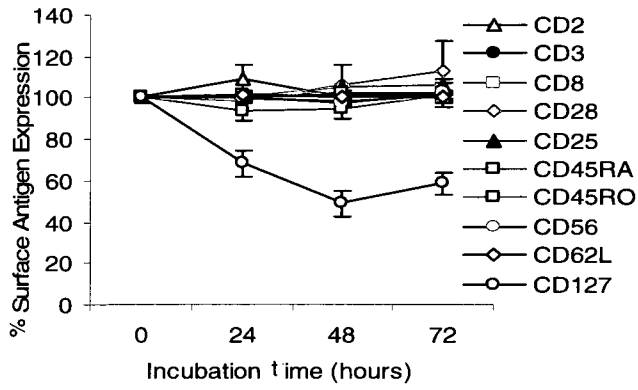
was maintained at over 90% irrespective of the presence of Tat. Apoptotic cells as identified by annexin V staining averaged 10.3% of gated cells and again was not different between CD8 T-cells exposed to Tat and media controls (figure 13B). In fact, cells incubated with Tat protein and analyzed within the lymphocyte gate consistently showed lower annexin V staining, although this difference never reached statistical significance. Since the greatest decrease in CD127 expression on purified CD8 T-cells occurred within the first 24 hours of exposure to Tat and since these cells remained viable for at least 72 hours in culture, I conclude that the changes in CD127 expression were not the result of cell death or apoptosis.

The HIV Tat protein specifically down regulates CD127.

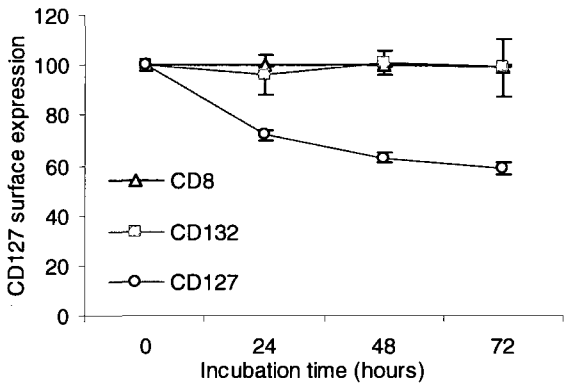
Since the HIV Tat protein is internalized by endocytosis, I next questioned whether exposure to Tat induces a generalized and nonspecific reduction in cell surface proteins on CD8 T-cells. To investigate this possibility, purified CD8 T-cells isolated from healthy donors (n=4 to 8) were incubated with 10 μg/ml of Tat protein, and expression of several cell surface proteins was followed over 72 hours by flow cytometry. As shown in figure 14A, Tat induced a down regulation of CD127 on CD8 T-cells, but had no effect on the expression of CD2, CD3, CD8, CD25, CD28, CD45RA, CD45RO, CD56 or CD62L. Expression of these cell surface proteins was unaffected both in terms of number of cells positive and in the extent of surface expression as indicated by mean channel fluorescence. It is notable that Tat did not affect surface expression of CD132, the common γ-chain that associates with CD127 to form the heterodimeric IL-7 receptor (figure 14B). Given that expression of CD25 and CD132

Figure 14: HIV-1 Tat specifically down regulates the IL-7 receptor α-chain. Purified CD8 T-cells were incubated with Tat protein (10 μg/ml) for 72 hours. **(A)**, percent change in positive staining CD8 T-cells compared to media controls for each of the surface proteins indicated (n=4). **(B)**, percent change in CD132 positive CD8 T-cells compared to media controls (n=6). Mean values are shown +/- SEM.

A


140
120
100
80
60
40
20
0
% Surface Antigen Expression
0
24
48
72
Incubation time (hours)
CD2
CD3
CD8
CD28
CD25
CD45RA
CD45RO
CD56
CD62L
CD127

B


120
100
80
60
40
20
0
CD127 surface expression
0
24
48
72
Incubation time (hours)
CD8
CD132
CD127

were both unaffected, it is unlikely that the effects of Tat on CD8 T-cells can be generalized to the family of common γ-chain cytokine receptors. These data then indicate that the HIV Tat protein specifically targets CD127 on CD8 T-cells.

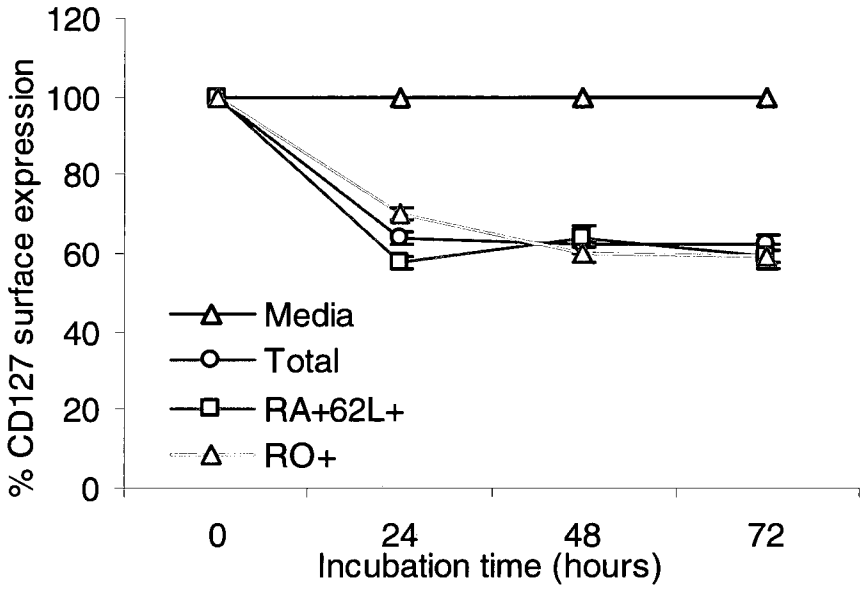
CD127 is down regulated by the HIV Tat protein on both naïve and memory CD8 T-cells.

Among T-cells, CD127 expression is primarily limited to the naïve and memory subsets (30, 41). I then questioned whether Tat down regulated CD127 on either or both of these subpopulations. CD8 T-cells were purified from healthy donors (n=4) and incubated with 10 μg/ml of Tat protein and then analyzed for CD127 expression on naïve (CD45RA+CD62L+) and memory (CD45RO+) cells. Both subsets were affected equally and mirrored the total CD8 T-cell population in terms of degree and rate of CD127 down regulation (figure 15).

Down regulation of CD127 by Tat is not the result of nonspecific CD8 T-cell stimulation.

CD8 T-cell stimulation induces a number of phenotypic changes including up regulation of CD25, HLA-DR and perforin, and down regulation of CD127 (41). I questioned then whether exogenous Tat could induce a nonspecific activation of purified CD8 T-cells resulting in the down regulation of CD127. To investigate this possibility, CD8 T-cells from healthy HIV seronegative donors were incubated in RPMI-20 with either purified Tat protein (10 μg/ml) or with anti-CD3 and anti-CD28 monoclonal antibodies. Cells stimulated through CD3 and CD28 ligation as well as Tat-treated cells both down regulated CD127 on the cell surface as anticipated, although the decrease in

Figure 15: Tat down regulates CD127 on both naïve and memory CD8 T-cells. Purified CD8 T-cells (n=4) were incubated with HIV-1 Tat protein (10 μg/ml) and stained at 24 hour intervals with anti-CD127-PE and either anti-CD45RO-FITC or anti-CD45RA-FITC plus anti-CD62L-ECD. Percent change in CD127 positive naïve (CD45RA+CD62L+) and memory (CD45RO+) cells compared to media controls is shown (mean +/- SEM).

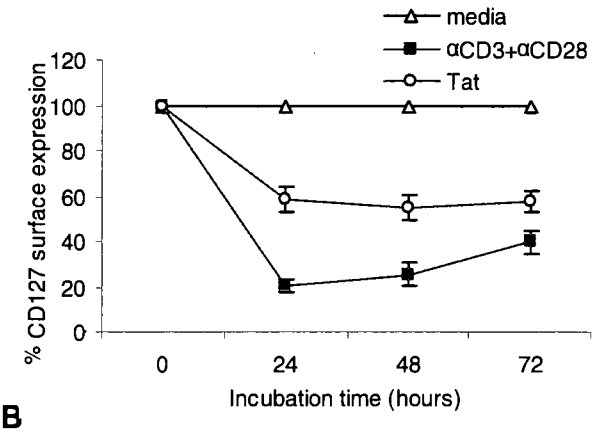

120
100
80
60
40
20
0
% CD127 surface expression
Media
Total
RA+62L+
RO+
0
24
48
72
Incubation time (hours)

expression was greater with anti-CD3 and anti-CD28 compared to Tat alone (25% +/- 4% versus 51% +/- 2% CD127+ respectively at 24 hours, figure 16A). As expected, stimulation of CD8 T-cells with anti-CD3 plus anti-CD28 monoclonal antibodies caused an increase in CD25 expression (1.7-fold) within 24 hours. CD8 T-cells incubated with purified Tat, however, showed no change in CD25 over 72 hours (figure 16B). Surface expression of HLA-DR and CD38 also appropriately increased following stimulation with anti-CD3 and anti-CD28 monoclonal antibodies but did not change at all following exposure to purified Tat protein (data not shown). This is in agreement with the data presented in figure 14A showing no change in the expression of other cell surface proteins in response to Tat including CD28 and CD62L which are normally down regulated, and CD56 which is normally up regulated following CD8 T-cell stimulation. Consistent with cell phenotype, exogenous Tat protein also did not stimulate CD8 T-cell proliferation. In the presence of Tat, incorporation of ^{3}H-thymidine remained at background levels while cells stimulated with anti-CD3 and anti-CD28 monoclonal antibodies showed marked proliferation (table 2).

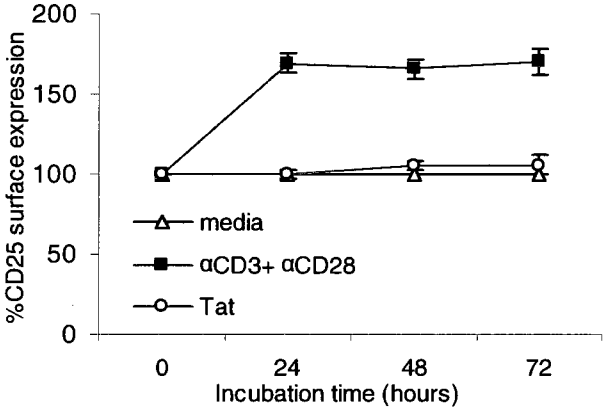
Once stimulated, CD8 T-cells mature into cytolytic effector cells and up regulate perforin. Smyth *et al.* (69) demonstrated several years ago that IL-7 causes an increased accumulation of perforin gene transcripts in human CD8 T-cells. Similarly, I have found that IL-7 induces an increase in intra-cellular perforin expression in CD8 T-cells and that accumulation of perforin is time-dependent. Exposure to Tat protein, however, did not induce perforin synthesis in CD8 T-cells (figure 16C). Given the stable phenotype, absence of perforin induction and lack of proliferation, I conclude that the decrease in

Figure 16: Down regulation of CD127 by Tat is not due to CD8 T-cell activation. Purified CD8 T-cells (n=4) were incubated for 72 hours with HIV-1 Tat protein (10 μg/ml), anti-CD3 and anti-CD28 monoclonal antibodies, or IL-7 (20 ng/ml) as indicated. **(A)**, percent change in CD127 positive CD8 T-cells compared to media controls. **(B)**, percent change in CD25 positive CD8 T-cells compared to media controls. **(C)**, percentage of total CD8 T-cells expressing perforin. Mean values are shown +/- SEM.

A


media
αCD3+αCD28
Tat
% CD127 surface expression
120
100
80
60
40
20
0
0
24
48
72
Incubation time (hours)

B


%CD25 surface expression
200
150
100
50
0
media
αCD3+ αCD28
Tat
0
24
48
72
Incubation time (hours)

C

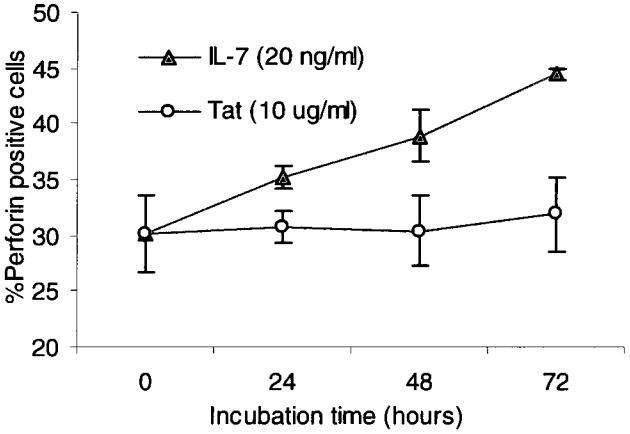

%Perforin positive cells
50
45
40
35
30
25
20
IL-7 (20 ng/ml)
Tat (10 ug/ml)
0
24
48
72
Incubation time (hours)

Table 2: Tat does not stimulate CD8 T-cell proliferation. CD8 T-cells were purified from healthy donors (n=3) and cultured in media alone, with Tat protein (10 μg/ml), or with anti-CD3 and anti-CD28 monoclonal antibodies. ^{3}H-Thymidine incorporation is shown in cpm with mean values +/- SEM.

Treatment Conditions	CPM (± SE)
Media	164 ± 10
HIV-1 Tat	107 ± 10
α-CD3 + α-CD28	48836 ± 8986

CD127 expression induced by exogenous purified Tat protein is not due to a nonspecific stimulation of CD8 T-cells.

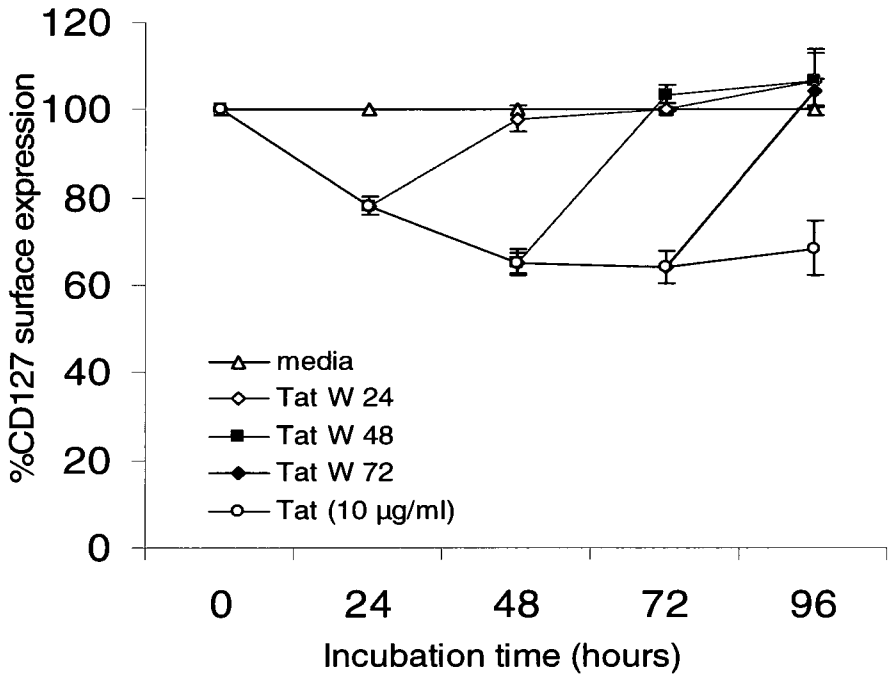
The effect of Tat on CD127 expression is reversible.

I questioned whether CD127 is irreversibly down regulated on CD8 T-cells by Tat or whether Tat is continuously required to maintain suppression. To investigate this, purified CD8 T-cells from healthy controls were cultured with Tat protein and at 24 hour intervals aliquots were removed, centrifuged at 1600 rpm and resuspended in fresh RPMI-20. As shown in figure 17, once Tat was removed from the culture media, CD127 surface expression recovered back to baseline within 24 hours and remained stable thereafter. Full recovery of CD127 was evident even on cells incubated with Tat for up to 72 hours. This suggests that Tat does not irreversibly affect CD8 T-cells but is instead continually required to maintain suppression of CD127. Of note, as can be seen in figure 17, cells cultured in the presence of Tat for up to 96 hours tended to show a slight rebound in CD127 expression. I attribute this to depletion of soluble Tat in our cultures. Indeed, if supplemental Tat protein was added at 48 or 72 hours this rebound was not evident (data not shown).

The concentration of Tat required to down regulate CD127 is dependent on cell concentration.

Relatively high concentrations of Tat protein (up to 10 μg/ml) were required to demonstrate a significant down regulation of CD127 expression on CD8 T-cells. This is

Figure 17: CD127 recovers on CD8 T-cells after HIV-1 Tat is removed from the culture. Purified CD8 T-cells (n=4) were incubated with purified Tat protein (10 μg/ml). At 24 (Tat W24), 48 (Tat W48) and 72 (Tat W72) hours cells were washed in PBS and placed in fresh media in the absence of Tat. Percent changes in CD127 positive CD8 T-cells compared to media controls are shown (mean +/- SEM).

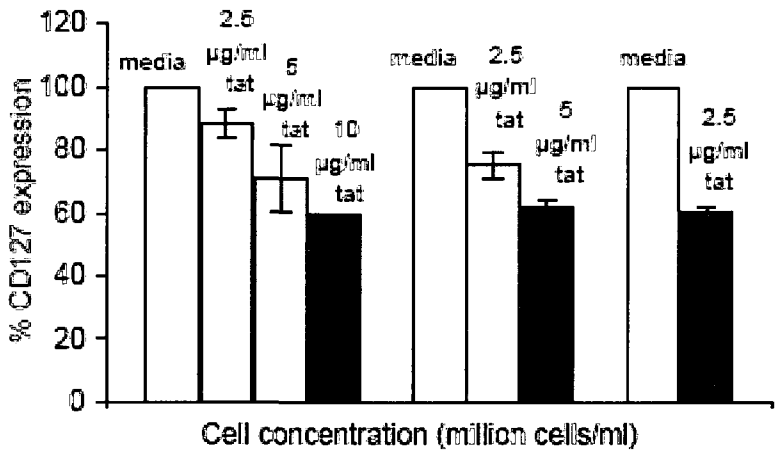

120
100
80
60
40
20
0
%CD127 surface expression
media
Tat W 24
Tat W 48
Tat W 72
Tat (10 µg/ml)
0
24
48
72
96
Incubation time (hours)

likely due to the lack of post-translational modification of Tat produced in E. coli, and the relative biological activity of a purified protein preparation. However, since Tat is taken up by individual cells in culture and so removed from the medium, one would also predict a stoichiometric relationship between cell concentration and concentration of Tat protein required in vitro to induce a decline in CD127. To investigate this possibility, CD8 T-cells were isolated from healthy donors and incubated in RPMI-20 at 1.0, 0.5 and 0.25 million cells per milliliter in the presence of decreasing concentrations of Tat protein. As predicted, less Tat was required to induce the same degree of CD127 down regulation when fewer cells were present in the culture (figure 18). Indeed, at 72 hours 2.5 μg/ml of Tat down regulated CD127 on CD8 T-cells by 11.5% +/- 4.5% in cultures of 1.0 million cells/ml, 24.8% +/- 4.0% in cultures of 0.5 million cells/ml, and 39.2% +/- 2.5% in cultures of 0.25 million cells/ml. Thus, the concentration of Tat required to induce a significant down regulation of CD127 on CD8 T-cells is determined at least in part by the concentration of cells present in the culture.

The presence of exogenous Tat protein impairs CD8 T-cell response to stimulation.

IL-7 plays an important role in the activation of cytotoxic CD8 T-cells. Signaling via the IL-7 receptor stimulates proliferation of CD8 T-cells and induces an up regulation of perforin. Since the HIV Tat protein down regulates CD127 on the surface of these cells, I questioned whether this effect was functionally significant. To examine this, purified CD8 T-cells (1.0×10^6 cells/ml) were pre-incubated in either medium alone or with Tat (10 μg/ml) for 72 hours, and then stimulated with anti-CD3 and anti-CD28 monoclonal antibodies +/- IL-7 (5 ng/ml). As shown in figure 19A, addition of IL-7

Figure 18: The concentration of Tat protein required to down regulate CD127 on CD8 T-cells is directly related to the concentration of cells in the culture. Purified CD8 T-cells (n=2) were cultured in RPMI-20 at 1.0(dark gey), 0.5 (light grey) and 0.25 (silver) million cells per milliliter each in the presence of 2.5, 5.0 or 10 μg/ml of Tat protein for 72 hours. Percent change in CD127 positive CD8 T-cells compared to media controls is shown (mean +/- SEM).

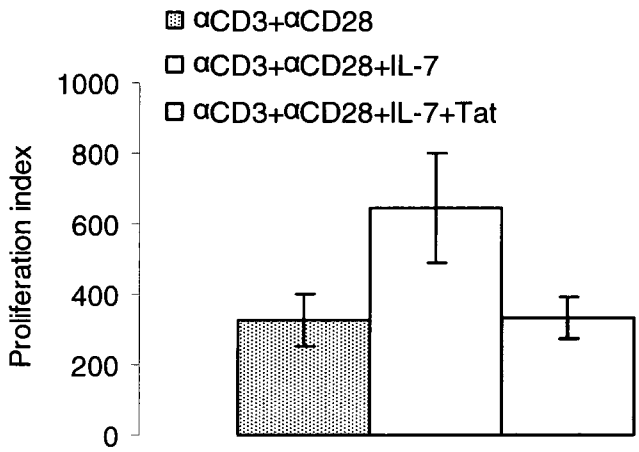

120
100
80
60
40
20
0
% CD127 expression
media
2.5 µg/ml tat
5 µg/ml tat
10 µg/ml tat
media
2.5 µg/ml tat
5 µg/ml tat
media
2.5 µg/ml tat
Cell concentration (million cells/ml)

enhances CD8 T-cell proliferation, with a proliferation index more than 2-fold greater than stimulation with anti-CD3 and anti-CD28 monoclonal antibodies alone. Pre-incubation with Tat, however, completely blocked the effects of IL-7. When stimulated with anti-CD3 and anti-CD28 monoclonal antibodies plus IL-7 (5 ng./ml), CD8 T-cells pre-treated with Tat proliferated to the same extent as untreated cells stimulated with only anti-CD3 and anti-CD28 antibodies. It would appear that by down regulating CD127, Tat is able to block stimulation with IL-7 and impair CD8 T-cell proliferation. Tat also inhibited CD8 T-cell proliferation to some extent in the absence of IL-7 suggesting additional pathways may be affected by this protein (data not shown). These additional effects may explain why the inhibition in cell proliferation was more complete than may have been anticipated given the fact that CD127 is not fully down regulated on all Tat-treated cells in our cultures.

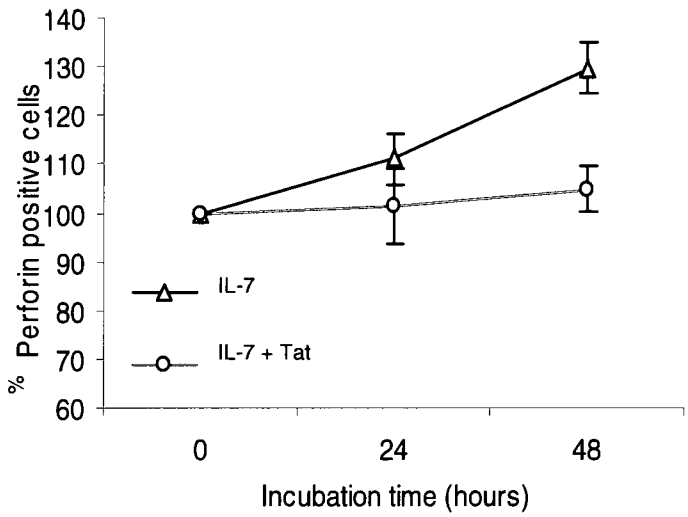
I also examined CD8 T-cells for their ability to synthesize perforin in the presence of exogenous Tat protein. While IL-7 (20 ng/ml) alone up regulates accumulation of intracellular perforin in CD8 T-cells, pre-incubation with Tat completely blocked this effect. As predicted, CD8 T-cells pre-treated with Tat showed no accumulation of perforin following addition of IL-7 to the culture (figure 19B). These data suggest that by down regulating CD127, Tat inhibits IL-7 induced accumulation of perforin in CD8 T-cells and thus likely impairs cytolytic activity.

Figure 19: Tat-induced down regulation of CD127 impairs CD8 T-cell proliferation and perforin synthesis in response to IL-7. Purified CD8 T-cells (n=6) were pre-incubated with Tat protein for 72 hours and then stimulated with either anti-CD3 and anti-CD28 monoclonal antibodies, IL-7 (20 ng/ml), or anti-CD3 and anti-CD28 monoclonal antibodies plus IL-7 (5 ng/ml). **(A)**, proliferation index as measured by ^{3}H-thymidine incorporation relative to non-stimulated media controls. **(B)**, percent change in perforin expressing CD8 T-cells compared to media controls. Mean values are shown +/- SEM.

A


αCD3+αCD28
αCD3+αCD28+IL-7
αCD3+αCD28+IL-7+Tat
Proliferation index
1000
800
600
400
200
0

B


% Perforin positive cells
140
130
120
110
100
90
80
70
60
IL-7
IL-7 + Tat
0
24
48
Incubation time (hours)

DISCUSSION

Impaired CD8 T-cell proliferation and function is well described during HIV infection. While declining CD4 T-cell help likely plays a role in reduced cell mediated immunity, direct effects on CD8 T-cells are also evident. Reduced expression of the IL-7 receptor α-chain on CD8 T-cells from HIV-infected patients with active viral replication has been shown by a number of groups (71). Here I show that the HIV Tat protein is likely responsible at least in part for this down regulation and that this down regulation results in impaired CD8 T-cell function.

Since Tat protein is secreted from infected cells during active HIV replication (105, 113) and in a paracrine fashion (88, 111) can alter protein expression in uninfected cells (90, 125-128), I questioned whether this protein exerted an effect on the expression of the IL-7 receptor α-chain on CD8 T-cells. Because infection of CD8 T-cells by HIV is likely a rare event, it seemed more biologically relevant to examine the effects of extracellular Tat on these cells. As predicted, purified Tat protein induced a significant decline in the expression of CD127 on CD8 T-cells isolated from healthy donors compared to cells maintained in medium alone. The effect was both dose and time dependent, and required the continual presence of Tat. Removal of Tat protein after up to 72 hours of incubation allowed full recovery of CD127 over the ensuing 24 hours. Interestingly, Tat down regulated CD127 expression equally on both naïve (CD45RA+CD62L+) and memory (CD45RO+) CD8 T-cells mirroring the bulk CD8 T-cell population in both kinetics and extent of decline. Prior treatment with Proteinase K, anti-Tat monoclonal antibodies and pre-incubation with heparin all abolished the ability of Tat to down regulate CD127. Further, purified HIV gp160, HIV Nef and whole killed

Candida had no effect on the expression of CD127. Taken together these data demonstrate that exogenous HIV Tat protein down regulates the IL-7 receptor α-chain on CD8 T-cells.

In view of the important role IL-7 plays in CD8 T-cell function, I questioned whether the reduction in CD127 expression induced by Tat was functionally significant. As expected, IL-7 significantly enhanced CD8 T-cell proliferation over stimulation with anti-CD3 plus anti-CD28 monoclonal antibodies. Pre-incubation with Tat, however, completely eliminated the effects of IL-7 and reduced proliferation back to levels similar to stimulation with anti-CD3 plus anti-CD28 antibodies alone. The inhibition of proliferation was more complete than anticipated suggesting Tat may have additional effects on cell cycle regulation. IL-7 also plays an important role in up regulating CD8 T-cell cytolytic activity specifically by increasing expression of perforin. Here again pre-incubating CD8 T-cells with Tat inhibited accumulation of intracellular perforin in response to IL-7. Thus, by down regulating CD127 expression on CD8 T-cells, HIV Tat is able to block the stimulatory effects of IL-7 and impair both CD8 T-cell proliferation and cytolytic capacity.

Interestingly, this is not the first time Tat has been shown to inhibit T-cell proliferation. Years ago Viscidi *et al.* (115) demonstrated purified Tat protein inhibited the proliferation of PBMC from healthy donors following stimulation with tetanus toxoid. The inhibitory effect was concentration dependent with 10 μg/ml Tat inhibiting proliferation by 81%. Similarly, Chirmule *et al.* (98) later demonstrated purified Tat protein (1 to 3 μg/ml) inhibited proliferation of CD8 T-cells cultured in the presence of autologous irradiated non-T cells and stimulated with anti-CD3 monoclonal antibodies.

As in our study, Tat did not affect cell viability nor did Tat affect the surface expression of CD25. Interestingly, these authors also showed Tat did not disrupt intracellular signaling following stimulation of the T-cell receptor (TCR) indicating the inhibitory effects of Tat were mediated through a different pathway. I suggest the inhibition observed in these studies was the result of Tat-induced down regulation of CD127 and the loss of IL-7 signaling in these mixed cell cultures.

While Tat down regulated CD127 on the surface of healthy CD8 T-cells, I found no effect on the overall phenotype of these cells or on cell viability. Several authors have suggested that during chronic viral infection persistent antigen leads to continuous CD8 T-cell activation resulting in decreased CD127 expression and cellular exhaustion. Indeed, in mice previously infected with a weakly replicating strain of LCMV, persistent gp33 antigen caused a decrease in CD127 expression on LCMV gp33-specific CD8 T-cells (124). Consistent with immune activation, these cells exhibited an effector phenotype with increased granzyme B expression, increased annexin V staining, and a gradual decline in number. In contrast, Tat does not appear to induce CD127 down regulation by activating CD8 T-cells. Exposure to Tat did not result in an up regulation of the activation markers CD25, CD38 or HLA-DR, nor did it stimulate cell proliferation or accumulation of perforin. In fact, no changes in the expression of a number of cell surface markers were detected suggesting a stable CD8 T-cell phenotype in the presence of purified Tat protein. Thus Tat does not appear to induce down regulation of CD127 through nonspecific stimulation of CD8 T-cells.

In our experiments Tat was required in nanomolar concentrations (2.5 to 10 μg/ml) to down regulate CD127 on CD8 T-cells. While this concentration is similar to

that used in most in vitro studies (98, 113, 115), it is about 10-fold higher than estimates of Tat concentration in patient sera (300-500 ng/ml)(114). There are a number of reasons why more protein may be required in vitro. First, I have demonstrated here that the concentration of Tat required to down regulate CD127 is proportional to the concentration of cells in the culture (figure 16). Second, the Tat protein used in our experiments is produced in bacteria and thus is unlikely to be post-translationally modified. Tat is known to associate with the eukaryotic acetyltransferases p300, PCAF and hGCN5 (134-136), and acetylation of Tat at Lys28 and Lys 50 both independently enhance Tat activity in transcriptional assays (134-136). It may be that lower concentrations of the more active acetylated form of Tat will be required to down regulate CD127 on CD8 T-cells in vitro. This is currently being investigated in our laboratory. Finally, other factors in addition to Tat may be involved in the down regulation of CD127 in vivo. IL-2, IL-4, IL-6, IL-7 and IL-15 all down regulate CD127 on CD8 T-cells isolated from mice (33). Thus it may be that Tat amplifies normal feed-back mechanisms regulating IL-7 receptor expression. Lower concentrations of Tat then may be required in vivo where Tat is appropriately post-translational modified and is not studied in isolation.

While CD127 expression is reduced on the majority of CD8 T-cells in untreated HIV-infected individuals (71), I have consistently found only 35-45% of CD8 T-cells from healthy donors significantly down regulate CD127 upon exposure to purified Tat in vitro. There are a number of possible explanations for this observation. First, the half-life of Tat protein in solution is estimated to be approximately 24 hours (113). It is possible then that in our in vitro experiments Tat becomes limiting. I doubt this is the case, however, as addition of supplemental Tat to our cultures at 48 and 72 hours did not result

in a further suppression of CD127 expression (data not shown). It is also possible that specific subpopulations of CD8 T-cells are unaffected by Tat. These cells may be depleted in HIV-infected patients yet comprise a significant proportion of the total population in healthy individuals and remain CD127-positive following exposure to Tat in culture. While I have not examined all CD8 T-cell phenotypes, down regulation of CD127 on both naïve and memory cells mirrors the total CD8 T-cell population with neither subset demonstrating a more limited effect. This possibility requires further investigation. A third possibility is that following the initial rapid decrease in CD127 on CD8 T-cells, Tat continues to induce a slow decline in CD127 expression beyond 72 hours. Certainly CD8 T-cells isolated from HIV-infected individuals will have been exposed to Tat for a considerably longer period of time. A final and perhaps more likely explanation is that Tat does not affect CD127 expression in isolation. Indeed, as I demonstrate in chapter 5, Tat and IL-7 act synergistically to down regulate CD127 to levels reflective of what is seen in patients. Thus, the combined effects of Tat and IL-7 in HIV-infected patients may have a greater effect in reducing CD127 expression than either alone.

CHAPTER 4 - THE HIV TAT PROTEIN MAINTAINS SUPPRESSION OF THE INTERLEUKIN-7 RECEPTOR ON CD8 T-CELLS ISOLATED FROM HIV+ PATIENTS

RATIONALE

CD127 is down regulated on CD8 T-cells in HIV+ patients and recovers following viral suppression with highly active antiretroviral therapy (HAART). (71-76) Interestingly, CD127 recovery is co-related with duration of viral suppression (time on HAART) (71). This suggests that continued HIV-1 viral replication plays a role in decreased CD127 expression on CD8 T-cells. It is not clear whether this recovery occurs on existing T-cells or reflects a repopulation of new cells during immune reconstitution. In the previous chapter I showed that soluble HIV Tat protein specifically down regulates CD127 on CD8 T-cells isolated from healthy HIV-negative volunteers.(118) Interestingly, Tat-induced down regulation of CD127 on the surface of CD8 T-cells required the continual presence of this viral protein. When Tat protein was removed from the culture media, CD127 returned to normal levels with 24 hours.(118)

In light of the fact that soluble Tat protein may be in part responsible for decreased CD127 expression on CD8 T cells, I questioned if CD8 T cells isolated from HIV infected patients maintained in culture media ex vivo would re-express CD127. If CD127 expression recovers, it would provide evidence that a soluble factor is responsible for maintaining suppression of CD127 expression on CD8 T-cells in HIV infected patients. Furthermore, in view of my previous findings demonstrating soluble HIV Tat protein specifically down regulates CD127 on the surface of CD8 T-cells isolated from healthy volunteers, I questioned whether this viral protein alone could maintain suppression of the IL-7 receptor on CD8 T-cells isolated from HIV+ individuals.

RESULTS

Patients

Patient characteristics and demographics (N=11) are shown in Table 3. The mean age was 39 years (range 21-56) and all were men. The mean CD3+CD4+ count was 355 cells/μl or 23% (range 203-573; 13-41%) with a mean CD3+CD8+ count of 1043 cells/μl or 60% (range 246-2411; 37-73%). The average CD4+/CD8+ ratio was 0.41. HIV viral loads ranged from 484 to 500,000 copies/ml with a mean of 94,023 copies/ml. Nine patients had never received antiretroviral therapy and 2 had been previously treated but had discontinued therapy five months prior to enrolment. The patients were otherwise in good health at the time of analysis.

CD127 recovers ex vivo on CD8 T-cells isolated from HIV+ individuals

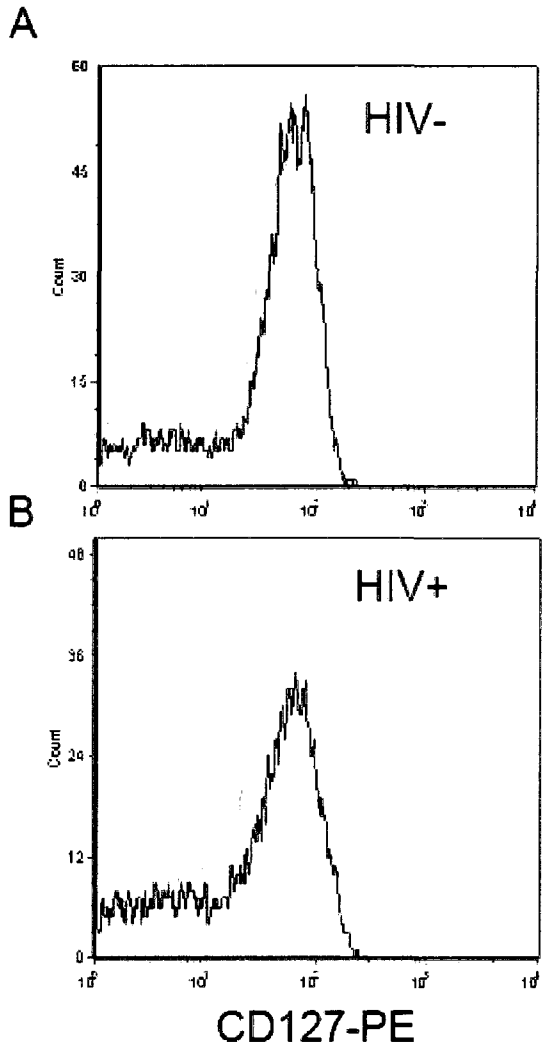
I have previously shown soluble HIV Tat protein down regulates CD127 on healthy CD8 T-cells in culture.(118) This down regulation is reversible and CD127 recovers back to normal levels once Tat is removed from the culture media. This prompted us to ask whether CD8 T-cells isolated from HIV+ individuals could also re-express CD127 when maintained in culture media ex vivo. To address this, CD8 T-cells were isolated from HIV-infected patients not on therapy and a portion of the cells were analyzed immediately for CD127 expression by flow cytometry. The remainder of the cells were washed in PBS and incubated for up to 72 hours in RPMI-20, and CD127 expression was monitored by flow cytometry at 24 hour intervals. Figure 20 shows typical flow histograms demonstrating CD127 expression on CD8 T-cells from an HIV-negative individual (panel A) and from an HIV+ individual (panel B) immediately

Table 3: Study Patients

Participant	Age (years)	Sex	CD3CD4 cells/ml (%)	CD3CD8 cells/ml (%)	CD4/CD8 ratio	Viral load (copies/ml)	Prior Rx
1	42	M	203 (16)	817 (64)	0.25	13,510	No
2	21	M	247 (17)	995 (69)	0.25	7,953	No
3	39	M	271 (18)	967 (65)	0.28	105,425	Yes
4	30	M	474 (28)	932 (54)	0.51	22,051	No
5	44	M	344 (22)	923 (59)	0.37	500,000	No
6	56	M	390 (24)	868 (53)	0.45	75,216	No
7	24	M	272 (41)	246 (37)	1.1	61,517	No
8	52	M	359 (21)	1091 (64)	0.3	484	No
9	43	M	530 (33)	850 (53)	0.6	27,158	No
10	43	M	573 (16)	2411 (66)	0.2	109,771	Yes
11	41	M	248 (13)	1376 (73)	0.2	111,174	No
Average	39		355 (23)	1043 (60)	0.41	94,023	

Abbreviations: M: male; %: percent lymphocytes; Rx: antiretroviral therapy.

Figure 20: CD127 recovers ex vivo to near normal levels on CD8 T-cells isolated from an HIV-infected individual. CD127 expression on purified CD8 T-cells is shown immediately following cell isolation (gray fill) and after 24 hours in culture medium (black line). Two representative histograms are shown, one from a healthy HIV-negative volunteer (panel A) and one from an HIV-infected patient with a CD4 count of 359 cells/μl and an HIV viral load (VL) of 484 copies/ml (panel B).


A
HIV-
Count
B
HIV+
Count
CD127-PE

following CD8 T-cell isolation (grey fill) and after 24 hours in culture media (black line). Consistent with previous reports, CD127 expression was significantly decreased on CD8 T-cells from the HIV+ individual immediately following isolation. However, when purified CD8 T-cells from this same individual were maintained ex vivo in RPMI-20, CD127 recovered over 24 hours to levels equivalent to that seen in the HIV-negative control. This was demonstrated in both percent positive staining cells and in mean channel fluorescence. In comparison, CD127 expression increased only marginally on CD8 T-cells isolated from the HIV-negative individual. Figure 22 shows similar data in composite form for seven HIV+ patients (participants 1-7). The average mean channel fluorescence for CD127 staining on CD8 T-cells increased from 3.45 +/- 0.19 (mean +/- standard error of the mean) at the time of isolation to 5.59 +/- 0.61 after 24 hours in culture media ($p=0.005$). I therefore conclude that suppression of CD127 on CD8 T-cells in HIV+ individuals is reversible and recovers within 24 hours ex vivo. This suggests a soluble factor or factors are responsible for the down regulation of CD127 in vivo.

HIV Tat protein maintains suppression of CD127 on CD8 T-cells isolated from HIV+ patients

In view of our previous findings demonstrating soluble HIV Tat protein specifically down regulates CD127 on the surface of CD8 T-cells isolated from healthy volunteers, I questioned whether this viral protein alone could maintain suppression of the IL-7 receptor on CD8 T-cells isolated from HIV+ individuals. To address this, CD8 T-cells were purified from HIV+ patients as described and incubated in either RPMI-20 alone or in media containing purified Tat protein. Figure 21 shows results from three representative patients. In all cases, CD127 expression increased within 24 hours on CD8

Figure 21 : Soluble HIV Tat protein maintains suppression of CD127 ex vivo on CD8 T-cells isolated from HIV-infected individuals. CD8 T-cells isolated from HIV-positive patients were cultured in media alone (panel A) or in media containing purified Tat protein at 2 μg/ml (panel B) or 10 μg/ml (panel C), or in media containing 200 pg/ml IL-7 (panel D) for 24 hours. Three representative HIV-positive patients are shown (Patient 5: CD4= 344 cells/μl and VL=500,000 copies/ml; Patient 6: CD4=390 cells/μl and VL=75,216 copies/ml; Patient 7: CD4=272 cells/μl and VL=61,517 copies/ml). CD127 expression on purified CD8 T-cells is shown immediately following cell isolation (gray fill) and after 24 hours in culture (black line).

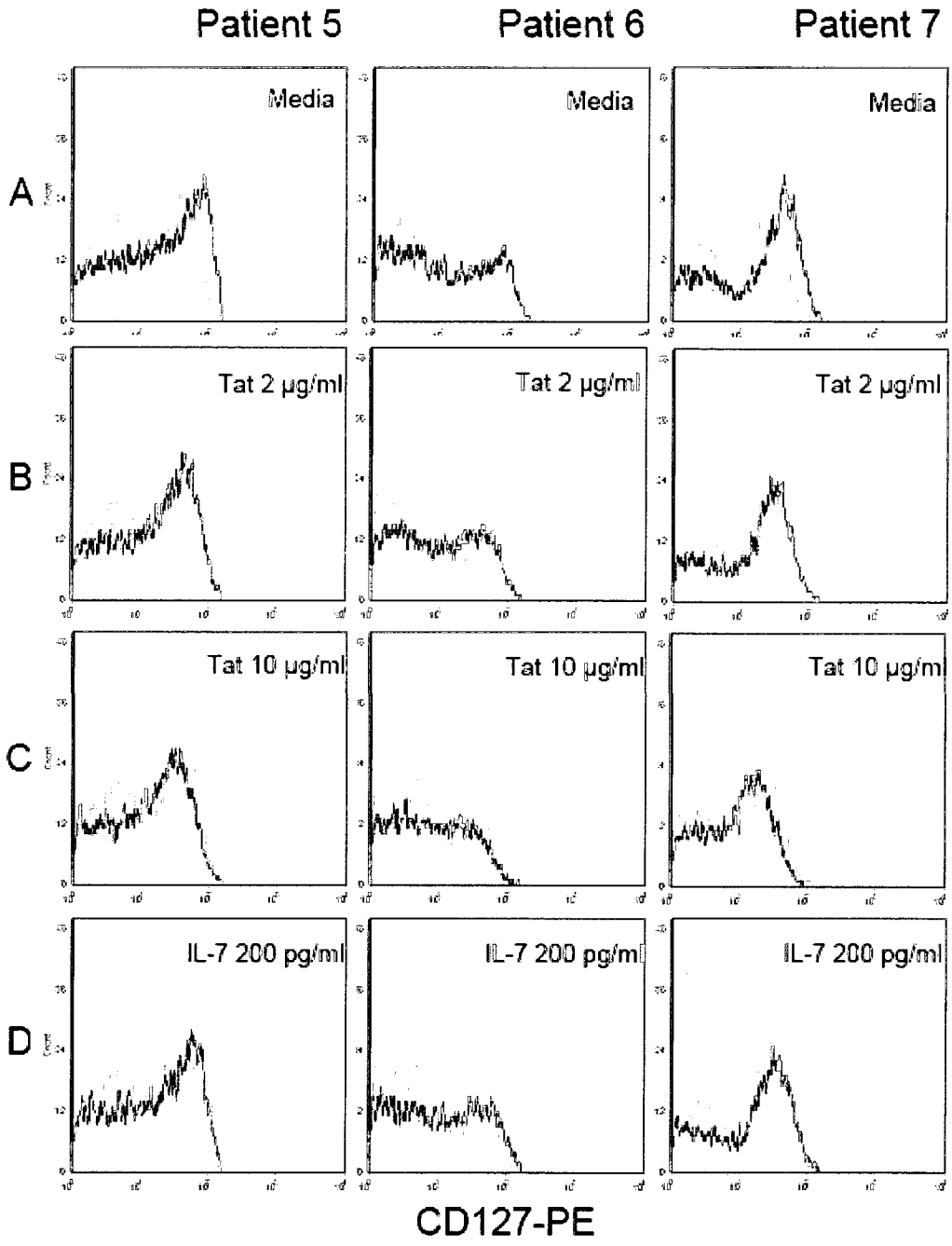
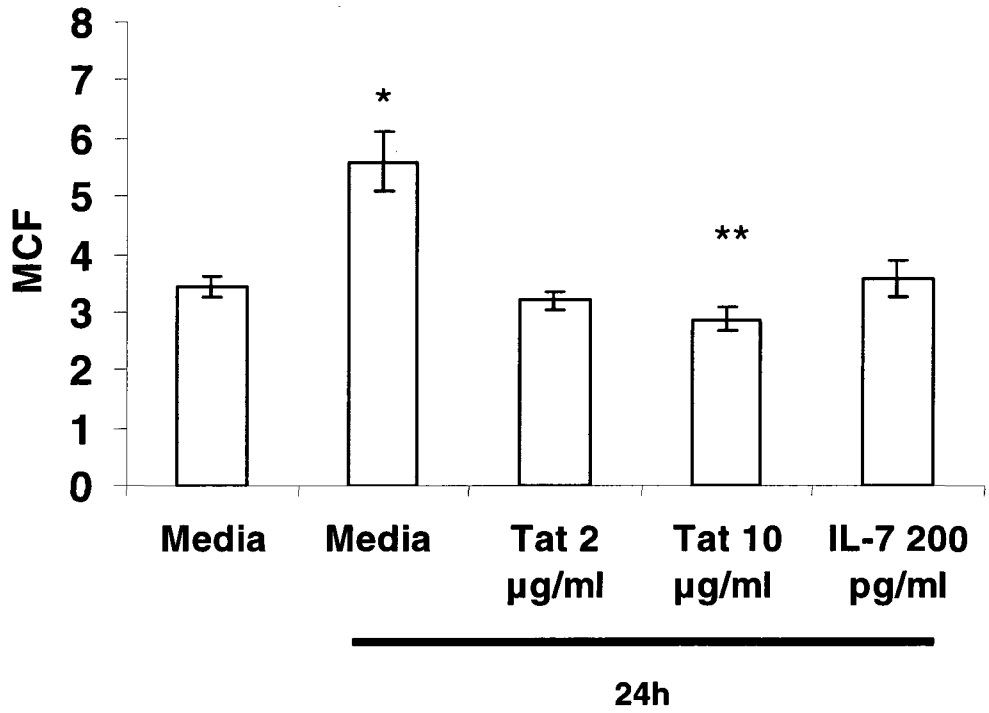

Patient 5
Patient 6
Patient 7
A
B
C
D
Media
Tat 2 µg/ml
Tat 10 µg/ml
IL-7 200 pg/ml
CD127-PE

Figure 22: While CD127 expression recovers on CD8 T-cells isolated from HIV+ patients and maintained in media alone, this recovery is prevented by soluble HIV Tat protein or IL-7. CD8 T-cells isolated from HIV-positive patients (N=7) were cultured in media alone, or in media containing purified Tat protein at 2 μg/ml or 10 μg/ml, or in media containing 200 pg/ml IL-7 for 24 hours. CD127 mean channel fluorescence increased on cells maintained in media alone for 24 hours compared to the time of isolation (* p=0.005), and decreased slightly when cultured in the presence of 10 μg/ml soluble Tat protein (** p=0.007). CD127 expression did not recover but remained low on CD8 T-cells cultured in 2 μg/ml Tat or 200 pg/ml IL-7. The CD127 mean channel fluorescence was not statistically different under these conditions compared to expression at the time of isolation (0h).


MCF
8
7
6
5
4
3
2
1
0
*
**
Media
Media
Tat 2 µg/ml
Tat 10 µg/ml
IL-7 200 pg/ml
24h

T-cells isolated from HIV+ individuals and maintained in media alone (panel A; grey fill, immediately following isolation; black line, after 24 hours in culture media). In contrast, when the cells were cultured in the presence of soluble Tat protein, CD127 expression remained low (figure 22, panel B). Indeed, Tat concentrations of 2 μg/ml maintained suppression of CD127 on CD8 T-cells with an average mean channel fluorescence of 3.20 +/- 0.21, essentially unchanged compared to the levels of CD127 at the time of isolation (p=0.39; figure 21). Interestingly, higher concentrations of Tat protein (10 μg/ml) were able to further suppress CD127 expression albeit slightly after 24 hours in culture (figure 22, panel C). Under these conditions, the average CD127 mean channel fluorescence decreased to 2.63 +/- 0.19 (p=0.007 compared to time of isolation; figure 21).

IL-7 has been shown to down regulate the expression of its own receptor.(33, 35, 76) I therefore also asked whether IL-7 could similarly maintain suppression of CD127 ex vivo. As shown in figure 21 panel D, IL-7 at 200 pg/ml did inhibit full recovery of CD127 on CD8 T-cells from HIV+ individuals. In the presence of IL-7, the average CD127 mean channel fluorescence remained low over 24 hours at 3.57 +/- 0.33, not statistically different from the level of CD127 at the time of isolation (p=0.75; figure 22).

To more fully describe the dynamics of CD127 recovery and its suppression by Tat and IL-7, CD8 T-cells isolated from six HIV+ individuals (participants 6-11) were followed over 72 hours. Figure 23 shows one representative patient (Participant 7) while composite data for all six patients are shown in figure 24. While time to peak recovery varied somewhat from patient to patient, CD127 expression increased on purified CD8 T-cells cultured in media alone achieving levels comparable to HIV-negative controls

Figure 23: Soluble HIV Tat protein maintains suppression of CD127 expression on CD8 T-cells isolated from an HIV-infected individual over at least 72 hours while IL-7 delays recovery. CD8 T-cells were isolated from an HIV-infected patient (Patient 7: CD4=272 cells/μl and VL=61,517 copies/ml) and incubated in media alone (panel A) or in media supplemented with 2 μg/ml Tat (panel B), 10 μg/ml Tat (panel C), or 200 pg/ml IL-7 (panel D) for 72 hours. CD127 expression on purified CD8 T-cells is shown immediately following cell isolation (gray fill) and after 24, 48 and 72 hours in culture (black lines).

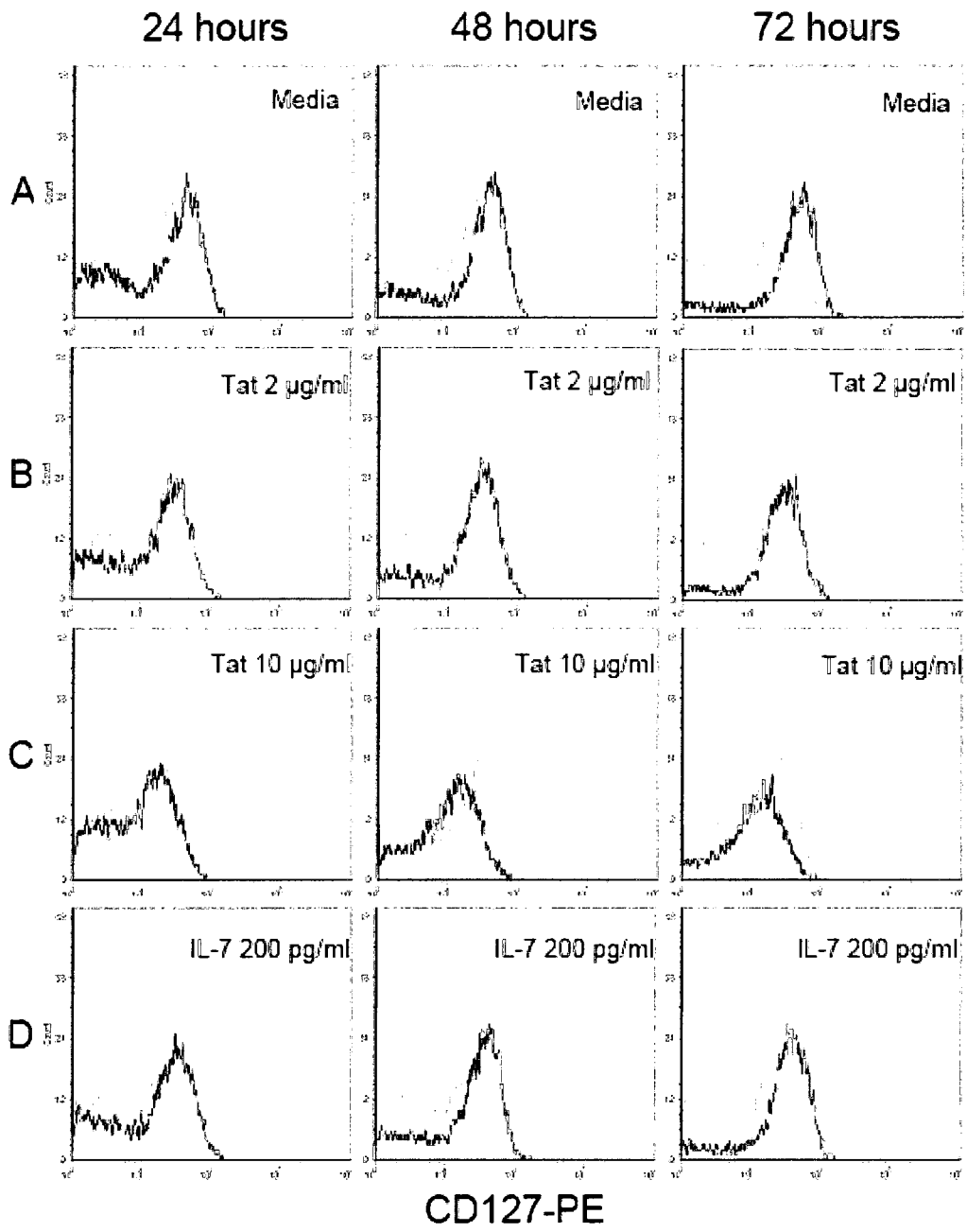
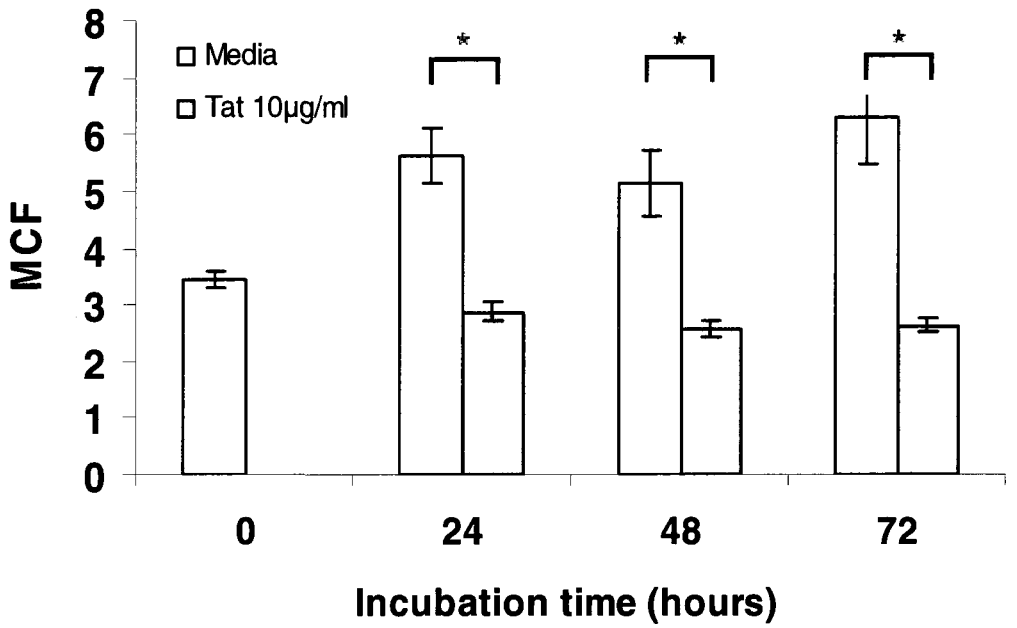

24 hours
48 hours
72 hours
A
B
C
D
Media
Tat 2 µg/ml
Tat 10 µg/ml
IL-7 200 pg/ml
CD127-PE

Figure 24: Soluble HIV Tat protein maintains suppression of CD127 expression on CD8 T-cells from HIV-infected individuals over at least 72 hours. CD8 T-cells were isolated from HIV-infected patients (N=6) and incubated in media alone, or in media containing 10 μg/ml Tat protein for 72 hours. For cells cultured in media alone, CD127 mean channel fluorescence increased within 24 hours and was maintained thereafter ($p \leq 0.01$ at 24, 48 and 72 hours compared to time of isolation). CD127 expression was not statistically different comparing cells cultured in media for 48 and 72 hours compared to 24 hours ($p=0.70$ and 0.98 respectively) When cells were cultured in the presence of Tat protein, CD127 expression remained low with mean channel fluorescence readings significantly lower compared to cells cultured in media alone.


8
7
6
5
4
3
2
1
0
MCF
Media
Tat 10µg/ml
*
*
*
0
24
48
72
Incubation time (hours)

within 24 hours and was maintained thereafter (figure 23, panel A). As shown in figure 24 and consistent with the data above, the average CD127 mean channel fluorescence increased from 3.52 +/- 0.19 at the time of isolation to 6.35 +/- 0.54 after 24 hours in culture media ($p=0.0003$), and then remained stable. When CD8 T-cells were cultured in media containing purified Tat protein, suppression of CD127 was fully maintained for up to at least 72 hours (figure 23, panels B and C). In the presence of 10 μg/ml Tat, CD127 mean channel fluorescence remained less than 3.2 over the 72 hours and well below recovered CD127 levels on cells maintained in parallel in media alone ($p \leq 0.003$). In contrast, recovery of CD127 on CD8 T-cells was simply delayed in the presence of IL-7 (200 pg/ml). At 24 hours, CD127 remained low in the presence of IL-7 but increased thereafter (figure 23, panel D).

DISCUSSION

Decreased expression of CD127 on the surface of CD8 T-cells in individuals with active HIV replication has been well described (71-76) and I have shown in chapter 3 that soluble HIV Tat protein specifically down regulates CD127 on the surface of CD8 T-cells isolated from healthy HIV-negative volunteers.(118) . I show here that once cultured ex vivo, purified CD8 T-cells from HIV+ patients are able to re-express CD127. This indicates reduced expression of the IL-7 receptor in vivo is not irreversible but instead is the result of active suppression by soluble factors in the patient serum. Similar recovery of CD127 on CD8 T-cells in mixed PBMC cultures from HIV+ patients has also been reported by Colle *et al.*(74) In their study, CD127 expression increased significantly after 3 days in culture on all CD8 T-cell subsets and most notably on naïve, central memory and effector memory cells. Rethi *et al.*(76) failed to detect CD127 recovery ex vivo but in their study they reported only percent low/negative T-cells and therefore may have missed an increase in cell surface receptor expression had they measured change in mean channel fluorescence. More importantly, however, these authors tried to measure CD127 expression on CD8 T-cells in mixed T-cell cultures from HIV+ patients where persistently infected CD4 T-cells may have been a source of ongoing Tat production. Indeed, lack of CD127 recovery under these conditions is consistent with our model presented here.

As noted in the previous chapter, suppression of CD127 on CD8 T-cells by Tat was maintained only if Tat was continuously present in the media. When Tat was removed from the media, CD127 recovered on the cell surface to pre-treatment levels within 24 hours.(118) I have shown here soluble HIV Tat protein alone also maintains

decreased CD127 expression on CD8 T-cells isolated from HIV+ individuals. In all patients examined, CD127 uniformly remained low in the presence of soluble Tat protein and essentially unchanged compared to the time of isolation. These data suggest soluble Tat protein may well play an active role in suppressing IL-7 receptor expression on CD8 T-cells in vivo. Interestingly, lower concentrations of Tat are required to maintain suppression of CD127 on CD8 T-cells isolated from HIV+ individuals compared to concentrations needed to down regulate this receptor de novo on CD8 T-cells isolated from healthy HIV-negative volunteers. In the previous chapter I found 2 μg/ml of Tat induce a 4 +/- 1% decrease in CD127 expression on healthy CD8 T-cells while 10 μg/ml induced a 39 +/-3% decline compared to cells maintained in media alone.(32) Here, Tat at 2 μg/ml was able to maintain full suppression of CD127 ex vivo on cells isolated from HIV+ patients. That lower concentrations are required to maintain receptor suppression is not surprising given the effects of Tat on CD127 appear to be stoichiometric. It is possible that protein-protein interactions between Tat and CD127 necessary for receptor down regulation mandate higher Tat concentrations in healthy CD8 T-cells where the density of CD127 on the cell surface is much higher, whereas lower Tat concentrations could be sufficient to prevent re-accumulation of CD127 on the surface of cells isolated from HIV+ patients. This stoichiometric relationship between soluble Tat protein and CD127 was also noted in chapter 3 with respect to Tat concentrations required to down regulate CD127 relative to cell density in culture.(118) The concentration of Tat protein required to down regulate CD127 on CD8 T-cells is also greatly influenced by the presence of IL-7. Indeed, as I will show in chapter 5 there is a synergistic effect between Tat and IL-7 in the down regulation of CD127, an effect dependent on JAK kinase

activity.(32) I also show here that IL-7 at 200 pg/ml is able to delay the ex vivo recovery of CD127 on the surface of CD8 T-cells isolated from HIV+ individuals but that this effect is not as durable as with Tat protein. The combined effects of Tat and IL-7 were not addressed in these experiments.

My observation that soluble HIV Tat protein alone is able to inhibit recovery of CD127 on the surface of CD8 T-cells isolated from HIV+ patients provides further evidence that Tat likely plays a major role in the suppression of CD127. This in conjunction with our previous data showing Tat induced down regulation of CD127 results in impaired CD8 T-cell proliferation and cytolytic potential lends additional support to the idea that soluble Tat protein likely contributes to poor lymphocyte responses and overall immune dysregulation in HIV+ patients.

CHAPTER 5 - INTERLEUKIN-7 AND THE HIV TAT PROTEIN ACT SYNERGISTICALLY TO DOWN REGULATE CD127 EXPRESSION ON CD8 T-CELLS

RATIONALE

IL-7 down regulates the expression of its own receptor in vitro. IL-7 has been shown to down regulate CD127 expression in murine lymph node T (LNT) cells (33) and human thymocytes (137). In addition, IL-7 has also been shown by several groups to down regulate CD127 mRNA transcripts and surface expression in resting primary CD4 and CD8 T-cells as early as 2 hours after exposure to IL-7(33). Whether IL-7 mediated down regulation of CD127 is physiologically relevant, however, is not clear. While HIV infected individuals have increased plasma concentrations of IL-7 compared to uninfected controls (45, 76, 82, 83), it is notable that concentrations in the range of 500 to 10,000 pg/ml are required in vitro to down regulate CD127 on purified CD8 T-cells isolated from healthy volunteers (33, 77). Since plasma IL-7 concentrations average 2.2 pg/ml in HIV-negative individuals and 12 to 55 pg/ml (45, 76, 82, 83) 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 SIV-infected macaques where administration of supra-physiologic concentrations of IL-7 exceeding 1000 pg/ml in plasma were required to down regulate CD127 on circulating CD8 T-cells (48). It therefore appears that elevated serum IL-7 concentrations alone may not be solely responsible for the low CD127 expression on CD8 T-cells in HIV-infected patients.

I have shown in chapter 3 that soluble HIV Tat protein down regulates CD127 on the surface of CD8 T-cells isolated from healthy HIV-negative volunteers (118). As with

IL-7, I found 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 (134-136). Alternatively, host factors may work in concert with Tat to down regulate CD127 in vivo. With that in mind, I questioned whether Tat and IL-7 together affect CD127 expression to a greater extent than either one alone.

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 (77) and mice (33). In agreement with this, I found IL-7 decreased CD127 on the surface of purified CD8 T-cells isolated from healthy volunteers in a dose-dependent manner. As shown in figure 25, cells incubated in RPMI-20 with increasing concentrations of IL-7 (100-1000 pg/ml) for 24 hours demonstrated an incremental decrease in CD127 expression. The effect was linear with 200 pg/ml inducing a 16.4% +/- 3.2% decline in receptor expression and 500 pg/ml causing a 33.2% +/- 6.9% decline compared to cells maintained in media alone (p=0.002).

I have previously demonstrated soluble HIV Tat protein also down regulates CD127 on the surface of CD8 T-cells (118). The effect was specific to CD127 and I 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-7 receptor (118). 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 lipopolysaccharide (LPS) or induction of apoptosis (118). As shown in figure 26 and consistent with our previous findings, Tat-induced down regulation of CD127 on the surface of CD8 T-cells is dose-dependent where 2 μg/ml soluble Tat protein induces a 4% +/- 1% decrease in CD127 expression

Figure 25: 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 hours, 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 pg/ml or 1000 pg/ml. **(B)** Composite data for n=8. Values represent percent positive CD8 T-cells expressing CD127 relative to media control +/- standard error of the mean (SEM). Bars marked with a * indicate values that are statistically different ($p<0.05$) from media control

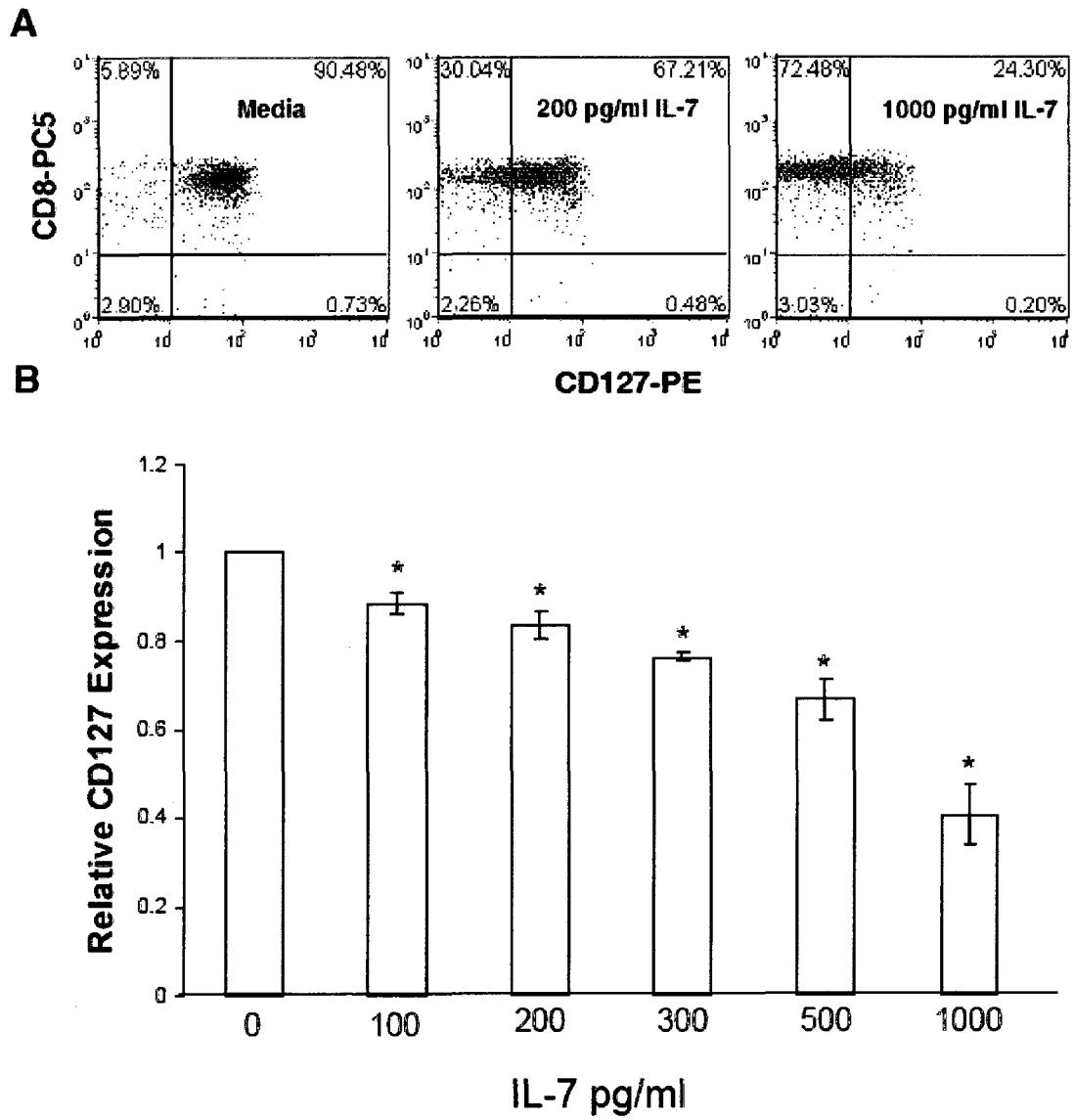
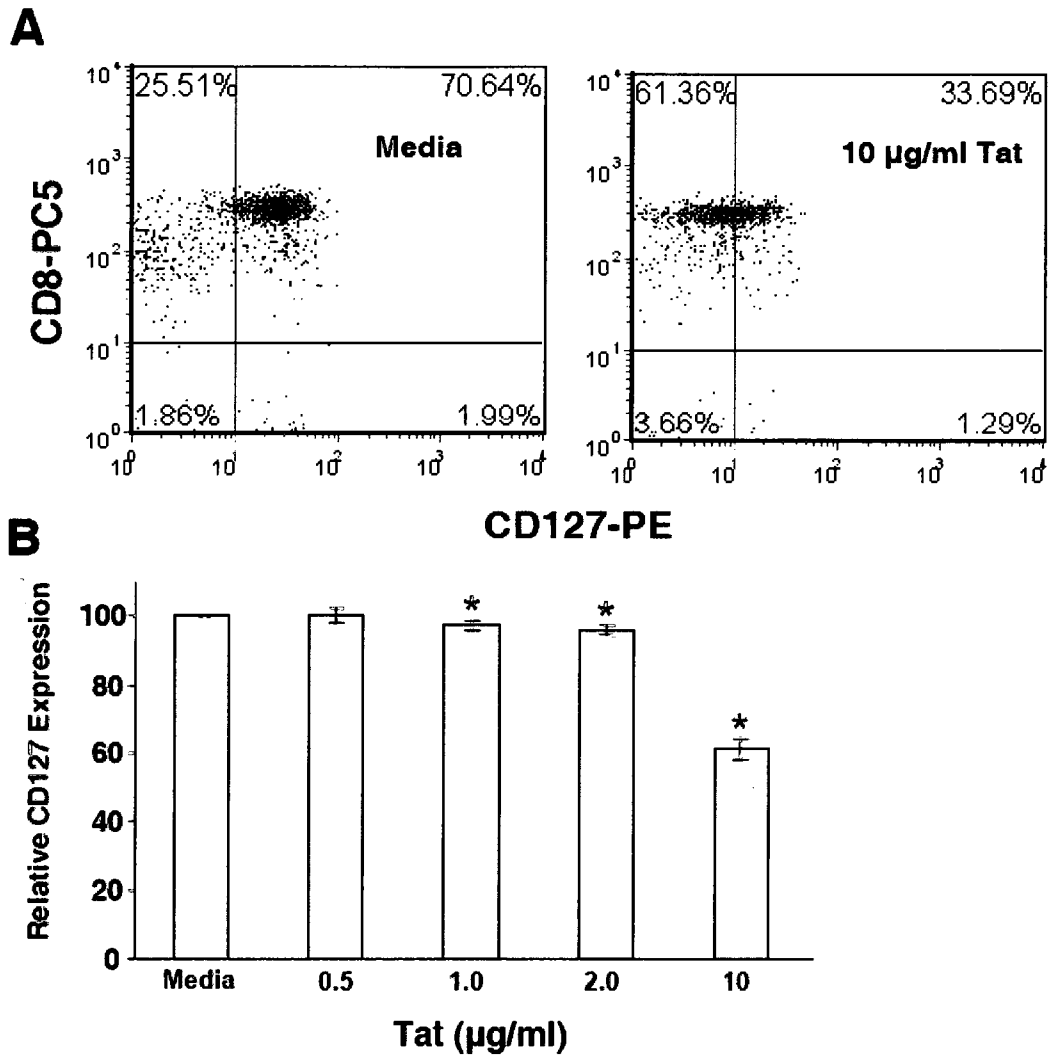

A
5.89%
90.48%
Media
2.90%
0.73%
30.04%
67.21%
200 pg/ml IL-7
2.26%
0.48%
72.48%
24.30%
1000 pg/ml IL-7
3.03%
0.20%
CD8-PC5
CD127-PE
B
Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
500
1000
IL-7 pg/ml

Figure 26: 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 hours, 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. (**B**) Composite data for n=4. Values represent percent positive CD8 T-cells expressing CD127 relative to media control +/- SEM. Bars marked with a * indicate values that are statistically different ($p<0.05$) from media control.


A
CD8-PC5
25.51%
70.64%
Media
1.86%
1.99%
61.36%
33.69%
10 µg/ml Tat
3.66%
1.29%
CD127-PE
B
Relative CD127 Expression
100
80
60
40
20
0
Media
0.5
1.0
2.0
10
Tat (µg/ml)

and 10 μg/ml Tat induces a 39% +/-3% decline compared to 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) to down regulate CD127 exceed the concentrations of IL-7 reported in the sera of both healthy controls (average 2.2 pg/ml) and in HIV-infected individuals (12-55 pg/ml) (45, 76, 82, 83). Likewise, Tat is required at nanomolar concentrations (5-10 μg/ml) in vitro to down regulate CD127, some 10 to 20-fold higher than estimates of Tat concentrations in patient sera (300-500 ng/ml) (114). In view of the fact that Tat and IL-7 each independently down regulate CD127 expression, I 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 of Tat has no effect on CD127 expression and 200 pg/ml 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 hours (figure 27A, Table 4). Synergy was evident over a range of concentrations and was observed whether Tat concentration was held constant relative to IL-7 or vice versa (figure 27B, Table 4).

Figure 27: 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 hours. 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 Tat, 200 pg/ml IL-7, or both. **(B)** Representative dot-plots of CD8 T-cells from a second individual incubated in media alone, 10 μg/ml Tat, 300 pg/ml IL-7, or both.

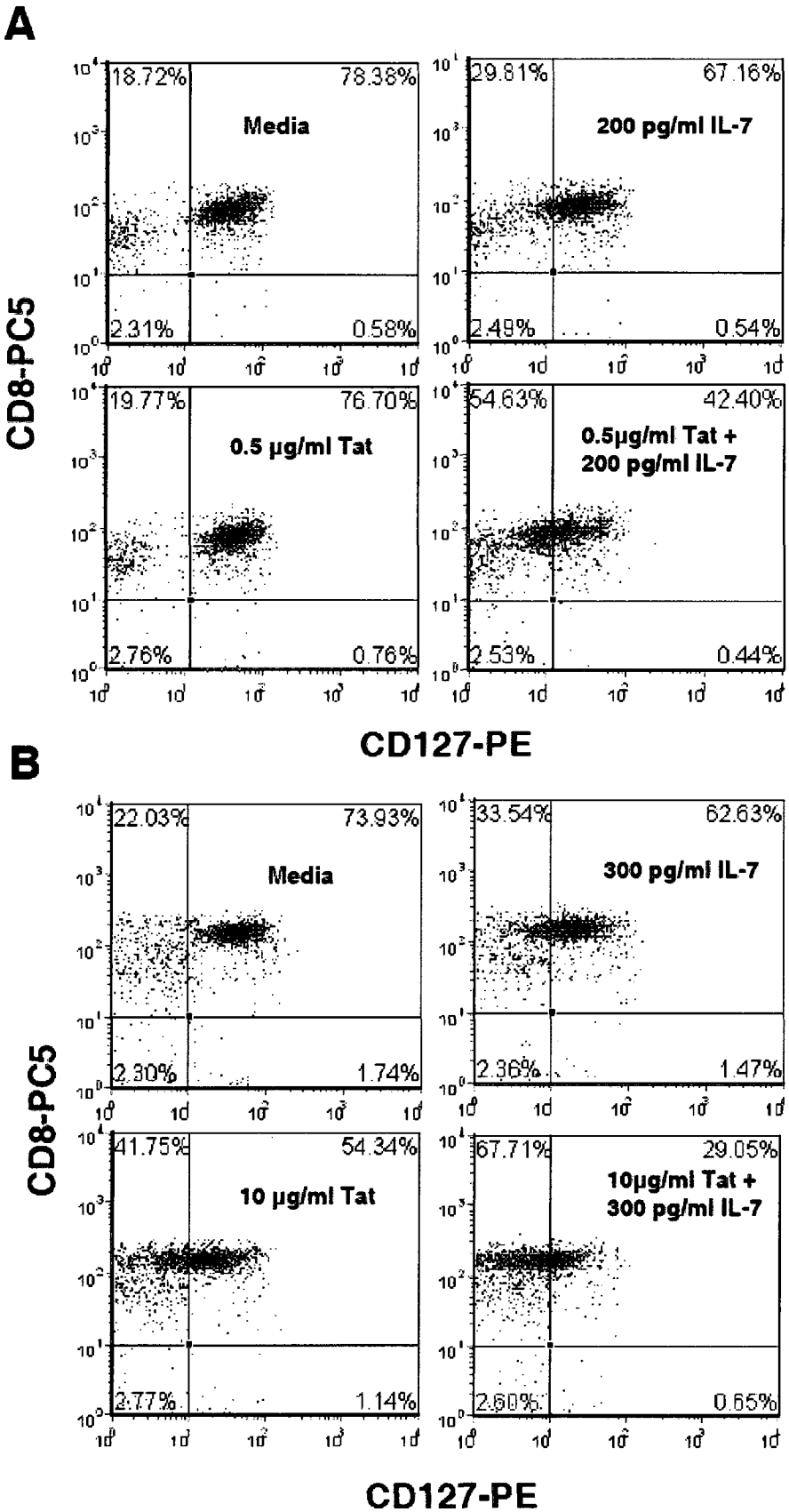

A
18.72%
78.38%
Media
2.31%
0.58%
29.81%
67.16%
200 pg/ml IL-7
2.49%
0.54%
19.77%
76.70%
0.5 µg/ml Tat
2.76%
0.76%
54.63%
42.40%
0.5µg/ml Tat +
200 pg/ml IL-7
2.53%
0.44%
CD8-PC5
CD127-PE
B
22.03%
73.93%
Media
2.30%
1.74%
33.54%
62.63%
300 pg/ml IL-7
2.36%
1.47%
41.75%
54.34%
10 µg/ml Tat
2.77%
1.14%
67.71%
29.05%
10µg/ml Tat +
300 pg/ml IL-7
2.60%
0.65%
CD8-PC5
CD127-PE

Table 4: HIV Tat and IL-7 act synergistically to down regulate CD127 on CD8 T-cells. Purified CD8 T-cells from healthy HIV-negative volunteers were incubated with purified Tat protein, IL-7 or both at concentrations indicated for 24 hours 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 a * indicate values that are statistically different ($p<0.05$) from media control.

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

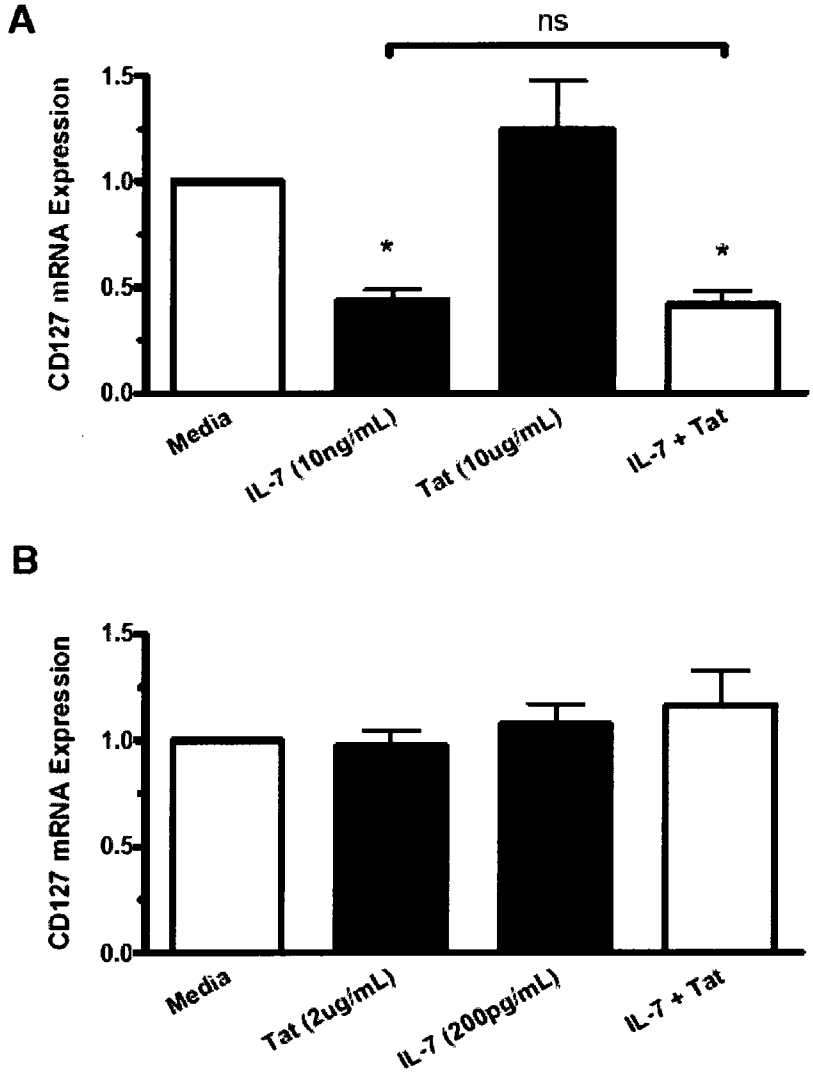
IL-7 induced decrease in CD127 gene transcripts is not affected by Tat

In mice, IL-7 has been shown to decrease CD127 gene expression (33). In view of this, I asked whether the synergy between Tat and IL-7 occurs at the level of transcription. As shown in figure 28A, IL-7 (10 ng/ml) induced a 60% +/- 10% decrease in the level of CD127 mRNA in CD8 T-cells within 24 hours. In contrast, Tat (10 μg/ml) 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. I 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 figure 28B, neither IL-7 at 200 pg/ml nor Tat at 2 μg/ml 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 kinase

Binding of IL-7 to its receptor results in heterodimerization of CD127 and the common γ-chain (CD132) bringing together the Janus kinases 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

Figure 28: 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 hours 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 +/- SEM. **(A)** Concentrations were IL-7: 10 ng/ml; Tat: 10 μg/ml (n=4). **(B)** Concentrations were IL-7: 200 pg/ml; Tat: 2 μg/ml (n=8). Bars marked with a * are statistically different ($p<0.05$) from media control. Values that are not statistically different are indicated by "ns".


A
ns
CD127 mRNA Expression
1.5
1.0
0.5
0.0
*
*
Media
IL-7 (10ng/mL)
Tat (10ug/mL)
IL-7 + Tat
B
CD127 mRNA Expression
1.5
1.0
0.5
0.0
Media
Tat (2ug/mL)
IL-7 (200pg/mL)
IL-7 + Tat

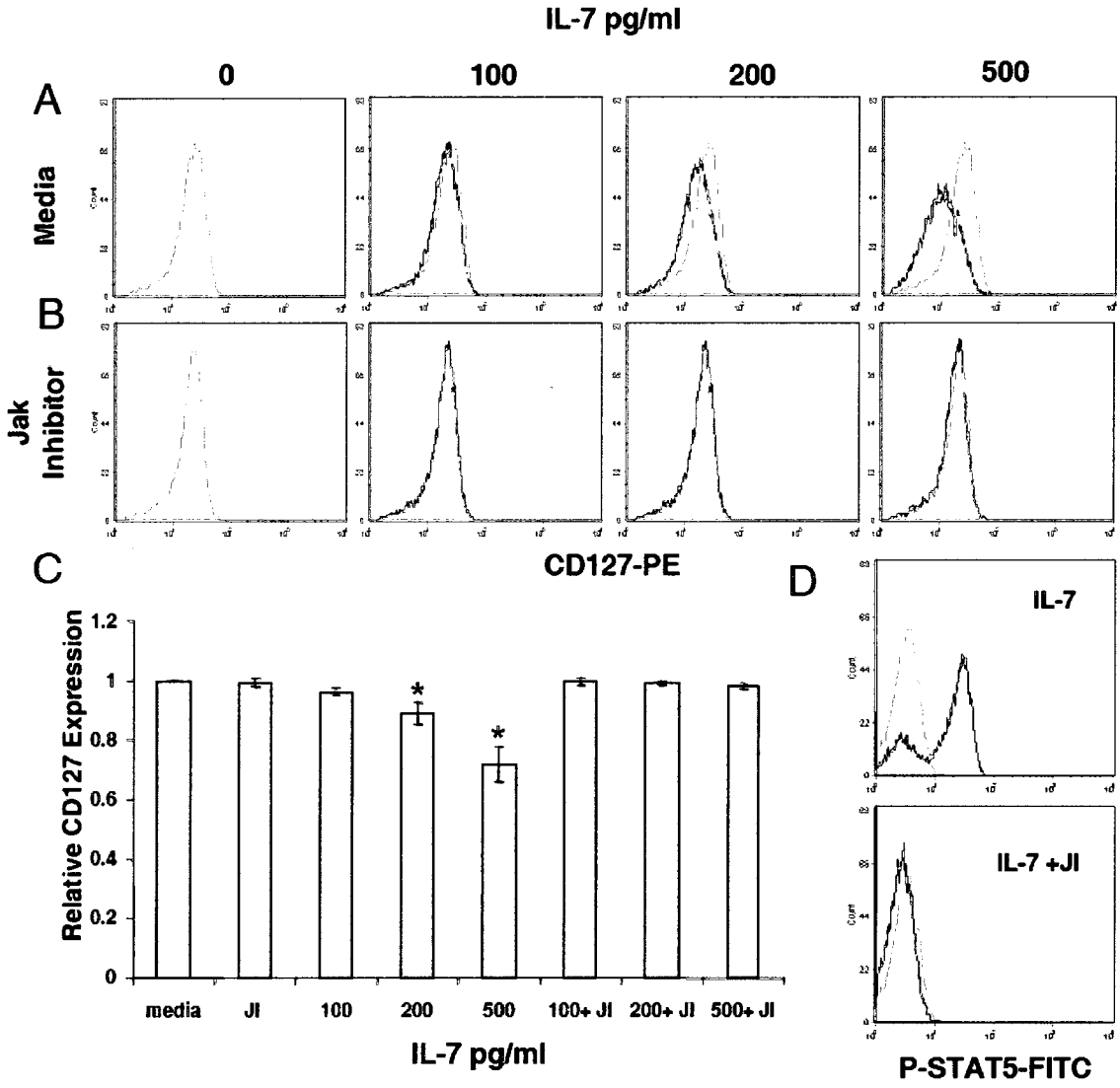
phosphorylation and signal transduction (29, 138). In view of this I questioned whether JAK kinase activity was required for CD127 down regulation by IL-7 and if so if this contributed to the synergy with Tat. To investigate this, CD8 T-cells were incubated in increasing concentrations of IL-7 in the presence or absence of the general JAK kinase inhibitor, JAK Inhibitor 1. As expected, surface CD127 expression decreased with increasing concentrations of IL-7 ranging from 200 to 500 pg/ml (figure 29A). Addition of the JAK kinase inhibitor, however, completely abolished IL-7's ability to down regulate CD127 (figure 29B and 29C). At concentrations of IL-7 up to 500 pg/ml, CD127 expression on CD8 T-cells remained unchanged in the presence of JAK Inhibitor 1 compared to cells maintained in media alone while IL-7 on its own at 200 pg/ml and 500 pg/ml induced a significant decline in CD127 compared to media controls ($p=0.04$ and $p=0.02$ respectively). As expected, inhibition of JAK kinase activity also blocked IL-7 induced STAT5 phosphorylation (figure 29D).

I next asked whether CD127 down regulation by HIV Tat was also dependent on the JAK kinases. To address this, CD8 T-cells were incubated with Tat (10 μg/ml) in the presence or absence of JAK Inhibitor 1. As seen in figure 30, inhibition of JAK kinase activity had no effect on Tat's ability to down regulate CD127. Taken together, these data indicate IL-7 and Tat down regulate CD127 surface expression through different mechanisms, one dependent on JAK kinases and the other not.

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

I next asked if JAK kinase activity plays a role in the synergy between IL-7 and Tat in down regulating CD127 surface expression. To address this, CD8 T-cells were

Figure 29: 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 hours, 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 +/- SEM. **(D)** Purified CD8 T-cells were incubated in media alone or media containing IL-7 (1 ng/ml) or IL-7 plus JAK inhibitor 1 (30 nM) for 15 minutes. 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 a * indicate values that are statistically different ($p<0.05$) from media control.


IL-7 pg/ml
0
100
200
500
A
Media
B
Jak Inhibitor
CD127-PE
C
Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
media
JI
100
200
500
100+ JI
200+ JI
500+ JI
IL-7 pg/ml
D
IL-7
IL-7 +JI
P-STAT5-FITC

incubated with soluble Tat and IL-7 in the presence of JAK Inhibitor 1. As shown in figure 30, inhibition of JAK kinase 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 Tat plus 200 pg/ml 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 2μg/ml; p=0.0002 Tat + IL-7 versus IL-7 only at 200 pg/ml). Addition of JAK Inhibitor 1 reduced the effect of Tat (2 μg/ml) plus IL-7 (200 pg/ml) to only 8% +/- 1%, equivalent to that seen with 2 μg/ml Tat alone (figure 31). This indicates that the synergy between Tat and IL-7 in down regulating CD127 requires the activity of JAK kinases.

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 JAK kinases, I questioned whether the synergistic effect with Tat was mediated through STAT5 signaling. Specifically, I 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 minutes (figure 32A). In contrast, Tat alone had no effect on STAT5 (figure 32B) and did not enhance STAT5 phosphorylation in the presence of IL-7 (figure 32C). 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 (figure 33A) or CD25 (figure

Figure 30: 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), JAK Inhibitor 1 (30 nM) or both. After 24 hours, 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 +/- SEM. Bars marked with a * indicate values that are statistically different ($p<0.05$) from media control.

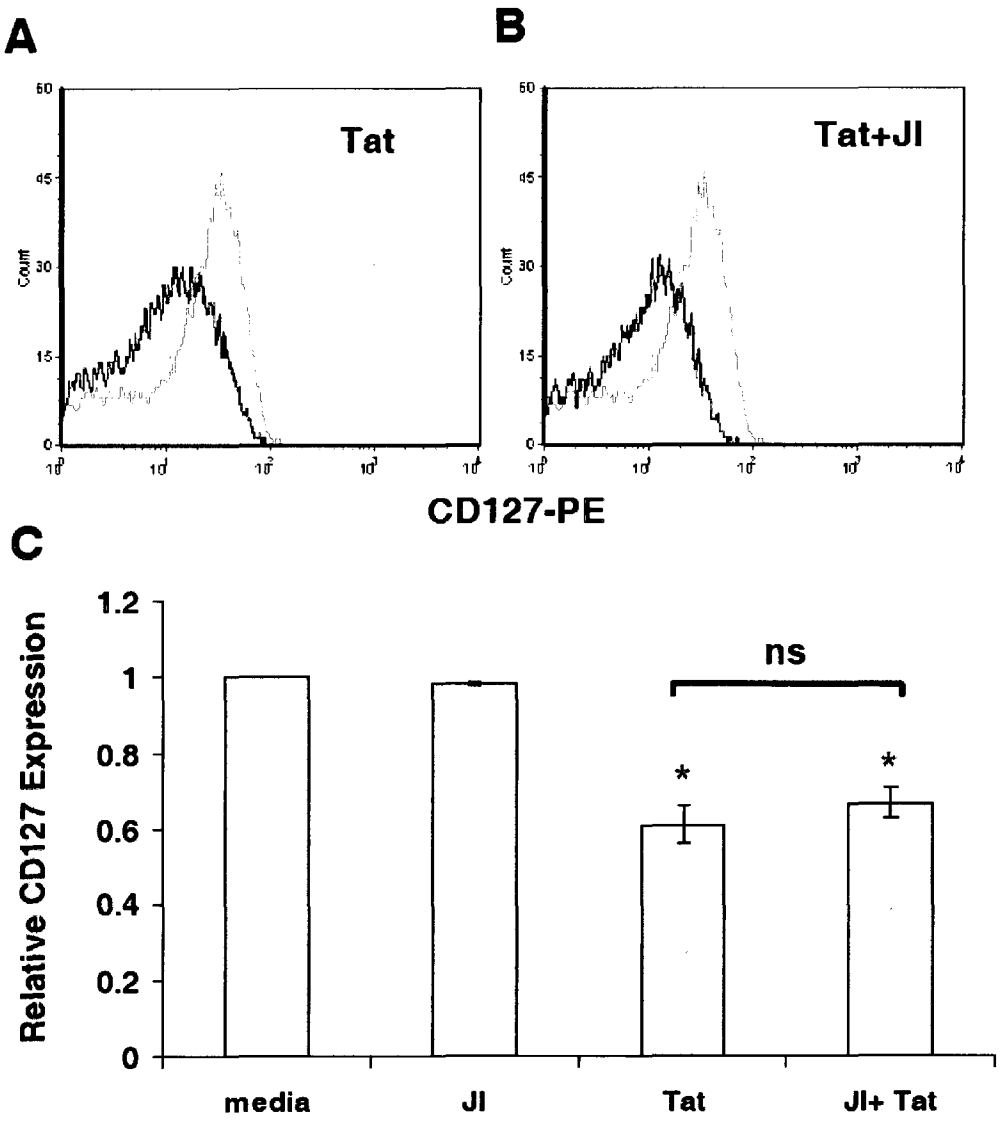

A
B
Tat
Tat+JI
Count
CD127-PE
C
Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
ns
*
*
media
JI
Tat
JI+ Tat

Figure 31: 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 hours, 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 Tat plus JAK Inhibitor 1 (black line), or **(B)** 2 μg/ml Tat with 200 pg/ml 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), IL-7 (200 pg/ml) or Tat (2 μg/ml) plus IL-7 (200 pg/ml) 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 +/- SEM. Bars marked with a * are statistically different ($p<0.05$) from media control. Values that are not statistically different are indicated by "ns".

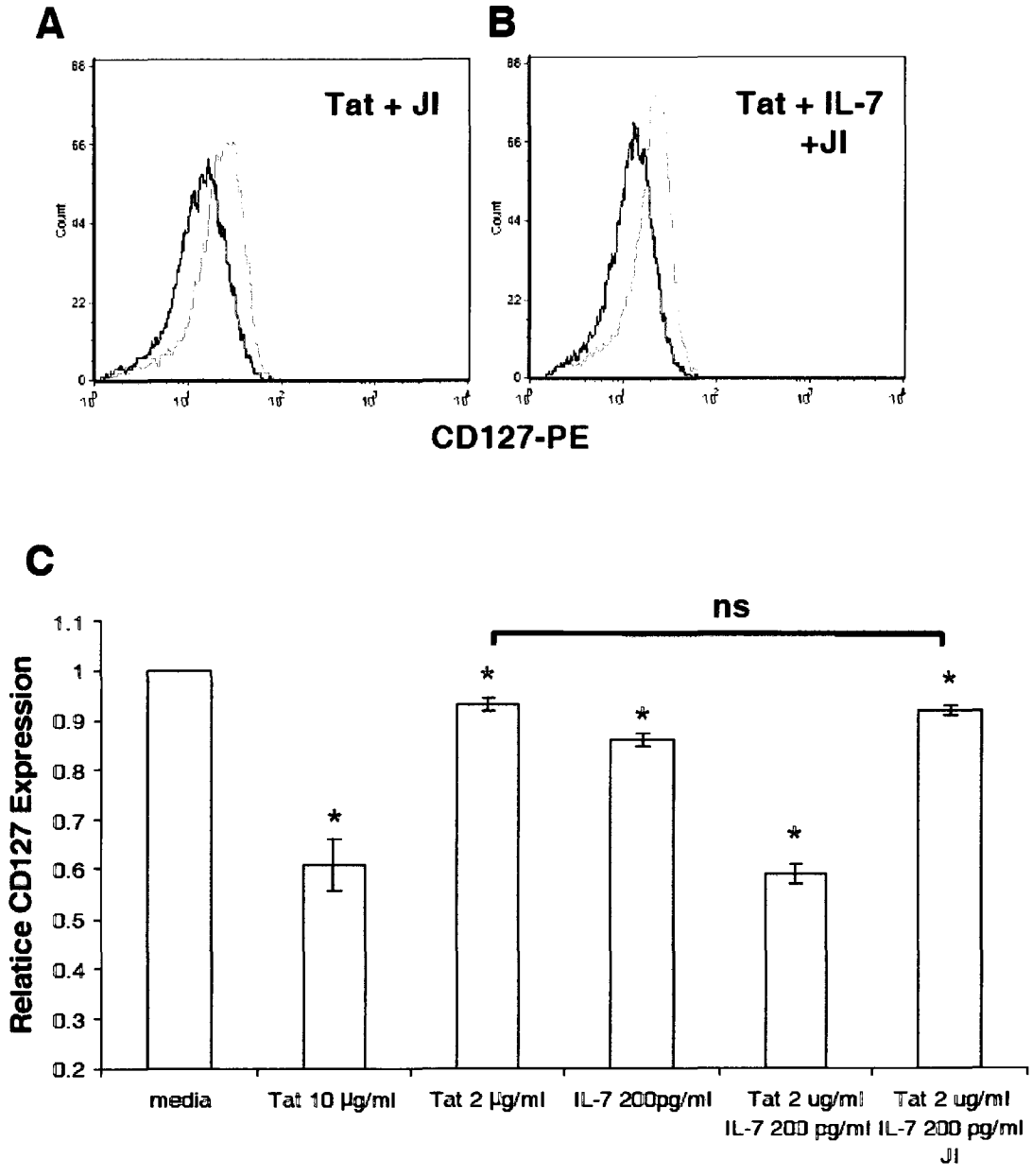

A
B
Tat + JI
Tat + IL-7
+JI
Count
CD127-PE
C
ns
Relatice CD127 Expression
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
media
Tat 10 µg/ml
Tat 2 µg/ml
IL-7 200pg/ml
Tat 2 ug/ml
IL-7 200 pg/ml
Tat 2 ug/ml
IL-7 200 pg/ml
JI

Figure 32: 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 IL-7 (black line), **(B)** 10 μg/ml Tat (black line), or **(C)** Tat plus IL-7 (black line) for 15 minutes. 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.

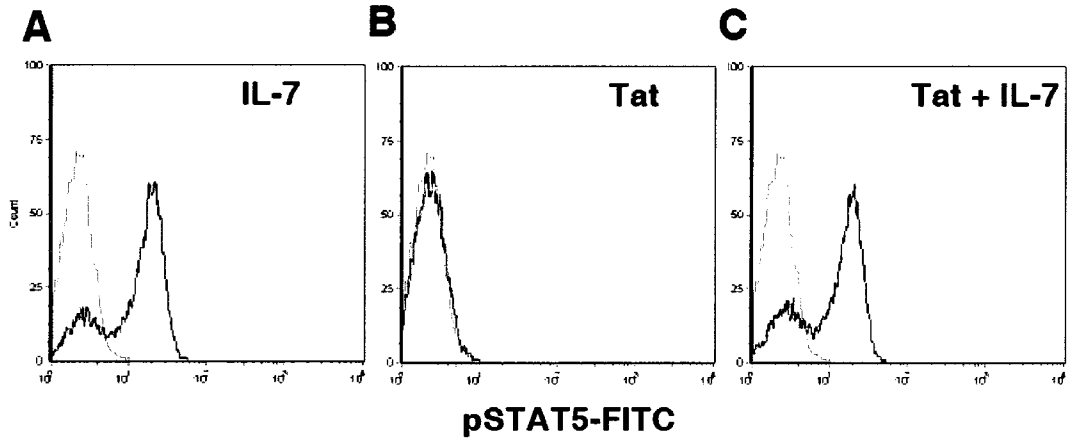
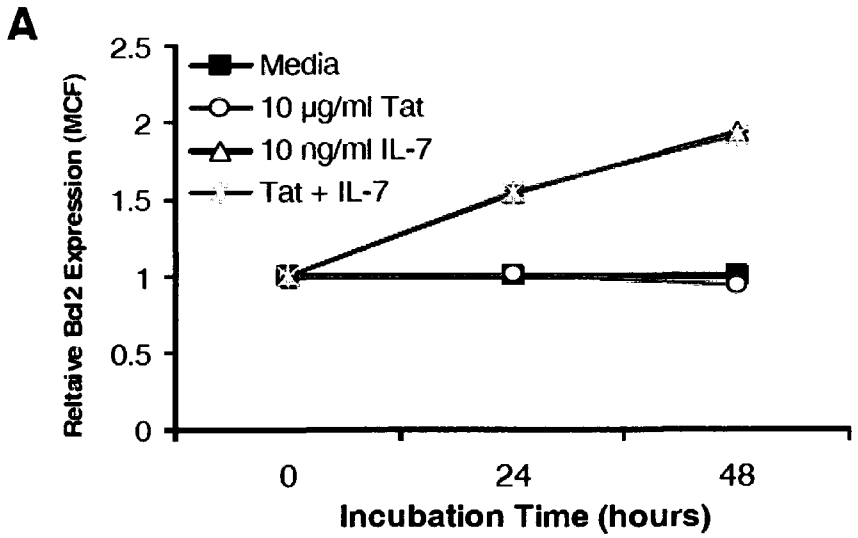
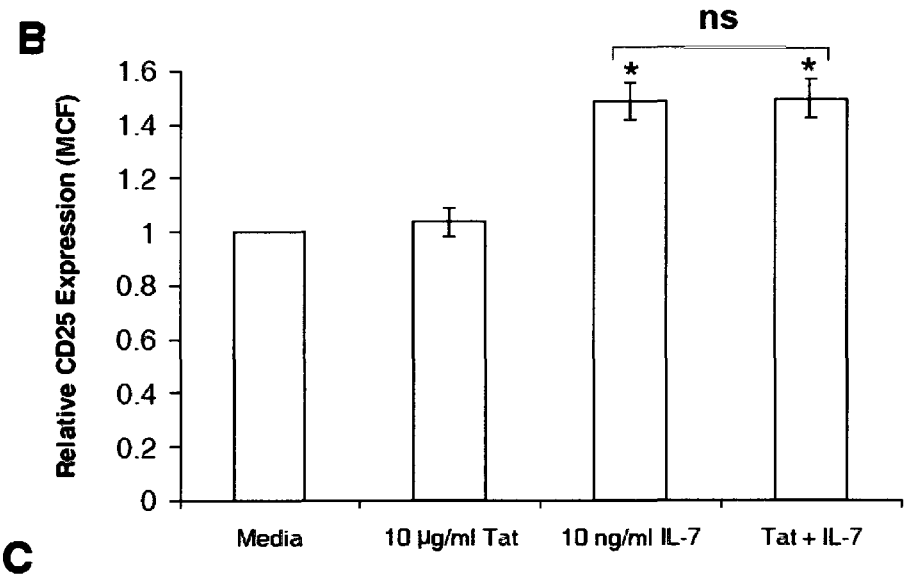
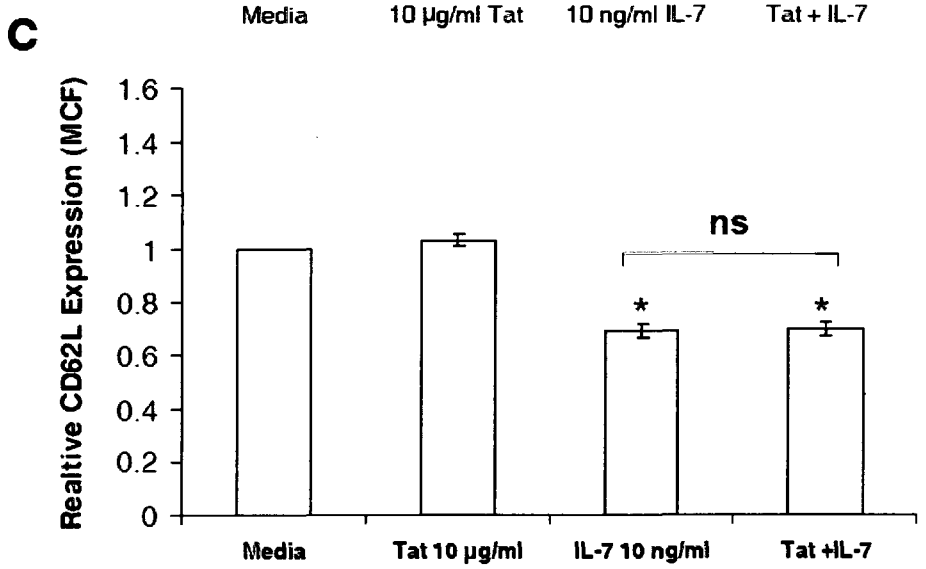

A
B
C
IL-7
Tat
Tat + IL-7
Count
pSTAT5-FITC

Figure 33: 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 Tat, 10 ng/ml IL-7, or both. After 24 hours, 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 +/- SEM. SEM for (A) range 0.02-0.03. Bars marked with a * indicate values that are statistically different ($p<0.05$) from media control.


A
2.5
2
1.5
1
0.5
0
Media
10 µg/ml Tat
10 ng/ml IL-7
Tat + IL-7
Reltaive Bd2 Expression (MCF)
0
24
48
Incubation Time (hours)


B
ns
*
*
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative CD25 Expression (MCF)
Media
10 µg/ml Tat
10 ng/ml IL-7
Tat + IL-7


C
ns
*
*
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Realtive CD62L Expression (MCF)
Media
Tat 10 µg/ml
IL-7 10 ng/ml
Tat +IL-7

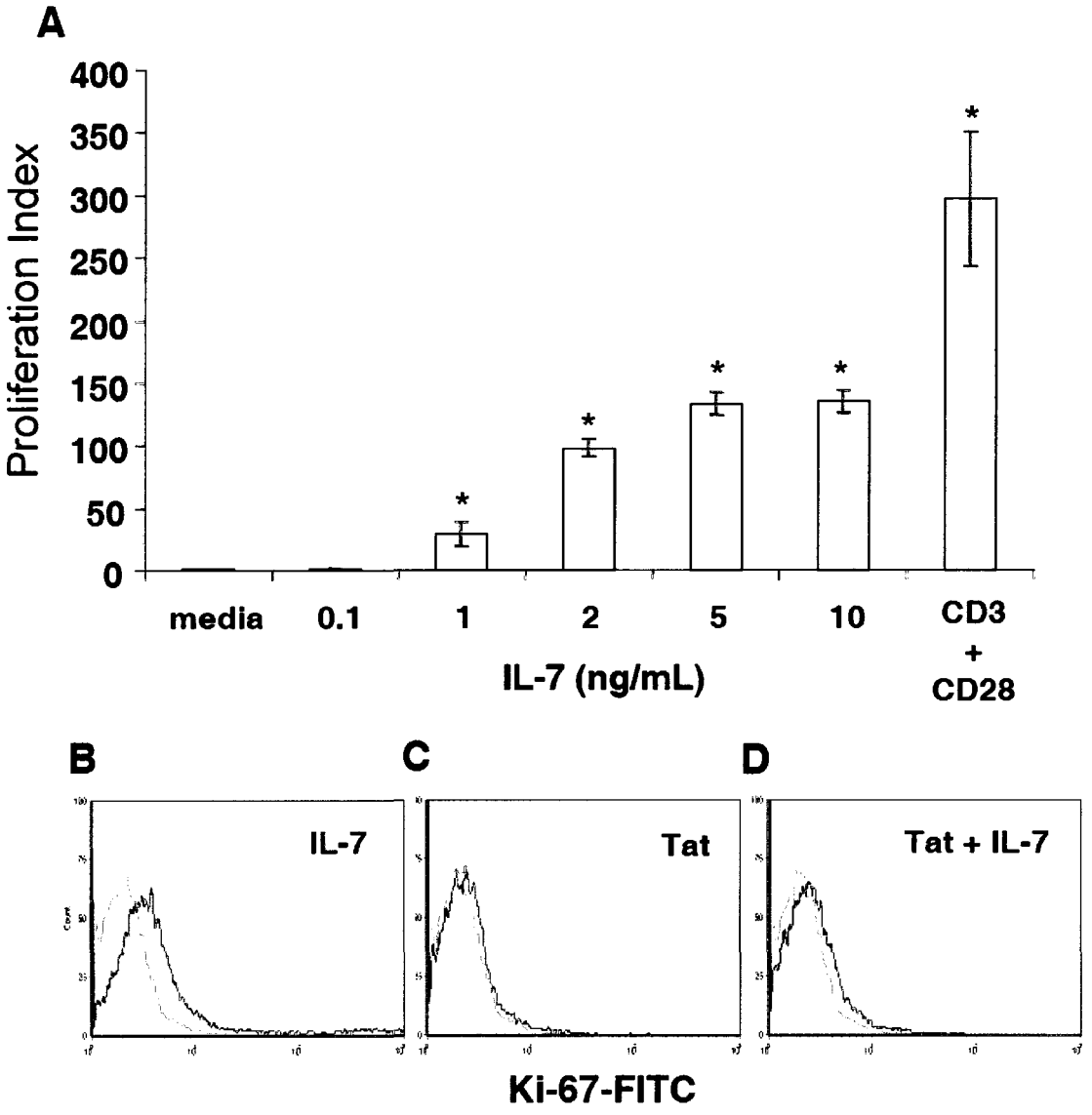
33B), or down regulation of CD62L (figure 33C) by IL-7. Thus, while JAK kinase activity plays an essential role in the synergistic down regulation of CD127 by IL-7 and Tat, I found no evidence to suggest this synergy was mediated by increased STAT5 phosphorylation.

Tat opposes IL-7 induced CD8 T-cell proliferation

IL-7 stimulates proliferation of both human and mouse CD8 T-cells in culture, (56-59) while T-cells from IL-7R-/- mice proliferate poorly with about half undergoing apoptosis (60, 61). While the pathway regulating cell proliferation by IL-7 has not been fully elucidated, stimulation with IL-7 appears to result in phosphorylation of the Retinoblastoma (Rb) protein thereby inducing progression from G1 to S phase of the cell cycle (29). Previously I 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 (118). Since Tat and IL-7 together down regulate CD127 on the surface of CD8 T-cells, I questioned what the simultaneous addition of these factors might have on cell division. I confirmed IL-7's ability to stimulate proliferation of primary CD8 T-cells in culture by both ^{3}H-thymidine incorporation and by induction of Ki-67. IL-7 had a dose-dependent effect on proliferation reaching a maximum at 5 ng/ml (figure 34A; p=0.006), and increased Ki-67 expression within 48 hours (figure 34B). Consistent with our previous report demonstrating Tat did not stimulate ^{3}H-thymidine incorporation in CD8 T-cells (118), Tat also did not induce Ki-67 expression (figure 34C). Perhaps somewhat surprisingly, I found Tat inhibited the induction of Ki-67 by IL-7 (figure 34D). 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-7 receptor requires further investigation.

Figure 34: 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 monoclonal antibodies (3 μg/ml and 2 μg/ml respectively) for 48 hours after which ^{3}H-thymidine incorporation was measured. Values represent proliferation indices relative to unstimulated media controls (mean +/- SEM). **(B-D)** Purified CD8 T-cells from healthy HIV-negative volunteers (n=4) were incubated in media alone (gray fill) or media containing either 10 ng/ml IL-7 **(B)**, 10 μg/ml Tat **(C)**, or both **(D)**. After 24 hours, cells were stained for Ki-67 and analyzed by flow cytometry. Flow histograms from one representative individual are shown. Bars marked with a * indicate values that are statistically different ($p<0.05$) from media control.


A
Proliferation Index
400
350
300
250
200
150
100
50
0
media
0.1
1
2
5
10
CD3
+
CD28
IL-7 (ng/mL)
B
IL-7
C
Tat
D
Tat + IL-7
Ki-67-FITC

DISCUSSION

In the previous chapters I demonstrated soluble HIV Tat protein down regulates surface CD127 expression on CD8 T-cells. To have an appreciable effect, Tat was required in vitro at concentrations some 20-fold higher than those found in patient sera. Here, in agreement with other reports (33, 77), I show IL-7 also down regulates CD127 on the surface of CD8 T-cells but like Tat is required in vitro at supra-physiologic concentrations. Here I have shown soluble HIV Tat protein and IL-7 at near physiologic concentrations act synergistically to down regulate CD127 on CD8 T-cells. While 10 μg/ml of Tat or 500 pg/ml of IL-7 were each required alone to induce a 40-50% decrease in CD127 surface expression, together only 2 μg/ml of Tat plus 200 pg/ml of IL-7 were able to achieve the same effect. While I have yet to fully elucidate the mechanism by which Tat and IL-7 together down regulate CD127, several possibilities may be considered. I 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 kinase activity. Morelon *et al.* (139) have suggested that phosphorylation of the common γ-chain (CD132) also marks this receptor subunit for endocytosis and degradation. While phosphorylation of neither CD127 nor CD132 has yet been demonstrated in IL-7 receptor endocytosis, I have found inhibition of JAK kinase activity completely blocks IL-7 induced down regulation of CD127 on the surface of CD8 T-cells. Whether JAK kinase directly phosphorylates CD127 marking it for removal from the cell membrane or phosphorylates other proteins or adaptors that mediate CD127 down regulation awaits further investigation. It may be, consistent with the suggestion of Morelon *et al.* (139), 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. As shown in the following chapter, I have demonstrated that Tat induces clustering of CD127 molecules on the cell surface and subsequent internalization (manuscript submitted). 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 (88, 109, 110) and is subsequently internalized in T-cells through clathrin-coated pits (112). Similarly, IL-7 like other cytokines has also been shown to interact with heparin sulfate proteoglycans on the cell membrane (140-142). This interaction may ensure 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 promote IL-7 signaling.

I 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 Tat amplifies a direct effect of JAK kinase 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. I have also found Tat directly inhibits IL-7 induced up regulation of Ki-67 and cell proliferation. These different responses likely reflect the cascade of intra-cellular 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 (143, 144). Similarly, Tat has also been shown to alter the activity of several acetyltransferases including Tip60 (145, 146), CBP/p300 (147, 148) and $TAF_{II}250$ (100). 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 PI3 kinase (29, 149-151). However, while extracellular Tat induces c-fos expression and AP-1 DNA-binding activity (152), IL-7 has in contrast been shown to suppress c-fos and c-jun gene transcription (153). 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, I have shown here that at supra-physiologic concentrations soluble HIV Tat protein and IL-7 each independently down regulate the IL-7 receptor α-chain on the surface of CD8 T-cells. Here I demonstrate for the first time 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. I 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 (118). The evidence I 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 kinase, the effects of Tat on CD127 are amplified. With decreased CD127 expression, CD8 T-cells no longer accumulate perforin (118). 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-7 receptor 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.

CHAPTER 6 - SOLUBLE HIV TAT PROTEIN REMOVES THE IL-7 RECEPTOR ALPHA-CHAIN FROM THE SURFACE OF RESTING CD8 T-CELLS AND TARGETS IT FOR DEGRADATION

RATIONALE

Interleukin (IL)-7 plays a pivotal role throughout the lifespan of a T-cell. It has been shown previously that CD127 is down regulated on the surface of CD8 T-cells isolated from HIV-infected individuals with uncontrolled viral replication.(71-77) Given the essential role of IL-7 in CD8 T-cell function, this decrease in IL-7 signaling likely contributes to the impaired cell mediated immunity and inefficient immunologic control of viral replication evident in HIV+ patients with progressive disease(15-18). I have shown in the previous chapters that this down regulation of CD127 on the surface of CD8 T-cells is mediated in part by soluble HIV Tat protein.(118) Acting in a paracrine fashion, secreted Tat protein is rapidly internalized by neighboring uninfected cells (88, 104, 105) and localizes to both the nucleus and the cytoplasm.(105, 154) Once inside the cell, Tat on its own at high doses or at lower doses in the presence of IL-7 down regulates CD127 at the cell surface. In view of these findings, I set out to determine the mechanism by which soluble HIV Tat protein down regulates expression of the IL-7 receptor alpha-chain on the surface of CD8 T-cells.

There are several possible mechanisms that Tat could employ to down regulate CD127 expression. Due to Tat's canonical role as a regulator of viral transcription, it is not surprising that Tat has been show to regulate a wide range of cytokine and cytokine receptor expression though transcriptional mechanisms (89-97). Surprisingly, I saw no effect of Tat on CD127 transcripts in resting CD8 T-cells (chapter 5). This suggests that Tat protein could be affecting CD127 protein translation, transport or stability.

Interestingly, Kalantari et al. (155) have shown that soluble Tat protein induces the internalization and degradation of the RON (recepteur d'origine nantais) receptor tyrosine kinase in monocytes and macrophages. As demonstrated by Kalantari et al.(155), soluble Tat induces the internalization and degradation of RON via the ubiquitin-proteasome pathway through a process dependent on the RON intracellular kinase domain. It is possible that Tat also directs the IL-7 receptor α-chain to the proteasome through ubiquitinylation. Notably, Tat was recently shown to induce Tip60 polyubiquitination and degradation through recruitment of p300 and the E3-ubiquitin ligase Mdm2.(146), Tat has also been shown to interact with subunits of the proteasome and increase the activity of the 26S proteasome.(156, 157) In addition to CD127 and RON, Tat has also been shown to induce the proteasomal degradation of microtubule-associated protein 2 (MAP2) (158) and postsynaptic density protein 95 (PSD95).(80)

To determine the mechanism of Tat mediated down regulation of CD127, I questioned if Tat affects CD127 protein translation, transport and stability. In light of the evidence that Tat has been shown to regulate protein expression by proteasomal degradation, I further questioned if Tat interacts with CD127 proteins and targets them for degredation by the proteasome.

RESULTS

Soluble HIV Tat protein increases the rate of decay of CD127 from CD8 T-cells

I first set out to determine the normal rate of turnover for the IL-7 receptor alpha-chain in resting CD8 T-cells. To do this, cells were maintained in media and new protein synthesis was blocked with 100μM cycloheximide. Cells were harvested at 24 hour intervals and total CD127 protein levels were analyzed by Western blot normalizing to β-actin, and surface CD127 expression was monitored by flow cytometry. As seen in figure 35A, cells incubated in media alone maintain a constant level of CD127 protein over at least 72 hours. When new protein synthesis was blocked with cycloheximide, total CD127 decayed slowly over several days (figure 35A). Composite data from three separate experiments are shown in figure 35B and demonstrate in resting CD8 T-cells CD127 is relatively stable with a half-life of approximately 72 hours ($p=0.04$). Over this same period in cycloheximide, I noted very little cell death likely indicative of the low metabolic activity of resting CD8 T-cells in culture. Similarly, when new protein could not be replenished in the presence of cycloheximide, CD127 decayed from the cell surface with roughly the same kinetics as total CD127 protein (figure 36A). Thus by both Western blot and flow cytometry, I found CD127 is stable and turns over slowly in purified resting CD8 T-cells. Similar data have been reported for the IL-2 receptor alpha-chain which has been shown to have a half-life on the surface of lymphoblastic T-cells of 35-40 hours (159, 160).

I have shown previously in chapter 3 that soluble Tat protein down regulates surface expression of CD127 on resting CD8 T-cells (118). This down regulation was not associated with T-cell activation and expression of other cell surface proteins including

Figure 35: CD127 has a prolonged half-life in resting CD8 T-cells. CD8 T-cells were isolated from healthy HIV-negative donors and incubated in media alone or in the presence of 100μM cycloheximide (CHX) to block new protein synthesis. **(A)** Western blot showing CD8 T-cell extracts harvested at the time points indicated and probed with a polyclonal anti-human CD127 antibody. CD127 is visualized at the expected molecular weight of 90 kdal. Blots were stripped and re-probed for β-actin as a loading control. **(B)** CD127 band intensities from three independent experiements were analyzed using an unsaturated image of the gel normalizing CD127 band pixel intensity to β-actin in each lane. Mean band intensities +/- standard error of the mean (SEM) are shown relative to media from each experiment. (* p=0.04)

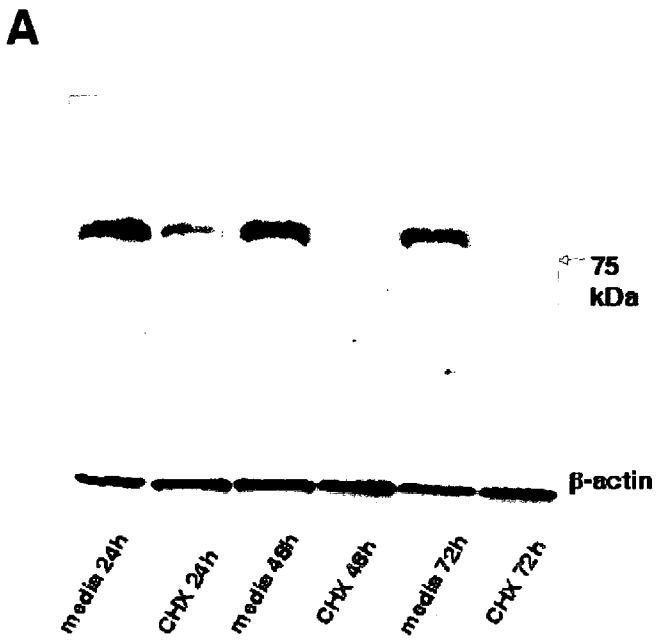
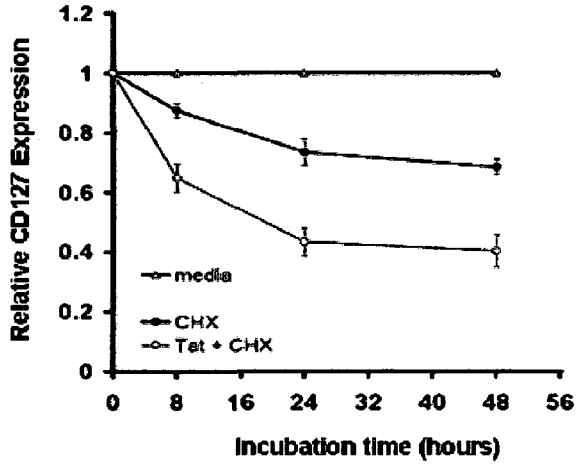
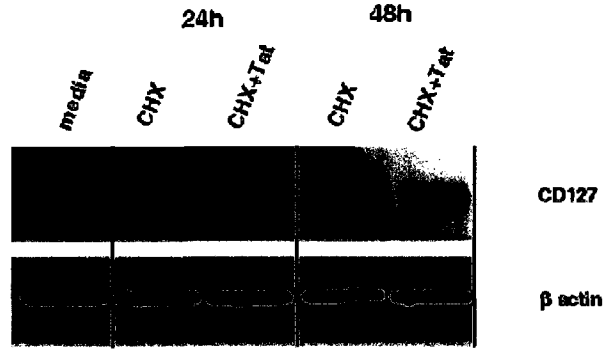

A
75
kDa
β-actin
media 24h
CHX 24h
media 48h
CHX 48h
media 72h
CHX 72h
B
Relative CD127 Protein
1.25
1.00
0.75
0.50
0.25
0.00
*
media
24h
48h
72h
CHX

Figure 36: Soluble Tat protein decreases the half-life of CD127 at both at the cell surface and as total protein. CD8 T-cells were incubated in media alone or media containing 100μM cycloheximide (CHX) or cycloheximide plus purified Tat protein (10μg/ml) for 48 hours. **(A)** CD127 surface expression was analyzed by flow cytometry. Percent change in CD127 expression relative to media control is shown (n=4). Mean values +/- SEM are indicated. **(B)** Western blot showing CD8 T-cell extracts harvested at the time points indicated and probed with a polyclonal anti-human CD127 antibody. Blots were stripped and re-probed for β-actin as a loading control.

A


Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
media
CHX
Tat + CHX
0
8
16
24
32
40
48
56
Incubation time (hours)

B


24h
48h
media
CHX
CHX+Tat
CHX
CHX+Tat
CD127
β actin

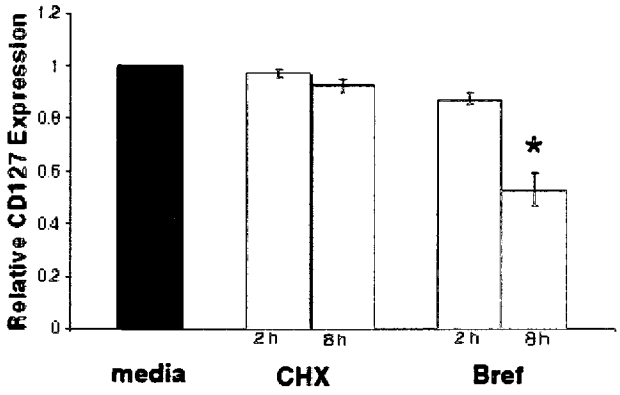
CD25, CD38 and HLA-DR remained unchanged in the presence of Tat.(118) Interestingly, Tat has been shown to affect the stability of other cellular proteins including microtubule-associated protein 2 (MAP2) (158) and postsynaptic density protein 95 (PSD95).(80) To determine whether Tat affects the stability of CD127, resting CD8 T-cells were treated with cycloheximide to block new protein synthesis and then incubated with soluble Tat protein. As shown in figure 36A, Tat increased the rate at which CD127 was lost from the cell surface. Indeed, in the presence of Tat, the half-life of CD127 at the cell membrane decreased 4.5-fold to approximately 16 hours. Analysis of total CD127 protein by Western blot provided similar data and demonstrated a more rapid decay of CD127 in the presence of Tat (figure 36B). Thus, the HIV Tat protein appears to increase CD127 turn over in CD8 T-cells. Further, the fact that Tat is able to decrease the levels of CD127 in the presence of cycloheximide indicates a direct effect and that new protein synthesis is not required for Tat to alter CD127 expression (figure 36A and B).

Tat enhances the rate at which CD127 is removed from the cell membrane.

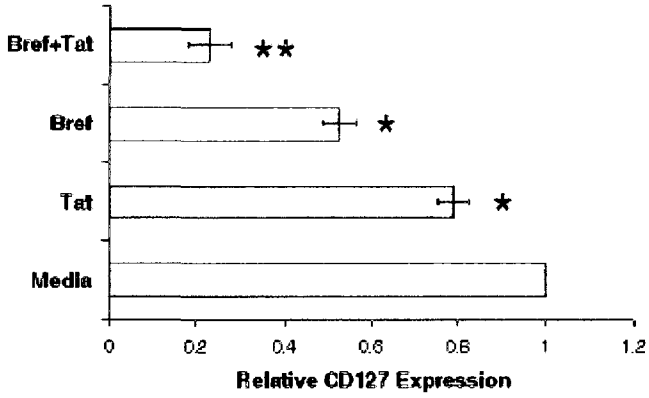
While CD127 is relatively stable in resting CD8 T-cells, it may yet have a high rate of turnover at the cell surface recycling through early endosomes. This has in fact been shown to be the case for the IL-2 receptor alpha-chain (160, 161). To investigate this for CD127, I used brefeldin A to block protein transport to the cell membrane. As shown in figure 37, CD127 expression on the surface of resting CD8 T-cells changes very little after 2 to 8 hours in cycloheximide. However, when cells were incubated with brefeldin A to prevent protein transport to the cell membrane, CD127 surface expression decreased by 48% +/- 6% within 8 hours ($p=0.001$; figure 37A). Since

Figure 37: Soluble Tat protein enhances the off rate of CD127 at the cell membrane. CD8 T-cells were incubated in media alone or media containing purified Tat protein (10μg/ml), brefeldin A (Bref; 10μg/ml), cycloheximide (CHX; 100μM) or Tat plus brefeldin A. After up to 8 hours, surface CD127 expression was analyzed by flow cytometry. **(A)** Surface CD127 expression relative to media control is shown for (A) cells incubated for 2 and 8 hours in cycloheximide or brefeldin A (* $p=0.001$ compared to media), and **(B)** cells incubated for 8 hours in Tat, brefeldin A or both (* $p \leq 0.05$ relative to media; ** $p \leq 0.04$ relative to Tat and brefeldin A individually). Mean values +/- SEM are indicated (n=4).

A


Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
2h
8h
2h
8h
*
media
CHX
Bref

B


Bref+Tat
Bref
Tat
Media
**
*
*
0
0.2
0.4
0.6
0.8
1
1.2
Relative CD127 Expression

the cycloheximide data indicate CD127 remains at the cell surface for a prolonged period without the replenishment of new protein, the loss of receptor from the cell surface in the presence of brefeldin A alone suggests CD127 continuously cycles off and then back to the membrane. Thus, while CD127 is turned over very slowly in resting CD8 T-cells, maintenance of stable levels at the cell surface is dependent on continual recycling of the receptor and transport through vesicles back to the cell membrane.

As shown above, Tat increases the rate at which CD127 is lost from the cell surface. This may be achieved by either slowing the rate at which CD127 is delivered to the cell membrane or increasing the rate at which it is removed. To determine whether Tat protein affects either the on or off rate of CD127 at the cell surface, resting CD8 T-cells were incubated with Tat, brefeldin A, or both. Since brefeldin A prevents protein transport to the cell membrane, if Tat also interferes with delivery of CD127 to the cell surface it should have little to no effect when incubated with brefeldin A. If on the other hand Tat increases the rate at which CD127 is removed from the cell membrane, the effects of Tat and brefeldin A should be roughly additive. As shown in figure 37B, after 8 hours soluble Tat protein alone induced only a 22% +/- 5% decrease in surface CD127 expression ($p=0.05$) while incubation in brefeldin A alone resulted in a 47% +/- 3% decline ($p=0.01$). When cells were treated with Tat and brefeldin A together, CD127 expression on the cell surface decreased by 77% +/- 1% ($p \leq 0.04$ compared to Tat and brefeldin A alone). Since soluble Tat protein and brefeldin A have an additive effect in decreasing CD127 expression at the cell surface, this would suggest Tat likely increases the rate at which CD127 is removed from the membrane.

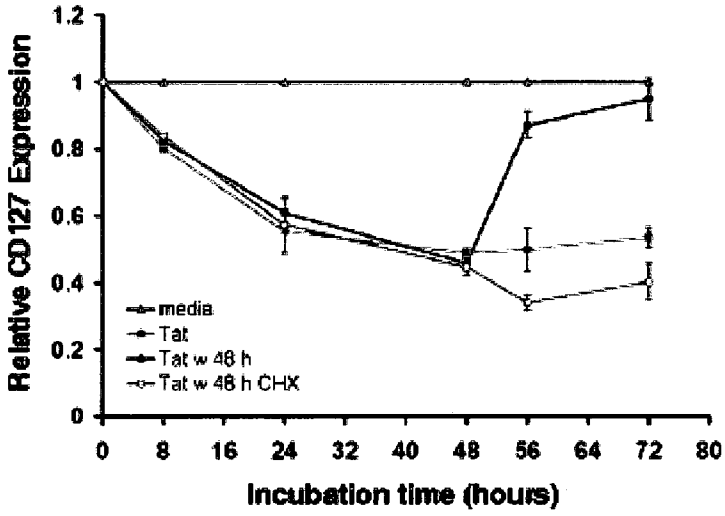
New protein synthesis is required to replenish CD127 lost in the presence of Tat protein.

I have shown previously in chapter 3 (figure 17) that Tat-induced down regulation of CD127 on CD8 T-cells requires the continual presence of this viral protein. When Tat protein is removed from the culture media, CD127 returns to normal levels with 24 hours.(118) To determine if this recovery of CD127 at the cell surface requires new protein synthesis or perhaps results from the release of sequestered protein, CD8 T-cells were first incubated with soluble Tat for 48 hours and then washed and re-incubated in media alone or media containing cycloheximide. As shown in figure 38, cells maintained in media containing Tat protein maintained suppression of CD127 for up to 72 hours while those washed and transferred to media alone after 48 hours recovered surface CD127 expression back to 87% +/- 4% within 8 hours. However, if after removing Tat protein from the media the cells were then incubated with cycloheximide, CD127 failed to recover (figure 38). Thus Tat induces an absolute loss of CD127 from the cell and new protein synthesis is required to replace the receptor once Tat is removed from the media.

Endosomal transport and subsequent acidification is required for Tat to down regulate CD127 on CD8 T-cells

Soluble Tat protein is rapidly internalized by a variety of cell types including lymphocytes.(88, 104, 105) Tat binds via its arginine-rich basic domain to heparan sulfate proteoglycans on the cell surface(88, 109, 110) and is then internalized by endocytosis. In Hela cells, internalization is mediated through caveolae while in lymphocytes, which are devoid of caveolin,(88, 111, 162) endocytosis occurs through clathrin-coated pits.(112) Once inside the cell, Tat progresses through early endosomes

Figure 38: Recovery of CD127 at the cell surface following treatment with Tat requires new protein synthesis. CD8 T-cells were incubated in media or media plus Tat protein (10μg/ml). After 48 hours, cells cultured in Tat were either left for an additional 24 hours, or washed in PBS and resuspended in media alone or media containing cycloheximide (CHX; 100μM). CD127 expression on CD8 T cells relative to media control is shown. Mean values +/- SEM are indicated (n=4).


Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
media
Tat
Tat w 48 h
Tat w 48 h CHX
0
8
16
24
32
40
48
56
64
72
80
Incubation time (hours)

to late endosomes and release into the cytoplasm is dependent on the acidification of these vesicles. Blocking endosomal acidification has been shown to prevent Tat from exiting the vesicle and entering the cytosol.(112)

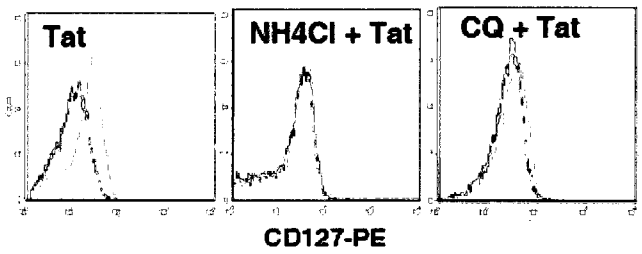
To determine if Tat's entry into the cell cytosol is necessary for it to down regulate CD127 surface expression, CD8 T-cells were incubated with Tat protein alone or in the presence of ammonium chloride or chloroquine, both of which have been shown to prevent endosomal acidification and trap Tat in early vesicles.(112) Figure 39A shows typical flow histograms demonstrating surface CD127 expression on CD8 T-cells, while figure 39B shows relative CD127 expression from four independent experiments. While neither ammonium chloride nor chloroquine alone had any effect on CD127 expression (data not shown), each blocked Tat's ability to down regulate the receptor ($p \leq 0.03$). Because ammonium chloride and chloroquine do not prevent Tat from binding to or entering the cell through endocytosis but do prevent Tat from entering the cytosol, I conclude Tat does not interact with the extracellular domain of CD127 and carry it into the cell during endocytosis but instead down regulates the receptor from within the cytoplasm.

Tat protein co-localizes with CD127 at the cell surface

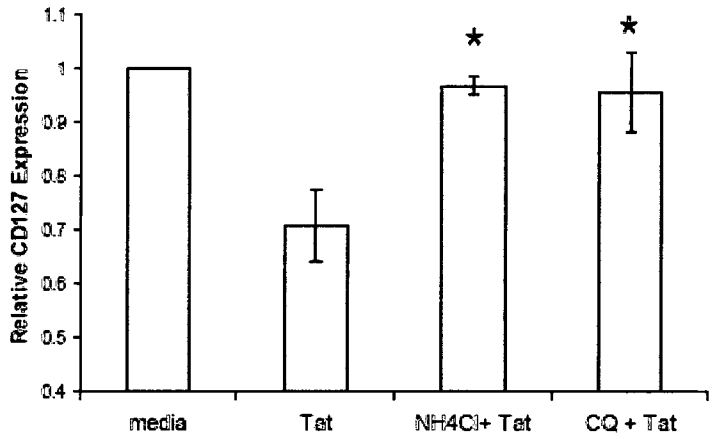
Since Tat down regulates CD127 expression from within the cell, I next used fluorescence microscopy to track the fate of CD127 in the presence of Tat protein. In resting CD8 T-cells maintained in media lone, CD127 can be seen over the entire surface of the cell. Interestingly, however, the distribution generally is not even and a higher density of CD127 is usually seen towards one pole (figure 40). Remarkably, when soluble Tat protein was added to the cells, it induced clustering of CD127 generally to

Figure 39: Tat-induced down regulation of CD127 on CD8 T-cells requires endosomal acidification. CD8 T-cell were incubated in media alone or media containing Tat (10μg/ml) with or without 200 mM ammonium chloride (NH_4Cl) or 200 μM cholorquine (CQ) for 24 hours after which CD127 surface expression was analyzed by flow cytometry. **(A)** Representative flow histograms for one individual. **(B)** Composite data for n=4. CD127 surface expression on CD8 T-cells relative to media control is shown (* $p \leq 0.03$ relative to cells incubated with Tat alone). Mean values +/- SEM are indicated

A


Tat
NH4Cl + Tat
CQ + Tat
CD127-PE

B


Relative CD127 Expression
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
media
Tat
NH4Cl+ Tat
CQ + Tat
*
*

one area on the cell membrane forming a cap (figure 40). Overlay of the fluorochromes demonstrated Tat co-localizes with CD127 within these caps at the cell surface. In a number of fields CD127 and Tat can be seen together in small structures surrounding these caps perhaps representing endocytic vesicles (figure 40B). To be absolutely certain the Tat-antibody complexes used in these experiments did not artificially change CD127 staining, I repeated these experiments with unlabeled Tat protein and re-examined CD127 surface staining. As seen in figure 40C, irrespective of methodology, Tat induced clustering of CD127 at the surface of CD8 T-cells. Taken together these data suggest that after being taken up by CD8 T-cells, Tat enters the cytosol and associates with CD127 at the cell membrane where it induces receptor capping and possibly internalization.

Tat binds directly to the cytoplasmic tail of CD127

Since Tat protein co-localizes with CD127 in CD8 T-cells, I wanted to know if Tat bound to the receptor. To address this, soluble Tat protein was added to whole CD8 T-cell extracts and CD127 was immunoprecipitated with polyclonal goat anti-CD127 antibodies. Following separation by gel electrophoresis, immune complexes were transferred to PVDF membranes and probed with an anti-Tat monoclonal antibody. As seen in figure 41A, Tat protein co-immunoprecipitated with CD127 and is identified as a 14 kda band running at the same size as recombinant Tat in the adjacent lane, thus demonstrating a clear interaction between Tat and CD127.

We next wanted to know to which domain of CD127 Tat bound. To investigate this I devised an ELISA assay where equimolar amounts of target proteins were first fixed to the bottom of 96 well plates and then, after blocking with BSA, were overlaid with purified Tat protein. Target proteins included soluble CD127 and a CD127-Fc

Figure 40: Tat co-localizes with CD127 and induces capping of the receptor on the surface of CD8 T cells. Fluorescence microscopy was used to track CD127 within the cell following addition of Tat protein. CD8 T cells were incubated in media or media plus Tat (10μg/ml) for 3 hours after which cells were fixed and permeabilized. **(A)** and **(B)** Tat was tagged with an FITC labeled anti-Tat antibody before being added to CD8 T-cells. Cells maintained in media (top panel) and cells treated with Tat (bottom panel) were stained with DAPI and anti-CD127-PE as indicated. **(C)** To ensure tagging Tat with antibody did not alter its function, CD8 T-cells were incubated in media or with purified Tat protein alone and then stained as above with DAPI and anti-CD127-PE. Scale bar: 10 μm.

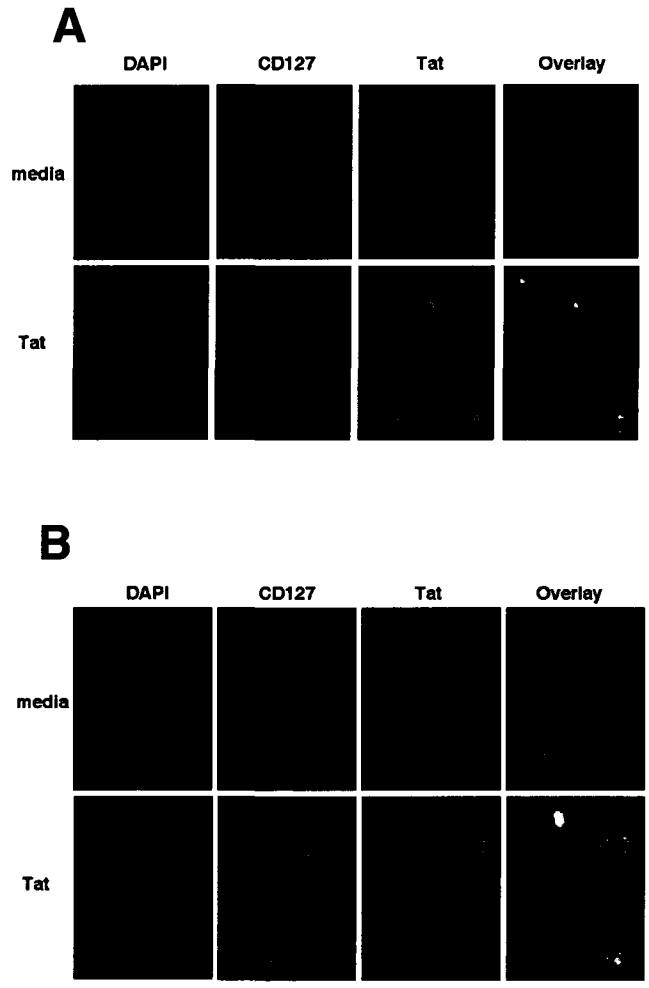
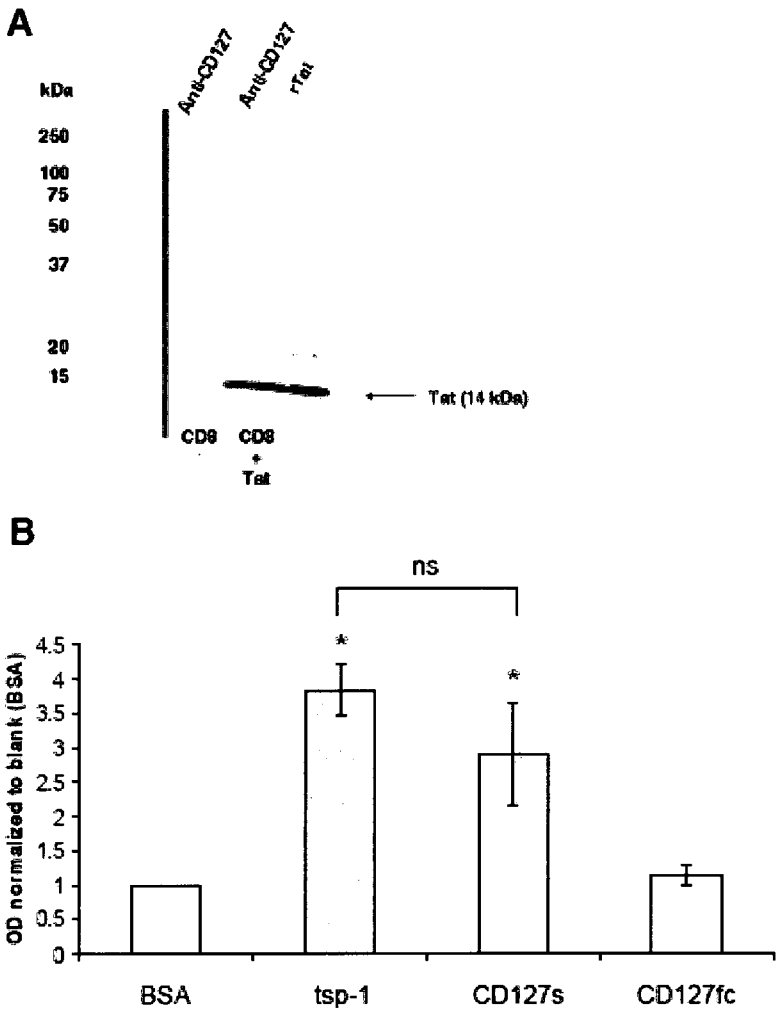

A
DAPI
CD127
Tat
Overlay
media
Tat
B
DAPI
CD127
Tat
Overlay
media
Tat
C
media
Tat

Figure 41: Tat binds directly to the cytoplasmic tail of CD127. **(A)** Tat co-immunoprecipitates with CD127 from CD8 T-cell extracts. Whole cell CD8 T-cell extracts were incubated for 1 hour with and without purified Tat protein (10 μg/million cells). CD127 was then immunoprecipitated with a goat polyclonal anti-CD127 antibody and immune complexes were separated by SDS-PAGE. After transferring to PVDF membranes, Tat was detected by Western blot using a monoclonal anti-Tat antibody. Lane 1: CD8 T-cell extract alone; Lane 2: CD8 T-cell extract containing Tat protein; Lane 3: purified recombinant Tat protein as positive control. **(B)** Tat binds to soluble CD127 (CD127 extracellular and cytoplasmic domains) but not to the CD127-Fc chimera (CD127 extracellular and transmembrane domains). Target proteins including bovine serum albumin (BSA; negative control), thrombospondin-1 (tsp-1; positive control), soluble CD127 (CD127s) and a CD127-Fc chimera (CD127fc) were fixed to the bottom of wells of a 96 well plate and then overlaid with purified Tat protein. After extensive washing, Tat bound to target proteins was detected with a biotinylated anti-Tat antibody and streptavidin-peroxidase. Optical density readings normalized to the negative control are shown (n=4). Mean values +/- SEM are indicated. * $p \leq 0.05$. Values not statistically different are indicated by "ns".

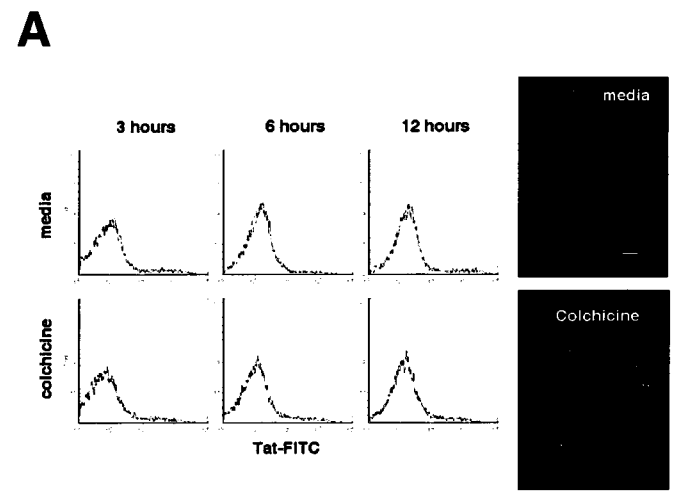

A
kDa
250
100
75
50
37
20
15
Anti-CD127
Anti-CD127
rTat
Tat (14 kDa)
CD8
CD8
+
Tat
B
ns
*
*
OD normalized to blank (BSA)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
BSA
tsp-1
CD127s
CD127fc

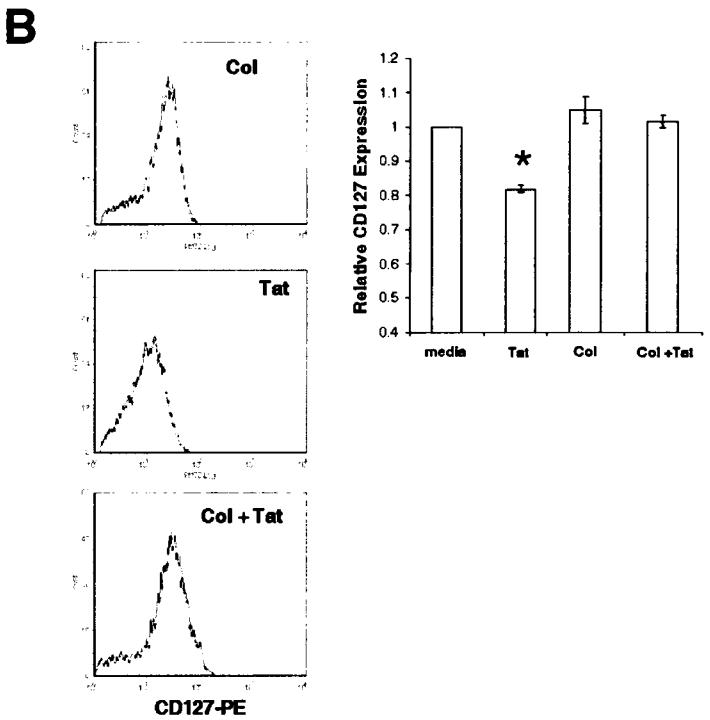
chimera. The soluble isoform of CD127 lacks the transmembrane domain but contains both the intracellular and extracellular domains of the receptor (38). The CD127-Fc chimera contains the extracellular and transmembrane domains of CD127 but lacks the cytoplasmic domain. Thrombospondin-1, an extracellular matrix protein previously shown to bind Tat with high affinity, was used as a positive control (123). As seen in figure 41B, Tat bound to the soluble isoform of CD127 ($p=0.05$) but not to the CD127-Fc chimera lacking the CD127 intracellular domain ($p=0.38$). Binding to soluble CD127 and thrombospondin-1 could be titrated over a range of Tat concentrations but no interaction with the CD127-Fc chimera was detected even at the highest Tat concentrations tested (40 ng/ml; data not shown). Taken together these data indicate Tat protein interacts directly with the cytoplasmic tail of CD127. Notably, these data are consistent with those in figure 39 indicating Tat down regulates CD127 from within the cytosol.

Down regulation of CD127 by Tat is dependent on microtubules

Cellular uptake of soluble Tat protein from the external environment is well described and consistent with this Tat can be seen entering CD8 T-cells both by flow cytometry and by fluorescence microscopy in a pattern resembling vesicular transport (figure 42A). The kinetics for Tat internalization into CD8 T-cells found here are similar to those reported previously.(112) Colchicine, a potent inhibitor of microtubule assembly, has no effect on Tat uptake and transport into the cell (figure 42A). In contrast, colchicine does block Tat's ability to remove CD127 from the cell surface (figure 42B). Representative flow histograms and composite data from four independent experiments demonstrate CD127 surface expression does not change in the presence of Tat when incubated with colchicine ($p=0.39$ compared to media). These data suggest that while Tat

Figure 42: Down regulation of CD127 by Tat is dependent on microtubules. CD8 T-cells were incubated in media or media containing colchicine (30μm; Col), Tat (10μg/ml), or both. **(A)** Representative flow histograms and fluorescence microscopy showing Tat accumulation in CD8 T-cells over 3 to 12 hours in both the absence and presence of colchicine. Histograms show media as grey fill and Tat as black line. Intracellular Tat was detected after 3 hours incubation by fluorescence microscopy using an FITC conjugated anti-Tat antibody counterstained with DAPI. **(B)** Representative flow histograms and composite data for n=4 showing CD127 surface expression in the presence of Tat, colchicine, and Tat plus colchicine. Histograms show CD127 expression on cells in maintained in media (grey fill) and following treatment with Tat +/- colchicine for 24 hours (black line). Composite data show CD127 surface expression relative to media. Mean values +/- SEM are indicated. * p=0.0005


A
3 hours
6 hours
12 hours
media
colchicine
Tat-FITC
media
Colchicine

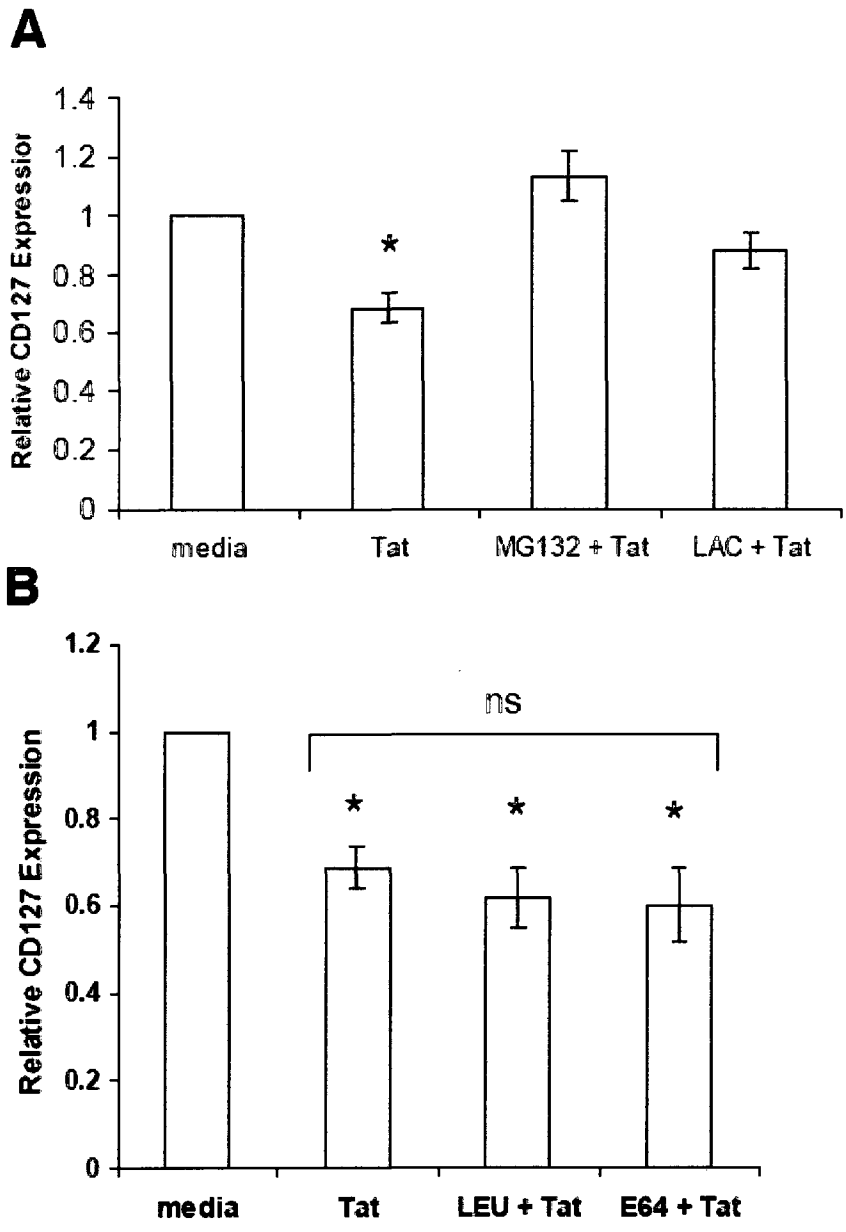

B
Col
Tat
Col + Tat
CD127-PE
Relative CD127 Expression
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
*
media
Tat
Col
Col +Tat

is able to enter the cell in the presence of colchicine, it's ability to remove CD127 from the cell surface is dependent on microtubules. Whether microtubule assembly is required to transport Tat from the endocytic vesicle back to the inner leaflet of the cell membrane where it interacts with CD127 or to allow CD127 capping and internalization following binding of Tat remains to be determined.

Tat promotes degradation of CD127 via the proteasome

Intracellular protein degradation occurs predominantly through lysosomes or through the ubiquitin-proteasome system. To determine whether Tat induces CD127 degradation by either of these pathways, CD8 T-cells were treated with soluble Tat protein in the presence of proteasomal or lysosomal inhibitors and CD127 expression was monitored by flow cytometry. As seen in figure 43A, the proteasomal inhibitors MG132 and lactacystin prevented a reduction in CD127 following the addition of Tat protein ($p \geq 0.29$ relative to media). In contrast, the lysosomal inhibitors leupeptin and E64 had no effect and Tat down regulated CD127 expression equally in the presence of these inhibitors as when added alone ($p \leq 0.03$ relative to media; figure 43B). Intracellular staining for CD127 showed the same effect (data not shown). These data suggest that upon binding to the cytoplasmic tail of CD127, Tat induces receptor capping and internalization and directs CD127 to the proteasome for degradation. These data are also consistent with those in figure 38 demonstrating new protein synthesis is required to replenish CD127 once Tat is removed from the culture media.

Figure 43: Proteasome inhibitors prevent Tat-induced decreases in CD127 expression. CD8 T-cells were incubated in media alone or in the presence of Tat (10μg/ml) or Tat plus inhibitors for 24 hours. Surface and intracellular CD127 expression was then analyzed by flow cytometry. **(A)** Surface CD127 expression relative to media control is shown for cells incubated in Tat or Tat plus proteasome inhibitors MG132 (5μM) or lactacystin (10μM; LAC). * $p=0.002$ ($p \geq 0.3$ for media versus Tat plus inhibitors) **(B)** Surface CD127 expression relative to media control is shown for cells incubated in Tat or Tat plus lysosome inhibitors E64 (10μM) or leupeptin (10 μM; LEU). * $p \leq 0.03$ relative to media. Values not statistically different are indicated by "ns". Mean values +/- SEM are indicated (n=4).

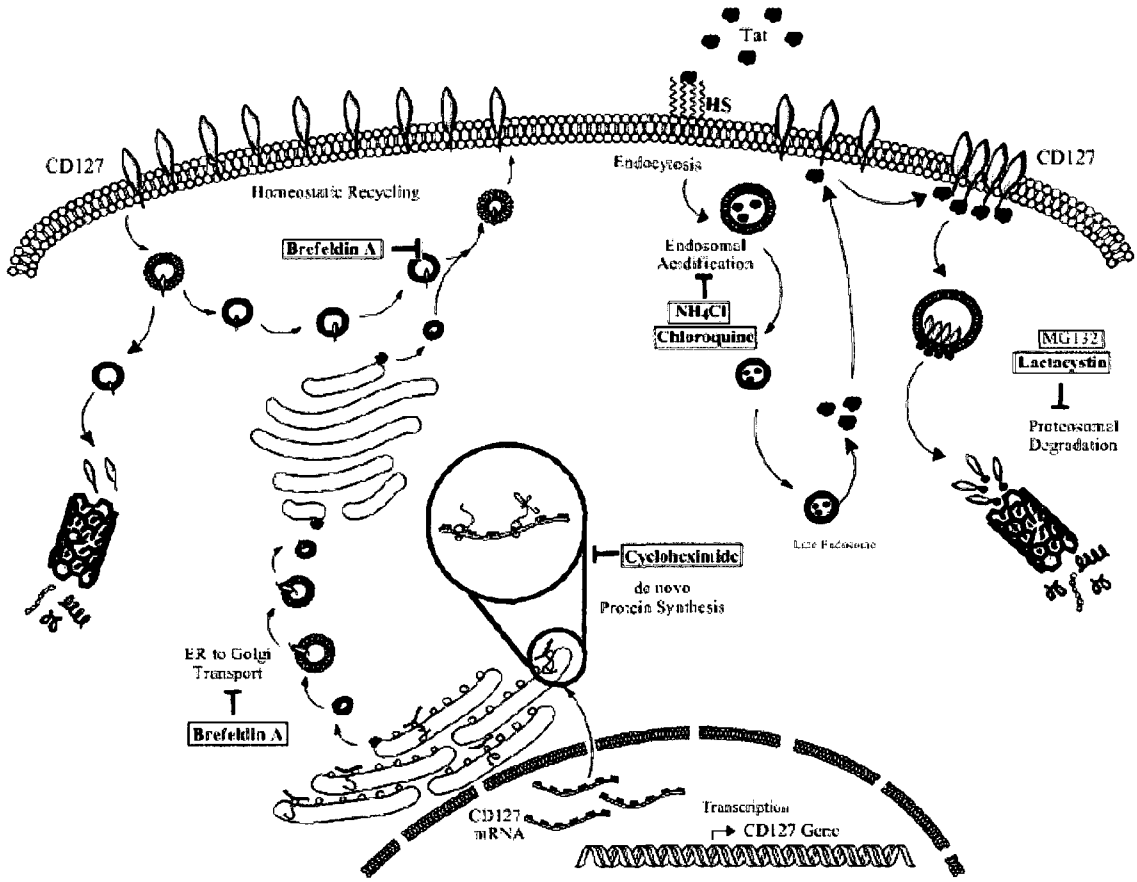

A
Relative CD127 Expressior
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
media
Tat
MG132 + Tat
LAC + Tat
B
Relative CD127 Expression
1.2
1
0.8
0.6
0.4
0.2
0
ns
*
*
*
media
Tat
LEU + Tat
E64 + Tat

DISCUSSION

IL-7 signaling is vital for optimal CD8 T-cell function and a number of groups including ours have shown decreased expression of the IL-7 receptor alpha-chain on the surface of CD8 T-cells during active HIV infection.(71-77). In chapter 3, I demonstrated this down regulation of CD127 is mediated in part by soluble HIV Tat protein.(118) Tat's effect on CD127 is both dose and time dependent, specific, reversible, and can be blocked with anti-Tat antibodies. Importantly, the decrease in CD127 induced by Tat results in deficits in CD8 T-cell function including reduced proliferation and impaired accumulation of intracellular perforin following stimulation with IL-7. Thus, by down regulating CD127 expression on CD8 T-cells, HIV Tat is able to block IL-7 signaling and impair both CD8 T-cell expansion and cytolytic capacity. I have also shown, unlike IL-7, Tat does not suppress CD127 gene transcription and down regulates the receptor independent of JAK kinase activity.(32)

In this chapter I have elucidated the overall mechanism by which soluble HIV Tat protein reduces CD127 expression on the surface of CD8 T-cells (figure 44). In resting CD8 T-cells, CD127 is evident over the entire surface of the cell although its distribution appears somewhat polarized. CD127 protein is quite stable in the absence of cell activation with a half-life of approximately 72 hours, and appears to continuously recycle on and off the cell membrane likely through early endosomes. A similar recycling at the plasma membrane has been described for both the IL-2 receptor alpha-chain (160, 161) and the IL-15 receptor alpha-chain. Recycling of the latter is induced by IL-15 and is dependent on the cytoplasmic domain of the receptor.(163) Consistent with previous studies, I have demonstrated soluble Tat protein enters CD8 T-cells and exits late

Figure 44: Proposed model showing homeostatic recycling of CD127 at the cell surface and down regulation of the CD127 by Tat protein. HIV Tat protein enters CD8 T-cells by binding via its basic domain to heparan sulphate proteoglycans (HS) on the cell surface followed by endocytosis. As early endosomes progress to late endosomes acidification of the vesicles allows Tat to exit the endosome and enter the cytosol. Once in the cytosol, Tat translocates to the inner leaflet of the cell membrane where it binds to the cytoplasmic tail of CD127. This association with Tat induces receptor aggregation and internalization and subsequent degradation by the proteasome.


Tat
HS
CD127
Homeostatic Recycling
Endocytosis
CD127
Brefeldin A
Endosomal
Acidification
NH4Cl
Chloroquine
MG132
Lactacystin
Proteasomal
Degradation
Late Endosome
Cycloheximide
de novo
Protein Synthesis
ER to Golgi
Transport
Brefeldin A
CD127
mRNA
Transcription
CD127 Gene

endosomes through a process dependent on the acidification of these vesicles. Once inside the cytoplasm, Tat translocates to the inner leaflet of the plasma membrane where it binds directly to the cytoplasmic tail of CD127. This interaction with Tat appears to induce receptor aggregation or capping and removal from the cell surface through a process dependent on microtubules. Data presented here suggest CD127 is internalized in the presence of Tat and directed to the proteasome for degradation.

The mechanism delineated here demonstrating Tat's effect on CD127 at the surface of CD8 T-cells is remarkably similar to that recently described by Kalantari et al.(155) who showed soluble Tat protein induces the internalization and degradation of the RON (recepteur d'origine nantais) receptor tyrosine kinase in monocytes and macrophages. Reminiscent of IL-7's effects on CD8 T-cells, RON plays a critical role in macrophage activation regulating cell proliferation, differentiation, phagocytosis and cell motility. As demonstrated by Kalantari et al.(155), soluble Tat protein is taken up by U937 monocytic cells as well as primary macrophages and induces the internalization and degradation of RON via the ubiquitin-proteasome pathway through a process dependent on the RON intracellular kinase domain. Similar to our previous data for CD127 expression in CD8 T-cells (32, 118), soluble Tat protein does not affect viability of monocytes and macrophages, does not regulate transcription of the RON gene, and does not require new protein synthesis to alter the expression of RON. Further, the effect of Tat on RON expression is specific with no change in the expression of other surface proteins including TrkA, CD14 or CD54.(155) These data suggest that soluble Tat protein, acting in a paracrine manner, may use a common mechanism to target receptors necessary for the activation of T-cells and macrophages and induce their removal from the cell surface and degradation through the proteasome.

The protein-protein interactions and protein modifications involved in Tat's down regulation of CD127 at the cell surface are currently under investigation in the lab. As proteins are removed from the cell membrane they are sorted along various endocytic pathways. This sorting is dependent on molecular signals which cause the tagged proteins to move from default pathways and be redirected to degradation compartments. This has been demonstrated for the IL-2 receptor where the β and γ chains are ubiquitinylated and degraded by the proteasome while the α chain recycles back to the cell surface (160, 164). It is possible that Tat binding to the cytoplasmic domain of the IL-7 receptor α-chain redirects the receptor from its normal recycling pathway at the cell surface to the proteasome through ubiquitinylation. Notably, Tat was recently shown to induce Tip60 polyubiquitination and degradation through recruitment of p300 and the E3-ubiquitin ligase Mdm2.(146) Tat's interaction with Mdm2 is intriguing in view of the role this ubiquitin-ligase plays in the endocytosis and degradation of other cell surface receptors.(165-167) Interestingly, Tat has also been shown to interact with subunits of the proteasome and increase the activity of the 26S proteasome (156, 157). In addition to CD127 and RON, Tat has also been shown to induce the proteasomal degradation of microtubule-associated protein 2 (MAP2) (158) and postsynaptic density protein 95 (PSD95) (80).

It is also possible that Tat could direct the proteasomal degradation of CD127 through phosphorylation. Although Tat does not possess intrinsic kinase activity, it has been shown to induce a variety of cellular kinases including Mitogen-Activated Protein Kinase (MAPK)(35), Protein Kinase C and the Extracellular Signal-Regulated Kinases (ERK) 1 and 2(168), as well as Phosphoinositide 3-kinae (PI3K) (95, 169, 170). Tat also

binds to and activates Protein Kinase R leading to phosphorylation of several protein targets including IkappaBα resulting in its subsequent proteasomal degradation (125, 126, 171, 172). While this pathway warrants investigation, I have already shown that unlike suppression of CD127 expression by IL-7, down regulation by Tat does not require at least JAK kinase activity (32). Along these lines I have also demonstrated synergy between soluble Tat protein and IL-7 in the down regulation of CD127 at the cell surface suggesting convergence of two distinct pathways. How and at what point these pathways converge requires further investigation.

While CD8 T-cells remain in the circulation at relatively normal frequencies in HIV-infected patients with advanced disease (19-23), in vitro studies have demonstrated functional deficits in these cells (15-18). Indeed, CD8 T-cells isolated from HIV+ patients proliferate poorly to their cognate antigens and fail to express normal levels of perforin or interferon (IFN)-γ, or demonstrate cytolytic activity in standard chromium release assays (20, 22-26). There is now abundant evidence demonstrating the negative effects of soluble Tat protein on lymphocyte function. While Tat has been measured in the serum of HIV+ patient at levels ranging from 40-550 ng/ml (114, 173), these concentrations in the peripheral circulation are likely low compared to concentrations in the lymph nodes and gut mucosa where the majority of HIV infected T-cells reside. Soluble Tat protein is readily taken up by infected cells including lymphocytes (88, 104, 105) and has been shown to impair lymphocyte proliferation following stimulation with tetanus toxoid, (115, 116) Staphylococcal enterotoxin B,(117) or anti-CD3 monoclonal antibodies (98). In chapter 3, I demonstrated that by down regulating CD127 expression on CD8 T-cells, the HIV Tat protein is able to impair both CD8 T-cell proliferation and

accumulation of intracellular perforin following stimulation with IL-7 (118). In this chapter, I demonstrate how Tat removes CD127 from the cell surface (174-176). Given the important role of IL-7 in CD8 T-cell function, down regulation of the IL-7 receptor alpha-chain by Tat likely contributes to the impaired cell mediated immunity and inefficient immunologic control of viral replication evident in HIV+ patients with progressive disease. The fact that Tat targets for degradation cell surface receptors important to immune cell activation including CD127 on CD8 T-cells and RON on macrophages makes this viral protein an important target itself in the development of therapeutic vaccines and other immune-based therapies.

CONCLUSION

It is well established that HIV infection results in a loss of CD8 T-cell activity. Although CD8 T cell counts remain at levels similar to healthy controls, over the course of HIV infection there is a progressive loss of CD8 T cell mediated immunity. CD8 T cells fail to respond to their respective antigens and loose cytolytic activities (20, 22-26).

Interestingly HIV patients with poor virologic control express lower surface levels of CD127 compared to healthy controls (71-77). In view of the importance of IL-7R, the established paracrine activity of HIV Tat protein and the established effects of Tat on cytokine and cytokine receptor expression, I hypothesized that this viral factor may also effect expression of the IL-7 receptor α-chain (CD127).

I have shown here that indeed soluble HIV Tat protein specifically down regulates surface expression of CD127 on purified CD8 T-cells and that this down regulation results in impaired CD8 T-cell proliferation and perforin synthesis following stimulation with IL-7. I have also shown that soluble HIV Tat protein alone is able to inhibit recovery of CD127 on the surface of CD8 T-cells isolated from HIV+ patients, providing further evidence that Tat likely plays a major role in the suppression of CD127. In chapter 6, I demonstrated that Tat does not affect CD127 protein translation or transport and directly impacts protein stability. I further demonstrate that Tat enters CD8 T cells by receptor mediated endocytosis and exits the endosomal pathway to enter the cytoplasm. Once in cytoplasm Tat interacts with the cytoplasmic tail of CD127 resulting in clustering and subsequent degradation by the proteasome. Finally, I have shown that Tat acts synergistically with IL-7 to down regulate CD127 on the surface of

CD8 T-cells through a process involving JAK kinase. In addition, Tat appears to have a negative effect on IL-7 induced entry into the cell cycle.

Taken together, these data suggest that by disrupting IL-7 signal transduction, Tat could impair cell mediated immunity, resulting in poor control of HIV replication leaving patients vulnerable to opportunistic infections. Data shown here provide an increased understanding of the mechanisms employed by Tat to suppress immune function, allowing HIV to evade immune control. This information may potentially lead to new strategies to repair immune deficiencies associated with HIV infection.

At present, HIV-1 vaccine strategies have been unsuccessful and have failed to prevent infection in vaccinated hosts. In order to control HIV infection, innovative strategies are required that go beyond the present understanding of vaccine development (177, 178). To further our understanding of how Tat interacts with CD127, our lab is currently characterizing CD127:Tat interactions using truncation and deletion mutations of the HIV-1 Tat protein. Once the specific site(s) of interaction have been identified, inhibiting Tat:CD127 interactions may provide a new strategy to promote prolonged CD8 T cell function in HIV patients.

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Elliott Faller - Curriculum Vitae

Education

Ph.D. in Microbiology and Immunology (Oral Defense Dec 16 2009)
Faculty of Medicine Department of Biochemistry, Microbiology and Immunology
University of Ottawa
Thesis Title: Hiv-1 Tat Protein Down Regulates Interleukin-7 Receptor On CD8 T Cells
Supervisors: Dr. P. MacPherson and Dr. J. Angel

Master of Science in Cell Biology (2003)
Faculty of Science, Department of Biology
University of Ottawa
Thesis Title: Modulation of Microtubule Dynamics by the Microtubule-Associated Protein MAP1a
Supervisor: Dr. D. Brown

Bachelor of Science in Biology Highest Honors (2000)
Faculty of Science, Department of Biology
Carleton University
Thesis Title: Non-Target effects of *Bacillus thuringiensis*
Supervisors: Dr. C. Wyndham and Dr. N. Cappuccino

Academic Awards

1. CIHR Doctoral Research Award, Graduate (Ph.D) Scholarship (2006-2009)
2. Fisher Scientific Award of Excellence (PhD, Faculty of Medicine) 2009
3. Kalpesh Oza New Investigator Award in Basic Sciences (CAHR 2009)
4. Graduate (Ph.D.) National Excellence Scholarship 2006-2009 (University of Ottawa)
5. Graduate (Ph.D.) Admission Scholarship 2005-2009 (University of Ottawa)
6. Graduate (M.Sc.) admission scholarship 2000-2001 (University of Ottawa)
7. NSERC summer research scholarship 2000 (Carleton University)
8. NSERC summer research scholarship 1999 (Carleton University)

9. Deans Honour List 1999-2001 (Carleton University)

10. J. Lorne Gray Scholarship 1999-2000 (Carleton University)

11. Canadian Association for AIDS Research Conference Basic Sciences Research Track Scientific Scholarship (2006 and 2009)

12. Young Investigator Award, 15th Conference on Retroviruses and Opportunistic Infections (2008)

13. Keystone Symposia Scholarship awarded by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (2008)

14. Canadian Scholarship, XVI International AIDS Conference (2006)

15. Travel Award, ISAC XXIII International Congress (2006)

16. Scholarship Award, 7th- 11 th Ontario HIV Treatment Network Conferences (2005 - 2009)

17. Travel Award from the Faculty of Graduate and Post Doctoral Studies (2007, 2008, 2009)

18. Travel Award from the Department of Microbiology/Immunology (2007, 2009)

19. Travel Award from the University of Ottawa GSA (2007, 2009)

20. 2nd Prize, OHRI Research Day Conference Poster Presentation (2009)

21. 2nd Prize, OHRI Research Day Conference Oral Platform Presentation (2005)

22. 2nd Prize university of Ottawa BMI Graduate student poster day (2006).

Additional Qualifications:

Introductory Flow Cytometry Course (2 days), ISAC XXIII International Congress (2006)

Paramedicine Diploma (1997) Algonquin College

Emergency Medical Care Assistant certificate for the practice of Para-medicine in Ontario (1997).

Publications:

Accepted Refereed Papers (4)

1. **Faller E**, Kakal J, Kumar R, MacPherson P. (2009) INTERLEUKIN-7 AND THE HIV TAT PROTEIN ACT SYNERGISTICALLY TO DOWN REGULATE CD127 EXPRESSION ON CD8 T-CELLS. *Int. Immunol. 21(3): 203-216.*

2. **Faller E**, McVey M, Kakal J, MacPherson P. (2006) INTERLEUKIN-7 RECEPTOR EXPRESSION ON CD8 T CELLS IS DOWN REGULATED BY THE HIV TAT PROTEIN. *Journal of Aquir. Immune Defic. Syndr. 43(3): 257-269.*

3. **Faller E,** Villeneuve T, Brown D. (2009) MAP1A ASSOCIATED LC3 STABILIZES MICROTUBULES BY SUPPRESSING MICROTUBULE DYNAMICS. *Molecular and. Cellular Neuroscience. 41(1): 85-93*

4. **Faller E**, Brown D. (2009) MODULATION OF MICROTUBULE DYNAMICS BY THE MICROTUBULE-ASSOCIATED PROTEIN MAP1A. *J. Neurosci. Res. 87: 1080-1089.*

Papers Submitted for Publication (5)

1. **Faller E**, McVey M, MacPherson P. THE HIV TAT PROTEIN MAINTAINS SUPPRESSION ON THE INTERLEUKIN-7 RECEPTOR ON CD8 T-CELLS ISOLATED FROM HIV+ PATIENTS. Manuscript submitted to *Journal of Biomedicine and Biotechnology*, December 2009.

2. **Faller E**, Sugden S, Mcvey M, Kakal J, MacPherson P. HIV IMPAIRS CD8 T-CELL FUNCTION BY REMOVING THE IL-7 RECEPTOR ALPHA-CHAIN FROM THE CELL SURFACE AND TARGETING IT FOR DEGRADATION. Manuscript submitted to *The Journal of Immunology*,
3. July 2009. Currently under revision.

4. Kakal J, Sugden S, **Faller, E,** MacPherson P. TRANSCRIPTIONAL REGULATION OF THE HUMAN IL-7 RECEPTOR ALPHA-CHAIN GENE IN CD8 T-CELLS. Manuscript Submitted to *Journal of Immunology,* December 2009.

5. **Faller E,** Villeneuve T, Brown D. IMPACT OF LC2 EXPRESSION ON MICROTUBULE INTERACTIONS WITH ASSOCIATED HEAVY (1A) AND LIGHT CHAIN (LC1, LC3) MOLECULES. Manuscript submitted to *Experimental Cell Research* December 2009.

6. **Faller E**, Ghazawi A, Sugden S, MacPherson P. REVIEW : THE ROLE OF IL-7 IN CTL FUNCTION AND VACCINE ADJUVANT. Invited review for the Journal of Biomedicine and Biotechnology. Manuscript submitted to *Journal of Biomedicine and Biotechnology*, December 2009.

Oral/Invited Presentations (11)

1. **Faller E**, McVey M, MacPherson P. (2009) HIV TAT BINDS TO THE CYTOPLASMIC TAIL OF THE IL-7 RECEPTOR ALPHA-CHAIN AND INDUCES RECEPTOR DEGRADATION VIA THE PROTEASOME. 18th *Canadian Association for HIV Research Conference,* Vancouver, BC. Presentation was awarded the Kalpesh Oza New Investigator Award.

2. **Faller E**, Ghazawi F, MacPherson P. (2009) JAK/STAT SIGNALING MEDIATES REGULATION OF IL-7R EXPRESSION ON CD8 T CELLS BY BOTH TRANSCRIPTIONAL AND NON-TRANSCRIPTIONAL MECHANISMS. *11th Ontario HIV Treatment Network Conference. Toronto, ON.*

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7. **Faller E**, McVey M, Kakal J, MacPherson P. (2006) HIV-1 TAT PROTEIN IMPAIRS CD8 T CELL FUNCTION BY DOWN REGULATING TRANSCRIPTION OF THE IL-7 RECEPTOR ALPHA CHAIN (CD127). *XVI International AIDS Conference. Toronto, ON.* Also presented at the *16th Canadian Association for HIV Research Conference.* Quebec City, QC.

8. Invited moderator for Immune Based Diseases Symposia. (2006) *6th Annual Ottawa Health Research Institute Research Conference. Ottawa, ON.*

9. Kakal J, **Faller E**, Prematunga C , MacPherson P. (2006) TRANSCRIPTIONAL REGULATION OF THE HUMAN IL-7 RECEPTOR ALPHA-CHAIN GENE IN CD8

T-CELLS. *15th Canadian Association for HIV Research Conference.* Quebec City, QC.

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Published Abstracts/Posters (28)

1. **Faller E**, McVey M, MacPherson P. (2009) HIV TAT BINDS TO THE CYTOPLASMIC TAIL OF THE IL-7 RECEPTOR ALPHA-CHAIN AND INDUCES RECEPTOR DEGRADATION VIA THE PROTEASOME. *Keystone symposia on HIV Immunobiology X4. Keystone, CO.*

2. **Faller E,** Alvarez G, Roth V, McCarthy A, MacPherson P. (2009) IS THE PHENOMENON KNOWN AS IRIS ACTUALLY A PERIOD OF IMMUNE SUPPRESSION? 18th *Canadian Association for HIV Research Conference,* Vancouver, BC.

3. Ghazawi F, **Faller E**, MacPherson P. (2009) EFFECTS OF INTERLEUKIN-7 ON NOTCH-1 EXPRESSION IN CD8 T-CELLS. *11th Ontario HIV Treatment Network Conference. Toronto, ON*

4. **E. Faller**, A. Weirich and P. MacPherson (2008) EXTRACELLULAR HIV-1 TAT STIMULATES STAT3 PHOSPHORYLATION IN CD8 T CELLS THROUGH INDUCTION OF A SOLUBLE FACTOR. 10th *Ontario Treatment Network Conference, Toronto, ON.*

5. **E. Faller,** J Kakal, MacPherson P. (2008) IL-7 AND HIV TAT ACT SYNERGISTICALLY TO DOWN REGULATE THE IL-7 RECEPTOR ALPHA-CHAIN ON CD8 T-CELLS THROUGH A PATHWAY INVOLVING JAK KINASE. *15th Conference on Retroviruses and Opportunistic Infections.* Boston, MA.

6. **E Faller**, McVey M, MacPherson P. (2008) THE HIV TAT PROTEIN MAINTAINS SUPPRESSION OF THE INTERLEUKIN-7 RECEPTOR ON CD8 T-CELLS ISOLATED FROM HIV+ PATIENTS. *Keystone symposia on HIV Vaccines X7, Banff, AB.*

7. **E. Faller,** MacPherson P. (2008) IL-7 ENHANCES CD8 T-CELL STIMULATION AND DIFFERENTIATION IN VITRO. 17th *Canadian Association for HIV Research Conference. Montreal, PQ. Also presented at the 9th Ontario Treatment Network Conference, Toronto, ON.*

8. J. Kakal, **E. Faller,** MacPherson P. (2008) REGULATION OF CD127 EXPRESSION IN CD8+ T-CELLS . *Keystone symposia on HIV Vaccines X7, Banff, AB.* Also presented at the 17th *Canadian Association for HIV Research Conference. Montreal, PQ. and the* 10th *Ontario Treatment Network Conference, Toronto, ON.*

9. **E Faller**, J Kakal, M Kryworuchko, MacPherson P. (2007) WHEN VIRUSES EXPLOIT PHYSIOLOGIC PATHWAYS. IL-7 AND HIV TAT ACT SYNERGISTICALLY TO DOWN REGULATE CD127 EXPRESSION ON CD8 T-CELLS. *Keystone symposia on HIV Pathogenesis X7. Whistler, BC.*

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16. McVey M, **Faller E**, MacPherson P. (2006) THE RATE OF IL-7 RECEPTOR α-CHAIN (CD127) DECAY FROM THE SURFACE OF CD8 T-CELLS IS ENHANCED BY THE HIV-1 TAT PROTEIN. 8th *Ontario HIV Treatment Network Conference.* Toronto, ON.

17. Kakal J, **Faller E**, Kumar R, MacPherson P (2006) TRANSCRIPTION OF THE CD127 GENE IS DOWN REGULATED BY IL-7 IN CD8 T-CELLS. *16th Canadian Association for HIV Research Conference. Toronto, ON. Also presented at the* 8th *Ontario HIV Treatment Network Conference.* Toronto, ON.

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21. *XXIII International Society for Analytical Cytology Conference.* Quebec City, QC.

22. **Faller E**, Brown D. (2006) USING LIVE CELL FLUORESCENT MICROSCOPY TO MEASURE THE MODULATION OF MICROTUBULE DYNAMICS BY THE MICROTUBULE-ASSOCIATED PROTEIN MAP1A IN VIVO. *XXIII International Society for Analytical Cytology Conference.* Quebec City, QC.

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25. Komsic-Vranjkovic A, **Faller E**, MacPherson P, Angel J (2004) REGULATION OF IL-7 RECEPTOR (CD127) ON CD8+ T CELLS BY IN VITRO HIV INFECTION.
26. *12th Conference on Retroviruses and Opportunistic Infections.* San Francisco, CA

27. Komsic-Vranjkovic A, **Faller E**, MacPherson P, Angel J (2004) DIFFERENTIAL REGULATION OF IL-7 RECEPTOR (CD127) ON CD8+ T CELLS: IMPLICATIONS FOR HIV IMMUNOPATHOGENESIS. *13th Canadian Association for HIV Research Conference.* Montreal, QC. Also presented at the 6th *Ontario HIV Treatment Network Conference.* Toronto, ON.

28. Hynes S, C Wyndham, N Auger, **E Faller**. (2001) SOIL MICROCOSMS AS ENVIRONMENTAL RESEARCH TOOLS FOR THE STUDY OF MICROORGANISM GENE TRANSFER IN SOIL ENVIRONMENTS. *44th Canadian Federation of Biological Societies Conference.* Ottawa, ON. Also presented at the 44th *Genetics Society of Canada Annual Meeting.* Kingston, ON.

Work Experience:

1. **Part Time Research Technician** (2005-Present) Ottawa Hospital Research Institute

2. **Full time Research Technician** (2003-2005) Ottawa Hospital Research Institute

3. **Graduate Biology Teaching Assistant/ Lab Demonstrator:** (2000-2003) University of Ottawa

4. **Undergraduate Research Assistant** (1999-2000) Microbiology Lab (Carleton University-Dr.Campbell Wyndham)

5. **Undergraduate Biology Teaching Assistant/ Lab Demonstrator:** (1999-2000) Carleton University

6. **Senior Resident at Carleton University:** (2003-2006) responsible for Upper Glengarry residence, responsible for counseling/mediation, supervision of residence fellows.

Community Involvement

1. **Presentations at the AIDS Committee of Ottawa (2009-Present)**

2. **Canada Wide Science Fair Judge** (2008) Health Sciences and Interdisciplinary sections

3. **Lets's Talk Science** (2008-Present)

4. **Volunteer Fire Fighter** (2005-Present) City of Ottawa Fire Service

5. **Carleton University Residence Community:** (2003-2006) Organizing and promoting student involvement in community events (AIDS walk, Big Brothers/Big Sisters, Ottawa Food Bank etc.)
6. **Manager/ Player for men's recreational soccer league** (1999-Present)

7. **Lifeguard team coach/volunteer/participant/judge** (1994-2001) at regional, provincial and national events.

8. **Royal Life Saving Society of Canada Instructor/Examiner for life guarding courses** (1995-2001)

8. **Aikido Instruction/Demonstrations at cultural events** (1999-2005)