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Expression of the Interleukin-7 Receptor Alpha-Chain is Down Regulated on CD4T-Cells by the HIV-1 TAT Protien

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EXPRESSION OF THE INTERLEUKIN-7 RECEPTOR ALPHA-CHAIN IS DOWN REGULATED ON CD4 T-CELLS BY THE HIV-1 TAT PROTIEN

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ABSTRACT

HIV infection elicits defects in CD4 T-cell homeostasis in both a quantitative and qualitative manner. Interleukin-7 (IL-7) is essential to T-cell homeostasis and several groups have shown reduced levels of the IL-7 receptor alpha-chain (CD127) on both CD4 and CD8 T-cells in viremic HIV+ patients. Our lab has demonstrated that soluble HIV Tat protein specifically down regulates cell surface expression of CD127 on human CD8 T-cells. Once taken up by CD8 T-cells, Tat enters the cytoplasm and interacts directly with the cytosolic tail of CD127 inducing receptor capping, endocytosis and degradation.

The purpose of this study is to determine if, similar to CD8 T-cells, HIV Tat down regulates surface CD127 expression on CD4 T-cells and if this down regulation affects IL-7 signaling. To investigate this, primary CD4 T-cells isolated from healthy HIV-negative volunteers were incubated in medium alone or with Tat protein (10 ug/ml) for up to 72 hours and then analyzed by flow cytometry.

As anticipated, soluble HIV Tat protein induces a significant decrease in surface CD127 expression on CD4 T-cells relative to cells cultured in medium alone. The effect was dose and time dependent, reversible, and could be blocked with anti-Tat antibodies or heparin. Tat down regulated CD127 equally on naïve and memory cells and did not require new protein synthesis to have its effect. Tat's down regulation of CD127 was not associated with cell activation as there was no change in overall cell phenotype including expression of CD25 and CD56. Further, expression of CD132, the common gamma-chain which associates with CD127 to form the IL-7 receptor, was also unaffected by Tat. Notably, down regulation of CD127 by Tat resulted in lower induction of the anti-apoptotic protein Bcl-2 following stimulation with IL-7.

In view of the important role IL-7 plays in CD4 T-cell proliferation, homeostasis and survival, this down regulation of CD127 by Tat may well play a role in HIV-induced immune dysregulation and CD4 T-cell decline. Understanding this effect could lead to new therapeutic approaches to reverse the CD4 T-cell loss evident during HIV infection.

for the most important people in my life...

Lisa- you picked me up when I was down

Lydia, Jillian & Mel - you push me to be a better man

and...

Mom- you gave me all I ever needed and more than I could ever want

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This work is not so much the culmination of my lab work but would more accurately be described as the acquisition and dispersal of the combined wisdom, experience and knowledge of all those I've encountered.

My supervisor, Dr. Paul MacPherson, created an environment in which kept me engaged by the techniques and subject as well as difficulties that go hand in hand with this type of research. I appreciate his ability to temper his high standards and sense of urgency with encouragement and patience. I feel lucky to have found a supervisor that was able challenge me to exceed my own expectations.

The students with whom I shared lab space were truly valuable resources. Elliott's constant source of input and ideas were invaluable along the way. I could always count on Juzer to take time out to help me when I needed it. Scott never ceased to amaze me with his encyclopedic knowledge, memory and outlook on life. I must thank Scott for helping me with his time and his perspective. Feras Ghazawi taught me more than I ever expected to learn in regards to using the resources available to me both in the lab and in life. Charlene regularly managed to drop what she was doing to help me out. Agatha was gracious enough to familiarize me with the flow cytometer. Angela helped me to keep my head up and eyes forward. I could always depend on Peter to motivate me to work harder and not give up... thanks for the laughs too.

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LIST OF ABBREVIATIONS

AIDS – Acquired Immunodeficiency Syndrome
ARV- Antiretroviral
Bad- Bcl-2 Antagonist of Cell Death
Bax- Bcl-2 Associated X Protein
Bcl- B-cell Lymphoma
BSA- Bovine Serum Albumin
c-ART- Combination Antiretroviral Therapy
CCR5- Chemokine Receptor 5
CD- Cluster of Differentiation
CFSE- Carboxyfluorescein Succinimidyl Ester
CHX- Cyclohexamide
CTL- Cyotoxic T-lymphocyte
CXCR4- Alpha Chemokine Receptor
DMSO- Dimethylsulfoxide
FACS- Fluorescent Activated Cell Sorter
FITC- Fluorescein Isothiocyanate
HAART- Highly Active Antiretroviral Therapy
HIV- Human Immunodeficiency Virus
Ig- Immunoglobulin
IL- Interleukin
kD- Kilodalton
LCMV- Lymphocytic Choriomeningitis Virus
MALT- Mucosa-associated Lymphoid Tissue
MHC- Major Histocompatability Complex
mRNA- Messenger Ribonucleic Acid
PBMC- Peripheral Blood Mononuclear Cell
PBS- Phosphate Buffered Saline
PC-5- Phycoerythrin Cyanin 5
PE- Phycoerythrin
PFA- Paraformaldehyde
PI-Propidium Iodide
RNA- Ribonucleic Acid
TAR- Transactivation Responsive Element
Tat- Transactivator of Transcription
TCR- T-cell Receptor
UNAIDS- Joint United Nations Programme on HIV/AIDS
USP- United States Pharmacopeia

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INTRODUCTION

1.1 The human immunodeficiency virus

In 1981, patients began presenting with a new disease manifested as an apparent deficiency in the immune system [1]. The appearance of unusual infections such as *Pneumocystis jiroveci* pneumonia and cancers such as Kaposi's sarcoma characterized this new syndrome [2-3]. The disease was termed acquired immune deficiency syndrome (AIDS) and, two years later, a virus isolated by Françoise Barré-Sinoussi *et al.* was determined to be the causative agent[4]. Electron microscopy revealed budding retrovirus morphology and the virus was later recognized as a lentivirus[5]. Three separate research groups isolated similar yet somewhat distinct retroviruses from AIDS patients and these viruses were categorized together as the human immunodeficiency virus (HIV)[6]. Another retrovirus recovered from AIDS patients in West Africa with up to a 40% genetic difference was discovered shortly afterwards and was named HIV-2 [3, 7-8]. The latest estimates put the global population of those living with HIV worldwide at 33.2 million (30.6-47.1 million) (UNAIDS/WHO 2007). An estimated 2.7 million people were newly infected in both 2007 and 2008 and 2 million AIDS-related deaths occurred in each of those years[8]. More than 60 million individuals have been infected with HIV-1 and more than 25 million have died due to complications resulting from AIDS since the pandemic began (UNAIDS/WHO, 2005).

HIV infects CD4+ T-cells and monocytes and results in the characteristic depletion of CD4 T-cells. This depletion occurs immediately following infection (the acute phase) at which time viral load is high and CD8 T-cells are highly activated. Virus replication occurs preferentially in CD4 T-cells expressing CCR5 (to be discussed) and a rapid net loss of these cells in the mucosa-associated lymphoid tissue (MALT) results [9-10]. Seroconversion follows and failure to completely clear the virus allows it to persist and replicate within monocytes and the CD4 T-cell pool. During the following 6 month to 20 year period, termed the asymptomatic phase, CD4 T-cell counts continue to decline until they reach critically low levels (<200 cells/μl) at which point the host is said to have AIDS. At this stage, the host has a compromised ability to fight off pathogens leading to an increased susceptibility to opportunistic infections[11].

The virus gains access to cells via binding with the CD4 receptor [12-13] as well as the coreceptors CCR5 or CXCR4 [14-16]. This permits infection of not only CD4 T-cells but also macrophages and dendritic cells which serve as primary antigen presentation cells [17]. This cell tropism allows for efficient spread of the virus throughout the host immune system. Once inside host CD4 T-cells, the virus is able to replicate itself by hijacking the host cell machinery facilitating its own replication and interfering with normal T-cell function [18]. The process begins with fusion of the viral envelope to the host cell. The viral core then enters the cell, uncoats and releases the viral RNA into the cytoplasm. The HIV enzyme reverse transcriptase converts the viral RNA into DNA. This DNA then moves into the nucleus and integrates into the host genome. Once integrated, the HIV DNA is known as provirus. The host can now produce viral particles which, when released from the cell, begin the process again [11, 19].

After over 20 years of research, AIDS has been transformed from a disease in which patients died within a few years of infection [20] to a treatable chronic disease. HIV treatment has evolved to utilize numerous anti-HIV drugs (called anti-retrovirals or ARVs) in combination. This technique is referred to as highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (c-ART). Over 20 ARVs had been developed for treatment by 2003[21]. Therapy has focused on inhibiting a number of the viral enzymes including reverse transcriptase [22-23], protease [24-25], and integrase necessary for insertion of the viral genome into the host chromosome [26]. Viral fusion inhibitors [27-28] and CCR5 binding inhibitors [29-30] have also been developed. By suppressing HIV replication, HAART has significantly reduced morbidity and mortality. However, treatment has led to problems concomitant with a multi-drug regimen; toxic side-effects and daily adherence to medication over many years are a couple of the current challenges. In a study by d'Arminion et al. a cohort of over 800 antiretroviral naïve HIV positive patients were evaluated for HAART discontinuation. After a median 45 week follow-up visit, more than 35% of the patients had discontinued therapy. The study indicated the probability of discontinuing HAART at 1 year was 25.5% [31]. The current approaches could be much improved. Toxic effects linked to ARVs impact a variety of organ systems. For example, ARVs have been shown to cause hepatotoxicity [32], accelerate bone loss [33] and lead to increased cardiovascular risk [34]. Difficulties also arise as a result of resistance to ARVs due to the generation of multiple variants. These variants result primarily from the low fidelity of reverse transcriptase with errors introduced during replication of RNA to DNA. The daily generation of 10^9 to 10^{10} virions results in significant viral variation within individuals [11]. This is further complicated by the emergence of recombinant forms [35].

Inasmuch as HAART has facilitated control of HIV infection, current avenues have failed to elicit long-term control of this virus without the potential of highly adverse effects. Despite nearly three decades of work, both a cure and vaccine remain elusive. It is clear that novel approaches in treatment and patient care are required.

1.2 CD4 T-cells

T-cells are crucial responders to viral infections [36]. The initial observation of two functionally distinct T-cell subsets was based on the expression of the surface antigens CD4 and CD8 [37-38]. The CD4 T-cell subset has since been further classified into T_H1, T_H2, T_H17 and T_{reg} subsets based on cytokine response profiles. The $CD4^+$ population is clearly heterogeneous in phenotype and function. It comprises from 50-70% of circulating T-cells [39] and is most notably associated with helper/inducer activity in the immune system [40]. Helper (T_H2) activity facilitates the humoral response (B-cell proliferation, class switching and antibody production). Inducer (T_H1) activity activates other T-cells leading to a cellular response (proliferation of cytotoxic CD8 T-cells and release of proinflammatory cytokines) [11, 41]. T_H17 activity enhances neutrophil response via the synthesis and release of cytokines which include members of the IL-17 family such as IL-17A and IL-17E [11]. T_H17 cells also produce IL-22 which, in cooperation with IL-17, acts as an amplifier of the acute inflammatory response at sites of early infection [11]. T_{reg} cells maintain tolerance in the immune response by mitigating the T_H1/T_H2 response [11]. The differential regulation of these CD4 T-cell responses is believed to be pivotal in the immune system response [42-43]. CD4 T-cells respond through the T-cell receptor to antigens presented on class II MHC

molecules [44]. This, in the presence of co-stimulatory molecules, activates the cells and stimulates the release of proliferative factors including IL-2. CD25, the alpha-subunit of the IL-2 receptor is also up regulated upon T-cell stimulation allowing IL-2 to act in an autocrine and paracrine fashion. Proliferation of T_H1 CD4 T-cells magnifies the release of these and other pleiotropic cytokines which in turn promotes the proliferation and development of CD8 T-cell effector cells. Without CD4 T-cell activation and signaling via IL-2, CD8 T-cells become anergic and undergo subsequent apoptosis [45]. In total, it is believed that a normal CD4 T-cell population assists the proliferation and activity of CD8 T-cells and also facilitates antiviral antibody responses [46]. In a healthy homeostatic state, CD4 T-cells balance proliferative, inflammatory, and cytotoxic activities within the immune system.

1.3 CD 4 T-cells and HIV

Explaining the progressive loss of CD4 T-cells is a major challenge in understanding HIV disease progression [47-48]. The direct infection and killing of CD4 T-cells can explain only a portion of loss. It is estimated that less than 0.2% of the peripheral CD4 T-cell population is productively infected [49-51] which is much lower than the number of cells lost from the circulation [52]. Production of CD4 T-cells is also likely diminished.

The CD4 T-cell population was shown to be crucial in clearance of chronic infection in a study involving lymphocytic choriomeningitis virus (LCMV). LCMV-specific CTL response was completely lost in CD4-depeleted mice chronically infected with LCMV [53]. CD4 T-cells are required for clearance of numerous viral infections including measles [54] and West Nile Virus [55] as well as HIV. As mentioned above, CD4 T-cell depletion is a

hallmark of HIV disease progression and, in fact, no other human viral infection has shown such profound CD4 T-cell loss. HIV depletes CD4 T-cells by numerous mechanisms including direct infection and killing of the cells, increasing susceptibility of infected cells to apoptosis, and targeting of cells to HIV-specific natural killer (NK) cells and CTLs which recognize the non-self antigens presented by infected CD4 T-cells [11, 56-57]. Population depletion has also been suggested to result from migration of the cells out of peripheral blood and retention in lymph nodes or non-lymphoid tissues (liver or lung) leading to the appearance of CD4 T-cell loss [58-60]. Chronic activation[61] and impaired production[62] have also been suggested to account for CD4 T-cell population decline. The estimated number of mature CD4 T-cells for a normal young (<30 year old) adult is dramatically diminished by the time the peripheral blood CD4 T-cell count falls to 200 cells/μl [63-64]. The fact that the depletion found in the CD4 T-cell compartment occurs over such a tremendously varied period of times (6 months-20 years), suggests this depletion is the result of multiple mechanisms.

CD4 T-cell depletion is compounded by HIV-induced qualitative changes in the population. Subsets within the pool are differentially depleted resulting in a reduction in the memory/naïve CD4 T-cell ratio [65-68]. Many complex signals regulate the memory subset including those received through the interleukin-7 receptor (IL-7R) [69-70] which is highly expressed on naïve and memory cells and also to a lesser extent on primary effector cells destined to become long term memory cells [71]. Numerous studies have shown decreased IL-7R levels in CD4 and CD8 T-cell populations of HIV-infected individuals [72-74] and loss of this receptor was shown to correlate with CD4 T-cell depletion [75]. Compared with healthy controls, HIV-infected individuals show an increase in $CD127^-$ cells relative to the

absolute decline in $CD127^+$ CD4 T-cells [67]. Changes in the fraction of CD4 T-cells expressing IL-7R have been associated with disease progression [67] and impaired responsiveness to IL-7 (especially in the memory subset) has been demonstrated in HIV-infected individuals [76]. The significance of IL-7R and the implications of homeostatic imbalance contributing to CD4 T-cell depletion during HIV infection warrant examination of the IL-7 pathway.

1.4 Interleukin-7

Interleukin-7 (IL-7) is a 25 kilodalton (kD) protein produced by a variety of cell types including endothelial cells, hepatocytes, keratinocytes, and neurons [77-79]. It is expressed in most organs including the brain [80] and is secreted by stromal cells in the thymus and bone marrow facilitating B and T-cell development in mice and T-cells in humans [81].

IL-7 plays an integral role in maintaining T-cell homeostasis. It has been shown to induce antiapoptotic factors, such as Bcl-2 and Bcl-xL [82], and inhibit the pro-apoptotic factors Bad and Bax, thus prolonging T-cell survival [83]. Human memory CD4 T-cells were found to proliferate in vitro in response to IL-7 and IL-15. The absence of IL-7 results in a substantial depletion of resting memory CD4 T-cells and failure to regenerate this subpopulation [70].

IL-7 also acts on CD8 T-cells to help initiate cellular responses. It facilitates anti-viral and anti-tumor cyotlytic activity in this subset [84-85]. Through activation of the transcription factor STAT5 [86], IL-7 upregulates perforin expression which is used by CD8 T-cells to lyse their targets [87].

In short, IL-7 is central in controlling CD4 T-cell homeostasis, thymopoiesis and proliferation of naïve T-cells and in mediumting CTL response [83, 88-92]. It is also required for the long term survival of memory T-cells, especially within the CD4 T-cell subset [70, 93].

IL-7 signals through IL-7R which is a heterodimeric complex comprised of two subunits: a unique α-chain (CD127) [94] and a common γ-chain (CD132) [95]. CD132 is a component of the IL-2, IL-4, IL-9, IL-15, and IL-21 receptor complexes [94, 96] putting IL-7R into the γc family of receptors. CD127 is highly expressed on all quiescent T-cell populations, including memory CD4 and CD8 T-cells [97-98]. IL-7 signal transduction requires both receptor components. It binds initially to CD127 which promotes heterodimerization with CD132 and subsequent activation of numerous signaling pathways. These pathways are still poorly defined but the proteins p56lck, p59fyn, JNK, p38, PI3K, STAT3 and STAT5 have all been implicated downstream in the signaling cascade [78]. T-cell activation induces rapid up regulation of CD132 [99] and, in contrast, down regulation of CD127 through the TCR or γc family cytokines including IL-7 [83, 100]. Differential regulation of the IL-7R chains is proposed to optimize utilization of γc family cytokines other than IL-7 while maximizing IL-7 stores for resting cells and promotion of naïve T-cell survival [101].

In short, IL-7 has been demonstrated to be important in homeostatic maintenance and activation of T-cells. This is reinforced by how tightly expression of the IL-7 receptor is controlled. These factors highlight the essential role of IL-7 signaling in cell mediated immunity.

1.5 Interleukin-7 and HIV

HIV-1 infection is associated with significant dysregulation of the IL-7 signaling pathway. Reduced CD127 surface expression on CD4 and CD8 T-cells has been associated with HIV disease progression [74-75, 102-103]. The consequences are varied and extensive. HIV infection activates the IL-7/CD4 T-cell regulatory loop leading to increased plasma IL-7 levels presumably in response to low CD4 T-cell counts [104-105]. Plasma IL-7 levels are inversely correlated with CD4 T-cell counts in this condition [78]. Viremia in untreated patients also results in ineffective IL-7-mediated homeostatic regulation despite elevated levels of IL-7 [106]. Decreased expression of the IL-7 receptor on the surface of CD4 and CD8 T-cells in viremic HIV+ patients results in poor responses to IL-7 including decreased induction of anti-apoptotic factors [73] and CD25 expression [107]. Together, these outcomes support the idea that disruption of the IL-7 signaling pathway during HIV infection has a significant effect on CD4 T-cell number and function and, ultimately, on the integrity of the immune system.

1.6 HIV Tat accessory protein

Of the nine genes encoded by the HIV genome [17], the accessory protein Tat is of particular interest as it has been shown to adversely affect neighboring cells [103, 108-110] and could contribute to the continual decline in the CD4 T-cell population seen during chronic infection.

HIV-1 Tat was originally characterized as a viral activator of gene transcription, or *trans*-activator of transcription. It is a 14-16 kD protein encoded by two exons and varies in length from 86-110 amino acids depending on the splice variant. HIV Tat in clinical isolates is found predominantly in its 101 amino acid form [111-112]. The transactivation process begins with the binding of Tat to the transactivation responsive element (TAR) on nascent viral RNA enhancing the processivity of RNA polymerase and ensuring synthesis of full-length HIV transcripts. The absence of Tat results in inefficient transcription and short abortive viral mRNA. Tat has additionally been shown to modulate the expression of cellular genes resulting in the up regulation of IL-2, -4,-6, -8, -10, CCR5 and CD25 in HIV-infected cells and T-lymphoblastic cell lines [113-115].

Tat is secreted by infected CD4 T-cells [116] and can be found in the medium of cultured HIV-infected cells as well as in the serum of HIV-infected patients [117]. Soluble Tat protein is internalized by uninfected cells via its association with heparin proteoglycans on the cell surface followed by endocytosis in T-cells by clathrin-coated pits. Acidification of the endosome allows Tat to translocate into the cytosol [118] where it elicits a variety of effects on cell function.

The HIV-1 Tat protein is comprised of six functional domains [119]. In addition to its transactivating properties, HIV-1 Tat has been shown to interact with other transcription factors including NF-κB [120], Sp-1 [121], and NF-IL-6 [122] thereby contributing to cellular dysregulation. Tat can damage the host by dysregulation of cytokine expression, induction of apoptosis in neuronal cells, and interaction with other cellular factors which could lead to development of a favorable environment for opportunistic infections [108].

The numerous cytokines influenced by the presence of HIV-1 Tat are modulated through the protein's association with transcription factors and activation of signaling pathways. Through more than one mechanism, including binding to IL-6 leader RNA [123] and activation of IκB degradation and nuclear translocation of NF-κB [124], Tat promotes IL-6 production. HIV-1 Tat activates Sp-1, CREB-1 [125-126] and also regulates histone H3 modifications on the IL-10 promoter [127]. Both of these interactions lead to induction of IL-10 production. The Tat-induced overproduction of IL-6 and IL-10 leads to the development of B-cell lymphoma, Kaposi's sarcoma, and AIDS-associated non-Hodgkin's lymphoma [128]. Tat has been demonstrated to induce TNF-α production both directly [110, 129] and through interaction with the costimulatory protein CD40 [130]. This induction has diverse consequences including activation of other cytokines, cell death, viral replication and even neurotoxicity [112]. HIV-1 Tat has also been shown to induce IFN-α expression in PBMCs [131-132] and perturb the IFN-γ-signaling cascade in primary blood macrophages [133]. Disruption of the IFN-γ-signaling cascade has been suggested to lead to inhibition of antigen presentation, compromise immune response to pathogen invasion and enhance intra-cellular pathogen replication [112]. Signaling pathways associated with disruption of these cytokines include upregulation of MAP kinases, protein kinase R (PKR), protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and suppression of STAT-1 phosphorylation [112]. Tat has even been shown to differentially disrupt the apoptosis pathway. Jurkat cells overexpressing low levels of Tat are less susceptible to apoptosis [134] but, conversely, high levels of Tat late in HIV infection have been shown to paradoxically cause induction of T-cell apoptosis [112].

Interaction with various coinfecting opportunistic pathogens during AIDS progression is yet another implicated characteristic of HIV-1 Tat. It interacts with herpes simplex virus-1 (HSV-1) to induce HIV-1 transcription in coinfected cells [135-136]as well as the Kaposi's sarcoma-associated herpes-virus (KSHV) to contribute to Kaposi's sarcoma development in AIDS patients [137]. HIV-1 Tat also interacts with human papillomavirus (HPV) leading to changes in gene expression profile and resulting in the development of cervical, anal and oral cancers [138-140]. Tat has been shown to interact with protozoa from the Trypanosomatidae family [141], various microbes in the *Mycobacterium* genus [142] and the fungal species *Candida albicans* [143]. These interactions not only lead to enhancement of opportunisitic infections but also contribute the growth and dissemination of both HIV and the coinfecting pathogens.

Asymptomatic HIV-infected patients have higher levels of anti-Tat antibodies than symptomatic patients [144]. In fact, the presence of anti-Tat antibodies in patients' sera is associated with a slower rate of AIDS progression [145]. Indeed, the presence of anti-Tat cytotoxic T-lymphocyte responses has been shown to be inversely correlated with less viremia and slower progression of AIDS [146]. In light of these data and the wide range of its deleterious effects, Tat represents a compelling direction for research in pursuit of novel vaccines and/or treatment for HIV patients.

1.7 Soluble Tat protein down regulates CD127 on the surface of CD8 T-cells

The MacPherson lab has examined the affects of HIV-1 Tat protein on CD8 T-cells. Reduced expression of the IL-7R α-chain (CD127) on CD8 T-cells from HIV-infected

patients with active viral replication was demonstrated [74] and this reduction is at least partly due to the HIV-1 Tat protein. Specific, dose- and time-dependent down regulation of CD127 surface expression was demonstrated and these effects could be blocked with anti-Tat antibodies as well as with heparin. This down regulation lead to inhibition of T-cell proliferation and perforin synthesis after stimulation with IL-7 [103]. Tat significantly decreases the half life of surface CD127. Tat is endocytosed by CD8 T-cells and is released into the cytosol upon endosomal acidification. It can then bind to the cytoplasmic tail of CD127 on the inner leaflet of the cellular membrane. Receptor aggregation and microtubule-dependent internalization follows and Tat appears to then target CD127 for proteosomal degradation [147]. The significant role IL-7 plays in cell-mediated immunity suggests a pivotal role for Tat-induced down regulation of CD127 and subsequent disruption of IL-7 signaling. The effect of HIV-1 Tat on CD127 expression may play a pivotal role in the HIV-induced immune dysregulation and progression to AIDS.

The effects of Tat on CD127 expression on CD4 T-cells have not yet been examined.

1.8 Hypothesis

I propose that HIV-1 Tat protein down regulates CD127 expression on the surface CD4 T-cells in a manner similar to that previously shown on CD8 T-cells.

MATERIALS AND METHODS

2.1 T-cell separation and treatment

2.1.1 Isolation of Peripheral Blood Mononuclear Cells

After obtaining informed consent, whole blood from healthy volunteers with no known risk factors for HIV infection was collected into 60mL syringes (BD Biosciences) containing 1mL of 100 USP U/mL Heparin Lock flush solution (Hospira) and mixed by inversion. Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood by density centrifugation using Ficoll-Paque PLUS (GE Healthcare). The blood was centrifuged at a 2:1 (blood: Ficoll) ratio in a Megafuge 1.0 centrifuge (Heraeus Instruments) at 443g for 35 minutes with the brake turned off. The plasma layer was then discarded and the PBMC-containing buffy coat was transferred into a sterile tube and washed twice in phosphate buffered saline (PBS) and centrifuged at 443g for 10 minutes after each wash. Cells were counted on a hemocytometer prior to the second wash.

2.1.2 Isolation of Primary CD8 T-Cells

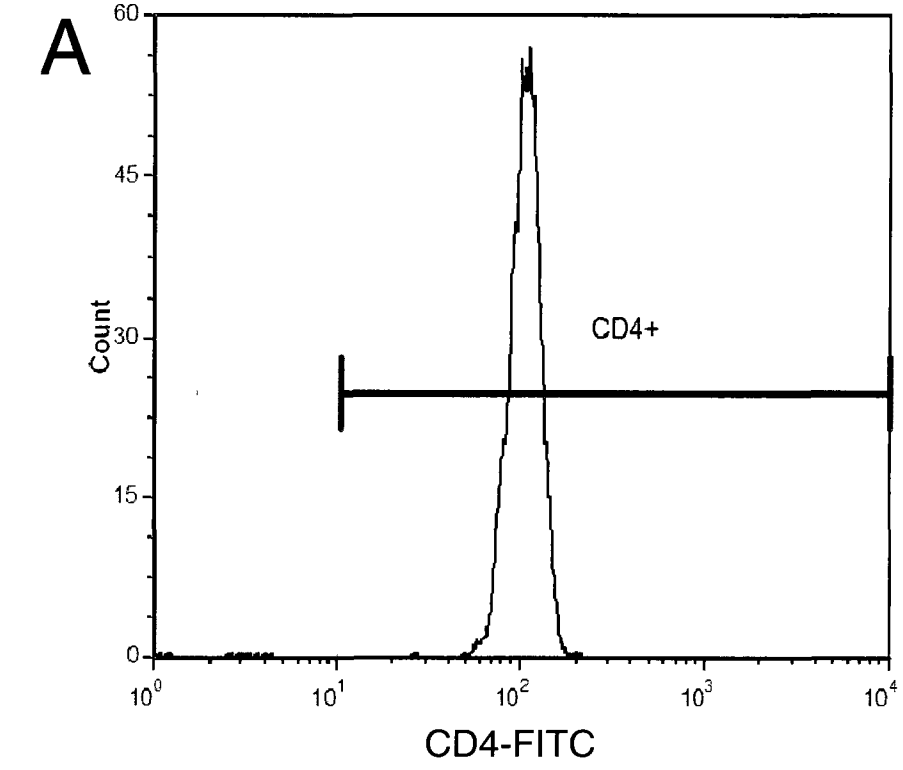
CD8+ T-cells were separated from the PBMC by magnetic cell sorting using Miltenyi's CD8 T-cell purification protocol as follows. PBMC were incubated with anti-CD8 antibody conjugated ferromagnetic beads at a concentration of 20 µl/10 million cells in 80 µl of PBS for 15 minutes at 4° C. The cells were then washed with PBS and the cell pellet was resuspended in 2 ml of MACS buffer (5% BSA and 2mM EDTA in PBS) and the cells were separated using the AutoMACS cell separator positive sensitive selection program

(Miltenyi Biotech). Purified CD8 T-cells were counted on a hemocytometer and resuspended at 10^6 cells/ml in RPMI 1640 (Hyclone) medium supplemented with 20% fetal calf serum (FCS; Cansera, Rexdale, Ont. Canada) plus penicillin (100 Units/ml), and streptomycin (100 µg/ml) (RPMI-20).

2.1.3 Isolation and Culture of Primary CD4 T-Cells

Before sorting for CD4+ T-cells, the negative cell fraction obtained from the CD8 T-cell isolation was suspended in RPMI-20 (25 ml/syringe) and incubated in a 250 ml polystyrene 75 cm^2 flask (BD Falcon™) at 37°C for 75 minutes. This step allows CD4+ monocytes to adhere to the flask. The medium containing nonadhered lymphocytes was then decanted into a new tube. CD4 T-cell separation was performed in the same manner as for the CD8 T-cells using anti-CD4 antibody conjugated ferromagnetic beads. In order to confirm purity and ensure that CD4+ monocytes had been successfully eliminated, the cells were analyzed for CD4 and CD14 (a monocytic marker). As shown in figure 1, the cells were consistently more than 97% pure CD4 T-cells.

Figure 1: Purity of the isolated CD4+ T-cell population.
(A) Representative flow cytometry histogram of purified CD4 T-cells stained with anti-CD4-FITC demonstrating 97.8% of the cells are contained within the single peak. Autofluorescence is represented by grey fill. (B) Staining for the monocyte-specific CD14 marker demonstrated only 0.3% of the CD4 T-cell preparation (left peak) was CD14+. Adherent monocytes were also stained separately for CD14 as a positive control (right peak).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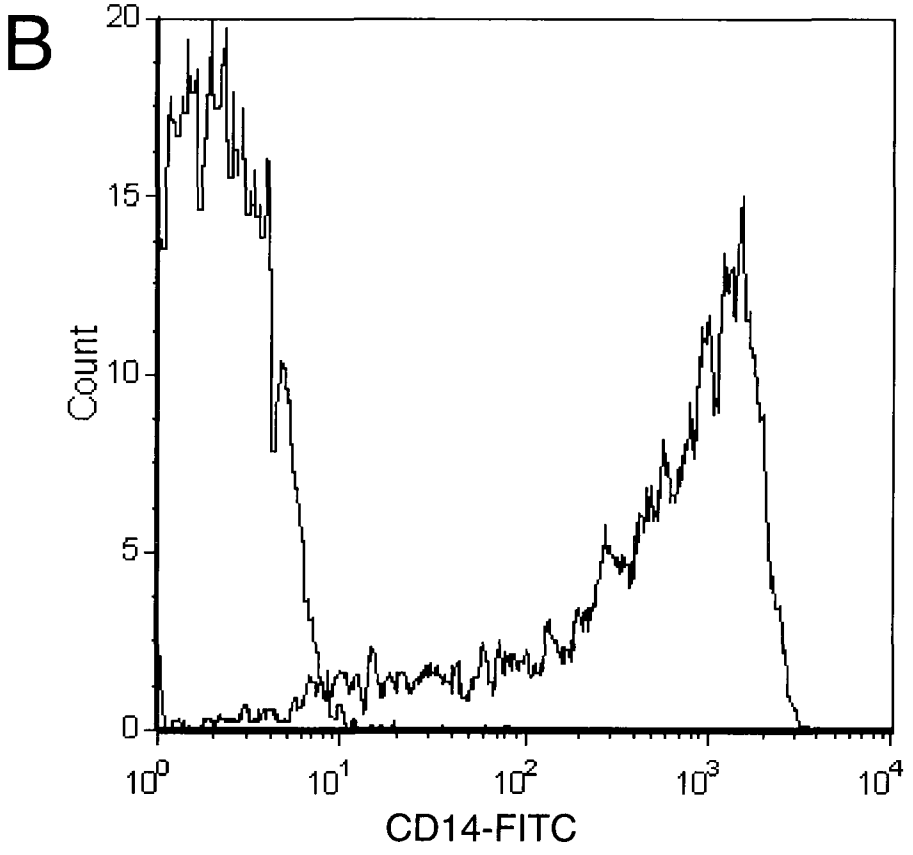
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CD14-FITC

The isolated T-cells were aliquoted and cultured at up to 10^6 cells/ml in FACS tubes (BD Biosciences; 5ml polystyrene round-bottom) in volumes not exceeding 500 µl/tube. Cells were maintained in medium alone or suspended in medium containing purified HIV-1 Tat protein (Advanced Bioscience Laboratories, Inc.) or recombinant human IL-7 (Invitrogen). Lyophilized Tat protein was resuspended in PBS containing 5mM dithiothreitol plus 0.1% BSA to give a final Tat concentration of 1 µg/µl. Cells in 500 µl of medium were treated with 10 µl Tat to give a final concentration of 20 µg/ml, 5 µl to give a concentration of 10 µg/ml and so forth. IL-7 was suspended in PBS with 0.1% BSA to give a concentration of 1 µg/ml and 5 µl were added to 500 µl of cells to give a final concentration of 10 ng/ml. Cells were then incubated in a 37°C, 5% CO_2 incubator for the indicated times.

Control experiments were also carried out where CD4 T-cells were incubated in RPMI-20 containing control standard endotoxin (CSE; Associates of Cape Cod Incorporated). A 0.5 µg vial of endotoxin was reconstituted in 500 µl PBS with 0.1% BSA to give a concentration of 1ng/µl. Of this 5 µl were added to 500 µl of CD4 T-cells (10^6 cells/ml) to give a final endotoxin concentration of 10 ng/ml. To block Tat's effect, purified Tat protein was incubated with heparin (Organon; 10 µl/ml to give final concentration of 100 USP units/ml) or rabbit anti-Tat polyclonal antibodies (Diatheva; ANT000) at a final concentration 100 µg/ml.

2.2 Flow cytometry

Surface staining by flow cytometry was used to verify phenotype and activation state as well as CD127 surface expression. Cells were incubated with the appropriate flourochrome-labeled antibodies for 20 minutes in the dark at room temperature and analyzed immediately using a Coulter Epics ALTRA flow cytometer (Fullerton, CA). Live cells were gated on the basis of side and forward scatter and at least 10,000 events were recorded for each sample. The following antibodies were obtained from Immunotech Beckman Coulter: anti-CD127-PE (R34.34), anti-CD3-FITC (UCHT1), anti-CD4-FITC (13B.2), anti-CD8-PC5 (B9.11), anti-CD14-FITC (RM052), anti-CD25-PC5 (B1.49.9), anti-CD28-PC5 (CD28.2), anti-CD45RA-FITC (ALB11), anti-CD45RO-FITC (UCHL1), anti-CD56-PC5 (N901(NHK-1), and anti-CD62L-FITC (DREG56). The IL-7R γ-chain was detected using anti-CD132-PE (555900) from BD Biosciences. All antibodies were used at saturating concentrations and the corresponding isotype controls were included.

2.3 Cell viability assays

Purified CD4 T-cells were incubated in medium alone or with Tat protein (10 μg/ml) in a humidified incubator at 37°C with 5% CO_2. Camptothecin (4mM; Sigma-Aldrich) was used as a positive control for apoptosis. At 24 and 72 hours, cells were stained with Annexin V-FITC and propidium iodide (PI) using the Apoptosis Detection Kit I (BD Biosciences: Pharmingen) according to the manufacturer's directions. In brief, cells were washed twice in cold PBS and resuspended in 1X binding buffer (provided) at 1 x 10^6 cells/ml. Cells were

then transferred to FACS tubes in 100 µl aliquots and stained with 5 µl of Annexin V-FITC and/or PI, gently vortexed, and incubated in the dark for 15 minutes at room temperature. Cells were then diluted with an additional 400 µl of binding buffer and immediately analyzed by flow cytometry.

2.4 Washout experiments

Purified CD4 T-cells were incubated in RPMI-20 at 1 x 10^6 cells/ml alone or in the presence of Tat protein (10 µg/ml) and/or cycloheximide (CHX 100 µM ; Sigma-Aldrich). Where indicated, cells were pre-incubated with cyclohexamide for two hours prior to the addition of Tat protein. At the times indicated, cells were washed twice in medium and resuspended in RPMI-20 alone or RPMI-20 containing cycloheximide. Cells were returned to 37°C for 24, 48 or 72 hours and then stained with anti-CD127-PE antibodies as described above and analyzed by flow cytometry.

2.5 Intra-cellular staining for BCL-2

Purified CD4 T-cells were pre-incubated at a concentration of 1 x 10^6 cells/ml either in RPMI-20 alone or with Tat protein (10 µg/ml) for 48 hours. Recombinant human IL-7 (10 ng/ml; Invitrogen) was then added and the cells were incubated at 37°C for 72 hours. Following treatment, the cells were fixed in 750 µl 2% paraformaldehyde in PBS (PFA) at room temperature for 10 minutes. The samples were then washed twice with PBS containing 1% BSA and permeabilized by resuspending in 500 µl cold methanol for 10 minutes at 4°C.

The cells were again washed twice as above and resuspended in 100 µl PBS with 1% BSA. The cells were then stained with FITC-conjugated mouse anti-human Bcl-2 monoclonal antibodies (Bcl-2/100; BD-Pharmingen) or FITC labeled mouse IgG, κ isotype control (MOPC-21; BD-Pharmingen) for 45 minutes at room temperature in the dark. The cells were then washed as above, resuspended in 200 µl PFA and immediately analyzed by flow cytometry.

2.6 Statistical analysis

All statistical analyses and graphing were performed using Microsoft Excel software. Statistical significance was measured by Students *t*-test for paired samples. Single-tailed tests were used for the samples treated with HIV-1 Tat and two-tailed *t*-tests were used for the controls. All flow cytometry data were analysed using the FCS Express 3.0 software (De Novo Software, Thornhill, ON, Canada).

RESULTS

3.1 Surface expression of the IL-7 Receptor alpha-chain is down regulated on CD4 T-cells in the presence of soluble HIV Tat protein

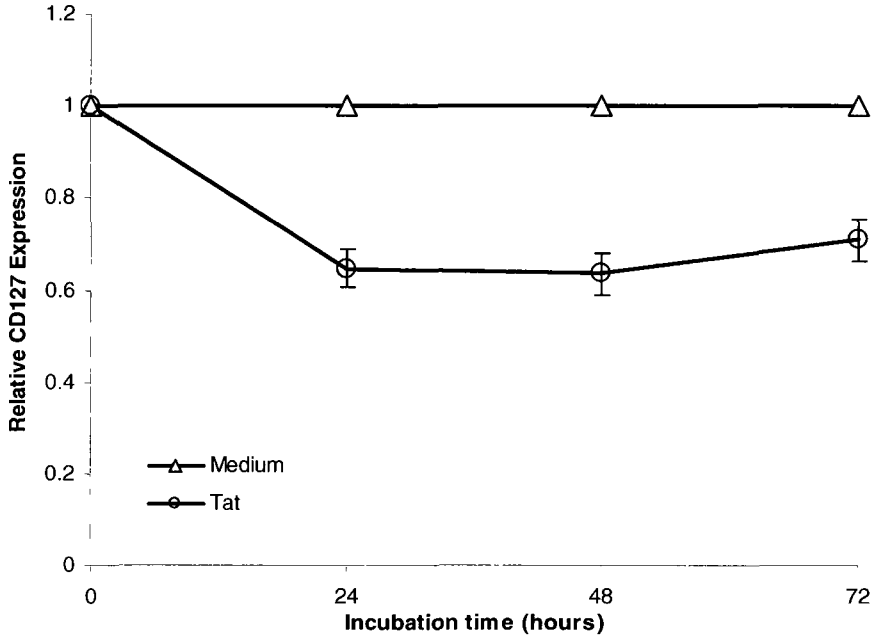
HIV disease progression has been associated with reduced surface expression of the IL-7 receptor α-chain (CD127) on CD4 and CD8 T-cells [65, 74]. The MacPherson lab has shown soluble HIV-1 Tat protein, an accessory protein required for efficient transcription from the viral LTR, directly induces a down regulation of CD127 at the cell surface of CD8 T-cells [103]. Tat was shown to specifically down regulate CD127 surface expression on CD8 T-cells in a dose- and time dependent manner. This effect is specific, reversible and can be blocked with anti-Tat antibodies and heparin. This down regulation also inhibited IL-7-induced proliferation and cytotoxic activity in CD8 T-cells [103]. The mechanism has been elucidated by which soluble Tat significantly decreases the half-life of surface CD127. Tat is taken up by CD8 T-cells, enters the cytoplasm upon endosomal acidification, binds to the cytoplasmic tail of CD127 at the inner leaflet of the cell membrane and induces receptor aggregation and microtubule dependent internalization. CD127 then appears to undergo proteosomal degradation [147]. Whether soluble Tat protein has the same effect on CD4 T-cells has yet to be explored.

To investigate the impact of soluble HIV Tat protein on the surface expression of CD127, CD4 T-cells isolated from healthy volunteers were incubated in RPMI-20 with or without purified Tat protein (10 μg/ml) and analyzed by flow cytometry at 24 hour intervals (n=17; Figure 2). CD127 surface expression varied little on cells cultured in medium alone

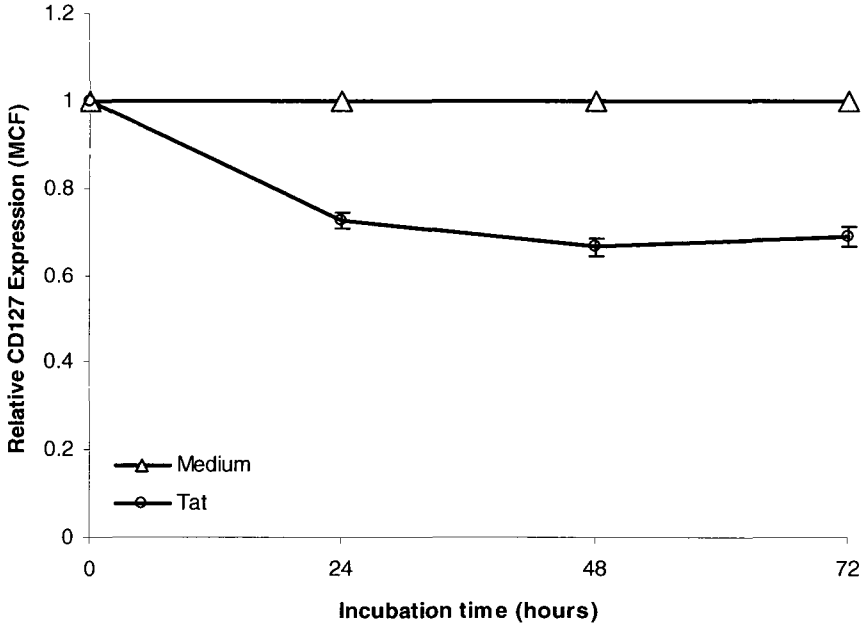
over at least 72 hours. In contrast, cells incubated in medium containing soluble Tat protein significantly down regulated CD127. In the presence of 10 μg/ml of Tat, there was a 35% ± 4% reduction in CD127 surface expression relative to controls at 24 hours. This decrease in receptor expression leveled off at 48 hours and showed some signs of recovery by 72 hours although CD127 still remained 30% ± 4.4% lower relative to controls (Figure 2A). The effects of Tat on surface CD127 expression were evident both in percent positive CD4 T-cells and in mean channel fluorescence indicating a reduction in the number of surface receptors on the total population. At 24 hours there was a 28% ± 1.8% reduction in MCF and at 72 hours this reduction was 31.2% ± 2.2% (Figure 2B).

Figure 2: HIV-1 Tat down regulates CD127 surface expression on CD4 T-cells.
(A) Percent change of CD127 positive CD4 T-cells cultured with Tat (10 μg/ml) compared with controls (n=17; $p<0.00003$). (B) Change in mean channel fluorescence relative to medium controls for the same cultures ($p<0.00000003$). CD127 expression is shown relative to medium controls. Graphs show mean values ± standard error of the mean (SEM)

A

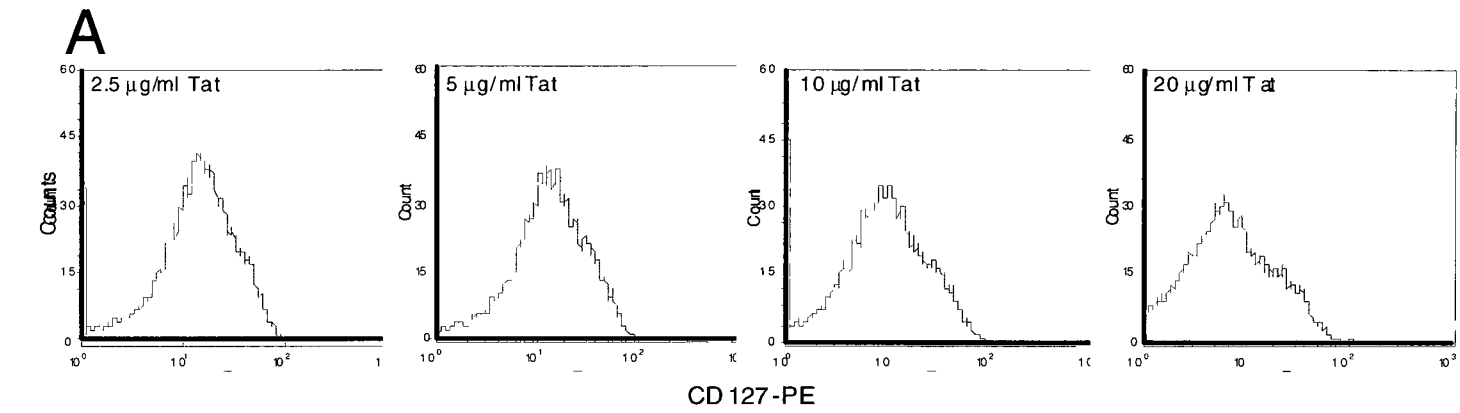

1.2
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression
Medium
Tat
0
24
48
72
Incubation time (hours)

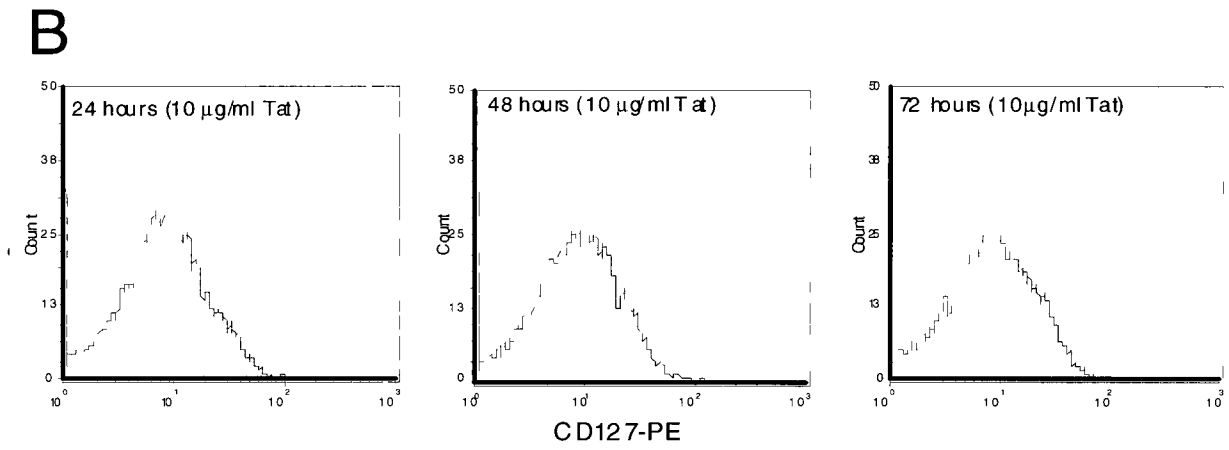
B

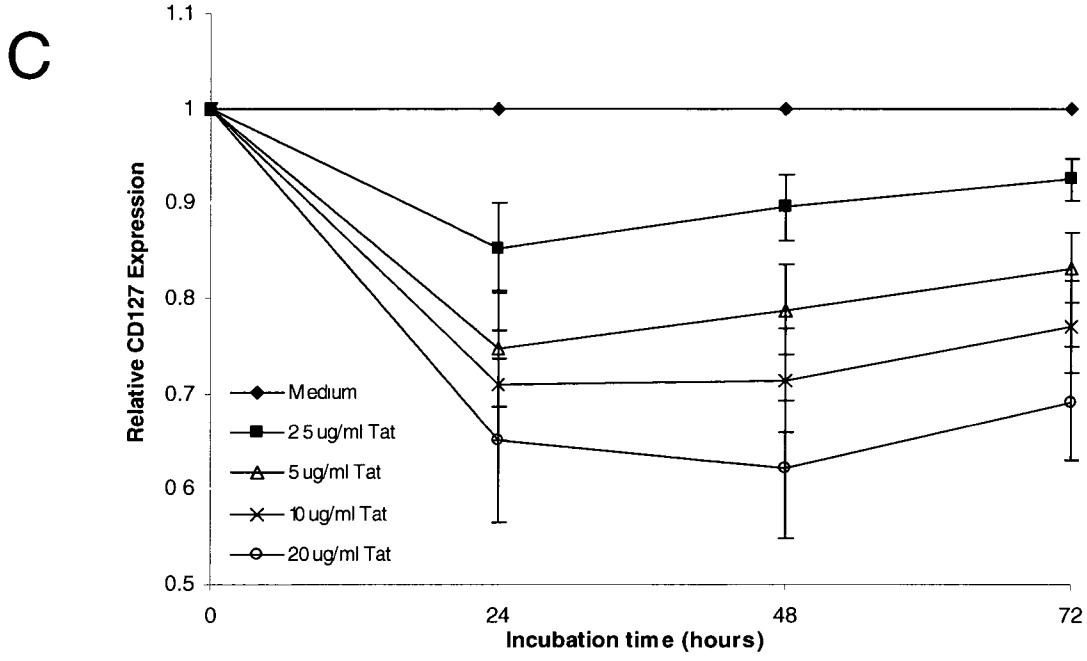

1.2
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression (MCF)
Medium
Tat
0
24
48
72
Incubation time (hours)

These effects of Tat on CD127 expression were both time and dose dependent. Increasing amounts of Tat from 2.5 to 20 μg/ml showed a progressively greater reduction in CD127 surface expression as measured by both mean channel fluorescence (Figure 3A) and percent positive cells (Figure 3C). At 24 hours, 2.5 μg/ml Tat induced a 14.7% ± 4.8% decrease in CD127 expression whereas 20 μg/ml induced a 35% ± 8.5% reduction. The effect of Tat was also time dependent (Figure 3B). Interestingly, peak suppression of CD127 occurred within 24 hours in the presence of 2.5, 5 and 10 μg/ml of Tat while peak suppression occurred at 48 hours in the presence of 20 μg/ml Tat. This may suggest Tat consumption plays a role in the recovery of CD127 at later time points (Figure 3C).

Figure 3: **Tat-induced down regulation of CD127 is dose and time dependent.** Purified CD4 T-cells from healthy HIV-negative volunteers were incubated with Tat protein or medium alone, stained for CD127 and analyzed by flow cytometry at the indicated times. (A) Representative flow cytometry histograms from the same individual showing progressive down regulation of CD127 surface expression at 48 hours with increasing amounts of Tat protein (black line) compared to medium controls (grey fill). (B) Representative flow cytometry histograms showing progressive CD127 down regulation over time with 10 μg/ml Tat protein. (C) Composite data ($n=6$; $p<0.02$) showing time and dose response to Tat compared to media controls (mean values ± SEM). CD127 expression is shown relative to medium controls.


A
2.5 µg/ml Tat
5 µg/ml Tat
10 µg/ml Tat
20 µg/ml Tat
Counts
Count
CD127-PE

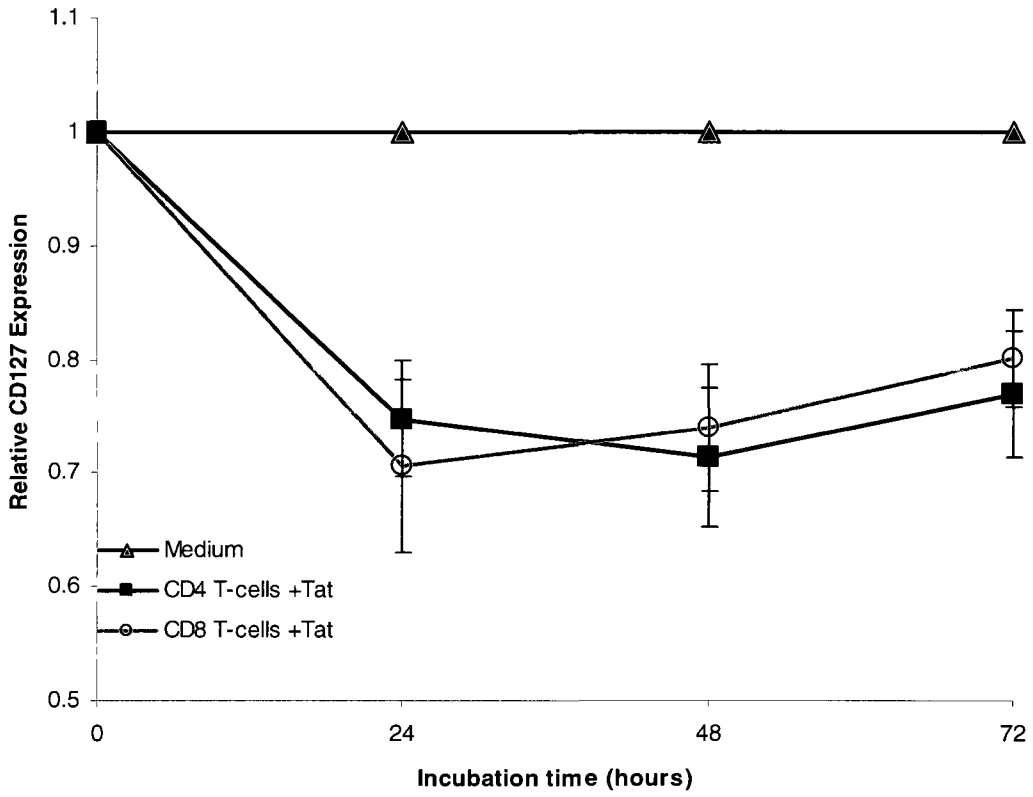

B
24 hours (10 µg/ml Tat)
48 hours (10 µg/ml Tat)
72 hours (10µg/ml Tat)
Count
CD127-PE


C
Relative CD127 Expression
1.1
1
0.9
0.8
0.7
0.6
0.5
Medium
2.5 ug/ml Tat
5 ug/ml Tat
10 ug/ml Tat
20 ug/ml Tat
0
24
48
72
Incubation time (hours)

3.2 HIV Tat protein down regulates CD127 equally on CD4 T-cells and CD8 T-cells

As mentioned, previous work in our lab examined the effects of HIV-1 Tat on CD8 T-cells and demonstrated down regulation of CD127 surface expression [103]. This finding prompted the question of how similar this effect is between the CD8 and CD4 T-cell subsets. In order to explore this, purified CD8 and CD4 T-cells from the same individuals (n=5) were incubated in RPMI-20 alone or with HIV-1 Tat (10 μg/ml) for up to 48 hours. Both subpopulations exhibited similar Tat-induced down regulation of CD127 surface expression. Within 24 hours, there was a 29.5% ± 7.5% CD127 surface down regulation on CD8 T-cells. As shown in figure 4, the effect on CD4 T-cells was similar to CD8 T-cells ($p=0.74$) and the difference between the subsets remains within one standard deviation throughout the 72 hours ($p>0.52$).

Figure 4: CD127 down regulation by Tat is similar in CD4 and CD8 T-cells.
Purified CD4 and CD8 T-cells from the same healthy HIV-negative volunteers were incubated for up to 72 hours with HIV-1 Tat protein (10 μg/ml) or in medium alone (n=5). Percent changes in surface CD127 expression compared to medium controls are shown (mean values ± SEM; $p<0.01$ for Tat-treated samples; $p>0.53$ between T-cell types). CD127 expression is shown relative to medium controls.

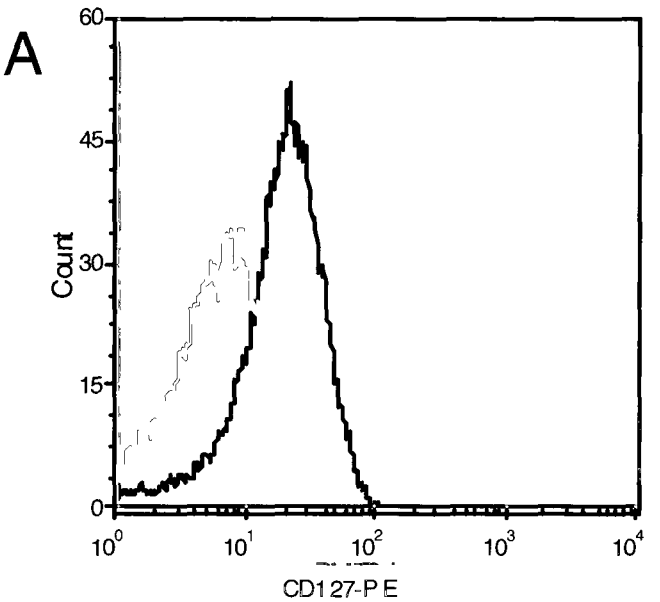

1.1
1
0.9
0.8
0.7
0.6
0.5
Relative CD127 Expression
Medium
CD4 T-cells +Tat
CD8 T-cells +Tat
0
24
48
72
Incubation time (hours)

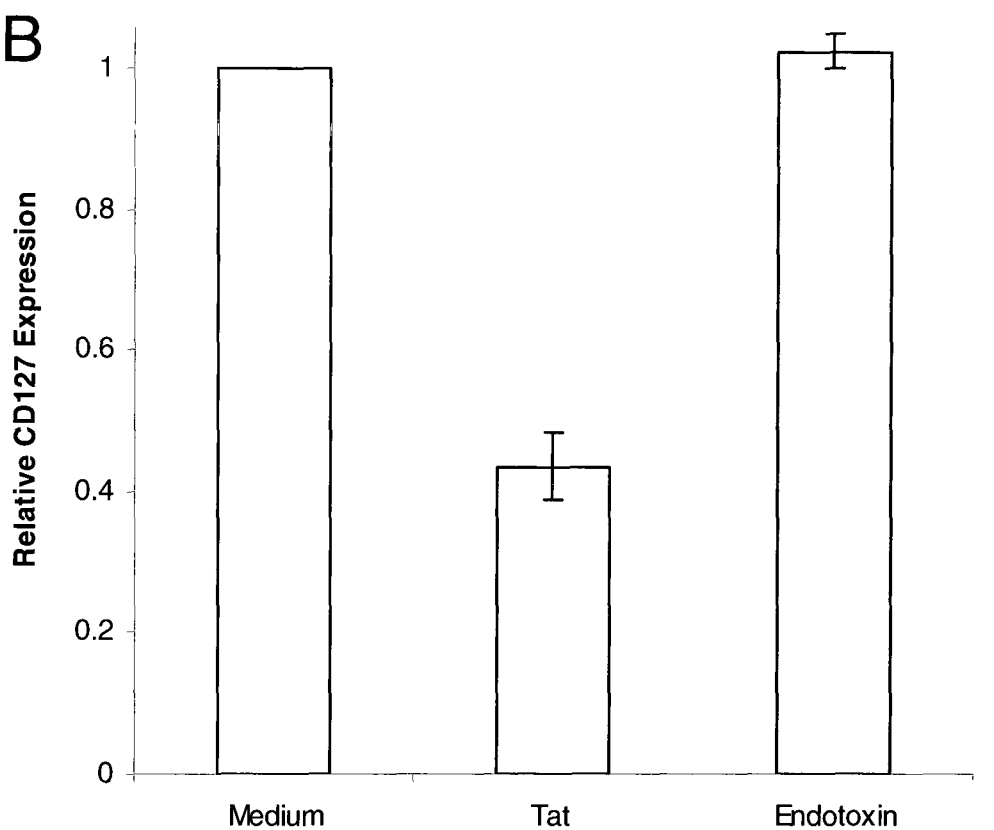
3.3 Bacterial endotoxins do not affect CD127 surface expression on CD4 T-cells

The recombinant HIV Tat protein used in these experiments was purified from *E. coli* and purchased from Advanced Bioscience Laboratories. According to the manufacturer, Tat is purified using affinity and reverse phase chromatography and is >90% pure. However, because the protein is purified from *E. coli* lysate, there was concern that bacterial endotoxins could be contaminating the Tat preparation and affect CD127 expression on CD4 T-cells. The maximum allowable amount of endotoxin per lot released by the manufacturer is 100 endotoxin units (EU; 10 ng/ml). To address the possibility that this amount could affect CD127 expression, purified CD4 T-cells were incubated in RPMI-20 alone, with purified HIV-1 Tat protein (10 μg/ml) or with 10 ng/ml control standard endotoxin. Cells were then analyzed by flow cytometry for CD127 surface expression at 24 hours. While the cells incubated in Tat protein showed a 56.5% ± 2.5% reduction in CD127 surface expression, bacterial endotoxin had no effect at all (±2.5% surface expression relative to media control; Figure 5). Thus any bacterial endotoxin that could be contaminating the Tat protein is not responsible for the down regulation of CD127 on CD4 T-cells. In a similar experiment, CD127 on CD8 T-cells was shown to be unaffected by LPS [103].

Figure 5: Endotoxin does not induce CD127 down regulation on the surface of CD4 T-cells.

Purified CD4 T-cells from healthy HIV-negative volunteers were cultured in medium alone, Tat protein (10 μg/ml) or control standard endotoxin (10 ng/ml) for 24 hours and then analyzed for CD127 expression by flow cytometry. (A) Representative histogram showing the effect of endotoxin (black line) and Tat (grey line) on CD127 surface expression relative to cells maintained in medium alone (grey fill). (B) Composite data (n=5) showing CD127 surface expression on CD4 T-cells cultured in the presence of Tat or endotoxin relative to medium controls (* p=0.0001, ** p=0.38). The relative changes are mean values ± SEM.


A
60
45
30
15
0
Count
10^0
10^1
10^2
10^3
10^4
CD127-PE

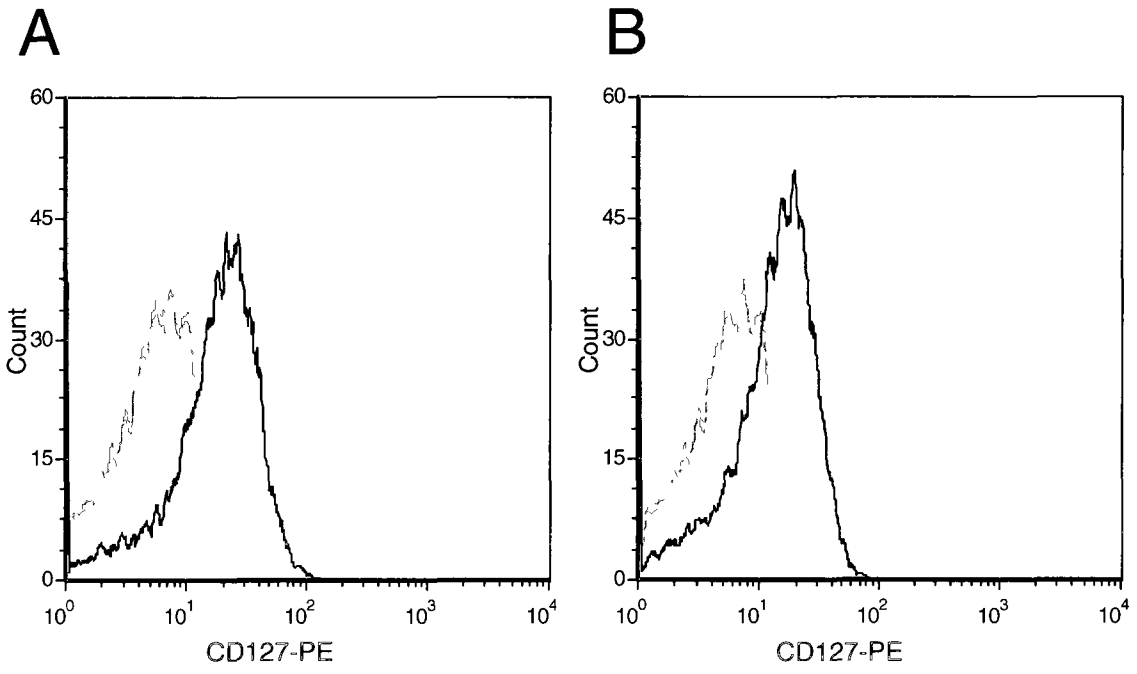

B
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression
Medium
Tat
Endotoxin

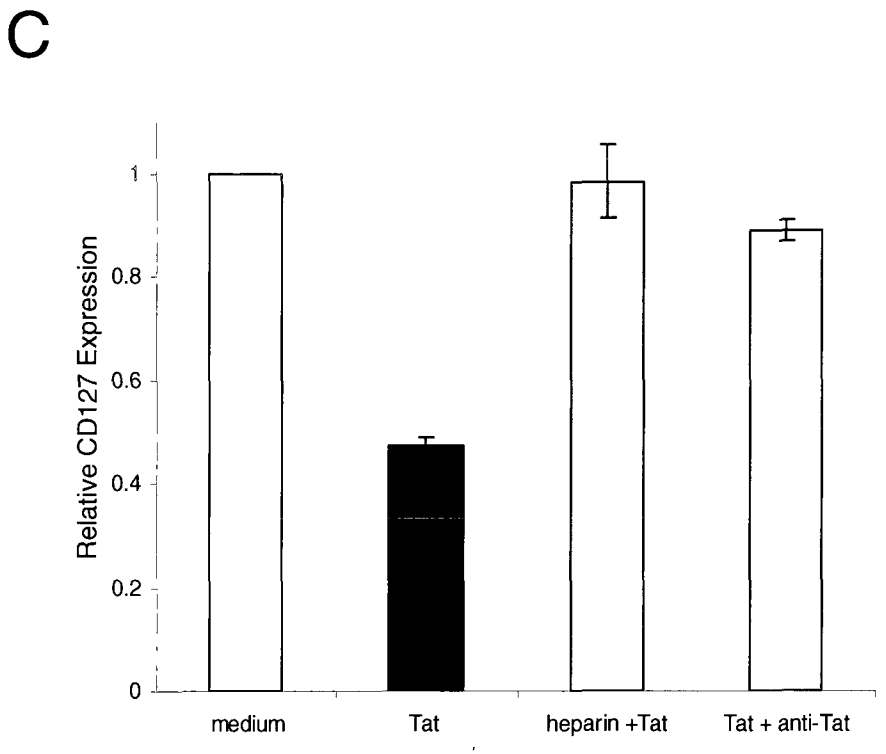
3.4 HIV-1 Tat-Induced down regulation of IL-7Rα surface expression can be blocked with heparin or anti-Tat antibodies

To determine if Tat's ability to down regulate CD127 surface expression was specific, Tat was incubated with either heparin or polyclonal anti-Tat antibodies. Heparin has been shown to bind directly to Tat protein and prevent its uptake by cells from the culture medium [148-149]. Purified soluble Tat protein was incubated for 30 minutes with an equimolar concentration of anti-Tat polyclonal antibodies (100 μg/ml) or with 100 USP/ml heparin The Tat preparations were then added to purified CD4 T-cells and the samples were incubated for 24 hours. Whereas cells incubated with heparin or anti-Tat antibodies alone demonstrated no change in CD127 surface expression, 10 μg/ml Tat protein induced a 52% ± 7% decrease in CD127. As seen in figures 6A and 6C, Tat protein pre-bound with heparin had no significant effect on CD127 expression (1.4% ± 2.1% decrease relative to media control; p=0.61). Similarly, polyclonal anti-Tat antibodies pre-bound to Tat also inhibited Tat's ability to down regulate CD127 (11% ± 5.4% reduction relative to media control; p=0.54). Since both heparin and anti-Tat antibodies block Tat's ability to down regulate CD127 on the cell surface, I concluded the effect on CD4 T-cells was specific to soluble Tat protein.

Figure 6: Tat-induced CD127 down regulation is blocked by heparin and anti-Tat antibodies.

Purified Tat protein (2 μg) was pre-incubated with 20 USP heparin or 20 μg anti-Tat polyclonal antibodies (equimolar ratio with Tat) for 30 minutes before being added to CD4 T-cells. Representative flow cytometry histograms show CD127 surface expression on CD4 T-cells after 24 hours incubation with (A) 10 μg/ml Tat (grey line), Tat plus heparin (black line) or in medium alone (grey fill); and (B) 10 μg/ml Tat (grey line), Tat plus anti-Tat antibodies (black line) or in medium alone (grey fill). (C) Composite data (n=5) showing CD127 expression relative to medium controls at 24 hours incubation (* $p=0.001$, ** $p>0.27$). The relative changes are mean values ± SEM.

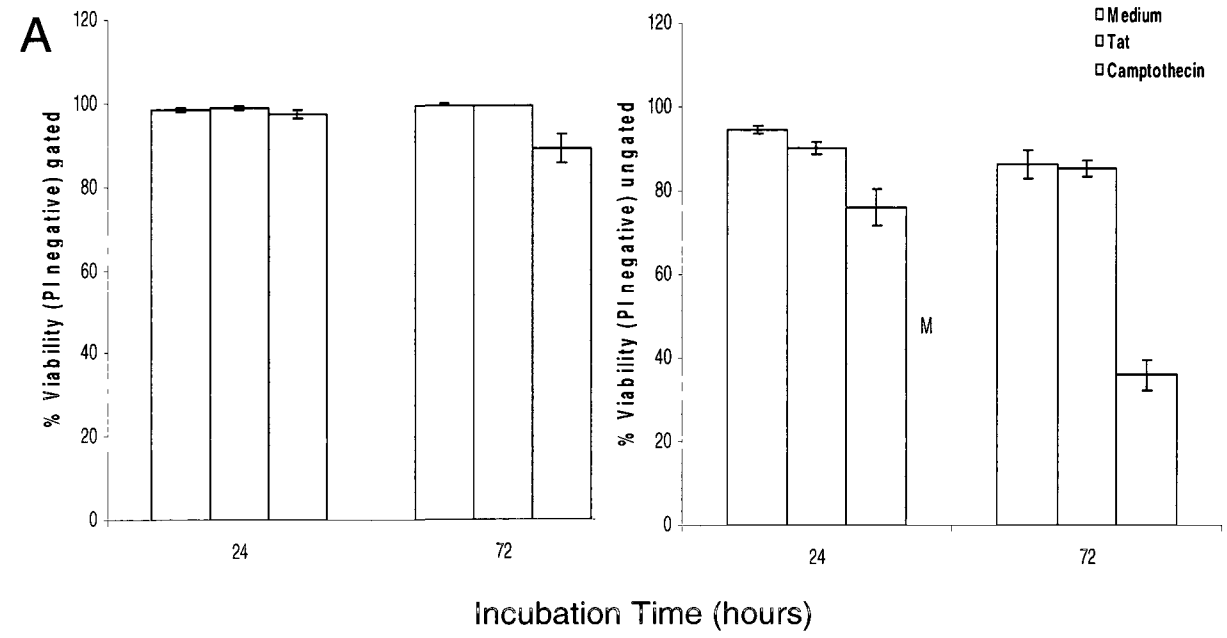

A
B
60
45
30
15
0
Count
10^0
10^1
10^2
10^3
10^4
CD127-PE

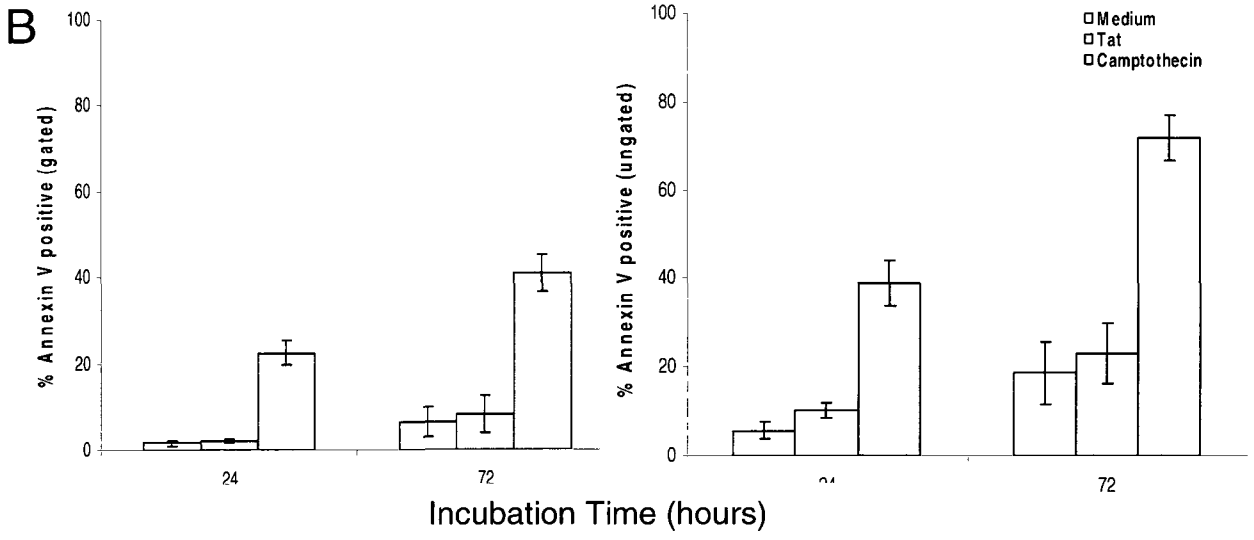

C
Relative CD127 Expression
1
0.8
0.6
0.4
0.2
0
medium
Tat
heparin +Tat
Tat + anti-Tat

3.5 HIV-1 Tat-Induced down regulation of IL-7Rα surface expression is not caused by cell death or apoptosis

There have been conflicting reports regarding the effect of HIV-1 Tat on apoptosis. It has been shown to promote as well as inhibit this cellular pathway in T-cells [150-152]. To ensure that the reduction in CD127 surface expression evident on Tat treated cells was not the result of apoptosis or cell death, purified CD4 T-cells were cultured in the presence of Tat protein (10 μg/ml) and stained with propidium iodide and annexin V at 24 and 72 hours. Cell viability as indicated by propidium iodide exclusion in the total ungated cell population was maintained at >85% ± 3.5% over 72 hours for cells incubated in RPMI-20 alone or in the presence of Tat protein. Within the live gate based on forward and side scatter viability was >99% ± 0.1% at 72 hours and again was not different comparing cells incubated in medium alone or in the presence of Tat protein (Figure 7A). Apoptosis as indicated by positive annexin V staining remained <10% ± 4.3% over 72 hours in the gated population regardless of the presence of Tat protein in the medium. In the ungated total population the difference in annexin V staining comparing Tat-treated cells to media control was only 4.5%, less than the standard error of 7.1% (Figure 7B). Thus, Tat-induced down regulation of CD127 surface expression is not the result of cell death or apoptosis.

Figure 7: Tat protein does not affect the viability of purified CD4 T-cells.
Purified CD4 T-cells from healthy HIV-negative volunteers (n=4) were incubated in medium alone or with Tat protein (10 μg/ml) for up to 72 hours and then stained with propidium iodide or annexin V-FITC and analyzed by flow cytometry. Analyses were performed on the total ungated population as well as on only live cells based on forward and side scatter. (A) Propidium iodide exclusion showed no significant difference in viability between cells cultured in Tat or medium alone. (B) Apoptosis as indicated by positive annexin V staining averaged <10% in the gated population at 72 hours and was no different comparing cells cultured in medium alone or with Tat protein. Camptothecin induces apoptosis and was used as a positive control. Graphs show mean values ± SEM.


A
% Viability (PI negative) gated
% Viability (PI negative) ungated
Medium
Tat
Camptothecin
M
24
72
Incubation Time (hours)

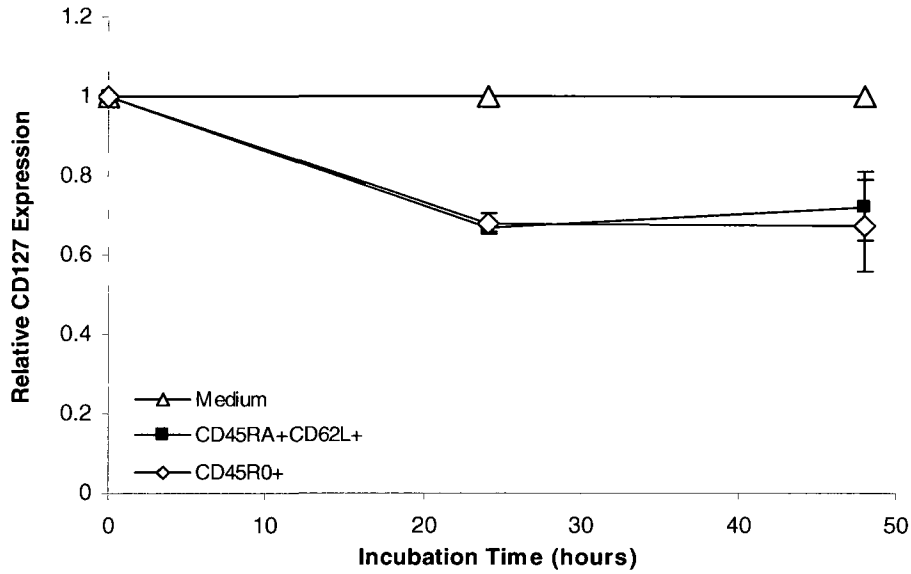

B
% Annexin V positive (gated)
% Annexin V positive (ungated)
Medium
Tat
Camptothecin
24
72
Incubation Time (hours)

3.6 CD127 surface expression is down regulated by HIV-1 Tat protein on both naïve and memory CD 4 T-cell subsets

The memory subset of CD4 T-cells has been shown to be particularly sensitive to CD127 down regulation in HIV-infected individuals [65-67]. This raised the question as to whether the HIV-1 Tat protein would have a more profound effect on this subset compared to naïve cells. Purified CD4 T-cells were incubated in RPMI-20 alone or with Tat protein (10 μg/ml) and CD127 expression was analyzed by flow cytometry gating on the different CD4 T-cell subsets. Naïve cells were defined as $CD45RA^{+}CD62L^{+}$ and memory cells were defined as $CD45RO^{+}$. As seen in figure 8, soluble HIV Tat protein down regulated CD127 surface expression equally on naïve and memory CD4 T-cells compared to controls. Both demonstrated a 32% ± 2.8% decrease in CD127 at 24 hours, equivalent to what was seen in the total CD4 T-cell population.

Figure 8: Tat down regulates CD127 surface expression equally on naïve and memory CD4 T-cell subsets.

Purified CD4 T-cells from healthy HIV-negative volunteers (n=4) were incubated in medium alone or with Tat protein (10 μg/ml) for up to 48 hours. CD127 surface expression was then measured by flow cytometry on naïve CD4 T-cells defined as CD45RA+ and CD62L+, and on memory cells defined as CD45RO+. There was little difference between the subsets ($p>0.33$). The changes relative to medium controls ($p<0.02$) are shown (mean values ± SEM).

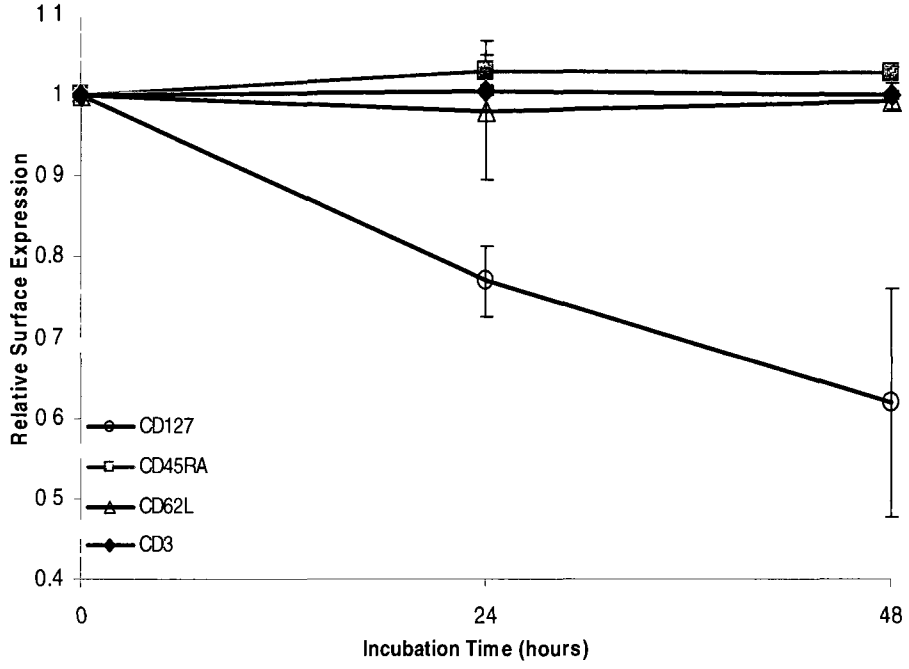

1.2
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression
Medium
CD45RA+CD62L+
CD45R0+
0
10
20
30
40
50
Incubation Time (hours)

3.7 HIV Tat specifically down regulates CD127 and does not affect other cell surface proteins

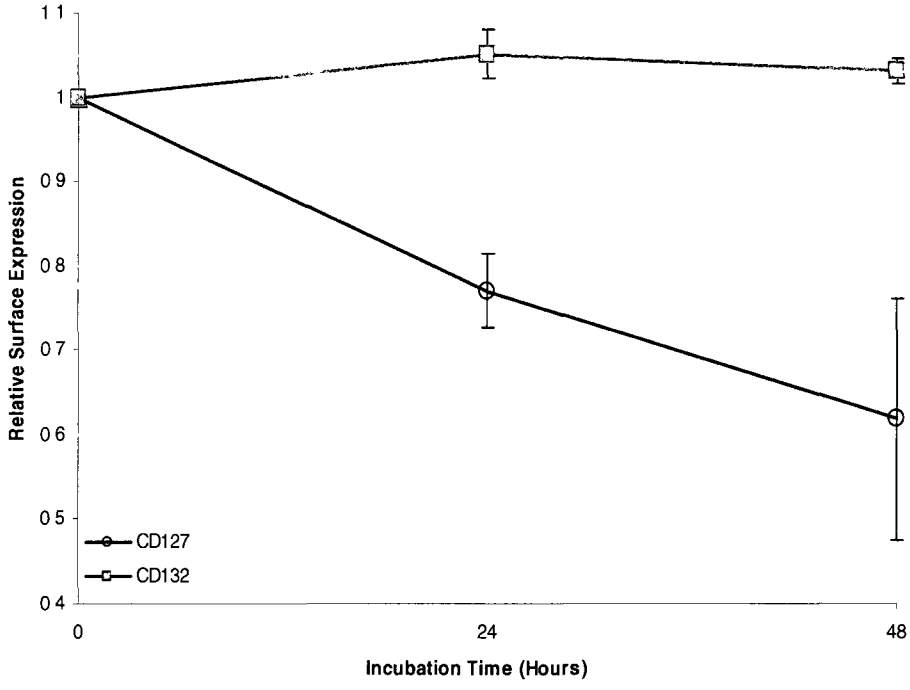
Internalization of soluble HIV-1 Tat protein by uninfected neighboring CD4 T-cells occurs via clathrin-mediated endocytosis [118]. The question arose as to whether transduction of Tat across the cell membrane could induce a nonspecific and generalized down regulation of cell surface proteins. To explore this possibility, CD4 T-cells were incubated in RPMI-20 alone or with Tat protein (10 μg/ml) and then analyzed by flow cytometry for expression of a series of cell surface markers. Cells were stained for CD127, CD45RA (a marker of naïve T-cells), CD62L (a homing receptor expressed on T-cells), and CD3 (an essential component of the T-cell receptor complex). Cells were also examined for the expression of CD132, the common γ-chain that associates with CD127 to form the IL-7 receptor heterodimer. Whereas Tat induced the down regulation of CD127 surface expression by 38.2% ± 14.2% at 48 hours relative to controls, it had no effect on the expression of the other cell surface proteins examined (Figure 9A). CD3 showed the maximum variance, only a 3.2% ± 2.3% difference relative to controls (Figure 9A). Similarly, Tat had no effect on the expression of CD132 (Figure 9B). After 48 hours in the presence of Tat, CD132 increased by only 3.1% ± 1.5% compared to controls. Thus it appears Tat specifically down regulates CD127 and does not affect at least a number of other cell surface proteins.

Figure 9: Tat does not affect overall CD4 T-cell phenotype.
Purified CD4 T-cells from healthy HIV-negative volunteers (n=4) were incubated in medium alone or with Tat protein (10 μg/ml) for up to 48 hours and then analyzed by flow cytometry. (A) Changes in positive staining for the indicated surface proteins relative to medium controls ($p>0.09$ for all proteins other than CD127 where $p<0.04$). (B) Change in CD132+ CD4 T-cells relative to medium controls ($p>0.12$). The changes are mean values ± SEM.

A


1 1
1
0 9
0.8
0 7
0 6
0 5
0.4
Relative Surface Expression
CD127
CD45RA
CD62L
CD3
0
24
48
Incubation Time (hours)

B

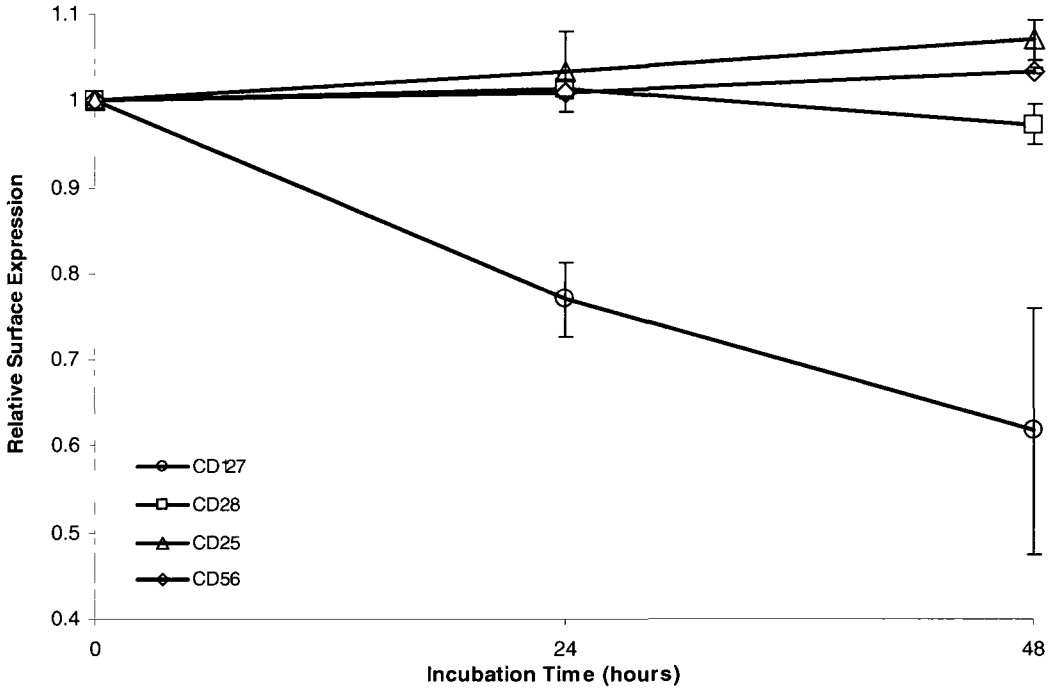

1 1
1
0 9
0 8
0 7
0 6
0 5
0 4
Relative Surface Expression
CD127
CD132
0
24
48
Incubation Time (Hours)

3.8 CD127 down regulation by HIV-1 Tat protein is not due to CD4 T-cell activation

T-cell activation has been shown to down regulate CD127 surface expression on CD8 and CD4 T-cells [75, 153-154]. Levels of $CD127^-$ T-cells were shown to be directly correlated with CD8 T-cell activation [75] and in a study of 42 HIV-infected individuals undergoing HAART, Benito et al. demonstrated CD127 down regulation was associated with T-cell activation in CD4 T-cells [154].

This raised the question as to whether Tat down regulates CD127 on the cell surface by activating CD4 T-cells. To address this issue, purified CD4 T-cells were incubated in RPMI-20 alone or with Tat protein (10 μg/ml) and were then examined by flow cytometry for expression of CD127 and established activation markers. In this study, CD28, CD25, and CD56 were analyzed. CD28 is constitutively expressed on naïve T-cells where it provides costimulatory signals and is down regulated upon T-cell stimulation. CD25 is the alpha-chain of the IL-2 receptor complex and is up regulated on activated T-cells. CD56 is an antigen found on activated T-cells. As seen in figure 10 and consistent with the previous data, Tat induced a significant down regulation of CD127 on the surface of CD4 T-cells. In contrast, CD28, CD25 and CD56 remained unchanged relative to controls in the presence of Tat protein. Thus Tat does not appear to induce a generalized activation of CD4 T-cells leading to the down regulation of CD127.

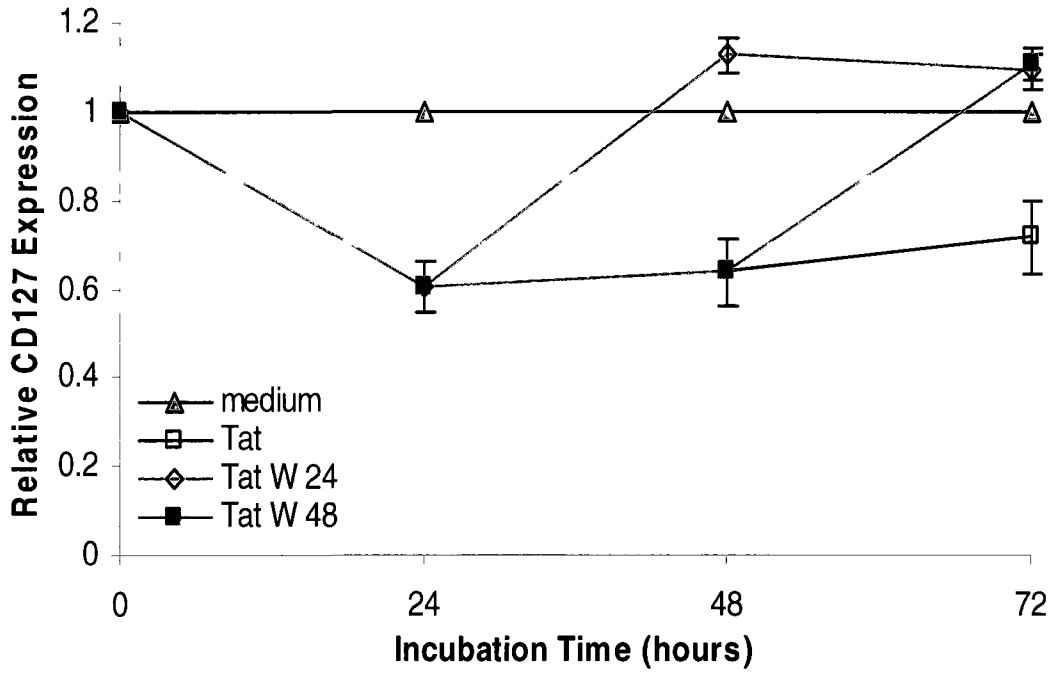
Figure 10: Tat-induced CD127 down regulation is not due to CD4 T-cell activation. Purified CD4 T-cells from healthy HIV-negative volunteers (n=4) were incubated in medium alone or with Tat protein (10 μg/ml) for up to 48 hours and then analyzed by flow cytometry. The changes in surface protein expression relative to medium controls are shown (mean values ± SEM; $p>0.19$ for all proteins other than CD127 where $p<0.04$).


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
Relative Surface Expression
CD127
CD28
CD25
CD56
0
24
48
Incubation Time (hours)

3.9 Tat-induced down regulation of CD127 surface expression is reversible

CD127 is down regulated on CD4 T-cells in HIV infected patients and partially recovers following viral suppression with antiretroviral therapy [102]. A cohort study of patients with primary, chronic and long-term nonprogressive HIV-1 infection showed that CD127 down regulation on CD4 T-cells was rapidly reversible and returned to normal levels less than 24 hours after incubation ex vivo in fresh medium [102]. This prompted me to ask whether CD127 down regulation by Tat was reversible or irreversible. To investigate this, purified CD4 T-cells were incubated in RPMI-20 alone or with Tat protein (10 μg/ml). At 24 and 48 hours, the cells were washed and resuspended in fresh RPMI-20 without Tat. As shown in Figure 11, once Tat was removed from the medium CD127 recovered on the cell surface within 24 hours. This clearly indicates Tat's effect on CD127 is reversible and that Tat is continually required to maintain suppression of CD127.

Figure 11: CD127 surface expression recovers after Tat is removed from the medium. Purified CD4 T-cells from healthy HIV-negative volunteers (n=3) were incubated in medium alone or with Tat protein (10 μg/ml). At 24 (Tat W 24) and 48 (Tat W 48) hours the cells were washed twice and resuspended in fresh medium in the absence of Tat protein. Cells were analyzed for CD127 surface expression by flow cytometry at the times indicated. The changes relative to medium controls are shown (mean values ± SEM).


1.2
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression
medium
Tat
Tat W 24
Tat W 48
0
24
48
72
Incubation Time (hours)

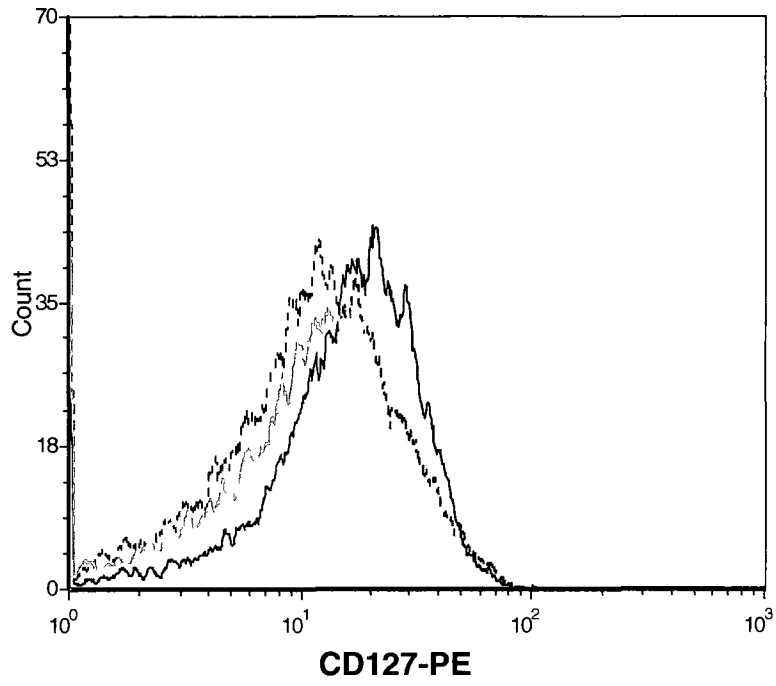
3.10 Tat has a direct effect on CD127 expression and does not require de novo protein synthesis

I next asked whether soluble HIV Tat protein had a direct effect on CD127 surface expression or whether induction of a second factor was required to down regulate the receptor. To investigate this, purified CD4 T-cells were incubated in RPMI-20 alone, with Tat protein (10 μg/ml), with cyclohexamide (CHX) or with Tat protein plus CHX. As seen in figure 12, cells incubated with CHX alone showed a 16.5% ± 7.3% decrease in CD127 surface expression over 24 hours. This then represents the normal decay rate of CD127 from the cell surface in the absence of new protein synthesis. Tat protein alone induced a 39.6% ± 5.6% decrease in CD127. When CHX was included with Tat, there was still a 46.5% ± 1.9% drop in CD127 surface expression indicating de novo protein synthesis is not required for Tat to have its effect.

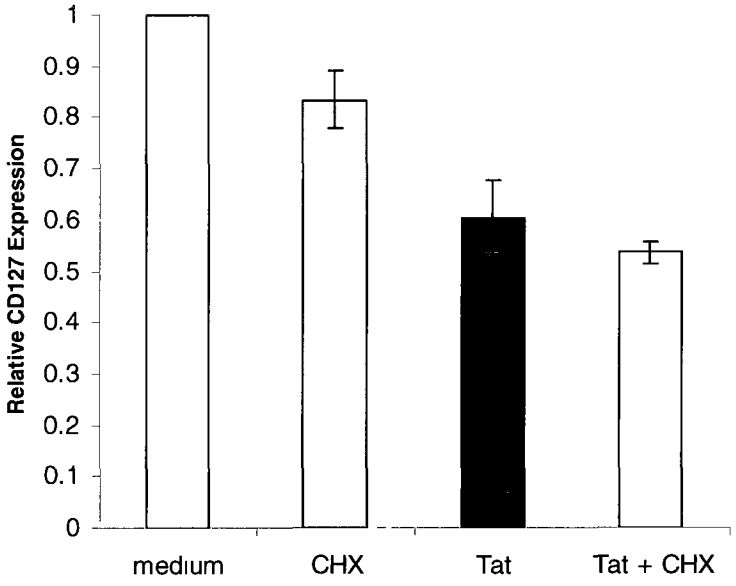
Figure 12: de novo protein synthesis is not required for Tat-induced down regulation of CD127.

Purified CD4 T-cells from healthy HIV-negative volunteers were incubated in medium alone, with cyclohexamide (100 μM; CHX), Tat protein (10 μg/ml) or Tat plus CHX for 24 hours and then analyzed by flow cytometry. (A) Representative histogram showing CD127 surface expression on CD4 T-cells cultured in the presence of CHX alone (black line), Tat alone (dashed line) and CHX plus Tat (grey line) compared to medium control (grey fill). (B) Composite data from n=3 demonstrating inhibition of protein synthesis with CHX does not prevent Tat-induced CD127 down regulation at the cell surface; CHX alone had no significant effect on CD127 expression ($p=0.07$). There was no significant variance of surface expression between Tat and Tat + CHX treatments ($p=0.38$) whereas both treatments showed significant down regulation relative to medium ($p<0.009$). The percent changes relative to medium controls are shown (mean values ± SEM). CD127 surface expression is shown relative to medium controls.

A


70
53
35
18
0
Count
10^0
10^1
10^2
10^3
CD127-PE

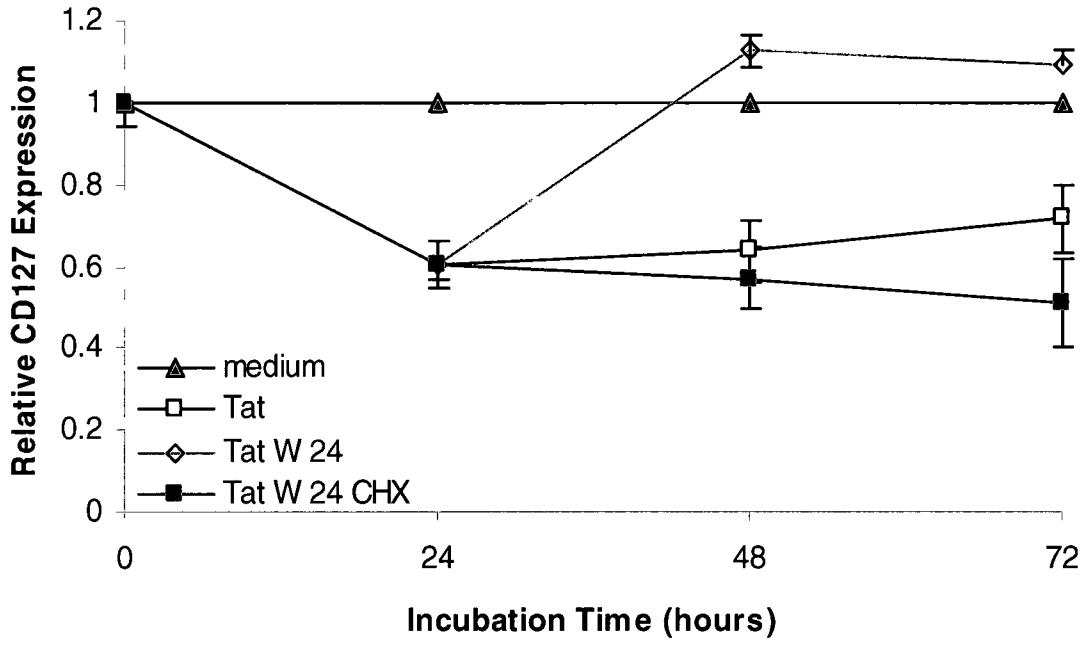
B


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative CD127 Expression
medium
CHX
Tat
Tat + CHX

3.11 New protein synthesis is required for CD127 recovery following down regulation by Tat

As shown above, CD127 recovers on the cell surface once soluble Tat protein is removed from the medium. To determine if this recovery of CD127 requires new protein synthesis, CD4 T-cells were first incubated with soluble Tat for 24 hours and then washed and re-incubated in medium alone or medium containing cycloheximide. As shown in figure 13, cells maintained in medium containing Tat protein maintained suppression of CD127 for up to 72 hours while those washed and transferred to medium alone after 24 hours recovered surface CD127 expression. However, if after removing Tat protein from the medium the cells were then incubated in cycloheximide, CD127 failed to recover. 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 medium.

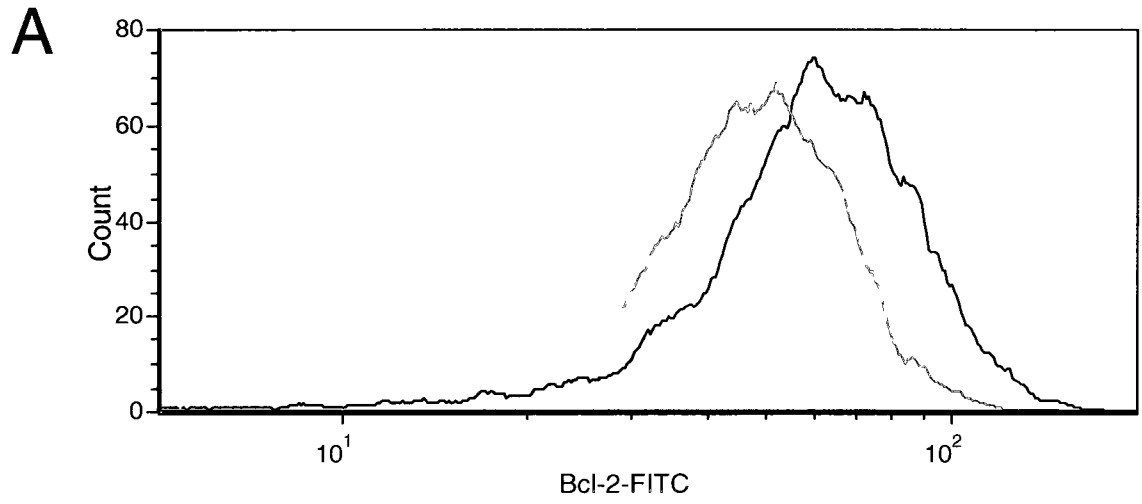
Figure 13: de novo protein synthesis is required for recovery of CD127 surface expression following Tat-induced down regulation.
Purified CD4 T-cells from healthy HIV-negative volunteers (n=3) were incubated in medium alone or with Tat protein (10 μg/ml). After 24 hours cells incubated with Tat were washed twice and resuspended in fresh medium alone (Tat W 24) or in medium plus 100 μM CHX (Tat W 24 CHX). Cells were analyzed for CD127 surface expression by flow cytometry at the times indicated. The changes relative to medium controls are shown (mean values ± SEM).

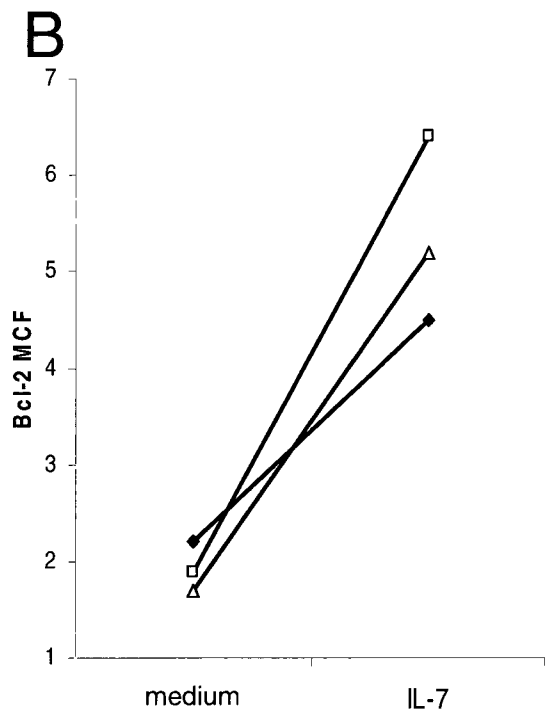

1.2
1
0.8
0.6
0.4
0.2
0
Relative CD127 Expression
medium
Tat
Tat W 24
Tat W 24 CHX
0
24
48
72
Incubation Time (hours)

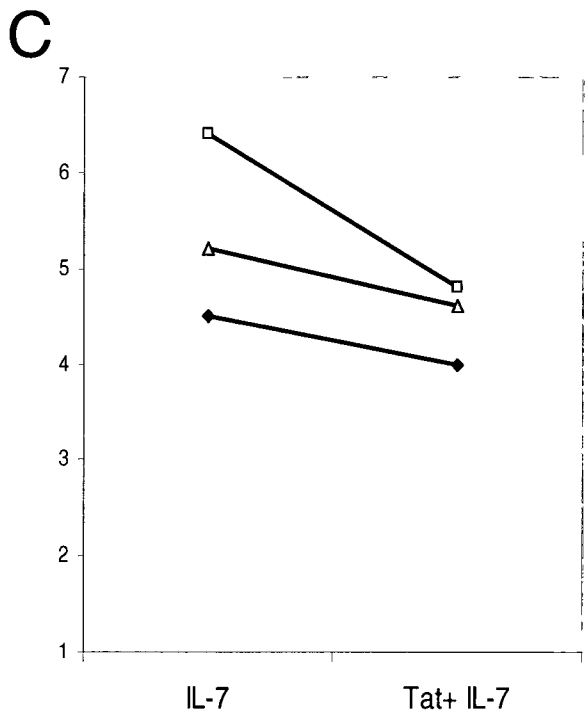
3.12 HIV-1 Tat protein inhibits IL-7-induced up regulation of Bcl-2

IL-7 signaling plays an essential role in CD4 T-cell proliferation and homeostasis and has been specifically shown to up regulate expression of the anti-apoptotic molecule B cell leukemia/lymphoma 2 (Bcl-2) [155-156]. Because Tat protein down regulates the IL-7 receptor alpha-chain on the surface of CD4 T-cells, I wanted to determine whether this effect was functionally significant. To investigate this, purified CD4 T-cells were pre-incubated in RPMI-20 alone, or with Tat protein (10 μg/ml) for 48 hours and then stimulated with IL-7 (10 ng/ml). While IL-7 as expected induced the expression of Bcl-2, pre-incubation with Tat protein significantly blunted IL-7's effect. The cells pre-incubated with Tat protein before administration of IL-7 showed diminished up regulation of Bcl-2 as demonstrated by mean channel fluorescence of Bcl-2 positive stained cells. The maximum effect is a 25% decrease in the MCF from cells treated with IL-7 alone compared to cells pretreated with Tat protein (Figure 14).

Figure 14: Soluble HIV-1 Tat protein inhibits IL-7 induced Bcl-2 expression. Purified CD4 T-cells from healthy HIV-negative volunteers were pre-incubated in medium alone or in medium containing Tat protein (10 μg/ml) for 48 hours. Cells were then stimulated with IL-7 (10 ng/ml) for an additional 72 hours and then fixed, stained for Bcl-2 expression and analyzed by flow cytometry. (A) Representative histogram showing Bcl-2 expression in untreated CD4 T-cells (grey fill), following IL-7 stimulation (black line), and following IL-7 stimulation of Tat pre-treated cells (grey line). (B,C) Bcl-2 expression as measured by mean channel fluorescence (MCF) in (B) cells treated with IL-7 compared to medium controls, and (C) cells pre-incubated in medium or with Tat prior to IL-7 stimulation (n=5; p=0.04).


A
80
60
40
20
0
Count
10^1
10^2
Bcl-2-FITC


B
7
6
5
4
3
2
1
Bcl-2 MCF
medium
IL-7


C
7
6
5
4
3
2
1
IL-7
Tat+ IL-7

DISCUSSION

The effect of HIV-1 on T-cell function and homeostasis throughout the course of infection leads to quantitative effects in CD4 T-cell populations, and qualitative effects in both CD4 and CD8 T-cell subsets. Homeostatic maintenance within the CD4 T-cell population is largely dependent upon IL-7 signalling. IL-7 prolongs T-cell survival through induction of the anti-apoptotic factors Bcl-2 and Bcl-xL as well as inhibition of pro-apoptotic factors Bad and Bax [83]. IL-7, in combination with IL-15, has also been shown to initiate proliferation in human memory CD4 T-cells and its absence results in substantial depletion and lack of regeneration in this subpopulation [70]. IL-7 is also important in T-cell development [81] and initiation of cellular responses [84-85].

IL-7 signalling is dependent upon heterodimerization of the IL-7 receptor complex consisting of the subunits CD132 and CD127 [94]. Down regulation of CD127 surface expression on CD4 and CD8 T-cells is associated with HIV infection [74-75, 102-103]. Viremia results in increased plasma IL-7 levels [104-105] which are inversely correlated with CD4 T-cell counts during HIV infection [78]. The decreased expression of CD127 on CD4 and CD8 T-cells in HIV+ patients results in poor responses to IL-7 including decreased induction of anti-apoptotic factors [73] and CD25 expression [107]. Effects on CD127 expression and subsequent IL-7 signaling would be expected to have significant effects on T-cell homeostasis and function.

Tat is secreted by infected cells and can affect uninfected bystander T-cells [116]. The MacPherson lab has shown that soluble Tat protein specifically down regulates CD127 expression on the surface of CD8 T-cells in a dose- and time-dependent manner. This leads

to inhibition of CD8 T-cell proliferation and perforin synthesis which normally accompany IL-7 stimulation. These effects could be blocked with anti-Tat antibodies as well as heparin. Surface expression of CD127 was also shown to recover once Tat was removed from the medium. Tat is initially internalized via endocytosis by the CD8 T-cell and, dependent upon endosomal acidification, exits the endosome. Tat then localizes to the inner leaflet of the cell membrane where it associates with the cytoplasmic tail of CD127. This association leads to receptor clustering, internalization and subsequent proteosomal degradation of CD127.

This thesis showing down regulation of CD127 by Tat on CD4 T-cells expands the range of influence for this accessory protein. Reduced CD127 surface expression in the presence of Tat was shown to be similar between CD4 and CD8 T-cells. This effect is dose- and time-dependent as well as reversible. Both the naïve and memory CD4 T-cell subsets showed equivalent CD127 surface down regulation. It was also verified that the observed effect was due specifically to Tat and not endotoxin, T-cell activation, or the result of cell necrosis or apoptosis. Tat was shown to selectively down regulate CD127 leaving the common γ-chain (CD132) and a number of other phenotypic markers unaffected. That Bcl-2 induction by IL-7 was impaired in cells pre-incubated with Tat suggests functional relevance.

Limitations of this research result from the *in vitro* and reductionist approaches taken in the experiments. There are numerous factors which cannot be accounted for in this type of set-up. The effects of countless soluble factors as well as *in vivo* microenvironments cannot be adequately predicted. While it is possible some contaminant in the Tat protein preparation induces a down regulation of CD127, this would seem unlikely since anti-Tat antibodies could specifically block the effect. In addition, I have shown bacterial endotoxin

has no effect on CD127 expression. The use of markers to delineate subpopulations of CD4 T-cells was also not exhaustive. It would be of interest to examine if the effect of Tat on CD127 surface expression was equivalent on T_H1, T_H2, T_H17, and T_{reg} cells. The effect of HIV-1 Tat on expression of the anti-apoptotic factor Bcl-2 was statistically significant ($p=0.04$) but the limitations of the experiment do not illustrate how substantial this effect could be *in vivo*. First of all, Bcl-2 expression was measured after less than 7 days of incubation with Tat. Secondly, although Tat has been measured in nanomolar concentrations in the sera of HIV-infected individuals [157], much more concentrated amounts of the protein could be found in lymphoid tissue microenvironments including the thymus, spleen and mucosal tracts. Not only this, but there is also a continuous source of Tat protein in infected individuals whereas in this experiment, Tat was only administered at 72 hour intervals. Therefore, the effects demonstrated here could be further compounded by the long term exposure to elevated concentrations and continuous source of Tat one would find in an individual living with the virus for decades.

The hallmark of HIV infection is a decline of the CD4 T-cell population which leads to the ultimate deterioration of immune function and susceptibility to opportunistic infections. Dysregulation of CD4 T-cell homeostasis and impairment of function are well described in HIV infection. It has been established that the IL-7 receptor alpha-chain is down regulated on T-cells of HIV infected patients [74, 102]. The HIV-1 Tat protein was shown to be at least partially responsible for this down regulation in CD8 T-cells and lead to functional impairment of this subset [103]. This study has added to these observations and shown that HIV-1 Tat may be at least partially responsible for the down regulation of IL-7 receptor alpha-chain surface expression on CD4 T-cells. This loss of surface expression

diminished IL-7-induced up regulation of the anti-apoptotic factor Bcl-2, a functional outcome that demonstrates how Tat can impair the maintenance of CD4 T-cell homeostasis.

The severity of CD4 T-cell depletion in HIV infection is clearly an area of great interest. As discussed earlier, direct destruction by virus cannot account for the total loss in the population. In quantitative terms, cell loss must be due to a combination of both direct and indirect means. To fully elucidate a model of disease progression, virally mediated destruction of CD4 T-cells must incorporate several factors into the model. This model will need to include HIV-1 Tat-induced disruption of the IL-7 signalling pathway. Numerous studies demonstrate the principal role IL-7 plays in homeostasis in a lymphopenic setting in general [158] and in HIV disease in particular [159-160]. It is apparent that HIV-1 Tat is not only a transcriptional factor, but also could be at a nexus between HIV infection and disruption of IL-7 signalling leading to homeostatic dysregulation and the ultimate depletion of the CD4 T-cell population.

The next direction to take in this area of research would be to investigate the mechanisms by which Tat leads to the surface down regulation of CD127 on CD4 T-cells. It would be expected to occur in a manner similar to that demonstrated on CD8 T-cells. Therapeutic advantages could also be explored in regards to HIV-1 Tat. In fact, based on the results of a phase I clinical trial using a Tat variant as a vaccine which showed safe induction of a balanced T_h1/T_h2 immune response and anti-Tat antibody production, a phase II clinical trial was in progress as of April 2010 [161]. From the data currently available, one might expect by blocking Tat, CD127 on CD4 T-cells could recover at least partially leading to increased CD4 function and an improvement in AIDS related symptoms.

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Publications

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Graduate Thesis

Expression of the Interleukin-7 Receptor Alpha-chain is Down Regulated on CD4 T-cells by the HIV-1 Tat Protein

Undergraduate Abstracts

Functional Analysis of a MATE gene in cluster roots of White Lupin Rick White, Denny McLaughlin, Alex Jobrack, Claudia Uhde-Stone, 2006, 2nd Pan American Plant Membrane Biology Workshop (May 17th-20th 2006 ; South Padre Island, Texas)

RT-PCR expression analysis of a nutrient stress-induced white lupin gene (*LaMATE)* in response to auxin and auxin inhibitor
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Awards

University of Ottawa – Excellence Scholarship
Ontario Graduate Scholarship in Science and Technology
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Ontario HIV Treatment Network – Conference Scholarship 2009
University of Ottawa FGPS – Travel Scholarship
University of Ottawa Dept. of Microbiology and Immunology – Travel Scholarship OHTN Research Day - 2nd Place oral presentation

Research Experience

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- Cell and bacterial culture
- Bacterial transformation
- Immunoblotting
- Silver staining
- ELISA
- Single-color flow cytometry
- DNA isolation from human and plant tissue
- RNA isolation from plant tissue
- PCR amplification of human and plant DNA
- Plant transformation using *Agrobacterium rhizogenes*
- Cloning/subcloning
- Cycle sequencing of PCR and plasmid templates
- Spectrophotometric quantification of RNA and DNA
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