Mutational Analysis of the HIV-1 Tat Protein and Its Role in Downregulating CD127 on CD8 T cells

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Abstract

HIV Tat protein downregulates surface expression of the interleukin-7 receptor alpha-chain (CD127) on CD8 T cells resulting in impaired T cell proliferation and cytolytic capacity. Once taken up by CD8 T cells, Tat binds directly to the cytoplasmic tail of CD127 inducing receptor internalization and degradation. Given the important roles of CD127 in proper immune function, the Tat/CD127 interactions were characterized and the mechanisms required to induce receptor loss from the surface of CD8 T cells were investigated.

Tat deletion mutants were generated each sequentially lacking a region of the protein. CD8 T cells isolated from HIV negative volunteers were exposed to exogenous or intracellular Tat proteins before surface CD127 expression was analyzed by flow cytometry. To characterize Tat/CD127 physical interactions, wild type Tat and Tat mutants were incubated with lysates from a $CD127^+$ Jurkat cell line followed by CD127/Tat co-immunoprecipitation. The effect of Tat on CD127 post-translational modifications was also investigated.

Removal of the N-terminus of Tat (aa 1-10 or aa 17-21) prevented Tat from downregulating CD127 and prevented Tat from binding CD127 as assessed by co-immunoprecipitation. Deletion of the basic region (aa 48-59) also prevented Tat from downregulating CD127 but did not prevent Tat from interacting physically as demonstrated by co-immunoprecipitation. Strikingly, endogenously expressed ΔBasic Tat acted as a dominant negative mutant, causing an accumulation of CD127 at the cell surface. These observations suggest that Tat may bind CD127 via its N-terminus to disrupt the normal recycling of the receptor, and then recruit cellular endocytic machinery to the receptor via it's basic region, to remove the receptor from the cell surface and target it for degradation. Furthermore, Tat encourages the ubiquitination of CD127 by recruiting the cytokine-inducible SH2 containing (CIS) protein to the receptor, possibly leading to accelerated CD127 internalization and proteasomal degradation. I propose a model whereby Tat binds CD127 via its N-terminal region then recruits CIS via its basic region. CIS in turn recruits a cellular E3 ubiquitin ligase to ubiquitin tag the receptor for internalization and proteasome degradation. This research may lead to novel treatments designed to maintain IL-7 signalling and strengthen CD8 T cell function in HIV^+ persons.

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i. List of Abbreviations

Amino Acid Codes

Amino Acid	3 letter code	1 letter code
Glycine	Gly	G
Alanine	Ala	A
Serine	Ser	S
Threonine	Thr	T
Leucine	Leu	L
Isoleucine	Ile	I
Valine	Val	V
Cysteine	Cys	C
Tyrosine	Tyr	Y
Phenylalanine	Phe	F
Tryptophan	Trp	W
Methionine	Met	M
Lysine	Lys	K
Arginine	Arg	R
Asparagine	Asn	N
Asparate	Asp	D
Glutamine	Gln	Q
Glutamate	Glu	E
Proline	Pro	P
Histidine	His	H

Tat Deletion Mutants

HIV2-N10—Tat-1 with the first ten aa substituted with the first 10 aa of Tat-2
ΔAcidic—Tat-1 lacking aa. 1-11
ΔCys—Tat-1 lacking aa 22-37
ΔCore—Tat-1 lacking aa 38-46
ΔBasic—Tat-1 lacking aa 48-59
ΔGln—Tat-1 lacking aa 60-72
ΔC-Term—Tat-1 lacking aa 72-86

Weights and Measures

M—Moles
g—grams
m—meters
L—Litres
λ —Wavelength
c—centi
m—milli
μ—micro
n—nano
p—pico

A
aa—Amino acid
APOBEC3G—Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
ADA— Adenosine deaminase
AIDS—Acquired immunodeficiency syndrome
Amp— Ampicillin
AP—Antarctic phosphatase
APCs—Antigen presenting cells
APS—Ammonium persulfate
ART—Anti-retroviral therapy

B
βTrCP—Beta-transducin repeat-containing protein
Bad—Bcl-2-associated agonist of cell death
Bax—Bcl-2–associated X protein
BCA—Bicinchoninic acid
Bcl-2—B-cell CLL/lymphoma 2
Bcl-xl—B-cell lymphoma-extra large
BglII—*Bacillus globigii* restriction enzyme II
Bim—Bcl-2-like protein 1
BSA—Bovine serum albumin

C
C—Carboxyl
c-CBL—Cellular casitas B-lineage lymphoma
c-Myb—Cellular myeloblastosis
CA—HIV Capsid protein, p24
CAF—CD8 antiviral factor
Cat. No.—Catalogue number
CCPs—Clathrin-coated pits
CCR5—CC chemokine receptor 5
CD—Cluster of differentiation
CD132—Common gamma chain; interleukin receptor
Cdk—Cyclin dependent kinase
CIS—Cytokine-inducible SH2 protein
CMV—Cytomegalovirus
CpG—Phopho-cytosine-guanidine islands
co-IP—Co-Immunoprecipitation
CRH1—Cytokine receptor homology class I
CTD—Carboxyl-Terminal Domain
CTL—Cytotoxic T Lymphocyte
Cul5—Cullin 5
CXCR4—CXC Chemokine Receptor 4

D
DC—Dentritic Cells
DC-SIGN—Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DCIR—Dendritic Cell Immunoreceptor
ddH_20—Double distilled H_20
de novo—"Anew"
DpnI—*Diplococcus pneumoniae* restriction enzyme 1
DNA—deoxyribonucleic acid
dNTP—deoxy Nucleotide Tri-Phosphates

E
EC—Elite controllers
ECD—Extracellular Domain
ECL—Enhanced chemiluminescence
EcoRI—*E coli* RY13 restriction enzyme RI
EDTA—Ethylenediaminetetraacetic acid
eGFP—Enhanced green florescent protein
EGTA—Ethylene glycol tetraacetic acid
Env—HIV envelop polyprotein
ER— Endoplasmic Reticulum
ERAD—Endoplasmic Reticulum Associated Degradation
ERK—mitogen-activated protein kinase 3
et. al.—"And others"

F
FADD—Fas-Associated protein with Death Domain
Fas—TNF receptor superfamily, member 6
FasL—Fas ligand
FCS—Fetal calf serum
Flk-1—Fetal liver kinase 1
FoxO1—Forkhead box protein O1
FoxP3—Forkhead box protein P3

G
g—Gravities
GABP—GA Binding Protein
Gag—Group-specific Antigen Protein
GALT—Gut-associated lymphoid tissue
GE—General Electric
Gfi-1—Growth factor independence-1
GFP—Green florescent protein
GLUT-1—Glucose transporter 1
Golgi—Golgi Apparatus
gp120—Glycoprotein: 120 KDa (HIV)
gp160—Glycoprotein: 160 KDa (HIV)

gp41—Glycoprotein: 41 KDa (HIV)
GRE—Glucocorticoid response element
GVHD—Graft versus Host Disease

H
HCl—Hydrochloric Acid
HCV—Hepatitis C virus
HDAC—Histone deacetylase complex
HindIII—*Haemophilus influenzae* Rd restriction enzyme III
HIV—Human immunodeficiency virus
HIV-2N10—HIV-1 Tat protein substituted with the first ten amino acid of HIV-2 Tat.
HPLC—High pressure liquid chromatography
HRP—Horseradish peroxidase
HS—Heparan sulphate
Hsp—Heat shock protein
HSPG—Heparan Sulphate Proteoglycan
HSV-8—Herpes Simplex Virus 8

I
i.e.—"That is"
IFN—Interferon
IL—Interleukin
IL-7R—Interleukin-7 receptor
IL-7Ra—Interleukin-7 receptor alpha chain
In vivo—"In life"
In vitro—"in glass"
Int—HIV Integrase
IPTG—Isopropyl β-D-1-thiogalactopyranoside
IRS—Insulin receptor substrate

J
Jak—Janus Kinase
Jaki—Inhibitor of Jak activity

K
Kb—Kilobase
KCl— Potassium chloride
K_D—Disassociation constant
KDa—Kilodalton
KIDR—Kinase insert domain receptor
Kip27—Kinase inhibitor 27 KDa
KS—Kaposi's Sarcoma
KSHV—Kaposi's Sarcoma Herpes Virus (Herpes Simplex Virus 8)

L
LB—Luria Broth

LC—Langerhans Cell
LFA—Leukocyte function associated antigen
LRP—Low Density Lipoprotein Receptor-Related Protein
LTNPs—Long term non-progressor
LTR—Long terminal repeat
luc—Luciferase

M
M-tropic—Macrophage tropic HIV
MA—HIV matrix protein
MACS—Magnet assisted cell sorting
MAP-2—Microtubule-associated protein 2
MAPK—Mitogen-activated protein kinase
Mcl—Myeloid cell leukemia
mDC—Myloid dendritic cells
MDDC—Monocyte-derived dendritic cells
Mdm-2—Murine double minute-2
MG132—Carbobenzoxy-Leu-Leu-leucinal
MHCI—Major histocompatibility complex I
MHCII—Major histocompatibility complex II
mRNA—Messenger RNA

N
N—Number of replicates
N-terminal—Amino-terminal
NaCl—Sodium chloride
NaH_2PO_4—Sodium dihydrogen phosphate
NC—Nucleocapsid protein
Nef—Negative regulatory factor
Neo—Neomycin
Ni-NTA—Nickel-nitriloacetic acid
NIAID—National Institute of Allergy and Infectious Diseases
NIH—National Institute of Health
NP-40-- Nonidet P-40

O
OD_{450}—Optical Density at 450 nm
OD_{600}—Optical Density at 600 nm
OHRI—Ottawa Hospital Research Institute

P
P/O—Promoter operator
p7—HIV nucleocapsid protein
p15—HIV ribonuclease H
p17—HIV matrix protein
p24—HIV capsid protein

p31—Integrase
p55—HIV Gag polyprotein
p51—HIV reverse transcriptase protein
$p56^{lck}$—Lymphocyte cell specific kinase, 56 KDa
p66—HIV reverse transcriptase protein with RNaseH subunit
p300/CBP—300 KDa protein/CREB-binding protein complex
PBMCs—Peripheral Blood Mononuclear Cells
PBS—Phosphate Buffered Saline
PCAF—p300/CBP-associated factor
Pck—Phosphoenolpyruvate carboxykinase
PCR—Polymerase Chain Reaction
PD-1—Programmed cell death 1
pDC—Plasmacoid Dendritic Cells
PE—Phycoerythrin
PE-PC7—Pphycoerythrin-phycoerythrin cyanin 7
PI-3K—Phosphoinositol-3-Kinase
$PI(4,5)P_2$— Phosphatidylinositol 4,5 phosphate
PIC—Pre-integration complex
PKA—Protein kinase A
PKC—Protein kinase C
PKR—Protein kinase R
Pol—Polymerase
Prod. No.—Product number
Prot— HIV protease
PRR—Pattern recognition receptor
pTEFb—Positive transcription elongation factor b
PU.1—Purine-rich box binding protein 1
PVDF—Polyvinylidine fluoride
pY449—Phosphorylated tyrosine at position of 449
Pyk2—Protein tyrosine kinase 2

R
Rbx—Ring box protein X
Rev—HIV regulator of virion expression protein
RIPA—Radio immunoprecipitation assay
RLU—Relative light units
RNA—Ribonucleic acid
ROD—Subtype A virus that was isolated from a Cape Verdian in 1986
RON—Recepteur d'origine nantais
RPMI—Roswell Park Memorial Institute (Media)
RPMI-10—Roswell Park Memorial Institute (Media) with 10% fetal calf serum
RPMI-20—Roswell Park Memorial Institute (Media) with 20% fetal calf serum
RRE—Rev response element
RT—HIV reverse transcriptase
RT-RNasH—HIV reverse transcriptase with RNasH subunit

RUNX1—Runt-related transcription factor 1

S
SOC—Super optimal broth with catabolic repressor
sCD127—Soluble CD127
SCID—Severe combine immunodeficiency
SCF—Skp, Cullin, F-box containing complex
SDM—Site-directed mutagenesis
SDS—Sodium dodecyl sulfate
SH2—Src homology domain 2
Shc—Src homology 2 domain containing
SIRT1—Sirtuin 1
SIV—Simian immunodeficiency virus
SOCS—Suppressor of cytokine signalling
Sp1—Specificity protein 1
SNP—Single nucleotide polymorphism
STAT—Signal transducer and activator of transcription
STI—Sexually transmitted infection

T
T—Thymus
TAR—Tat response element
Tat—Transactivator of transcription
Tat-1—HIV-1 Tat
Tat-2—HIV-2 Tat
Tat-6xHis-- HIV-1 Tat with C-terminal polyhistidine tag
Tat2-6xHis—HIV-2 Tat with C-terminal polyhistidine tag
TBST—Tris-buffered saline with Tween-20
T_{CM}—Central memory T
TCR—T cell receptor
T_{EM}—Effector memory T
TEMED—Tetramethylethylenediamine
TGF-β--Tumour growth factor beta
Tip60—Tat interactive protein, 60kDa
TLR—Toll-like Receptor
TNF—Tumour Necrosis Factor
Treg—Regulatory T cell
tRNA—Transfer RNA

U
U—International Standard Unit
Ub—Ubiquitin

V
vDNA—Viral DNA
VEGF—Vascular endothelial growth factor

Vif—Viral infectivity factor
VLA-4—Very late antigen 4
Vpr—Viral protein R
Vpu—Viral protein U

W
WCL—Whole cell lysis
WHO—World Health Orgainzation

Y
y449—Tyrosine at position of 449

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1. Chapter 1: General Introduction

1.1 HIV-1

The Human Immunodeficiency Virus-1 (HIV-1), the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS), has infected more than 40 million people in the last 30 years with a world wide estimated prevalence of 0.8% (World Health Organization; WHO). Currently, 31.6- 35.1 million people are living with HIV-1, with 2.4-2.9 million new infections occurring in 2010 alone (WHO).

In Canada, 53 000 – 83 000 are estimated to be living with HIV, and 2,417 newly diagnosed infections were reported in 2009 alone (WHO) with an estimated 2600 to 4300 occurring annually. Despite recent efforts [1-3], a protective vaccine or cure for HIV-1 remain elusive.

1.1.1 Epidemiology

Natural HIV-1 infection occurs via body fluid transmission to a mucosal membrane of a recipient. The vast majority of new infections are a result of sexual intercourse (approx 85%-95%) [4, 5] or the sharing of drug paraphernalia. Vertical transmission from mother to child continues to be a problem in low resource countries, and is dependent on maternal viral loads. HIV-1 infection during breast feeding can also occur [6].

Epidemiological and laboratory experiments have shown that several other sexually transmitted infections (STI) increase the risk of HIV-1 transmission. These include, *Treponema pallidum* (syphilis), *Neisseria gonorrhoea*, and *Chlamydia trachomatis* [7].

Sub-Saharan Africa has the highest prevalence of HIV-1 infection worldwide, followed by the Caribbean, and Eastern Europe /Central Asia. The vast majority of HIV-

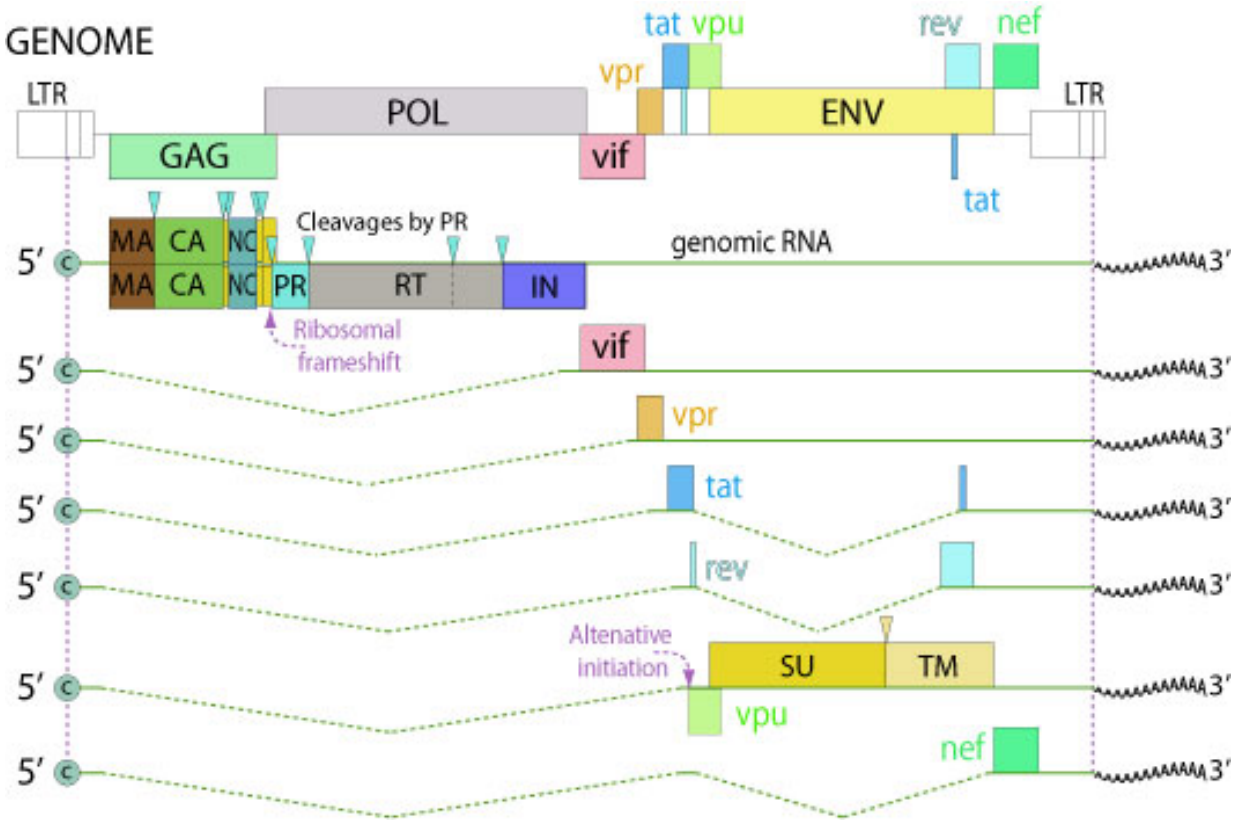
infected individuals reside in Sub-Saharan Africa (WHO). Indeed, Sub-Saharan Africa accounts for 14% of the world's population and 70% of HIV^+ individuals (WHO).

1.1.2 The Virion

HIV is a single stranded, positive-sense RNA virus species of the *lentivirus* genus in the *Retroviridae* family. Each particle is a spherical enveloped virion of 100 to 120 nm in diameter. The genome is 9.8 kb in size and encodes for 10 functional genes which transcribe into over 30 mRNAs. These mRNAs produce at least 16 functionally distinct proteins via alternative splicing, post-translational protease cleavage and polymerase stuttering which results in a frameshift during transcription.

Each virion contains two positive sense copies of the genome. Glycoprotein spikes protrude from the envelope of the virion composed of gp120 and gp41 hetro-trimers. Three structural proteins are embedded within the lipid envelope and are all encoded by the *gag* gene: the matrix protein (MA, p17), the capsid protein (CA, p24), and the nucleocapsid protein (NC, p7). These proteins are originally translated as one large polyprotein which is subsequently cleaved by the viral protease. The *pol* gene encodes for HIV-1's enzymatic proteins. These proteins include the HIV-1 protease (Prot), reverse transcriptase (RT), the HIV-1 RNase (p15) and the HIV-1 integrase (p31; Int). These proteins are again produced initially as a single polyprotein which is cleaved post-translationally into the component proteins, often via intramolecular protease activity [8]. The viral RT is a heterodimer composed of p66 (RT-RNaseH) and p51 (RT). See figure 1 for the HIV-1 genome organization.

Figure 1: The HIV-1 genome. The HIV-1 genome codes for three large structural polypeptides (Gag, Pol and Env) and 6 accessory proteins (Vpr, Tat, Vpu, Rev, Nef and Vif). Once inserted into the host genome, a series of multiply spliced, single spliced and unspliced mRNAs are produced. Nef, Tat and Rev are translated first from multiply spliced mRNAs. Vif, Vpr, Vpu and Env are translated from singly spliced mRNAs. The structural polypeptides Gag and Pol are translated later in the infection cycle by unspliced mRNAs. The surface glycoproteins gp120 and gp41 are originally translated as the gp160 polypeptide and cleaved by furin-like host proteases in the Golgi apparatus. The Gag and Pol polypeptides are cleaved into the individual mature proteins by the viral protease upon viral maturation. http://viralzone.expasy.org/all_by_protein/7.html


GENOME
LTR
GAG
POL
vif
vpr
tat vpu
ENV
rev nef
LTR
tat
MA CA NC
MA CA NC PR RT IN
Cleavages by PR
Ribosomal frameshift
genomic RNA
5′
3′
vif
vpr
tat
rev
Altenative initiation
SU
TM
vpu
nef

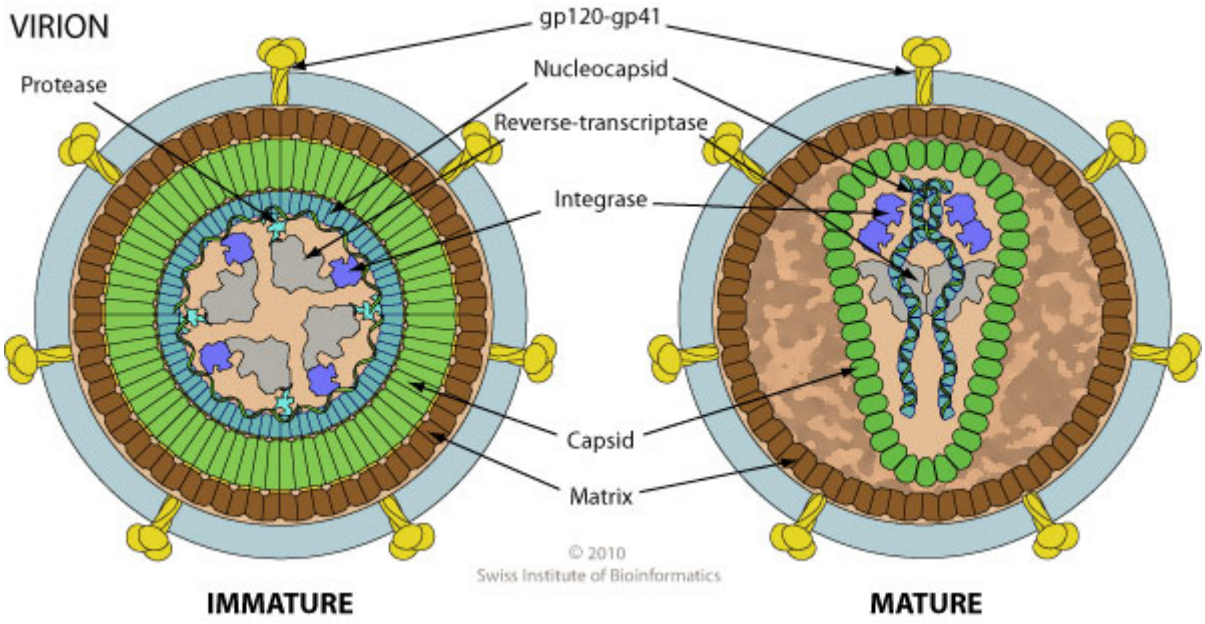
Within the mature virion, MA forms a spherical shell just underneath the lipid bilayer. CA forms the conical shell separating the MA protein from the RNA genome. MA units associate with the inner leaflet of the viral membrane and the gp41 tails. NC interacts with and stabilizes the RNA genome within the capsid core. Env trimers protrude from the surface of the virions to resemble spikes. Two copies of the RT and IN enzymes are packaged into the viral core bound to the two genome vRNAs. See figure 2.

Lastly, six non-structural mRNAs encode for the auxiliary proteins. These gene products are usually separated into regulatory proteins (Rev, Tat) which play a role in temporal and spacial control of viral gene transcription/translation, or accessory proteins (Vif, Vpr, Vpu, Nef) which promote infection by facilitating viral egress, interfering with host immunoregulatory mechanisms, and/or optimizing intracellular conditions to support viral growth.

1.1.3 Transmission Events

During the sexual transmission of HIV through the exchange of bodily fluids, the first cells to be infected are most likely to be associated with mucosal membranes. Within the female genital tract, transmission is believed to occur at the mucosal epithelium of the vagina, ecotocervix, and endocervix. In men, both epidemiological studies and circumcision trials suggest that the mucosal lining of the foreskin may be a gateway for transmission [9-12]. Indeed, HIV-1 infection through the inner foreskin epithelium has recently been described in molecular detail [13]. Transmissions through the simple rectal and intestinal epitheliums have also been documented [14]. Presumably, this is the major route of infection during anal sexual intercourse.

Figure 2: Structure of the HIV-1 virion. The HIV-1 virion contains in its core two single stranded copies of the positive-sense viral RNA genome. This genomic material is associated with repeating units of nucleocapsid (NC) protein and is encased within a conical shell composed of a p24 capsid protein (CA) lattice. Also contained within the p24 capsid shell are the viral enzymes: integrase and reverse-transcriptase. The p24 capsid is in turn encased within a lipid bilayer taken from the host cell during viral egress. A protein lattice composed of matrix protein (MA) is found associated with the inner leaflet of this bilayer. Numerous receptor "spikes" composed of gp120-gp41 glycoprotein hetero-trimers protrude from the virions surface. http://viralzone.expasy.org/all_by_protein/7.html


VIRION
gp120-gp41
Protease
Nucleocapsid
Reverse-transcriptase
Integrase
Capsid
Matrix
© 2010
Swiss Institute of Bioinformatics
IMMATURE
MATURE

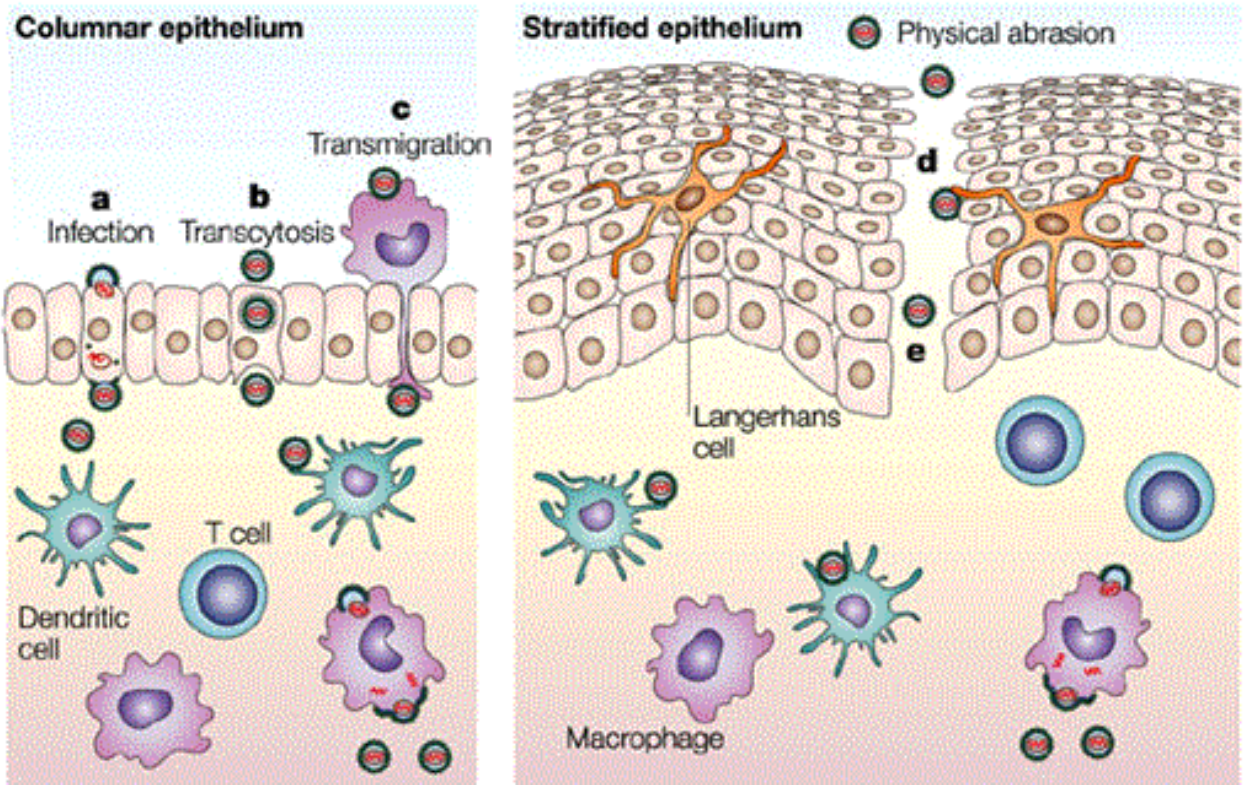
Whether disseminating through simple monolayer or stratified multi-layer epithelium, virus-infected cells in bodily fluids are likely a major source of HIV-1 transmission. HIV^+ cells are found in much higher numbers than free virus in vaginal and seminal fluids [15].

Stratified mucosal layers found in the vagina and foreskin contain APC integrated within them such as Langerhans cells (LCs), dendritic cells (DCs) and macrophages, as well as T lymphocytes. Original work by three different groups showed that LCs/DCs could be infected *in situ* or *ex vivo* and subsequently transmit HIV to autologues CD4 T cells [16-20]. Therefore these APCs were thought likely to be the first cells infected [16-20], perhaps through direct sampling of the local environment [21].

In the case of simple epithelial monolayers, evidence exists that HIV-1 may be selectively carried through the cell to be released intact at the basolateral membrane via transcytosis [22-24], where it is readily taken up by cells resident within the lamina propria [24] (see figure 3).

Once captured within DC/LC, HIV-1 induces the migration of these cells to local lymphoid structures. In this way whole HIV-1 virions are transported from mucosal sites protected within cellular endosomes to secondary lymph nodes where DC:T cell interactions can facilitate the infection of activated $CD4^+$ T cells [25]. Resident DCs found under the rectal mucosa have been shown to transport whole virions to intestinal and peripheral blood lymphocytes [26] and present the virus in *trans* to disseminate the infection systemically. Hence, DCs and LCs appear to present whole HIV-1 particles to $CD4^+$ cells in lymphoid structures in both *cis* and *trans*.

Figure 3: HIV-1 transmission across mucosal epithelium. HIV-1 virions cross both columnar and stratified epithelium by numerous mechanisms. **A)** HIV may directly infect columnar epithelial cells. These cells may subsequently directionally bud *de novo* virus through the basolateral membrane. **B)** HIV-1 may transcytose through columnar epithelial cells by usurping vesicle compartments. **C)** Furthermore, HIV-1 may cross the columnar epithelium associated with HIV-1^+ cells found in infected bodily fluids. **D)** HIV-1 can also cross stratified epithelium by binding to Langerhan cells and dendritic cells. HIV-1 binds to langerin or DC-SIGN on the surface of Langerhan cells and dendritic cells, respectively. These cells then traffic to lymphoid structures where they can present HIV-1 to CD4 T cells in both *cis* and *trans*. **E)** HIV-1 crosses stratified epithelium through mechanical breaks in this barrier to infect macrophages and CD4 T cells underneath. Figure reprinted with permission from Macmillan Publishers Ltd. Shattock, R. J. & Moore, J. P. Inhibiting sexual transmission of HIV-1 infection. Nat Rev Microbiol 1, 25-34 (2003).


Columnar epithelium
c
Transmigration
a
Infection
b
Transcytosis
T cell
Dendritic
cell
Stratified epithelium
Physical abrasion
d
e
Langerhans
cell
Macrophage

More recent work from the Haase lab and others has challenged this established paradigm. This group and others have present compleing evidence that HIV-1 infection begins when a local population of approximately 40 to 60 CD4 T cells at the mucosal site become infected [27-29], beginning from a single founder virion [30]. Once this local "beach head" is established, CD4 T cells from this original population enter the lymphatic system to disseminate infection to local draining lymph nodes [31]. These studies are based on high-dose, highly pathogenic simian immunodeficiency virus (SIV).

1.1.3.1 Cellular Infection

HIV-1 first binds CD4 on the surface of T cells via gp120, which is expressed on the virion's surface as a part of gp120/gp41 heterotrimers. Subsequently, gp120 binds a co-receptor molecule (CCR5 or CXCR4) to form a CD4-gp120-co-receptor ternary complex. This complex initiates conformational changes in gp41 resulting in the insertion of the highly hydrophobic N-terminal "fusion peptides" of the gp41 molecules into the host cell plasma membrane [32-34].

Although a variety of fringe co-receptors have been identified [35], HIV-1 fusion with CD4 expressing cells usually depends on one of two co-receptors. Some HIV-1 virions use the integrin receptor CCR5, whereas others use the CXCR4 molecule. Strains that employ the CCR5 co-receptor are referred to as R5 viruses. CCR5 is expressed on macrophages and numerous CD4 T cell populations. Of note, the CCR5 molecule is also expressed on mucosa-associated memory CD4 T cells, which constitute the majority of $CD4^+$ T cells found in the gut [36-39]. CXCR4 is expressed on T cells, and viruses which employ this co-receptor are referred to as T-tropic.

Most natural person-to-person transmissions involve R5 strains. The importance of the CCR5 co-receptor for natural infection is highlighted by the fact that humans homozygous for a deletion mutant variant of CCR5 (CCR5 Δ32) are largely impervious to HIV-1 infection [40], and higher levels of CCR5 ligand protect against infection [41]. Use of the CCR5 co-receptor on gut-associated T cells may account in part for the massive depletion of this T cell population very early in infection.

Although 40% to 50% of individuals progress to late stages of infection maintaining R5 viruses [42], in most individuals the predominant isolatable virions change from R5 viruses to X4 or dual (X4R5) strains through mutations in the gp120 molecule prior to development of AIDS [43, 44]. These mutations may in part be brought about by immune system pressure [45]. Since the less transmissible X4 strains are able to infect an expanded repertoire of T cell subsets when compared to R5 virions, X4 viruses allow for greater dissemination through the body [46].

Regardless of the co-receptor used, fusion of the viral envelope with the cell membrane results in viral uncoating and injection of the p24-encapsulated core particle into the cytosol. Reverse transcription is catalyzed by the viral RT enzyme and is initiated within the cytosol prior to nuclear import. The viral DNA (vDNA) genome is imported into the nucleus as a part of the pre-integration complex (PIC) [47]. Once the PIC complex enters the nucleus, the HIV-1 integrase catalyses the insertion of the HIV-1 genome into a host chromosome, preferably in regions of active transcription [48-52].

1.1.3.2 Gene Expression

Once integrated into the host genome, HIV-1 gene transcription is driven off a promoter region found within the long terminal repeat (LTR) sequence, upstream of the protein coding region. Originally, only multiply spliced viral mRNAs encoding for Tat, Nef or Rev are released into the cytosol. Translated Tat protein returns to the nucleus where it acts to effectively increase HIV-1 gene transcription by increasing RNA polymerase prossessivitiy [53, 54]. Rev protein also returns to the nucleus were it binds the Rev response element (RRE) found within *gag*, *pol* and *env* transcripts, and the full length viral RNA genome (all of which are incompletely spliced), stabilizing them for nuclear export. The *pol* transcript is generated from a polymerase stuttering event occurring within the *gag* coding sequence, which occurs at a frequency of 5-10%.

1.1.3.3 Particle Assembly

The uncleaved Gag precursor polypeptide (p55) appears to be the major keystone of viral particle assembly. This peptide is translated in the cytosol and traffics to the host cell plasma membrane where it interacts specifically with the membrane, gp160 and other p55 molecules [55, 56]. Recruitment of the HIV-1 genomic RNA to the p55 molecules at the plasma membrane is dependent on the Ψ sequence found near the 5`end of the RNA [57].

The HIV-1 surface glycoproteins are originally synthesized in the rough ER as the polyprotein gp160 from the *env* transcript as a type I transmembrane precursor protein with an approximately 150 aa cytosolic tail [58]. While in the ER, disulphide bond formation results in creation of gp160 homotrimers. In the Golgi apparatus, the gp120 portion of the

receptor becomes highly glycosylated and gp160 is cleaved by host furin-like membrane-associated proteases to generate the mature gp41 and gp120 molecules.

1.1.3.4 Virus Budding and Particle Maturation

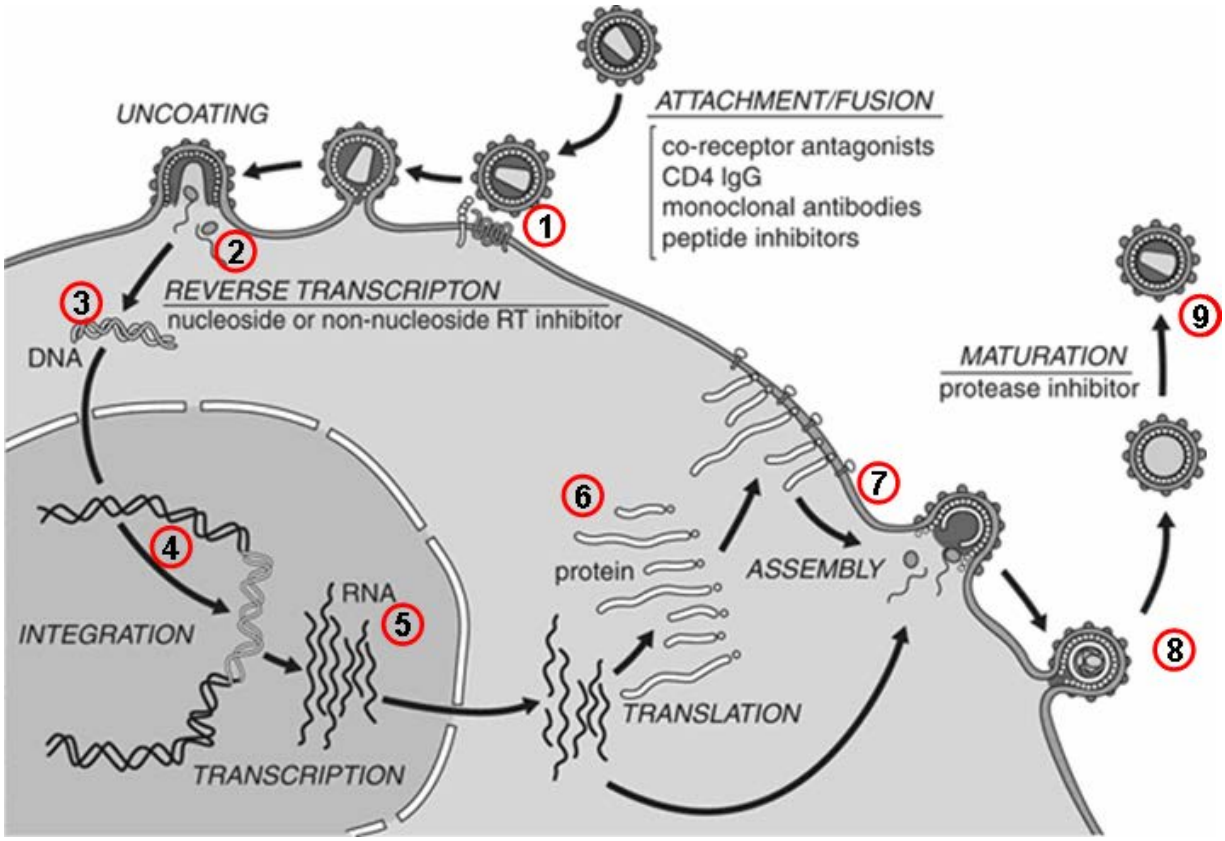
HIV-1 particle budding occurs at the host membrane and is a highly organized, active process which depends on p6 [59, 60] and the coordinated action of numerous host proteins [61]. During or slightly after the virion is released from the cell membrane, the viral PR cleaves the p55 and Pol precursors to generate mature HIV-1 proteins. See figure 4 for clarification of the virus live cycle.

1.1.4 Pathology

HIV-1 infection is classically characterized by a gradual depletion of CD4 T cell numbers, leading over the course of years to a loss of immunocompetence, increased incidences of opportunistic infections and increased rates of tumourigenesis [62].

Symptoms of acute retroviral infection, although commonly overlooked or misidentified, usually present themselves within the first 2 months after infection and often resemble the flu [63-68]. Fever, swollen lymph nodes, rash and sore throat are common [37]. Gastrointestinal symptoms and mucus membrane ulceration are not uncommon [37, 69-71]. HIV-1 viral loads often reach $1x10^6$ virions/mL by 8 to 12 weeks after infection, and then decrease dramatically as the adaptive immune system begins to mobilize against the infection [72]. During acute infection CD4 T cell populations in the gut are drastically reduced [38, 39]. The gut represents the body's largest reservoir of lymphocytes, many of which express both $CXCR4^+$ $CCR5^+$ [37-39]. By the end of the acute phase (i.e. when host

Figure 4: The HIV-1 cellular life cycle. **1)** HIV-1 particles bind to the host cell via interactions between the gp120 surface spike and CD4. **2)** gp120 interactions with the CCR5 or CXCR4 co-receptors results in viral uncoating. **3)** vRNA is transcribed cytosolically into vDNA by the viral reverse transcriptase. **4)** vDNA then enters the nucleus and integrates into the host genome using viral integrase. **5)** Viral RNAs and proteins are transcribed and **6)** translated, respectively, by host machinery. The viral glycoproteins are translated in the ER and processed by host machinery to produce the mature gp120 and gp41 units. Other viral proteins are translated cytosolically. **7)** Viral structural proteins congregate at the inner leaflet of the cell membrane and assemble into immature virions. **8)** Virions derive their envelope from the host lipid bilayer and bud directly off the cell membrane. **9)** Once free of the cell, HIV-1 proteases packaged into the immature virions cleave numerous polypeptides to yield the matured virion. Figure reprinted with permission from John Wiley & Sons, Inc. Munk, C. & Landau, N. R. Production and use of HIV-1 luciferase reporter viruses. Curr Protoc Pharmacol Chapter 12, Unit12 5 (2003).


UNCOATING
ATTACHMENT/FUSION
co-receptor antagonists
CD4 IgG
monoclonal antibodies
peptide inhibitors
1
2
3
REVERSE TRANSCRIPTON
nucleoside or non-nucleoside RT inhibitor
DNA
MATURATION
protease inhibitor
9
6
7
4
protein
ASSEMBLY
RNA
5
INTEGRATION
8
TRANSLATION
TRANSCRIPTION

immunity begins to contain HIV-1 infection), 80% of the gut associated lymphoid tissue (GALT) CD4 T cells have been depleted [37, 73]. Unlike CD4 T cell counts in other parts of the body which rebound with the decline of acute symptomology, T cell counts in the GALT never recover completely, even with successful ART therapy. As GALT T cells account for 60% of the body's T cell supply, the loss of this population represents a substantial reduction in whole body CD4 T cell numbers.

Interestingly, evidence suggests that it is at this point that viral reservoirs are established [74-76]. These reservoirs are contained within a population of approximately $1x10^6$ long lived, non-dividing memory CD4 T cells typically found in lymph nodes and peripheral tissues [76, 77]. Although these cells contain fully integrated, replication competent HIV-1 genomes, they to not produce viral gene products, and are hence difficult for the adaptive immune system to detect [77, 78]. Furthermore, this population of cells appears stable, even under effective ART therapy [79, 80].

After the clearance of acute symptoms, HIV-1 infection enters a period of clinical latency in which an individual can remain asymptomatic for many years, although certain signs may occur (i.e. thrush, shingles). Viral replication continues during this phase, and HIV-1 can be isolated from peripheral blood mononuclear cells (PBMCs) [67], blood serum [81], cerebral spinal fluid [82], bone marrow [83], seminal fluid [84] and vaginal secretions [85]. HIV-1 infection causes a gradual decrease in peripheral CD4 T cell counts over the course of many years with an average decrease of 50 to 75 cells/μL per year, with the rate of CD4 T cell decline accelerating as an individual approaches AIDS [86]. It is estimated that during the clinically latent phase only a small percentage of CD4 T cells are infected at any give time. Hence, the rate of CD4 T cell loss cannot be accounted for by direct infection

alone [87]. The mechanisms by which HIV-1 infection results in CD4 T cell destruction are not completely understood and remain under investigation.

Without drug treatment, the duration of the clinically latent phase is highly variable. Approximately 10% of people will progress to AIDS in two years without drug intervention, whereas 80% will show symptoms within ten years of contracting the disease. A select group do not show any symptoms and do not progress or progress very slowly to AIDS over the course of many years. Some individuals retain high CD4 T cell counts despite active HIV replication, and are termed long-term non-progressors (LTNP). Others retain undetectable viral loads and healthy CD4 T cell counts despite a lack of ART intervention, and are termed elite controllers (EC).

Gradually, most individual's CD4 T cell count declines until the body is no longer able to mount a proper immune response. When an individual's T cell count drops below 200 cells/μL of blood, he or she is defined as having AIDS. Lymph node fibrosis is often observed at late stages. Lymph node architecture becomes almost completely destroyed with an almost complete loss of follicular dendritic cells. Peripheral leukocyte populations including mDCs, pDCs and CD8 T cell counts decline sharply [88-90]. At this point, viral loads rise dramatically and an individual may no longer be able to cope with numerous ubiquitous, and normally innocuous, pathogens. Many individuals experience the reactivation of latent infections such as *Pneumocystis jiroveci*, *Toxplasma gondii*, *cytomegalovirus* (CMV) *and* HSV-8 (Kaposi sarcoma). The acquisition of other pathogenic infections such as *Mycobacterium avium*, *Cryptosporidium*, *Cryptococcus neoformans*, and various bacterial infections are also common [91]. Tumours, particularly non-Hodgkin's B-cell lymphomas and Kaposi's sarcoma also occur at a higher frequency in AIDS patients.

Pneumocystis jiroveci pneumonia is the major AIDS-defining illness in North America, Europe and Australia, whereas *Mycobacterium tuberculosis* and *Mycobacterium avium* are the major AIDS-defining illnesses in Thailand [92] and Sub-Saharan Africa [93].

1.2 Adaptive Immune Response

The adaptive immune response, in contrast to the innate immune response, is specific for a given pathogen. Although acitivated in part by the innate immune system, the adaptive immune system is based on the recognition of *specific* foreign antigens, often becomes more effective over the course of infection, and leads to the development of immune "memory".

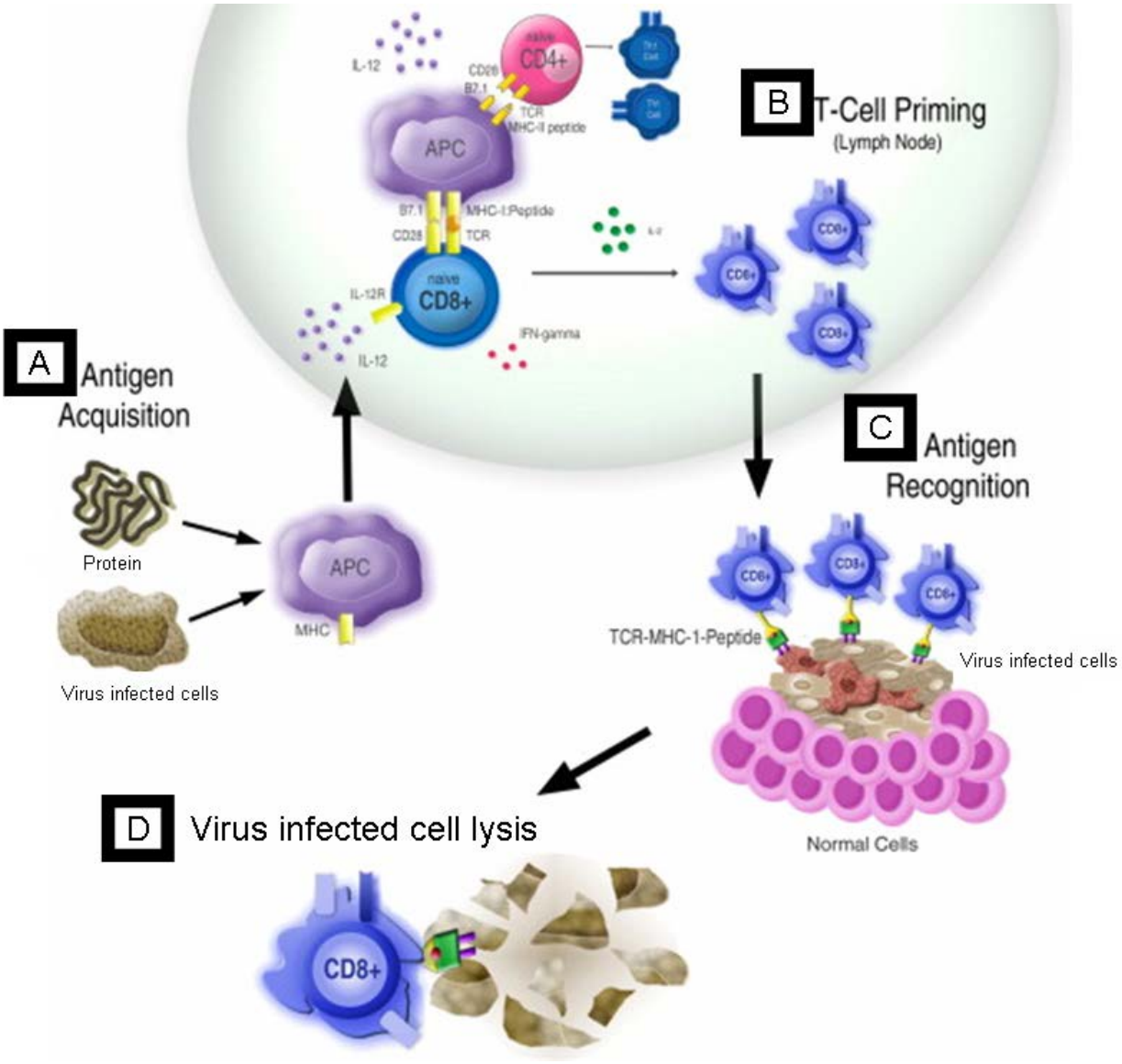
In the context of viral infections, an adaptive immune response is initiated when activated APCs at the source of infection traffic to secondary lymphoid structures. These cells display viral antigens in the context of MHCII molecules which are recognized by resident CD4 T cells through engagement of antigen specific surface T cell receptors (TCRs) as well as the CD4 co-receptor. These APCs also display activating co-receptor molecules such as CD80 and CD86 and produce cytokines such as interleukin (IL)-12 to promote the differentiation of CD4 T cells into a"T_{H1}" phenotype. When CD4 T cells engage APCs in the context of the proper secondary signals and cytokine environment, they become activated, differentiate into an effector phenotype and undergo clonal expansion. These CD4 T cells secrete a variety of cytokines such as IL-2, tumour necrosis factor (TNF)-β and interferon (IFN)-γ which support the differentiation and cytotoxic activity of CD8 T cells. Activated CD4 T cells can also help to strengthen humoural responses by releasing "T_{H2}" cytokines such as IL-10 and tumour growth factor beta (TGF-β), and through direct interactions with naïve B-cells via TCR:MHCII and CD40L:CD40 interactions.

CD8 T cells recognize foreign antigens presented in the context of MHC class I molecules on the surface of infected cells or APCs. In the context of proper secondary signals (i.e. CD80, CD86) and local cytokine environment, these CD8 T cells then undergo clonal expansion and differentiate into cytotoxic effector T lymphocytes (CTLs). Effector CD8 T cells traffic out of the lymph node and into peripheral circulation, homing to the site of infection. See figure 5 for an overview of the adaptive immune response.

Effector CD8 T cells identify infected cells through recognition of foreign antigens displayed on the infected cell's surface in the context of an MHCI molecule via their TCR and CD8 co-receptor. This forms a cell to cell contact known as an "immunological synapse". Calcium-dependent signalling [94, 95] originating from the immunological synapse results in the release of exocytic vesicles containing perforin, granzymes and granulysins directly onto the infected cell. Perforin is an amphipathic molecule which is able to form homomeric channels through the plasma membrane of target cells. These channels allow granzymes and granulysin to enter the target cell. Granzyme A promotes DNA damage by cleaving histone H1 [96] while Granzyme B enters the cytosol to initiate apoptotic cascades by cleaving pro-caspase 3 [97] as well as inducing the permeabilization of mitochondrial membranes [98]. A series of other granzymes containing proteolytic functions which also promote apoptosis have also been identified [99-102]. Granulysin has been shown to disrupt cell and mitochondrial membranes by generating perforin-like pore structures [103, 104], as well as inducing caspase-3 dependent apoptosis [103].

In addition to cytolytic exosome release, cytotoxic CD8 T cells induce target cell apoptosis via engagement of Fas trimer complexes found on the surface of target cells with the Fas ligand expressed on the cytotoxic CD8 T cell. Fas/Fas ligand interaction induces the

Figure 5: The adaptive anti-viral immune response. **A)** APCs at the site of infection acquire viral antigens through phagocytosis or direct infection. **B)** APCs traffic to lymph nodes where they activate CD4 T cells through presentation of foreign antigens in the context of MHCII molecules in combination with second signals such as B7. CD4 T cells respond by differentiating into an effector phenotype and then secrete cytokines such as IL-2, IFN-γ and IL-6. Lymphoid CD8 T cells bind foreign antigens on the surface of antigen presenting cells expressed in the context of MHCI via their T cell receptor. CD8 T cells are also stimulated by second signal through CD28/B7 interactions, inducing their clonal expansion and effector differentiation. IL-2, IFN-γ and IL-6 released from activated CD4 T cells and IL-12 released from APCs further encourage CD8 T cells to divide and develop into an effector phenotype. **C)** Effector CD8 T cells then traffic out to the sites of infection. **D)** Here, TCRs on CD8 T cells recognize foreign peptides displayed in the context of MHCI. This results in CD8 T cell-mediated destruction of the virally infected cell. Figure reprinted with permission from Elsevier Inc. Tucker, Z. C., Laguna, B. A., Moon, E. & Singhal, S. Adjuvant immunotherapy for non-small cell lung cancer. Cancer Treat Rev 38, 650-61 (2012).


IL-12
CD28
B7.1
naive CD4+
TCR
MHC-II peptide
APC
B7.1
MHC-I Peptide
CD28
TCR
naive CD8+
IL-12R
IL-12
IFN-gamma
B T-Cell Priming
(Lymph Node)
CD8+
A Antigen Acquisition
Protein
APC
MHC
Virus infected cells
C Antigen Recognition
TCR-MHC-1-Peptide
Virus infected cells
Normal Cells
D Virus infected cell lysis
CD8+

extrinsic apoptotic cascade via recruitment of Fas-associated death domain (FADD) proteins. This initiates caspase cascades resulting in DNA cleavage and cytochrome C release from the mitochondria [105, 106].

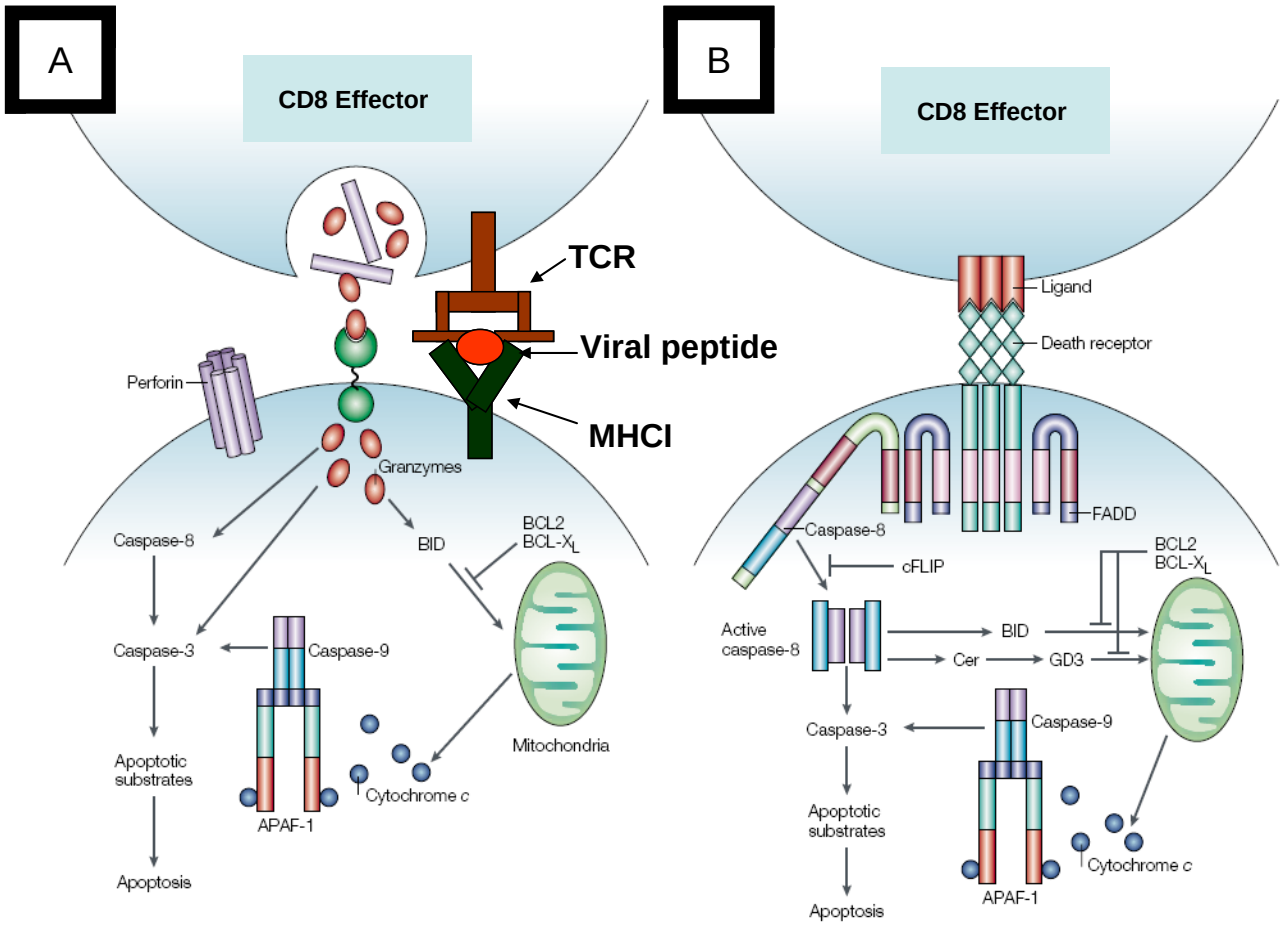
Upon destruction of a single target cell, the activated CD8 T cell is freed to identify new infected cells. In this manner, activated cytotoxic CD8 T cells act as "serial killers"; each cytotoxic CD8 T cell killing numerous target cells. See figure 6 for details of CTL killing.

In the majority of infection scenarios, the microbial threat is either completely eradicated or effectively contained, and the active infection is resolved. Upon resolution of an infection, the vast majority of clonally-expanded T cells undergo apoptosis. A minority of microbe-specific lymphocytes remain alive however, and differentiate into memory cells. Memory T cells can either reside in the peripheral tissues as "effector memory" T cells (T_{EM} cells), or home back to lymphoid structures were they are referred to as "central memory" T cells (T_{CM} cells). Although differences between these two populations exist [107], the main feature of all memory T cells is their readiness to respond to subsequent infections. Memory T cells require less antigen stimulation before differentiating to an effector phenotype, and produce larger amounts of IFN-γ more readily in response to TCR engagement. For example, many memory T cells may be activated without secondary signals via CD28.

1.2.1 CD8 T Cell Responses to HIV-1 Infection

Numerous studies have shown that $CD8^+$ CTLs are major contributors to the control of HIV-1 infection. A drastic reduction of HIV-1 viral loads is concurrent with an expansion of $CD8^+$ T cells upon resolution of acute symptomatology [108, 109]. Furthermore, a large

Figure 6: CD8 T cell cytolytic killing of virus infected cells. $CD8^+$ CTLs engage virally infected cells by binding to viral peptides displayed on the surface of infected cells in the context of MHCI molecules, via their TCR receptors. TCR/MHCI: peptide interactions trigger two cytolytic pathways within the infected cell. **A)** Cytolytic T cells excrete vacuoles containing perforin, granzymes and granulysin onto the surface of infected cells. Perforin forms homo-multimeric pores in the membrane of the target, resulting in loss of ionic balance in the infected cell. Furthermore, granzymes and granulysin enter the infected cell through these perforin pores. Once cytosolic, these molecules interact with pro-caspases and digest cellular DNA to trigger the intrinsic apoptosis cascade. **B)** FasL expressed on the surface of activated cytotoxic CD8 T cells binds Fas expressed on the surface of infected cells, triggering apoptosis through the caspase-8 dependent extrinsic cascade. Figure reprinted with permission from Macmillan Publishers Ltd. Stassi, G. & De Maria, R. Autoimmune thyroid disease: new models of cell death in autoimmunity. Nat Rev Immunol 2, 195-204 (2002).


A
CD8 Effector
TCR
Viral peptide
MHCI
Perforin
Granzymes
Caspase-8
BID
BCL2
BCL-XL
Caspase-3
Caspase-9
Apoptotic substrates
Mitochondria
Cytochrome c
APAF-1
Apoptosis
B
CD8 Effector
Ligand
Death receptor
FADD
Caspase-8
cFLIP
BCL2
BCL-XL
Active caspase-8
BID
Cer
GD3
Caspase-3
Caspase-9
Apoptotic substrates
Cytochrome c
APAF-1
Apoptosis

diversification of the HIV-1 genomes occurs as the virus is forced to evolve to escape CTL immune pressure [110, 111]. Mutations in the HIV-1 genome which allow the virus to escape CTL detection result in increases in viral load [112]. Furthermore, genetic variability at the HLA class I allele is highly associated with protection/susceptibility to disease in humans [113]. The HLA alleles HLA-B27 and HLA-B57 are found in high rates amongst viral controllers [114]. Polyfunctional CD8 T cells (i.e. display lytic activity, IFN-γ and IL-2 secretion) are found more often in LTNPs [115, 116]. In the gut, where initial T cell depletion is the most profound, strong polyfunctional Gag-specific CD8 T cell responses correlate with CD4 T cell counts and inversely correlates with viral load [117]. Lastly, new evidence from rhesus macaque studies strongly suggests that certain domainate CTL epitopes [118] and vaccines designed to induce strong CTL immunity at the mucosal sites of initial infection (i.e. vaginal mucosal) [119] may provide effective containment of viral spread prior to systemic dissemination throughout the body and the establishment of viral rersevoirs.

1.2.2 CD8 T Cell Impairment in HIV-1 Infection

Although cell mediated immunity becomes severely impaired over the course of progressive HIV-1 infection [120-122], CD8 T cells are not depleted like their $CD4^+$ counterparts [123]. Still, it is clear that HIV-1 directly affects CD8 T cell function. Initially CD8 T cells display impaired function before CD4 T cells become depleted to an appreciable extent [63, 124, 125]. CD8 T cells isolated from HIV-1 positive individuals display aberrant surface marker phenotypes, as well as display less lytic activity and antigen induced clonal expansion [126]. Low perforin expression has been detected in lymph nodes of HIV^+ persons [127]. Recent studies have correlated HIV-specific CD8 T cell anergy with the

upregulation of PD-1 on the surface of HIV-1 specific T cells [128, 129]. Higher PD-1 has been associated with faster disease progression [130, 131], and anti-PD-1 or PD-1 ligand blocking antibodies enhance HIV-1 specific CD8 T cell function [128, 129, 131].

Notably, loss of function is not restricted to HIV-specific CD8 T cells. Van Baarle et al. demonstrated that EBV-specific CD8 T cells from HIV^+ patients become dysfunctional and display aberrant phenotypes before a clinically significant degree of CD4 T cell depletion [132]. Also, people with HCV-HIV co-infection produce fewer peripheral effector CD8 T cells than people with HCV infection alone, regardless of CD4 T cell count [133]. These clinical investigations are supported by a series of *in vitro* studies showing that CMV and HIV-1 specific primary CD8 T cells can be isolated at normal frequencies [126, 134, 135], but fail to upregulate perforin and IFN-γ in response to cognate antigens, and are hence unable to destroy target cells [125, 135-137]. Loss of cell-mediated immunity therefore appears more complex than can be explained by a loss of CD4 T cell help alone. Since CD8 T cells are not infected by HIV, the virus must possess auxiliary functions which result in CD8 T cell dysfunction.

1.3 Interleukin-7

IL-7 is a non-redundant cytokine essential for the differentiation, proliferation and maturation of lymphocytes. In mice, IL-7 is required for the production of both B and T cells [138]. In contrast, humans with mutations in the IL-7 receptor lack T cells but produce B cells normally [139]. IL-7 is released primarily from lymph node T zone fibroblastic reticular cells, bone marrow stromal cells, both intestinal and thymic epithelial cells, as well

as follicular dendritic cells, keratinocytes and neurons [140, 141]. IL-7 is found in the blood at a normal concentration of 1-3 pg/mL [65].

1.3.1 IL-7 and Thymopoesis

IL-7 is essential for the proper maturation of thymocytes as well as proper thymic architecture [142]. Mice transgenically altered to over express IL-7 developed lymphoproliferative disorders, most notably an abundance of immature B-cells found circulating in the periphery [143-145]. Mice treated with anti-IL-7 antibodies and mice containing deletions of the IL-7 or the IL-7 receptor (IL-7R) genes are essentially athymic [146-148] with reduced thymic cellularity [149] and impaired T cell development [150, 151]. In humans, loss of function mutations in the *il-7ra* gene results in arrest of T cell development in the thymus and subsequent severe combined immunodeficiency (SCID) syndrome [138, 139, 152]. Gain of function mutations are found in various leukemias [153-156] as well as other cancers [156-158].

Without IL-7 signalling, T cell development is blocked at the triple negative stage. IL-7 signalling promotes the expression of anti-apoptotic Bcl-2 family members which play a definitive role in thymopoeisis [159-161]. IL-7R is expressed on thymocytes at several stages of T cell development including the double negative stage where it is involved in T cell survival and V(D)J rearrangement at the β, γ, and δ loci [149, 160, 162]. IL-7R is removed from the cell surface at the double positive stage. Loss of IL-7R expression during positive selection is believed to ensure that all T cell survival signalling occurs through TCR engagement. IL-7R is expressed again at the single positive stage to promote cell survival

during negative selection. IL-7R is then expressed and maintained as nascent T cells exit the thymus [162-164].

1.3.2 IL-7 and Homeostatic Regulation of T Cell Numbers

Outside of thymopoesis, IL-7 is required for the proper homeostatic regulation of peripheral naïve and memory T cell numbers in both humans and mice [165, 166]. As mentioned above, IL-7 signalling promotes cell survival by increasing the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-X_L while simultaneously confining the pro-apoptotic proteins Bad, Bax and Bim to the cytosol via post translational modification [166-168].

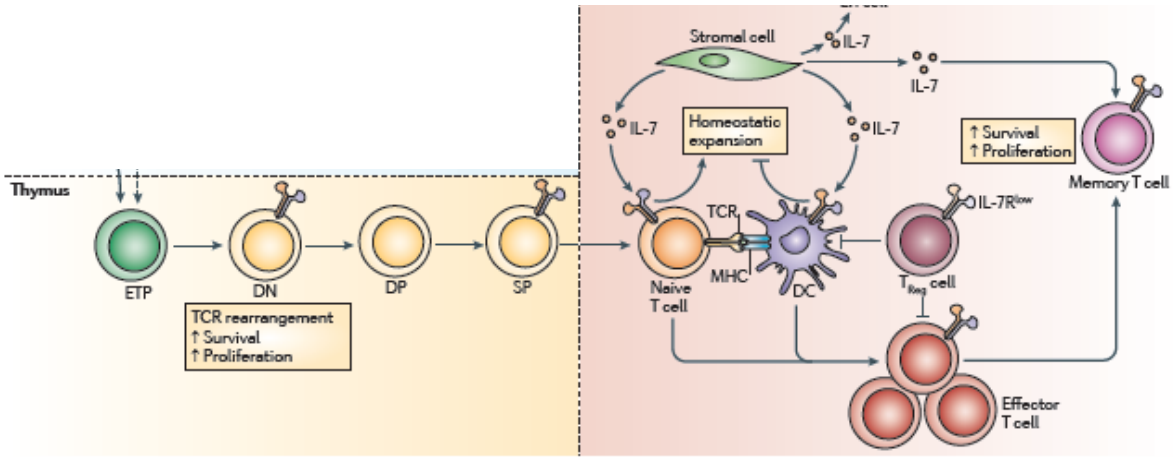
Along with increased survival, IL-7 signalling also drives proliferation of T cells by promoting cell cycle progression from G_0 to S_1, mainly through enhanced phosphorylation of Cdk molecules and degradation of Kip27 [164, 169, 170]. Of note, IL-7's effect on proliferation is more marked in CD8 T cells than in CD4 T cells [171]. IL-7 signalling also enhances the metabolic rate of T cells by promoting the translocation of the glucose transporter GLUT-1 to the cell membrane [133, 169] and thus increasing glucose uptake. By promoting cell survival and proliferation, IL-7 plays a central role in maintaining T cells in the peripheral circulation. IL-7 can also work in concert with self-antigen-induced TCR signalling to increase survival and proliferation of the peripheral T cell pool [172]. TCR/IL-7 signalling synergy plays a role in the survival of both naïve and memory T cells as it is well established that slight, repetitive TCR signalling in response to self peptides is important for homeostatic proliferation of these T cell populations [173].

1.3.3 IL-7 and the Immune Response

In addition to its role in normal homeostatic T cell regulation, IL-7 also plays an important role as an acute phase cytokine during an active immune response. IL-7 is required for both the proliferation and differentiation of CD4 and CD8 T cells from the resting naïve and memory phenotypes to the active effector phenotype [166, 174, 175]. A recent report has shown that hepatic IL-7 levels increase greatly in response to type I interferons during acute infection in mice [176].

IL-7 is specifically needed for the proper function of CTLs. IL-7 achieves this effect by promoting clonal expansion through the upregulation of telomerase in activated human CD8 T cells [177] and promoting the transition of CD8 T cells through S-phase of the cell cycle [170]. IL-7 also promotes CD8 T cell differentiation to an effector phenotype by upregulating the cytolytic molecule perforin [178]. Increased glucose uptake via GLUT-1 translocation to the cell membrane also provides cells with the extra nutrition required to meet the metabolic needs of effector differentiation and clonal expansion. IL-7 upregulates the second signal molecule CD69 as well as the CD25 component of the IL-2 receptor [179-181]. As IL-2 is a potent pro-CTL cytokine, this effect further encourages CD8 T cell effector functions. IL-7 signalling also increases production of IFN-γ following TCR stimulation to further support cell-mediated immune function [176, 182]. Furthermore, IL-7 in concert with TCR stimulation may promote CTL responses against weaker MHCI-restricted microbial and tumour epitopes, at least in part through the IL-7-induced upregulation of CD8 surface expression [180]. Upon resolution of infection, IL-7 signalling may promote the transition of some CD4 and CD8 T cells to a memory phenotype [183-186]. See figure 7 for a review of IL-7 physiology.

Figure 7: The role of interleukin-7 in T cell development, homeostasis and activation. In the thymus, interleukin-7 is expressed on thymocytes during the double negative stage of development where it plays a role in TCR rearrangement and T cell survival during negative selection. CD127 is downregulated during the double positive stage and re-expressed again at the single positive stage. In the periphery, IL-7 participates in T cell homeostasis. During an adaptive immune response IL-7 promotes CD8 T cell effector differentiation by upregulating the production of perforin and enhancing clonal expansion. Furthermore, IL-7 encourages the establishment of memory T cell populations. Figure reprinted with permission from Macmillan Publishers Ltd. Mackall, C. L., Fry, T. J. & Gress, R. E. Harnessing the biology of IL-7 for therapeutic application. Nat Rev Immunol 11, 330-42 (2011).


Thymus
ETP
DN
DP
SP
TCR rearrangement
↑ Survival
↑ Proliferation
Stromal cell
IL-7
IL-7
IL-7
IL-7
Homeostatic expansion
↑ Survival
↑ Proliferation
Memory T cell
TCR
MHC
Naive T cell
DC
IL-7Rlow
TReg cell
Effector T cell

1.3.4 Regulation of IL-7 Production

Although it is commonly believed that IL-7 output from IL-7 producing cells is kept constant and not affected by extrinsic factors [140, 187], a 2009 report published in Nature indicated that IL-7 is not produced at a stable and constitutive rate in humans [165]. Guimond *et. al.* presented a relatively simple feedback loop in humans where higher IL-7 levels in the body cause stromal cells and APCs to downregulate IL-7 mRNA transcription. Other groups have shown that IL-7 production is indeed induced in response to IFN and IL-6 signalling, and can be inhibited by TGF-β, representing what is likely a complex regulatory mechanism [176, 188, 189].

1.3.5 Interleukin 7 Signalling Dysregulation in HIV Pathology

Blood plasma IL-7 levels are increased during HIV-1 infection (from an average of 2.2 pg/mL in uninfected individuals to 18 pg/mL in HIV^+ persons) [190, 191] and CD127 expression on T cells is downregulated [192-201]. There has been considerable debate over the causes and consequences of these observations. It is believed by some that increased IL-7 during HIV infection represents a compensatory mechanism by which the body is attempting to augment CD4 T cell production in response to HIV-1 induced lymphopenia. There is some precedence for this assumption as injection of supraphysologic doses of IL-7 into lymphopenic individuals will induce lymphogenesis and homeostatic proliferation.

1.4 The IL-7 Receptor

IL-7 signalling is mediated through its high affinity binding (K_D = 60 nM) to the IL-7 IL-7R in a 1:1 stochiometric ratio [202]. The IL-7R is a heterodimer composed of two transmembrane polypeptides. One, the common gamma chain (CD132), is shared with the

IL-2, 4, 9, 15 and 21 interleukin receptors. The second polypeptide is the IL-7 receptor specific alpha chain (CD127). CD132 and CD127 are expressed independently on the cell membrane. IL-7 binding to the CD127 subunit promotes dimerization with CD132 thus forming the IL-7 receptor complex and activating signal transduction [203]. Janus kinase (Jak) 1 constitutively associates with CD127 [204] while Jak3 constitutively associates with CD132 [205, 206].

CD127 is expressed highest on memory T cells, and is also found at relatively high levels on naïve T cells. CD127 is downregulated on effector cells, but may remain high on a small subset which may be destined to become memory cells [174]. The immuno-attenuating T reg population is IL-7R^{low} [170, 207]. Dendritic cells, monocytes and macrophages also express CD127 at a lower level compared to naïve and memory T cells [187, 208].

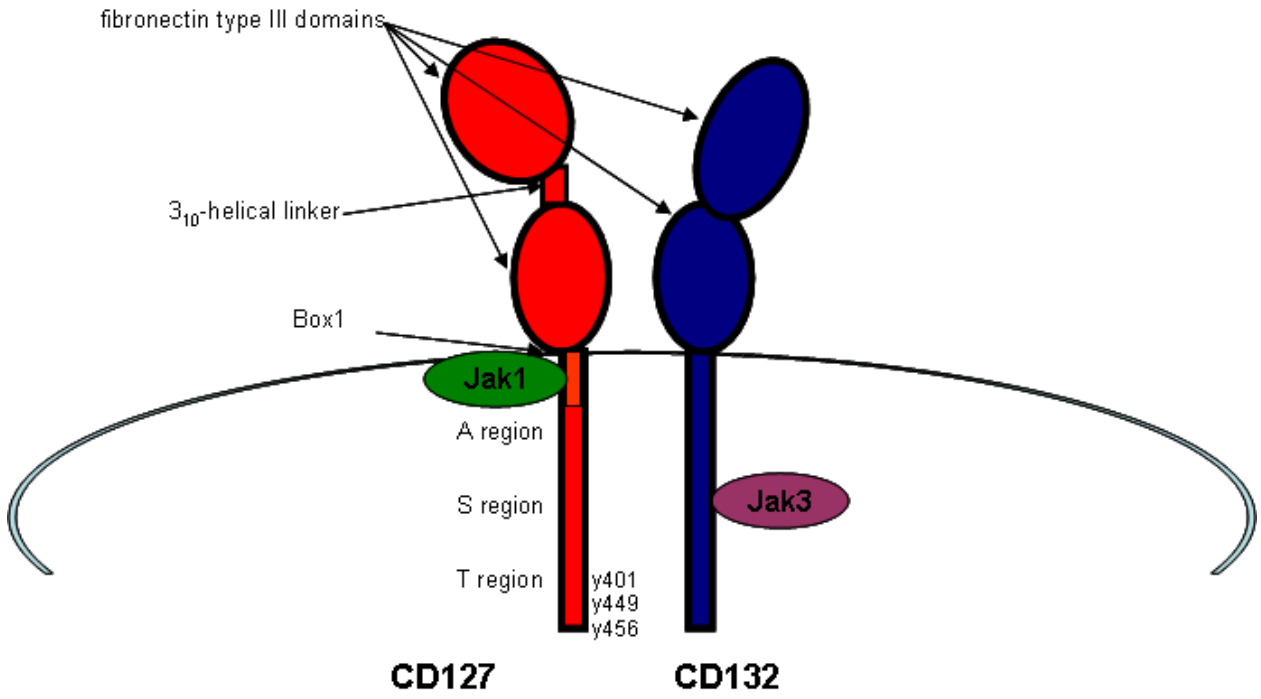
CD127 is a 439 amino acid, type I transmembrane glycoprotein with a 219 amino acid (aa) extracellular domain (ECD), a 25 aa transmembrane domain, and a 195 aa cytosolic tail [206]. The CD127 ECD belongs to the cytokine receptor homology class I (CRH1) family. It consists of two fibronectin type III domains connected by a 3_{10}-helical linker [209]. CD127 contains three internal di-sulphide bonds and six N-linked glycosylation sites [202]. Five different mutations in the first fibronectin type III domain of human CD127 have

been associated with SCID [210]. Interestingly these mutations do not map to the IL-7 binding interface.

The intracellular domain of CD127 consists of four distinct regions: an eight amino acid region called the Box1 motif which is found close to the cell membrane, a region rich in acidic residues (A region), a serine rich region (S region) and a distal region (T region) which contains three tyrosine residues Tyr401, Tyr449 and Tyr456 [203]. The Box1 domain as well as Y449 are highly conserved between humans and mice, and are of particular importance to CD127 function [211]. The Box1 domain binds Jak1 and is found in all type I cytokine receptors [211]. Y449 becomes phosphorylated in response to IL-7 binding and forms an important docking site for SH2 domain-containing proteins [203]. Both the Box1 domain and Y449 have been shown to be critical for thymocyte development in studies where IL-7R$^{-/-}$ mice were retrovirally reconstituted to express various CD127 mutant constructs [203].

Glycosylation of CD127 increases IL-7 binding affinity 300-fold by stabilizing receptor/ligand interactions [212]. This feature makes CD127 unique among common γ-chain receptors which, although glycosylated, do not show large changes in ligand binding affinity following glycosylation [140, 213-215]. CD127 glycosylation alters conformation minimally and does not participate directly with ligand binding. Instead, glycosylation is hypothesized to stabilize interactions with CD132, and thus promote ligand:receptor binding [210]. See figure 8 for IL-7R structure.

Figure 8: Structure of the interleukin-7 receptor. The interleukin-7 receptor is composed of two transmembrane peptides. Shown in red is the specificity-providing component of the receptor: CD127. CD127 is a 439 amino acid, type I transmembrane glycoprotein with a 219 amino acid (aa) extracellular domain, a 25 aa transmembrane domain, and a 195 aa cytosolic tail. CD127's extracellular domain consists of two fibronectin type III domains each composed of seven β-strands connected by a 3_{10}-helical linker. The intracellular portion of CD127 consists of four distinct regions: an eight amino acid region called the Box1 motif which is found near the membrane and binds Jak1, a region rich in acidic residues (A region), a serine rich region (S region) and a distal region (T region) which contains three tyrosine residues Tyr401, Tyr449 and Tyr456. The Box1 domain as well as Y449 are highly conserved between humans and mice, and are of particular importance to CD127 function. In blue is shown the common gamma chain. This peptide is shared with the receptors for other cytokines: IL-2, IL-4, IL-9, IL-15 and IL-21. This peptide also contains 2 extracellular fibronectin type III domains. The Jak3 molecule binds to the cytosolic tail of CD132.


fibronectin type III domains
3_{10}-helical linker
Box1
Jak1
A region
S region
T region
y401
y449
y456
Jak3
CD127
CD132

1.4.1 CD127 Signalling

Upon binding of IL-7 to the IL-7R, Jak enzymes transphosphorylate each other as well as phosphorylate the cytoplasmic tails of CD127 and CD132, creating scaffolding sites for the SH2 domain of STAT5 [216]. Jak1 is believed to also phosphorylate the recruited STAT5, leading to STAT5 activation via homodimerization [170]. STAT5 complexes then translocate to the nucleus where they act as transcription factors. Perforin and Bcl-2 are two of the many genes upregulated through STAT5 signalling [170, 217]. During thymopoiesis, STAT5 association with the TCR promoter has been implicated in proper V(D)J rearrangement through histone acetylation [217-219].

Phosphorylation of the IL-7R cytosolic tails and Jak1 activation also promotes PI-3K association with the receptor. PI-3K in turn phosphorylates and activates Akt [142, 211]. PI-3K dependent IL-7 signalling regulates the expression of Cdks which drive proliferation [164], sequester the pro-apoptotic molecules Bad and Bax [203, 219, 220], and induce the translocation of GLUT-1 to the cell membrane [133, 169]. In humans, a tyrosine at position 449 at the end of the CD127 cytosolic tail is essential to both STAT5 [142, 221] and PI-3K [221, 222] phosphorylation by Jak1. Y449, once phosphorylated, is therefore likely to provide a docking site for STAT5 binding via STAT5's SH2 domain. It is not surprising therefore that point mutation of Y449 prevents IL-7 driven proliferation [223], without lowering surface CD127 expression levels [148]. While IL-7Ra$^{-/-}$ mice transgenically altered to express CD127 develop normally, IL-7Ra$^{-/-}$ mice transgenically altered to express CD127 lacking its tyrosine containing T domain retain an under developed thymus [224].

CD127 interacts with several other signal transducing kinases of the *src* family: p56^{lck} and p59^{fyn} [225, 226], possibly mediating cross-talk between IL-7 and TCR signalling

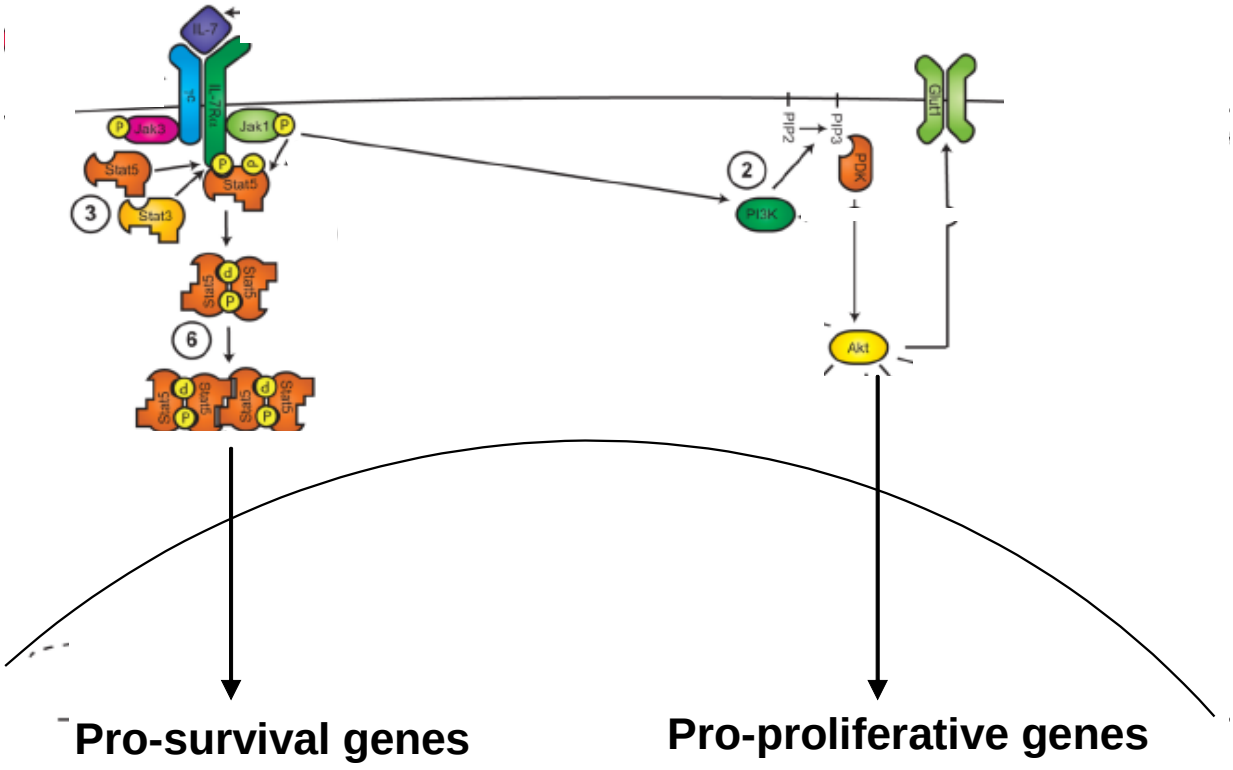
[173, 227]. CD127 is known to recruit other kinases in response to IL-7. Protein tyrosine kinase 2 (Pyk2) [204], Src homology 2 domain containing (Shc) [228] and insulin receptor substrate (IRS) [228] proteins have all been shown to compete for Y449 binding. However the effects of these associations and their relevance in humans *in vivo* are not yet clear. See figure 9 for a review of canonical IL-7 signal transduction pathways.

1.4.2 Mechanisms of CD127 Downregulation in Response to IL-7

Without stimulation, CD127 constitutively recycles on and off the cell membrane through the early endosomal system with a half-life of around 55 hours [229]. The majority of CD127 returns to the surface while a small percentage of CD127 is shunted to the lysosome for degradation. Degraded protein is replaced by *de novo* synthesis and surface CD127 levels remain relatively constant.

CD127 expression is tightly regulated in both B and T cells. Glucocorticoids and synthetic dexamethasone upregulate the expression of CD127 mRNA and protein [230-232]. The glucocorticoid response element (GRE) has been identified in mice and humans upstream of the transcriptional start site [233]. TCR signalling in the periphery also downregulates CD127 expression at the level of transcription [162, 174, 230], providing an additional layer of intricacy to IL-7:TCR crosstalk. CD127 transcripts are also suppressed by other members of the γ-chain family such as IL-2, IL-4, and IL-15, as well as the pro-inflammatory IL-6 [234]. Late phase nocturnal sleep also increases CD127 levels [235]. Perhaps the most important regulator of CD127 expression is its cognate ligand IL-7. We and others have shown that IL-7 signalling downregulates surface CD127 protein, directing it to the proteasome for degradation [193, 194, 196, 229, 236-238]. CD127 is downregulated in

Figure 9: Interleukin-7 signalling pathways. IL-7 binding to CD127 results in association of CD127 with CD132. This allows for CD127-associated Jak1 and CD132-associated Jak3 to come into close proximity resulting in phosphorylation of the cytosolic tails of the IL-7 receptor complex as well as Jak1 and Jak3. This in turn recruits STAT5 molecules to the cytosolic tail of the receptor and induces their phosphorylation. STAT5 phosphorylation results in its dimerization and nuclear translocation where it acts as a transcriptional activator for numerous pro-survival genes including *bcl-2*. Jak1 activation also induces the phosphorylation of PI-3K, resulting in the activation of pro-proliferative genes, activation of the cell cycle and the upregulation of the glucose transporter GLUT-1 on the cell surface. Figure reprinted with permission from the Chinese Society of Immunology. Palmer, M. J. et al. Interleukin-7 receptor signalling network: an integrated systems perspective. Cell Mol Immunol 5, 79-89 (2008).


IL-7
γc
IL-7Rα
Jak3
Jak1
Stat5
Stat5
Stat3
3
6
2
PI3K
PIP2
PIP3
PDK
Akt
Glut1
Pro-survival genes
Pro-proliferative genes

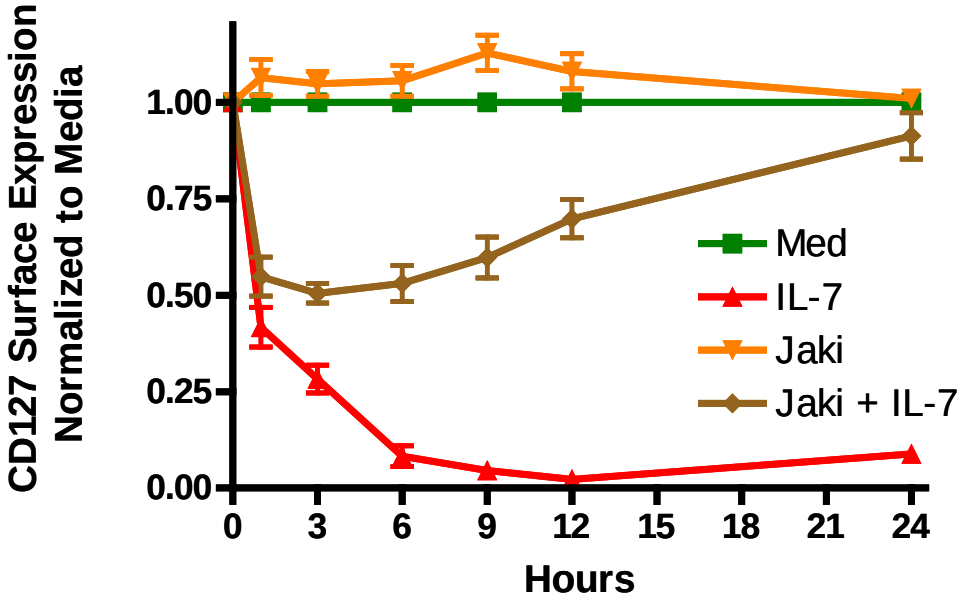
response to IL-7 binding by two distinct mechanisms. At lower concentrations of IL-7, CD127 surface protein is downregulated from the surface of T cells in a transient manner. At higher IL-7 concentrations, CD127 downregulation is sustained for prolonged periods with CD127 gene transcription being also shut off to inhibit the production of *de novo* protein.

1.4.2.1 Downregulation of Surface CD127 Protein

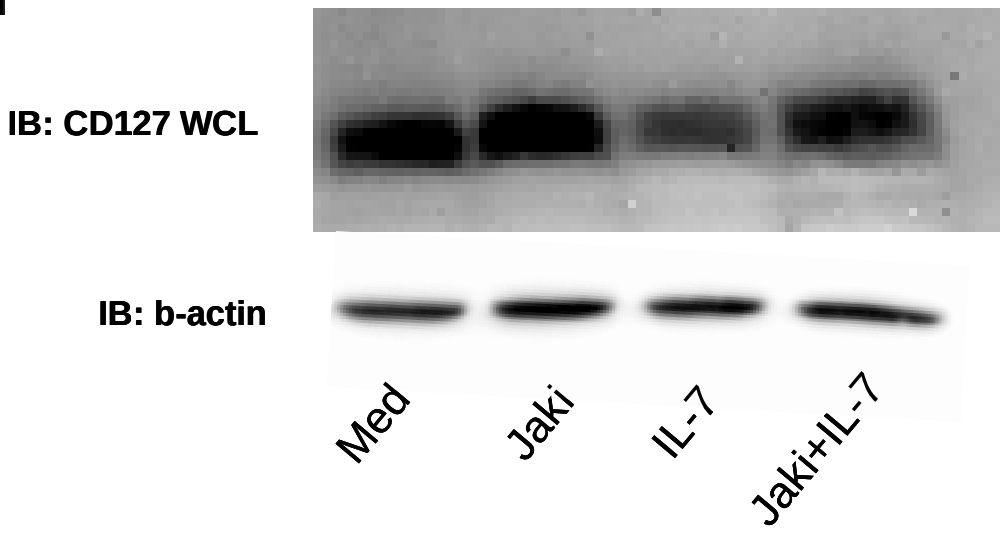
The cellular mechanisms responsible for CD127 downregulation from the surface of cells in response to IL-7 have yet to be completely elucidated, although several of the critical components have been identified. I have shown previously that when human CD8 T cells are pre-treated with Jak inhibitor (Jaki) followed by IL-7, CD127 is removed from the surface of the cell over the course of six hours and then slowly recovers to its original expression level by 24 hours, without any change in whole cell CD127 protein levels (see figure 10) [239]. This suggests that IL-7 can induce the internalization of CD127 transiently through a Jak independent mechanism without promoting CD127 degradation. A second mechanism is Jak-dependent and results in the targeting of CD127 for proteasomal degradation. Our lab has gone further to show that IL-7-induced targeting of CD127 for proteasomal degradation depends on the recruitment of suppressor of cytokine signalling (SOCS) 2 and cytokine-inducible SH2 containing (CIS) protein in primary human T cells. We have shown that IL-7 signalling upregulates the production of CIS and to a lesser extent SOCS2 through increased transcription. These proteins bind to CD127 to attenuate IL-7 signalling and target the receptor for proteasomal degradation through a ubiquitin-mediated process (unpublished observation, Feras Ghazawi). This observation is inline with previous

Figure 10: Jak is required for sustained repression and degradation of CD127 in response to IL-7. **A)** Primary human CD8 T cells were treated with IL-7 with our without Jaki, or left untreated. Surface CD127 expression was measured on the cell's surface at various time points over the following 24 hours. **B)** Cells were treated as shown in part A. At six hours post-treatment, cells were lysed and analyzed via Western blot for whole cell CD127 protein levels. Figure published by Nature Publishing Group: Immunology and Cell Biology (2013) 91, 149–158.

A


CD127 Surface Expression
Normalized to Media
1.00
0.75
0.50
0.25
0.00
0
3
6
9
12
15
18
21
24
Hours
Med
IL-7
Jaki
Jaki + IL-7

B


IB: CD127 WCL
IB: b-actin
Med
Jaki
IL-7
Jaki+IL-7

work demonstrating that SOCS proteins directly inhibit Jak1-mediated signalling [240], as well as recruit cellular E3 ligases to the cytosolic tails of numerous receptors inducing their ubiquitination and subsequent proteasomal degradation [241-244].

1.4.2.2 CD127 Transcriptional Regulation

The core promoter region of CD127 is within the first 197 base pairs upstream of the ATG translation start site. This region has 75% homology between humans and mice [187], and contains conserved binding sites for PU.1 and RUNX1. Mouse PU.1 is required for CD127 expression in pro-B cells [245, 246]. T cells do not express PU.1 however. In mice, GA-binding protein (GABP) transcription factor seems to bind the PU.1 site to promote CD127 expression in thymocytes and peripheral T cells [247]. In contrast, human GABP has not yet been shown to act on the human *cd127* promoter. The transcriptional repressor forkhead box (Fox)P3 protein has now been identified as a major effector molecule responsible for CD127 transcriptional repression [248]**.** Interestingly, FoxO1, a member of the same transcription factor family, upregulates CD127 expression [249, 250] by competing for the same binding site [248].

Along with downregulating the receptor from the cell surface, higher doses of IL-7 can shut off CD127 transcription. In mouse CD8 T cells, but not CD4 cells, transcriptional attenuation is mediated through upregulation of the transcriptional repressor growth factor independence (Gfi)-1 [251, 252]. Gfi-1 achieves repression by antagonizing GABP binding to the *cd127* promoter through the recruitment of histone deacetylase (HDAC) 1, which induces epigenetic alterations to chromosome structure [252]. Like GABP, Gfi-1 has not been shown to interact with the *cd127* promoter in humans. Recent work however, has

associated high Gfi-1 expression with low CD127 surface expression and signalling in human CD8 T cells isolated from HIV-1^+ individuals [253].

Our lab has recently shown that the transcription factor cellular myeloblastosis (c-Myb) may be responsible for the repression of the *cd127* gene transcription in response to IL-7 in primary human CD8 T cells (unpublished observation, Feras Ghazawi). Whether this is a cell type specific or universal mechanism has yet to be elucidated.

1.4.3 IL-7 Signalling in Disease

Given the essential role of IL-7 signalling in proper T cell function, it is not surprising that disruption of IL-7 homeostasis occurs in multiple human diseases, and is often prognostic of disease severity. IL-7 has been implicated in the pathogenesis of rheumatoid arthritis [254-256], juvenile idiopathic arthritis [255, 257], colitis [258], heart disease [259, 260], hepatitis C virus (HCV) [261], and sepsis [262]. IL-7 deficient mice do not develop graft versus host disease (GVHD) and anti-IL-7 antibody treatment dramatically improves GVHD [263, 264]. In humans, IL-7 levels predict the severity of GVHD [265]. Several SNPs in the CD127 gene have been associated with multiple sclerosis [266]. One SNP in particular is found in the transmembrane coding region and is believed to either disrupt an exonic splicing silencer resulting in decreased surface CD127 expression and an increase in the soluble form of the receptor [266-270], or alternatively this SNP may stabilize CD127/CD132 interactions resulting in sustained signal transduction [271].

1.4.4 IL-7 Receptor Dysregulation in HIV Infection

We and others have shown that CD127 expression is lowered on T cells during HIV-1 infection [193, 194, 196, 236-238]. *In vitro* assays have established that $CD127^{low}$ $CD8^{+}$ cells isolated from HIV infected individuals are unable to undergo clonal expansion and activation to mature effectors in response to HIV antigens and IL-7 [272, 273]. The cause of lowered CD127 expression in HIV infection is a matter of some debate. Certain groups believe that lowered CD127 is a direct consequence of increased IL-7 levels [197, 274]. The support for this comes from mouse studies showing injections of supraphysiological dosages of IL-7 will cause a downregulation of CD127 on peripheral T cells [275]. Raised IL-7 levels are unlikely to be the sole cause of CD127 downregulation however, as numerous studies have failed to observe a direct correlation between IL-7 levels and CD127 expression *in vivo* [194-196, 200, 276].

Chronic T cell activation may contribute to CD127 downregulation, and indeed, increased surface expression of the exhaustion marker PD-1 is inversely correlated with surface CD127 expression. However, activation induced CD127 downregulation would be expected to affect only HIV-specific effector and memory cells, whereas CD127 downregulation has been observed on bulk naïve cells [277].

Given the importance of CD127 expression for proper function of CD8 T cells, downregulation of this receptor may be responsible for the impaired function of this cell type in HIV infection. Indeed, lowered CD127 expression is linked to decreased anti-HIV CTL activity [278], and CD8 T cells isolated from HIV^{+} persons display less capacity to proliferate and produce perforin, both of which are promoted by IL-7 signalling. It is therefore possible that treatments designed to maintain the integrity of the IL-7 signalling

axis may have beneficial effects on CD8 T cell function during the course of HIV-1 infection.

1.5 HIV-1 Transactivator of Transcription (Tat)

1.5.1 Tat Transactivating Function

HIV-1 encodes for Tat protein which is required for HIV-1 gene transcription [279]. This molecule is produced early in HIV's replicative cycle and promotes the transcription of viral RNAs. Tat binds to a secondary hairpin structure formed at the 5' end of the nascent HIV RNA termed the trans-activation response (TAR) element. Tat-TAR interaction results in the recruitment of the positive transcription elongation factor b (pTEFb) complex, composed of cyclin T1 and Cdk 9. Cdk9 in turn hyper-phosphorylates the C-terminal domain of RNA-polymerase II resulting in greatly enhanced prossessivity [280]. Without Tat, HIV-1 transcription is initiated by host cell RNA polymerase II holoenzyme, but is unable to proceed with transcriptional elongation past the first 60 nucleotides [220]. Tat's trans-activator functions are absolutely essential to HIV replication; no productive infection is observed with Tat-deficient strains of HIV.

Although recruitment of the pTEFb complex is essential, Tat promotes transcription from the HIV-1 LTR in several other manners. Tat mediates the activation of Sp1 by promoting its phosphorylation, and several binding sites for Sp1 are found in the HIV-1 LTR [281, 282]. Tat also interacts with host cell acetylation machinery and is acetylated itself at Lys50 by p300/CBP and at Lys28 by PCAF [283]. Acetylation of Tat causes it to dissociate from the pTEFb complex. This allows pTEFb to be transferred to the elongating polymerase complex where it can continue to promote phosphorylation of the carboxyl-terminal domain

(CTD), as well as frees Tat to initiate a new round of transcription [284, 285]. Tat is deacetylated by SIRT1, leading to increased transactivation [286].

1.5.2 Tat and Immune Dysfunction

Tat is secreted from infected CD4 T cells by a Golgi-independent, non-traditional mechanism [287, 288] which relies on Trp11 of Tat interacting with $PI(4,5)P_2$ phospholipids at the cell membrane [289]. Free Tat protein can be found in tissue [281, 290] and in the blood plasma of HIV-infected persons at concentrations between 4 nM and 550 nM [291-293]. *In vitro* culture supernatants from HIV-1 infected monocytes have also been shown to contain 10 pM to 100 pM of Tat protein [294, 295]. A recent report showed that two-thirds of the Tat protein produced in an infected cell may be secreted [289]. Circulating Tat mediates a series of immunodysregulatory and pathogenic effects through interactions with $CD4^+$ and $CD4^-$ bystander cells.

Circulating Tat can affect bystander cells extracellularly through specific interactions with surface receptors. Interactions with the surface receptors CD26 [296], CXCR4 [292], heparan sulfate proteoglycans (HSPG) [297], the VEGF receptor KDR/flk-1 [298] and low-density lipoprotein receptor-related protein (LRP) [208] have all been documented, amongst others [295, 298-301].

Free Tat is also readily taken up by many different cell types via endocytosis including DCs, macrophages, CD8 T cells, B-cells and monocytes [287, 302-304]. Once in the cytosol, Tat can affect post-translational modification of proteins [76, 305], induce signalling cascades, induce protein degradation, and alter surface receptor expression levels. Cellular Tat readily transduces into the nucleus where it affects the transcriptional regulation

of host genes [217, 306-315] and alters mRNA splicing [316]. Tat disrupts nitric oxide production in macrophages by inhibiting nitric oxide synthase gene transcription [317]. This theoretically leads to dysfunction of the macrophage respiratory burst mechanism, rendering the host further susceptible to opportunistic infections [317].

Importantly, many of the molecular effects of Tat result in alterations to the cytokine and chemokine environments. Tat is able to alter the expression of many important immunological receptors such as MHCI [318], β2-microglobulin [311] and CD25 [319], and upregulate the expression of several cytokines such as IL-2, IL-6 and IL-10, TNF-β and TGF-β [217, 302, 307, 308, 313-315]. In particular IL-10 and TGF-β upregulation is associated with Tat-mediated T cell inhibition [320, 321]. Tat treatment increases the expression of the HIV-1 co-receptors CCR5 and CXCR4 on the surface of PBMCs [322], which correlates with increased viral infectivity [306].

Tat may also induce the migration of monocytes and macrophages towards infected cells by acting as a chemotactic gradient [323]. Tat contains the CC, CXC and CXXC aa motifs found in most chemokines. Evidence suggests that Tat acting as a chemokine promotes the chemotaxis of macrophages and monocytes toward infected cells and facilitates their activation, leading to greater permissibility to infection [323-325].

Interestingly Tat has been shown to cooperate synergistically with non-HIV proteins to promote the replication of other viruses by increasing viral gene transcription [326-328], particularly Kaposi's Sarcoma Herpes Virus (KSHV) the etiological agent of KS [329].

1.5.3 Tat Protein Structure

NMR studies suggest Tat is an intrinsically unstructured molecule, adapting different conformations to bind different targets [330, 331]. Tat completely lacks secondary structural elements [217, 276, 295, 332] and it is likely that this unstructured state is responsible for the lack of X-ray crystallography data available for free Tat. This lack of intrinsic structure may also explain how a single 14 KDa protein is able to bind so many different cellular factors. Indeed, it is commonly believed that Tat's conformation is altered specifically upon binding to its targets [304].

1.5.3.1 Regions of Tat

Tat is comprised of six well described regions. The amino terminal region (aa 1-21) is proline rich. This region is required for the release of Tat from infected cells [289] as well as transduction of Tat across the membrane of acidifying endosomes into the cytosol [333]. The amino-terminal region contains a portion of the pH sensor required for endosomal escape, comprised of Met1, Glu2, Trp11, Arg55, Arg 56, and Arg 57 [333]. The second region (aa 22-37) is cysteine rich; it contains 7 conserved cysteine residues at aa positions 22, 25, 27, 30, 31, 34, and 37. These cysteines have been shown to participate in zinc ion binding while associated with the p-TEFb complex [334]. Deletion of any of these cysteines with the exception of Cys 31 results in a loss of Tat-mediated HIV-1 transcription. It is of note that this region contains the CC, CXC, and CXXC motifs discussed above. The third region (aa 38-48) is known as the core region. It contains the conserved ^{41}KGLGISYG^{48} sequence. ^{45}ISYG^{48} is part of the minimal region required for transcriptional transactivation [335]. The basic region (aa 49-59) is composed of a string of highly basic residues (see

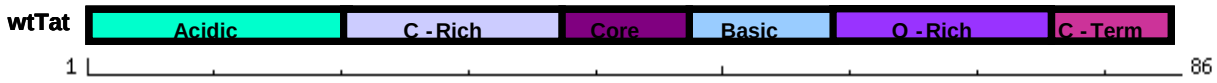
figure 11). This region is required for the interaction of extracellular Tat with HSPG at the surface of the cell prior to endocytosis, the translocation of Tat to the nucleus from the cytosol [336], and the binding of Tat to the TAR element [337]. Many post-translational modifications have been mapped to this region including acetylation [285], ubiquitination [338] and methylation [339-341]. The fifth region (aa 60 to 72) is rich in glutamine residues. This region is involved in Tat's ability to stabilize microtubules by binding to tubulin [342]. The three phosphorylation sites of Tat are located in this region (S62, T64 and S68) [343]. Phosphorylation of Tat has been shown to increase its ability to induce LTR transactivation. The sixth region is referred to here as the C-terminal region. It is either 13 or 28 amino acids in length depending on the Tat isolate (aa73-86 or 101). This region contains the conserved RGD domain found on extracellular matrix molecules, allowing Tat to adhere to cells expressing the integrins $\alpha_5\beta_1$ and $\alpha_5\beta_3$ as well as other surface integrins [287, 290, 344, 345]. A conserved ESKKVE sequence is involved in HIV-1 mediated NF-κB activation [346]. This region is also involved in disruption of cytoskeleton structure [347], T cell activation in combination with CD28 signalling [348], viral infection of and replication in MDDCs [349], and promotes the transduction of Tat into brain cells inducing their apoptosis [350, 351].

1.6 Tat and CD127

Purified Tat protein is able to downregulate the expression of CD127 from the surface of human CD8 T cells isolated from healthy individuals [352]. This observation provides explaination for our earlier observation that CD8 T cells isolated from the blood of HIV^+ persons have lower CD127 expression than age-matched HIV^- controls [193]. Tat's

Figure 11: HIV-1 protein is composed of six regions. **A)** Linear cartoon representation of wild type Tat protein. Individual regions are shown in different colours for emphasis. **B)** One letter code of Tat6xHis amino acid sequence. Individual regions are coloured as above. Polyhistidine tract is shown in blue.

A



B

M E P V D P R L E P W K H P G S Q P K T A C T N C Y C K K C C F H
C Q V C F I T K A L G I S Y G R K K R R Q R R R P P Q G S Q T H Q V
S L S K Q P T S Q S R G D P T G P K E R S H H H H H H

ability to downregulate CD127 is time and dose dependent, specific for CD127, can be blocked using anti-Tat antibodies, and can be reversed by removing Tat from the culture media [352]. Tat-induced CD127 downregulation is not the result of cell activation and does not induce CD8 T cell death.

As this receptor is absolutely required for proper CD8 T cell function, it is not surprising that Tat-mediated CD127 downregulation causes functional impairment of CD8 T cells. CD8 T cells pre-treated with IL-7 proliferate more robustly and produce more perforin upon TCR stimulation than cells that are TCR stimulated alone [352]. Pre-treating cells with Tat to lower surface CD127 prior to IL-7 treatment and TCR stimulation results in cells that are no longer able to proliferate or increase perforin production in response to IL-7 [352]. This mimics the CD8 T cell phenotype observed during HIV infection *in vivo*, in which CD8 T cells proliferate less and produce less perforin [127].

1.6.1 Mechanism of Tat-Induced Downregulation of CD127

We have begun to elucidate the mechanisms involved in Tat-induced CD127 downregulation. Briefly, Tat interacts with HSPG via its positively charged basic region on the cell surface and is internalized by host endosomal machinery [353, 354]. Tat has been shown to exploit caveolae preferentially over clathrin coated pits (CCPs) in HeLa cells [355, 356], and use CCPs exclusively in T cells which do not express caveolin [288]. Tat escapes from the endosomes using a pH-sensitive triggered spring mechanism that is dependent on Trp11 and interactions between Tat's basic region and the lipid membrane [333], and is facilitated by heat shock protein (Hsp) 90 [288]. Once in the cytosol, Tat binds CD127 directly to induce receptor aggregation. Tat then accelerates CD127 internalization using a

mechanism dependent on host microtubules, targeting the receptor for proteasomal degradation [229]. Degradation of CD127 is independent of *de novo* protein synthesis. Furthermore Tat does not affect the rate of CD127 transcription or the transport of CD127 from the Golgi apparatus to the cell membrane [229].

1.7 General Hypothesis

In light of the essential role of IL-7 for proper CD8 T cell function, and the observation that Tat induces CD127 downregulation leading to CD8 T cell dysfunction *in vitro* similar to that seen in people living with HIV, we hypothesize that CD127 downregulation may play a significant role in the development of CD8 T cell anergy observed in HIV-1 infection, and that Tat-mediated downregulation of CD127 from the surface of CD8 T cells is an important contributor to HIV-1 pathogenesis.

1.8 Specific Hypothesis

I hypothesize that soluble Tat binds the cytosolic tail of CD127 and acts as a bridging molecule between the receptor and host endocytic/degradation machinery.

1.9 Summary

A more thorough understanding of the mechanism by which Tat accelerates CD127 internalization at the plasma membrane will potentially lead to new targets for therapeutic treatment. I undertook a systematic investigation into the molecular details of CD127/Tat interactions. I show here that the core, glutamine-rich and carboxyl-terminal regions of Tat are dispensable for CD127 downregulation, whereas the N-terminal and basic regions are

required for CD127 downregulation. Furthermore, I show that the N-terminus of Tat is required to interact physically with CD127 whereas the basic region is not. Tat recruits CIS to the cytosolic tail of CD127 and induces the ubiquitination of the receptor. CIS is known to bind cellular E3 ligase complexes and we have shown previously that Tat-mediated CD127 degradation requires a functional proteasome. I therefore propose a model whereby cytosolic Tat protein is recruited to the cytosolic tail of CD127 via its N-terminal region. Once bound, Tat recruits CIS to the receptor's tail. CIS in turn recruits yet unidentified components of the cellular ubiquitin ligase machinery to the receptor which ubiquitinates the cytoplasmic tail resulting in the accelerated internalization and subsequent proteasomal degradation of CD127.

2. Chapter 2: Production of Wild Type and Mutant Tat Proteins

2.1 Introduction

We have shown previously that Tat protein downregulates CD127 from the surface of primary human CD8 T cells [352]. Exogenous Tat protein will enter human CD8 T cells through host endocytosis and transduce the endosomal membrane to the cytosol where it co-localizes with CD127 and induces its aggregation (i.e. capping). Tat then mediates the accelerated internalization and proteasomal degradation of CD127.

In HIV-1 infection, CD8 T cells display aberrant surface phenotypic markers and do not function optimally. Given the important role of CD127 in proper CD8 T cell function, coupled with our observation that CD127 is downregulated *in vivo* from the surface of CD8 T cells during HIV-1 infection, treatments designed to sustain CD127 surface expression levels on CD8 T cells during HIV-1 infection could result in improved CTL function. Hence, given our previous observations that Tat binds directly to CD127 to downregulate the receptor, a better understanding of the molecular details of Tat/CD127 interactions could lead to the identification of novel drug targets and the subsequent development of novel drugs therapies able to interrupt this interaction, possibly resulting in improved or sustained CTL immune function.

In order to characterize Tat/CD127 physical interactions, a series of Tat deletion mutant proteins were generated each lacking a successive region of the wild type protein. A series of 8 deletion mutant coding sequences were created and inserted into a bacterial plasmid expression system to allow for the production of recombinant Tat protein in *E. coli*. At the same time, the same series of coding sequences were subcloned into a eukaryotic expression vector for transduction of human CD8 T cells. These mutant proteins and

plasmids were used in subsequent chapters to treat primary CD8 T cells in order to characterize which regions of Tat may be essential to CD127 surface downregulation.

First, however, a large scale system capable of producing wild type and mutant Tat proteins needed to be developed. To do this, a protocol to produce recombinant Tat from *E. coli* was optimized. Several methodologies were attempted including heparan sulphate affinity binding, cationic exchange, cobalt metal co-ordinate bonding columns, size-exclusion chromatography, and high pressure liquid chromatography (HPLC). Ultimately an optimal method using nickel-nitriloacetic acid (Ni-NTA) columns was developed.

In this chapter, I describe a method to produce pure, biologically active, recombinant Tat protein. The identity of this protein was confirmed by numerous methods including Western blot and mass spectrometry fingerprinting. This Tat was biologically active with regards to LTR activation and CD127 downregulation. It could be stored stably for many months at -80^{o}C. In the following chapters, I use the recombinant proteins generated here to further elucidate the mechanisms of Tat/CD127 interaction and subsequent Tat-mediated CD127 downregulation.

2.2 Materials & Method

2.2.1 Plasmids

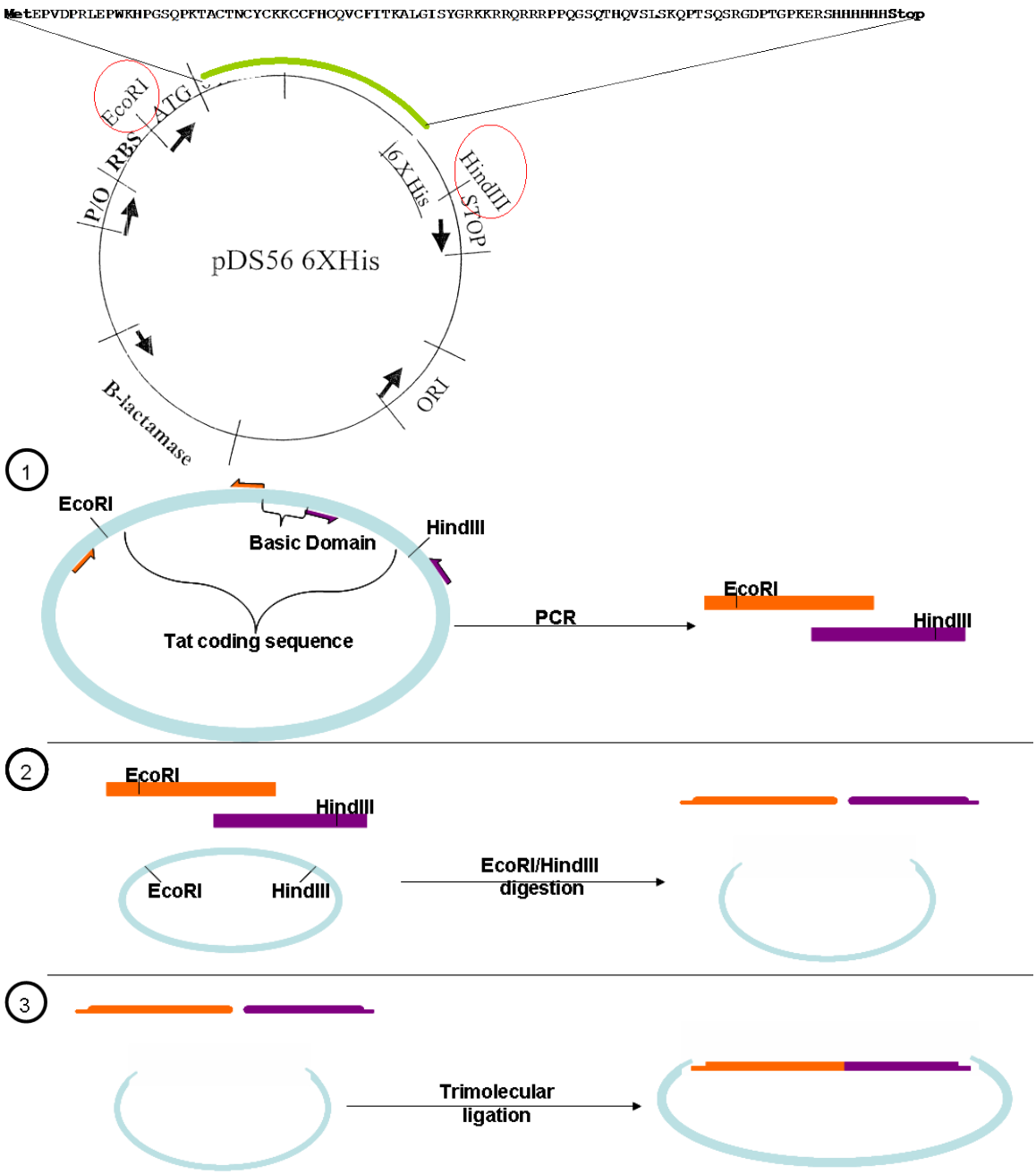
2.2.1.1 pTatC6H-1 Deletion Mutants

The pTatC6H-1 plasmid was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Abhay Patki and Dr. Michael Lederman. This vector has a pDS56 plasmid backbone and encodes for the B clade Tat variant BH10 linked to six histidine residues at its C-terminal end. Tat expression is driven

by a T5 bacteria phage promoter under the control of the lac operon promoter/operator (P/O) system (see figure 12). Standard molecular techniques were used to systematically delete portions of the Tat coding sequence. Two separate rounds of polymerase chain reaction (PCR) were used to amplify the coding sequences both upstream and downstream of the sequence to be deleted. The upstream PCR amplicons included an EcoRI restriction enzyme site upstream of the Tat start codon, and the downstream PCR amplicons included a HindIII restriction enzyme site downstream of the polyhistidine tract. Primers were 5'end phosphorylated to ensure proper downstream ligation reactions. PCR reactions were carried out by adding 25 μL of Taq MasterMix™ (Qiagen) to 1 μg of template pTatC6H-1 plasmid and 5 mM of each primer. Double distilled H_20 (ddH_20) was added to a total volume of 50 μL. PCR was carried out on an eppendorf-AG thermocycler machine using the following steps: an initial 7 minute denaturing step at 95°C; then 30 cycles at 95°C for 30 seconds, 53°C for 30 seconds and 72°C for 40 seconds; then a final elongation step at 72°C for 4 minutes was carried out to finish the reaction. PCR products were then stored at 4°C.

Upstream PCR amplified products were then digested with EcoRI and downstream products were digested with HindIII. To do this, 37.5 μL of each PCR product was add to 5 μL of 5x NEB Buffer 2 (New England BioLabs) 100 μg/mL bovine serum albumin (BSA), and 2 μL of EcoRI (New England BioLabs) or 2 μL of HindIII (New England BioLabs) for upstream or downstream PCR products respectively, in a final volume of 50 μL. Digestion mixtures were placed at 37°C overnight. Digestion was stopped the following day by incubating samples at 85°C for 20 minutes. The pTatC6H-1 vector was opened with these same two restriction enzymes. Ten mg of parent plasmid was incubated with 2 μL of EcoRI

Figure 12: Cloning strategy for pTat6CH-1 mutant plasmids. The pTatC6H-1 plasmid encodes for Tat protein on a pDS56 vector backbone. **1)** PCR was used to amplify the coding sequence regions upstream and downstream of the region to be deleted. PCR amplicons were designed to include the EcoRI and HindIII restriction enzyme cut sites located on the plasmid backbone upstream and downstream of the Tat coding sequence, respectively. **2)** The PCR products were digested with either EcoRI or HindIII as appropriate. The parent plasmid was double digested with both these enzymes. **3)** A tri-molecular ligation was used to insert the digested PCR products into the pDS56 backbone to generate plasmids lacking the deleted region.


MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHHStop
EcoRI
ATG
P/O
RBS
6XHis
HindIII
STOP
pDS56 6XHis
B-lactamase
ORI
1
EcoRI
Basic Domain
HindIII
Tat coding sequence
PCR
EcoRI
HindIII
2
EcoRI
HindIII
EcoRI
HindIII
EcoRI/HindIII
digestion
3
Trimolecular
ligation

and 2 μL of HindIII in 5x NEB Buffer 4 in a total reaction of 100 μL overnight at 37°C. The pDS56 plasmid backbone was separated from the wild type Tat coding sequence by agarose gel electrophoresis and pDS56 plasmid backbone was extricated from gel using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Linearized vector was treated with 2 μL of antarctic phosphatase (AP) (New England BioLabs) with six μL of 10x AP buffer (New England BioLabs) in a final reaction volume of 60 μL overnight at 37°C to remove terminal phosphates from the ends of the vector to prevent intramolecular ligation. Post-treatment, phosphatase activity was destroyed by placing the reaction at 85°C for 20 minutes.

PCR-amplified segments were then inserted into the vector via trimolecular ligation reactions. Each ligation was carried out in a 20 μL final volume containing 2 μL of T4 ligase (New England BioLabs), 2 μL of 10x ligation buffer (New England BioLabs), 100 ng of linearized, phosphatase treated vector backbone, and both coding sequence insert fragments (upstream and downstream of deleted region) at a 1:5 molecular ratio (vector:insert). Ligation reactions were carried out for 20 hours at 15°C. See figure 12 for clarification.

2.2.1.2 HIV-2N10 Tat

Amino acids 1-10 of the HIV-1 Tat (Tat) coding sequence were exchanged with the first ten amino acids of HIV-2 Tat ROD strain (Tat-2) to generate the HIV-2N10 Tat coding sequence. PCR was used as described above to amplify the region beginning at aa 11 of the Tat coding sequence and ending downstream of the HindIII cut site on pTatC6H-1. A short DNA sequence encoding for Tat-2 aa 1-10 upstream of an EcoRI cut site was purchased

from Invitrogen. A tri-molecular ligation reaction was used to merge the Tat-2 amino terminus coding sequence to the Tat-1 sequence between the EcoRI/HindIII cut sites of pTatC6H-1 as described above (see figure 13 for clarification).

2.2.1.3 Site-Directed Mutagenesis

The cysteine residues of Tat were systematically mutated using the QuickChangeII site-directed mutagenesis (SDM) kit (Stratagene). Primers were designed to alter 2 or 3 cysteines to serines in a single reaction by introducing a single thymidine to adenosine mutation at position one of the cysteine codon (TGT to AGT or TGC to AGC). Primers are shown in Table 1. The QuickchangeII SDM kit (Stratagene) was used as per the manufacturer's instructions. Briefly, 25 ng of parental pTatC6H-1 was combined with 125 ng of each primer, 10 μL of the 5x reaction buffer (proprietary), 1 μL of dNTP mix (proprietary) and 2.5 U of *Pfu Turbo* DNA polymerase in a final reaction volume of 50 μL. PCR was carried out on an eppendorf-AG thermocycler for 18 cycles using the following steps: denature at 95^{o}C for 30 seconds, anneal at 55^{o}C for 1 minute, elongate at 72^{o}C for 3 minutes and 42 seconds. PCR products were treated with 1 μL of DpnI restriction enzyme overnight at 37^{o}C to digest the original template plasmids.

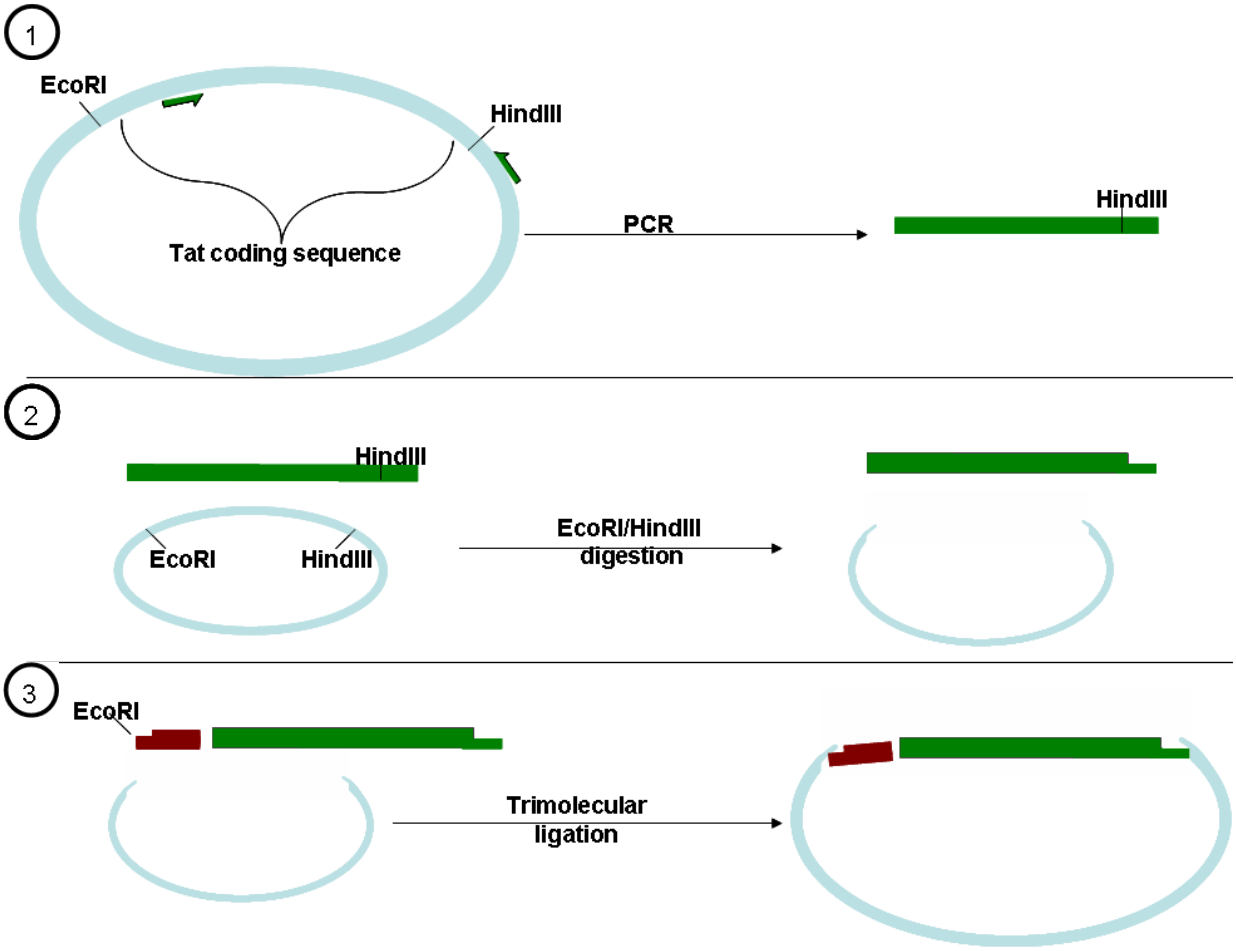
2.2.1.4 His-tagged HIV-2 Tat (Tat-2)

A pGEX2T plasmid encoding Tat-2 from the ROD strain was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Andrew Rice. PCR was used to amplify the Tat-2 coding sequence. Primers were designed

Primers	Forward	Reverse
Δ17-21 N-term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-GGAACCCGGGTGTTTCCAC-3’
Δ17-21 C-term	5’-Pi-TGCACCAACTGTTACTGTAAAAAGTG-3’	5’-ATCAGGCGGGCAAGAATGG -3’
Δ22-26 N-Term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-TGCAGTTTTCGGCTGGGAAC-3’
Δ22-26 C-Term	5’-Pi-TGTAAAAGTGTTGCTTCCACTGTC-3’	5’-ATCAGGCGGGCAAGAATGG-3’
ΔCore N-Term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-ACAAACTTGACAGTGGAAGCAACA-3’
ΔCore C-Term	5’-Pi-CGTAAGAAACGTAGACAGCGC-3’	5’-ATCAGGCGGGCAAGAATGG-3’
ΔBasic N-term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-ACCGTAGGAGATACCCAAAGC-3’
ΔBasic C-Term	5’-Pi-CCACCGCAAGGTTCTCAGA-3’	5’-ATCAGGCGGGCAAGAATGG-3’
ΔGln N-Term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-ACGTCTGCGCTGTCTACG-3’
ΔGln C-Term	5’Pi-CCGACCTCCCAATCTCGC-3’	5’-ATCAGGCGGGCAAGAATGG-3’
ΔC-term N-Term	5’-CACTCGTGCACCCAACTGAT-3’	5’-Pi-AGCTTAGTGATGGTGATGAGATCT-3’
ΔC-term C-Term	5’-Pi-AGATCTCATCACCATCACCATCACTA-3’	5’-ATCAGGCGGGCAAGAATGG-3’
SDM-1	5’-AGCCGAAAACTGCATCCACCAA CTCTTACTCTAAAAAGTGTTGCTTC-3’	5’-GAAGCAACACTTTTTAGAGTAAG AGTTGGTGGATGCAGTTTTCGGCTG-3’
SDM-2	5’-CACCAACTGTTACTGTAAAAAGTCTTC CTTCCACTGTCAAGTTTGTTTC-3’	5’-GAAACAAACTTGACAGTGGAAGGAA GAACTTTTTACAGTAACAGTTGGTG-3’
SDM-3	5’AAAAAGTGTTGCTTCCACTCTCAAGTTTCTT TCATCACCAAGGTTTG-3’	5’-CAAAGCCTTGGTGATGAAAGAAACT TGAGAGTGGAAGCAACACTTTT-3’
HIV-2N10 C-term	5’-Pi-TGGAAACACCCGGGTTCCC-3’	5’–ATCAGGCGGGCAAGAATG G–3’
pGEX2T HIV-2 Tat subcloning	5’-CCGAATTCATGGAGACACCCTTGAAGGC-3’	5’-CCAGATCTCTATCGGCCAGGGCCAG-3’
HIV-2N10 synthetic N-term	5-GCTCTCTGGCGCCTTCAAGGGTGTCTCCATAGTTAATTTCTCCTCTTTAATTG-3’ 5’-AATTCATTAAAGAGGAGAAATTAACTATGGAGACACCCTTGAAGGCGCCAGAGAGC-3’	
pcDNA3.1 subcloning	5’-CACTCGTGCACCCAACTGAT-3’	5’-ATCAGGCGGGCAAGAATGG -3’

Table 1: List of Primers

Figure 13: HIV-2N10 cloning strategy. **1)** PCR was used to amplify a region of the pTatC6H-1 plasmid from aa 11 to past the HindIII cut site. **2)** This PCR product was digested with HindIII. The parental plasmid was double digested with EcoRI and HindIII, as done previously. **3)** A short DNA sequence encoding for aa 1-10 of HIV-2 Tat attached to an upstream EcoRI cut site was purchased. A tri-molecular ligation strategy was used to insert the coding sequence for aa 1-10 of Tat-2 upstream of the coding sequence for aa 11-86 of the Tat-1.


1
EcoRI
HindIII
Tat coding sequence
PCR
HindIII
2
HindIII
EcoRI
HindIII
EcoRI/HindIII
digestion
3
EcoRI
Trimolecular
ligation

to add EcoRI and BglII restriction sites to the 5' and 3' ends respectively. Primers were phosphorylated at their 5'end to ensure proper downstream ligation reactions. PCR reactions were carried out by adding 25 μL of Taq MasterMix™ (Qiagen) to 1 μg of template pTatC6H-1 plasmid and 5 mM of each primer in a total volume of 50 μL. PCR was carried out on an eppendorf-AG thermocycler as follows. An initial 7 minute denaturing step was carried out at 95°C. Then, DNA amplification was carried out with five cycles at 95°C for 30 seconds, 54°C for 30 seconds and 72°C for 40 seconds, followed by 30 cycles at 95°C for 30 seconds, 63.5°C for 30 seconds and 72°C for 40 seconds. A final elongation step at 72°C for 4 minutes was carried out to finish the reaction. PCR products were stored at 4°C. Tat-2 PCR product was exchanged for Tat -1 in the pTatC6H-1 backbone. The pTatC6H-1 backbone was digested using EcoRI and BglII enzymes.

2.2.1.5 pcDNA3.1 Tat

The wild type and mutant Tat coding sequences generated above were removed from the pTatC6H-1 plasmid using EcoRI/HindIII restriction enzymes and transferred into the pcDNA3.1$^{(-)}$ multiple cloning site. Ten mg of parent pTatC6H-1 plasmid was incubated with 2 μL of EcoRI and 2 μL of HindIII (New England BioLabs) in 5x NEB EcoRI Buffer (New England BioLabs) in a total reaction volume of 100 μL overnight at 37°C. The Tat coding sequences were separated from the plasmid backbones by agarose gel electrophoresis and wild type or mutant Tat coding sequences were extricated from the gels using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey Nagel). Coding sequences were inserted into pcDNA3.1$^{(-)}$ vectors opened with the same two restriction enzymes by digestion with 2 μL of EcoRI and 2 μL of HindIII in 5x NEB EcoRI Buffer in a total reaction of 100 μL

overnight at 37°C. Ligation reactions were carried out overnight as described above (see figure 14). This strategy preserved the C-terminal polyhistidine tag for use in downstream applications.

2.2.1.6 Plasmid Identification and Sequencing

After molecular manipulations were carried out, plasmids were transformed into DH5α *E. coli* using a standard heat shock method: 50 μL of competent DH5α *E. coli* was added to 3 μL of putative construct mix, placed at 42°C for 30 seconds, then ice for 2 minutes, then 250 μL of super optimal broth with catabolic repressor (SOC) media was added and transformed cells were shaken at 37°C for 1 hour. 200 μL of cells were then plated onto a 9 cm agar plate containing 100 μg/mL of ampicillin (amp; Sigma). The following day, colonies were picked and inoculated into 5 mL of Luria broth (LB) with 100 μg/mL amp, and placed at 37°C overnight with shaking. The following day, mutant plasmids were isolated using the plasmid DNA purification kit (Macherey Nagel). All sequences were verified by Sanger sequence analysis at the Stemcore facility at the Ottawa Hospital Research Institute (OHRI). All primers were purchased from Invitrogen using their custom oligo service. See figure 15.

2.2.2 SDS-PAGE and Western Blotting

All SDS-PAGE was carried out on 1.0 mm thick, 14% polyacrylamide gels (37.5:1 Acrylamide:bis-arcylamide) in resolving buffer (375 mM Tris-HCl, 0.1% SDS, 0.0005% TEMED, 0.001 % APS, pH 8.8). Stacking buffer was composed of 4% acrylamide gels

Figure 14: Strategy for subcloning of wild type and mutant Tat coding sequences into the pcDNA3.1$^{(-)}$ vector. The wild type and mutant Tat coding sequences were removed from the parent pTatC6H-1 vectors by digestion using EcoRI and HindIII. Agarose gel electrophoresis was used to separate Tat coding sequences from the parent plasmids. Standard ligation techniques were employed to insert the coding sequences into the multiple cloning site of the pcDNA3.1$^{(-)}$ eukaryotic expression vector.

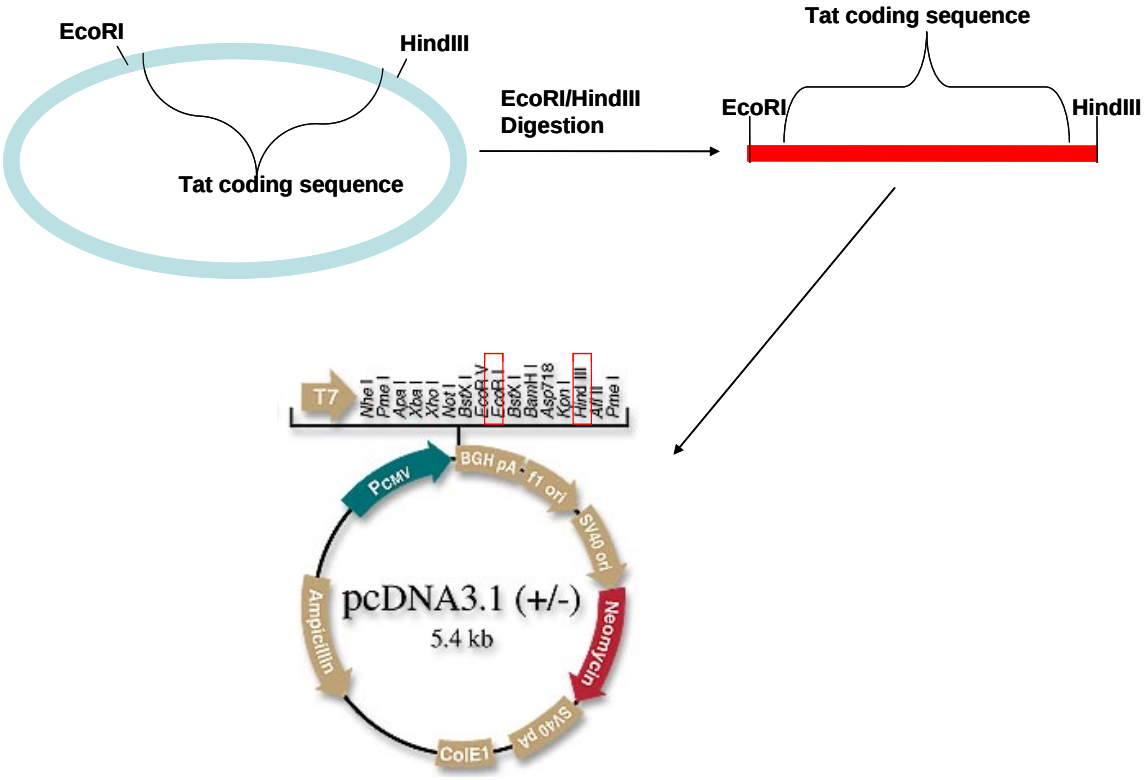
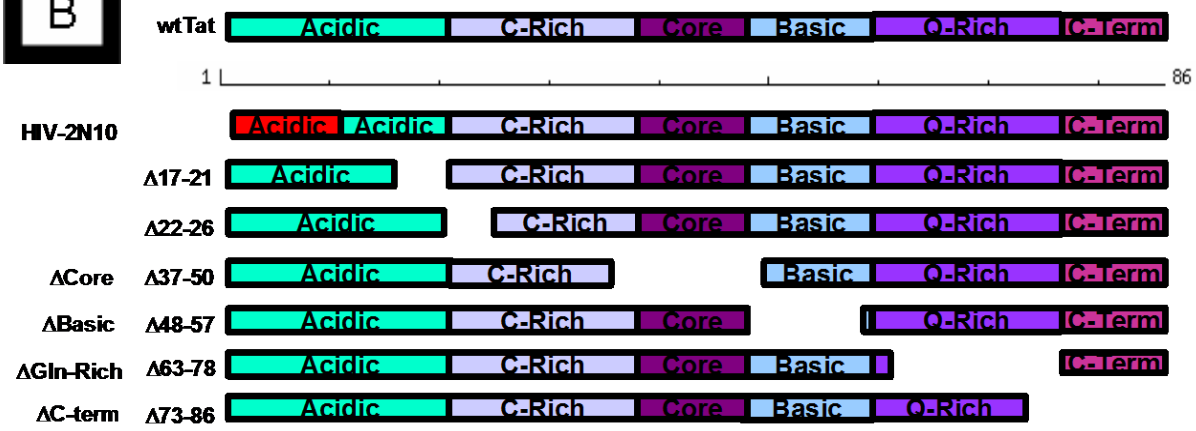

EcoRI
HindIII
Tat coding sequence
EcoRI/HindIII
Digestion
Tat coding sequence
EcoRI
HindIII
T7
Nhe I
Pme I
Apa I
Xba I
Xho I
Not I
BstX I
EcoR V
EcoR I
BstX I
BamH I
Asp718
Kpn I
Hind III
Afl II
Pme I
PCMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
ColE1
Ampicillin
pcDNA3.1 (+/-)
5.4 kb

Figure 15: Tat mutant plasmids. Standard molecular techniques were used to generate a series of Tat6xHis mutant proteins. **A)** One letter code of wild type and mutant Tat amino acid sequences. **B)** Linear cartoon representations of wild type and mutant Tat proteins. Red region denotes coding sequence from HIV-2 Tat. Individual regions are shown in different colours for emphasis.

A

```
MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  Wild Type
MetETPLKAPESWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  HIV-2N10
MetEPVDPRLEPWKHPGS-----CTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  Δ17-21
MetEPVDPRLEPWKHPGSQPKTA-----CKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  Δ22-26
MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQV-------------QKRRQRRRPPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  ΔCore
MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYG---------PPQGSQTHQVSLSKQPTSQSRGDPTGPKERSHHHHHH  ΔBasic
MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPTSQS--------------RGDPTGPKERSHHHHHH  ΔGlu
MetEPVDPRLEPWKHPGSQPKTACTNCYCKKCCFHCQVCFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQS------------RSHHHHHH  ΔC-Term
```

B



(37.5:1 acrylamide:bis-arcylamide) in 125 mM Tris-HCl, pH 6.8. SDS-PAGE was carried out using the Mini-PROTEAN Tetra Cell apparatus (BioRad) at 140 volts.

All gels were transferred onto polyvinylidine fluoride (PVDF) membranes by semi-dry transfer using a Trans-Blot SD Semi-Dry electorphoretic transfer cell (BioRad). PVDF membranes were preincubated in methanol with shaking for 5 minutes. Then PVDF, polyacrylamide gels, and Whatman paper were incubated in transfer buffer (25 mM Tris, and 192 mM glycine. pH 8.3) containing 20% methanol (v/v) for 15 minutes with shaking. Transfer was carried out at 17 volts for 15 minutes per gel. After transfer, all blots were blocked for 1 hour in 5% ECL Advance™ blocking reagent (GE Healthcare) dissolved in tris-buffered saline with Tween-20 (TBST: 137 mM NaCl, 20 mM Tris pH 7.6, and 0.1% Tween-20).

Blots were incubated in 2% ECL Advance™ blocking reagent (GE Healthcare) in TBST supplemented with the appropriate antibody overnight at 4^{o}C with mild agitation. The next day, blots were washed 3 times for ten minutes each in TBST and secondary labelling was achieved using the appropriate secondary antibody at a 1:10 000 dilution in 2% ECL Advance™ blocking reagent dissolved in TBST for one hour at room temperature with mild shaking. After secondary treatment, blots were washed again 3 times for ten minutes in TBST then detected using the Amersham™ ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.2.3 Polyacrylamide Gel Silver Staining

Tat6xHis and Tat2-6xHis proteins were separated on 14% polyacrylamide gels as described above and placed in fixing buffer (40% ethanol, 10% acetic acid, and 50% ddH_2O)

for 30 minutes with shaking at room temperature. Gels were then washed once for 10 minutes in rinse buffer (50% ethanol and 50% ddH_2O), and then twice in ddH_20 for 10 minutes. Gels were sensitized in 0.02% sodium thiosulfate for 1 minute then washed twice for 5 minutes in ddH_20. Gels were then placed in cold staining solution (0.1% silver nitrate) for 30 minutes with shaking before being developed using developer solution (3% sodium carbonate and 0.03% formalin). The developing reaction was quenched with 1% acetic acid.

2.2.4 Recombinant Tat Protein Purification

Recombinant Tat proteins were produced in the XL2 Blue strain of *E. coli* and isolated over Ni-NTA columns. XL2 Blue *E. coli* were transformed with pTatC6H-1 plasmids. Bacteria were grown in 500 mL of Luria Broth (LB) with 100 μg/mL of amp at 37^oC with aggitation to an OD_{600} =0.2-0.4 (approximately 12-14 hours) before Tat production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Bacteria were pelleted and resuspended in 10 mL of sonication buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) supplemented with 2 mg/mL of chicken egg white lysozyme (Sigma) and 10 mM β-mercaptoethanol, and incubated on ice for 1 hour to allow digestion of bacterial cell walls. Bacterial lysates were then sonicated using a vibe-cell machine (Sonics and Materials Inc) at 30% amplitude. Lysates were centrifuged at 10 000 g for 45 minutes at 4 ^oC. The soluble fraction was then passed through a Ni-NTA Superflow column (Qiagen). Columns were washed with a minimum of ten column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM β-mercaptoethanol, pH 8.0) with increasing amounts of imidazole (0-60 nM). Histidine tagged Tat protein (Tat6xHis) was eluted using 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM

NaCl, 250 mM imidazole and 10 mM β-mercaptoethanol, pH 8.0). The pH of the eluates was adjusted to 7.4 using 1N hydrochloric acid. Proteins were separated into 500 μL aliquoits in 1.5 mL siliconized microfuge tubes (Sigma) and frozen slowly using an isopropanol jacket and stored at -80^{o}C. Mock proteins were isolated exactly as done for true proteins using a pTatC6H-1 vector variant lacking the Tat-1 coding sequence.

The identity of wild type Tat-1 was confirmed by Western blot analysis as described above. PVDF membranes were probed for Tat using monoclonal anti-Tat (Immunodiagnostics; Prod. No. 1102) and monoclonal anti-polyhistidine (Sigma; clone HIS-1) antibodies. Goat anti-mouse-horseradish peroxidase (HRP) (R&Dsystems; cat. no. HAF007) was used as a secondary antibody. Protein bands on Western blots were detected using the Amersham ECL Advance Western Blotting Detection Kit (GE Lifesciences) as per the manufacturer's instructions. The production of mutant Tat proteins was confirmed by Western blot with antibodies for anti-polyhistidine (clone HIS-1; Sigma), as described above. Protein purity was confirmed by silver stain analysis.

Tat-2 was produced and isolated as done for Tat-1 with the exception that Tat-2 was produced in BL21 *E. coli*. The identity of Tat-2 was confirmed by Western blot using rabbit anti Tat-2 sera obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Bryan Cullen. Secondary labelling was achieved using mouse anti-rabbit-HRP antibodies, as described above.

All recombinant Tat proteins were quantified via densitometry of silver stained polyacrylamide gels using commercially purchased Tat as a standard. Tat protein was concentrated to 300-500 μg/mL using Amicon ultra-4 concentrator columns (Millipore) as required.

2.2.5 Mass Spectrometry Fingerprinting

Recombinant putative Tat6xHis eluates were separated via SDS-PAGE as described above under ultra sterile conditions. Samples were then silver stained and the single 14 KDa band was sent out for mass spectrometry fingerprinting at the laboratory of Daniel Figeys (Ottawa Institute of Systems Biology, University of Ottawa). Peptide fingerprint results were compared against a database of those for viral and bacterial proteins.

2.2.6 Endogenous Tat Detection in Jurkat-CD127 Cells

A Jurkat cell line engineered to ectopically express CD127 and enhanced green florescent protein (eGFP) from a bicistronic mRNA molecule driven from a CMV promoter (Jurkat-CD127) was provided as a generous gift from Dr. Rene van Lier (Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands). The bicistronic expression of both proteins ensured that GFP^+ cells are also $CD127^+$. Jurkat-CD127 cells were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.2 M L-glutamine (RPMI-10) and maintained at a density between $5x10^5$ and $2x10^6$ cells/mL. Jurkat-CD127 cells were kept at 37^oC and 5% CO_2 in a humidified incubator.

Empty $pcDNA3.1^{(-)}$ plasmids, or $pcDNA3.1^{(-)}$ plasmids encoding for wild type Tat or mutant Tat were transfected into Jurkat-CD127 cells using nucleofection technology (Lonza). Per condition, $2.5x10^6$ Jurkat-CD127 cells were resuspended in 100μL of cell line V nucleofection solution containing 1.5 μg of wild type or mutant Tat-expressing $pcDNA3.1^{(-)}$, or empty vector control, and 0.25 μg of DsREDExpress2 plasmid (Clontech). Samples were transfected with the Nucleofector II device using protocol X-005. Following

nucleofection, Jurkat-CD127 cells were placed in serum free RPMI-1640 for 4 hours at a concentration of $2.5x10^6$ cells/mL. Cells were then transferred to RPMI-10 at a concentration of $1x10^6$ cells/mL.

Forty-eight hours post- nucleofection, Jurkat-CD127 cells (approximately $4x10^6$ cells per condition) were lysed in 50 μL of radio immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Nonidet P-40 (NP-40), 1% SDS, and 2.5 mM sodium pyrophosphate) supplemented with cØmplete protease inhibitor (Roche) for 45 minutes on ice. Samples underwent one freeze-thaw cycle at -80°C before being cleared by centrifugation at 10 000 g for 5 minutes. Protein concentrations were standardized using a bicinchoninic acid (BCA) assay kit (Pierce) as per the manufacturer's instructions. Briefly, 5 μL of protein extract was mixed with 200 μL of BCA reagent and placed at 37°C for 2 hours. The OD_{450} was then measured using a SpectraMax 190 (Molecular Devices) 96 well plate reader.

Per condition, 28 μg of protein cell lysates were separated by SDS-PAGE. Tat proteins were detected by Western blot using mouse anti-polyhistidine and goat anti-mouse-HRP, as described above.

2.2.7 Luciferase Assays

The CEM.NKR-CCR5-Luc cell line was acquired from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John Moore and Dr. Catherine Spenlehauer. This cell line contains the firefly luciferase gene under the control of the HIV-2 long terminal repeat (LTR) promoter. CEM.NKR-CCR5-Luc cells were maintained in RPMI-10 supplemented with 800 μg/mL of G418 to maintain integration of

the HIV-2 LTR. Cells were grown to a density of $1x10^6$ cells/mL, and then $4x10^5$ cells were treated with 10 μg/mL of either wild type Tat6xHis, Tat2-6xHis or a volume-matched mock control. After 24 hours, cells were lysed in 100 μL of reporter lysis buffer (Promega) for 45 minutes on ice. Lysates underwent one freeze-thaw cycle at -80°C and debris was removed by centrifugation at 11 000 g for 5 minutes. Lysate protein content was quantified by BCA assay as described above. Approximately 20 μL of protein normalized lysates were added to 100 μL of luciferase assay reagent (Promega) and luminescence was measured using a LumiStar OPTIMA machine (BMG BioTech).

2.2.8 CD8 T Cell Isolation

PBMCs were isolated from consenting healthy volunteers by ficoll paque centrifugation and primary CD8 T cells were purified via automated magnet activated cell sorting (autoMACS; Miltenyi). Thirty mL of venous blood was gently layered over 15 mL of ficoll-paque™ PLUS (GE Healthcare) and centrifuged for 35 minutes at 16 000 rpm with the breaking mechanism disengaged. The PBMC layer was removed with a transfer pipette and washed twice in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, and 2.7 mM KCl. pH of 7.4). Cells were then pelleted and resuspended in 800 μL of MACS buffer (PBS with 2 mM EDTA and 0.5% BSA) per $1x10^5$ cells. Two hundred μL of CD8 MicroBeads (Miltenyi Biotec) were added per 800 μL of resuspended cells and mixtures were placed at 4°C for 20 minutes with shaking. Labelled PBMCs were washed twice in PBS, resuspened in 3 mL of MACS buffer and sorted using the autoMACS cell sorter running program “possel_s”. Isolated cells were washed once more with PBS and resuspended in RPMI-1640 (HyClone) media supplemented with 20% fetal calf serum (FCS;

HyClone), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 0.2 M L-glutamine (Gibco) (RPMI-20) at $1x10^6$ cells/mL. Isolated CD8 T cells were greater than 95% pure by flow cytometry (data not shown). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

2.2.9 Treatment of CD8 T Cells with Soluble Tat-1 or Tat-2 and Flow Cytometry

CD8 T cells were isolated as described above and cultured overnight in RPMI-20 to allow recovery of CD127 expression on the cell surface. The following day, cells were treated with Tat6xHis, Tat2-6xHis, or mock eluate. At 24 hour intervals, CD127 expression on CD8 T cells was measured by flow cytometry.

Per condition, 2 μL of anti-CD127-PE antibody (Beckman Coulter IO Test; Prod. No. IM1980U) was added to 100 μL of CD8 T cells ($1x10^5$ cells) and cells were incubated in the dark for 20 minutes to allow for antibody binding. Cells were then analyzed by flow cytometer and forward scatter versus side scatter gating was used to identify live cells. Flow cytometry was performed using the Cytomics FC500 flow cytometer (Beckman Coulter). Ten thousand events were captured per condition. Auto-florescence from unstained CD8 T cells was used to set the cut-off gate for $CD127^+$ staining.

2.2.10 Statistical Methods

For figures 19, 20 and 21, each treatment condition was analyzed for statistical difference against the CEM cell line alone (figure 19), or the mock treatment (figures 20 and 21) using a paired two-tailed student t-test. Results with p-values ≤ 0.05 were deemed significantly different.

2.3. Results

2.3.1 Production of Tat Plasmids

2.3.1.1 Tat Deletion Mutants

In this project I aimed to elucidate the mechanisms by which Tat downregulates CD127 from the surface of CD8 T cells. To accomplish this, I first constructed a series of deletion mutant proteins. Wild type and mutant recombinant HIV-1 Tat proteins were produced in *E. coli* with polyhistidine tags and purified over nickel columns. Originally, a series of six deletion mutants was constructed. Each mutant lacked one of the previously described regions of Tat. Plasmids encoding Tat lacking the N-terminal acidic region (aa 1-11; ΔAcidic), the cysteine-rich region (aa 22-37; ΔCys) the core region (aa 38-46; ΔCore), the basic region (aa 48-59; ΔBasic), the glutamine-rich region (aa 60-72; ΔGln), or the carboxyl-terminal region (aa 72-86; ΔC-Term) were successfully created. These plasmids were transformed into XL2 Blue bacteria, grown up in Luria broth with 100 μg/mL of amp and wild type or mutant Tat proteins were isolated by running bacterial lysates through Ni-NTA columns.

Interestingly, whereas wild type, ΔCore, ΔBasic, ΔGln, and ΔC-term Tat could all be produced at an acceptable yield, ΔAcidic and ΔCys proteins could not be detected by PAGE silver stain analysis (data not shown). These proteins were also not detected in the insoluble portion of the bacterial lysates (data not shown). In addition, point mutations of the cysteine residues also destroyed protein stability (data not shown). Previous studies have also found these cysteines to be essential to Tat stability [357-360]. In an attempt to make stable deletions within the first 40 aa of Tat, an additional series of six more mutants was generated. These constructs contained more refined deletions within the first 40 amino acids

of Tat in the hopes that removing smaller pieces of the protein would help retain protein stability. Whereas deletion of aa 17-21 or aa 22-26 allowed for an appreciable amount of Tat production, removal of either aa 1-5, 6-11, 12-16, 27-31 or 32-36 again resulted in a dramatically reduced protein yield.

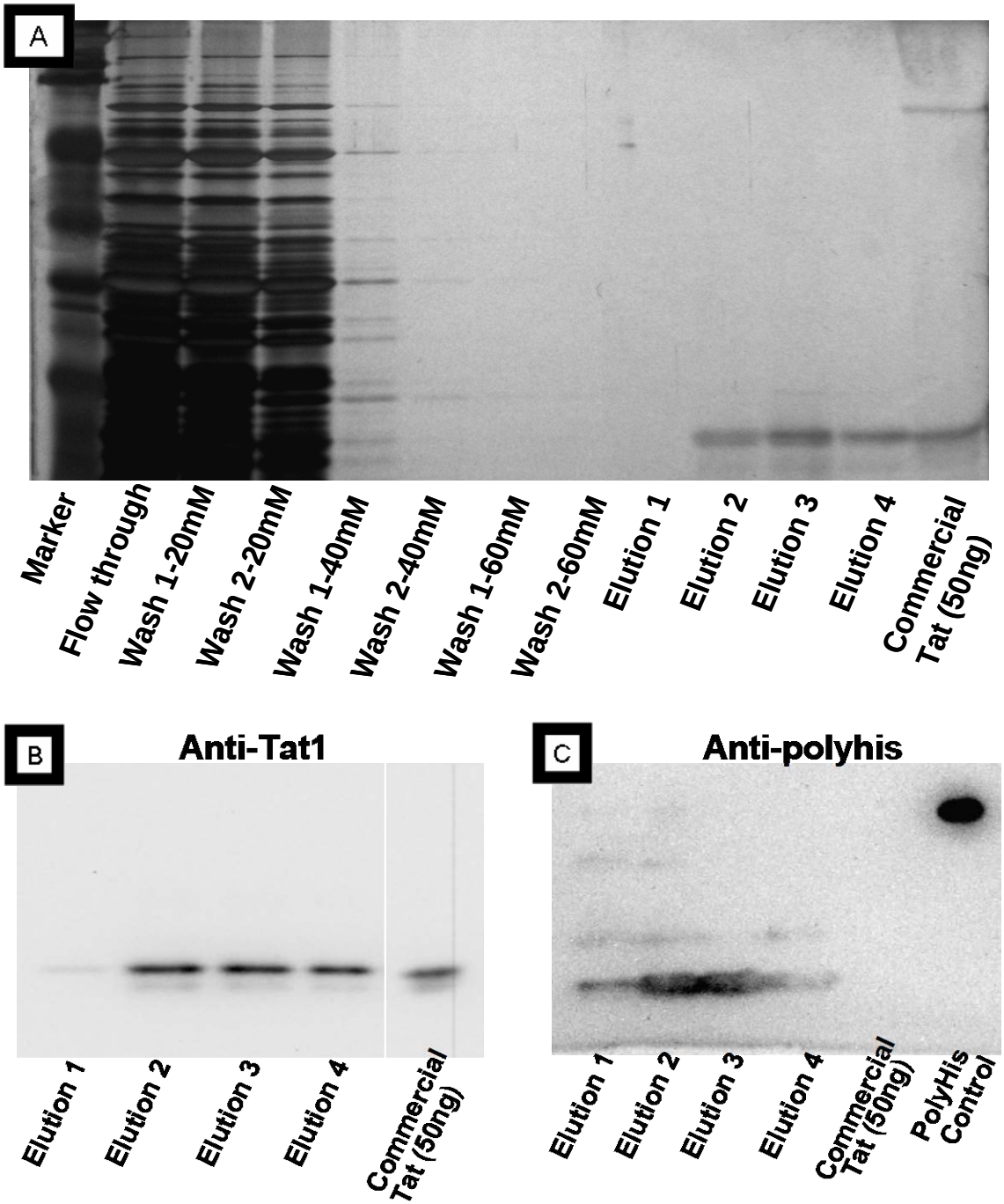
2.3.1.2 HIV-2N10 Plasmid

Since a stable N-terminal deletion of Tat could not be successfully produced, a chimeric mutant was generated in which the N-terminus of Tat-1 was replaced with the N-terminus from HIV-2 Tat (Tat-2) to generate a chimeric protein (HIV-2N10). The N-terminus of Tat-2 was chosen as this protein is similar to Tat-1 with regards to its role in the virus lifecycle and pathogenesis, yet shows a significant difference in the primary sequence of the first ten amino acids (see figure 15A). Ten amino acids were chosen in order to preserve the essential tryptophan found at position 11 of Tat [303]. HIV-2N10 Tat could be isolated at an appreciable yield, suggesting this protein is relatively stable when produced in bacteria.

2.3.2 Purification of Recombinant Tat-1 and Tat-2

pTatC6H-1 variants encoding for either wild type Tat-1 or Tat-2 were transformed into *E. coli* and purified by nickel-affinity using Ni-NTA columns. Using this method, a single 14 KDa band could be purified to homogeneity and visualized by silver stain analysis (see figure 16A).

Figure 16: Recombinant HIV-1 Tat can be produced and purified from *E. coli*. The pTatC6H-1 plasmid encoding Tat linked to a six histidine track (Tat6xHis) was transformed into *E. coli* and purified over Ni-NTA columns. **A)** Representative silver stain analysis showing purity of Ni-NTA column eluates. Milli-molar values denote the concentration of imidazole in the wash steps. **B&C)** Western blot analysis of Ni-NTA column eluates using either **(B)** anti-Tat or **(C)** anti-polyhistidine antibodies.


A
Marker
Flow through
Wash 1-20mM
Wash 2-20mM
Wash 1-40mM
Wash 2-40mM
Wash 1-60mM
Wash 2-60mM
Elution 1
Elution 2
Elution 3
Elution 4
Commercial Tat (50ng)
B
Anti-Tat1
Elution 1
Elution 2
Elution 3
Elution 4
Commercial Tat (50ng)
C
Anti-polyhis
Elution 1
Elution 2
Elution 3
Elution 4
Commercial Tat (50ng)
PolyHis Control

2.3.2.1 Identification of Tat Protein

Although wild type Tat protein isolated from bacteria is known to run at 14 KDa, I sought to confirm the identity of the protein as HIV-1 Tat. To this end, Ni-NTA eluates were investigated by Western blot with both anti-Tat and anti-polyhistidine antibodies. As shown in figure 16B and 16C, both anti-Tat and anti-polyhistidine antibodies identified a single band at 14 KDa by Western blot analysis. This confirmed the identity of the single 14 KDa protein as Tat.

To more definitively prove the identity the 14 KDa band as Tat, this band was excised from a silver stained gel and analyzed by mass spectrometry fingerprinting. By this approach, the strongest candidate protein identified was Tat-1 (see figure 17).

2.3.2.2. Identification of Tat-2 Protein

The wild type Tat-2 coding sequence was cloned into the pDS56 plasmid backbone, transferred into XL2 *E. coli* and Tat-2 protein linked to a polyhistidine track (Tat2-6xHis) was purified over Ni-NTA columns as done previously for Tat6xHis. As shown in figure 18A, Tat2-6xHis eluates produced a single 18 KDa band on a silver stained polyacrylamide gel. The identity of Tat-2 was confirmed by both anti-Tat-2 (figure 18B) and anti-polyhistidine (figure 18C) Western blot analysis, both of which produced a single 18 KDa band.

Figure 17: The identity of HIV-1 Tat-1 protein was confirmed by mass spectrometry fingerprinting. Recombinant Tat6xHis was visualized and isolated using sliver staining and analyzed via mass spectrometry fingerprinting. A list of the most probable proteins matching the peptide fingerprints is shown.

Mascot Search Results

User	: Julian
Email	: jvasiles@uottawa.ca
Search title	: Low Band - SG (Virus databases)
MS data file	: d:\PRC Customer Samples\2009\Jan112009\lowbandrun.mgf
Database	: NCBInr 20070216 (4626804 sequences; 1596079197 residues)
Taxonomy	: Viruses (379125 sequences)
Timestamp	: 14 Jan 2009 at 17:58:18 GMT
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 2 Da
Fragment Mass Tolerance	: ± 0.8 Da
Max Missed Cleavages	: 1
Instrument type	: ESI-TRAP
Number of queries	: 8110
Protein hits	: gi|7416448 Tat protein [Human immunodeficiency virus 1]
	gi|3319266 tat protein [Human immunodeficiency virus type 1]
	gi|115298529 77.2 kDa [Spodoptera frugiperda ascovirus 1a]
	gi|28863971 envelope glycoprotein [Human immunodeficiency virus 1]

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring | MudPIT scoring | Ions score or expect cut-off 40 | Show sub-sets 0

Show pop-ups | Suppress pop-ups | Sort unassigned Decreasing Score | Require bold red

1. gi|7416448 Mass: 8585 Score: 87 Queries matched: 2 emPAI: 0.48

Tat protein [Human immunodeficiency virus 1]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1091	588.3923	1174.7701	1176.4362	-1.6661	0	46	0.054	1	K.TACTNCYCK.K
3774	553.9963	1658.9669	1658.6496	0.3173	0	42	0.25	1	K.CCMHCQVCFLTK.G

---

2. gi|3319266 Mass: 8811 Score: 85 Queries matched: 2 emPAI: 0.46

tat protein [Human immunodeficiency virus type 1]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1091	588.3923	1174.7701	1176.4362	-1.6661	0	46	0.054	1	K.TACTNCYCK.K
1104	590.0244	1178.0342	1177.4202	0.6140	0	46	0.056	1	K.TACTDCYCK.K

Proteins matching the same set of peptides:

gi|54126328 Mass: 8597 Score: 85 Queries matched: 2

tat protein [Human immunodeficiency virus 1]

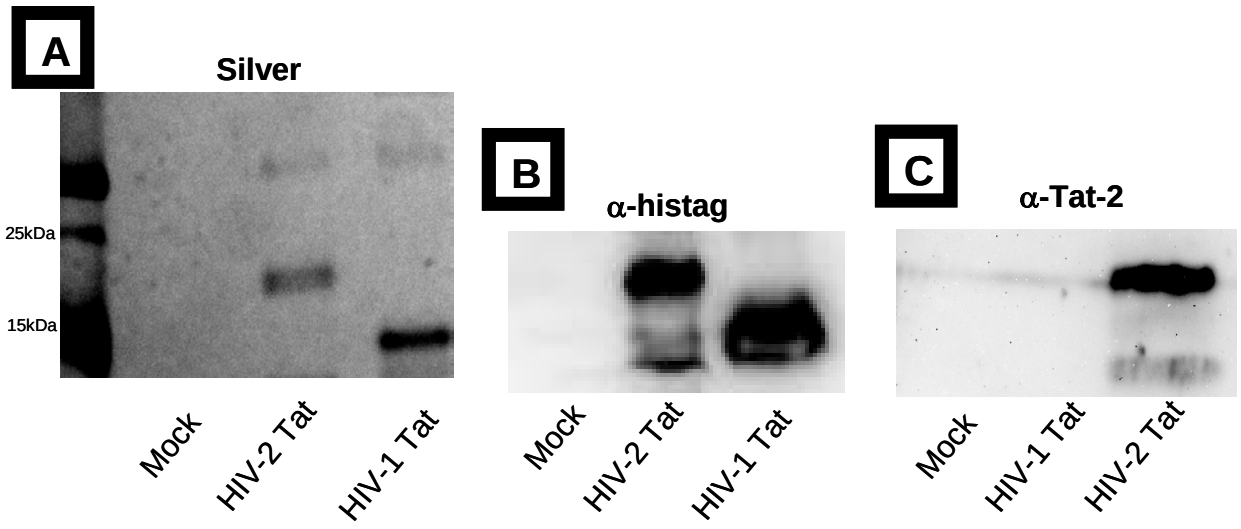
gi|54126344 Mass: 8646 Score: 85 Queries matched: 2

tat protein [Human immunodeficiency virus 1]

gi|112497905 Mass: 11521 Score: 85 Queries matched: 2

tat protein [Human immunodeficiency virus 1]

Figure 18: HIV-2 Tat-2 can be produced and isolated from *E. coli*. HIV-2 Tat protein tagged to a polyhistidine tract (Tat-26xHis) was generated in *E. coli* and isolated over Ni-NTA columns. **A)** Silver stain analysis showing the purity of Tat6xHis and Tat-26xHis isolates. **B&C)** Western blot analysis of Ni-NTA column eluates using either **(B)** anti-polyhistidine antibodies or **(C)** anti-Tat-2 rabbit sera to confirm the identity of the 18 KDa band as Tat-2.


A
Silver
25kDa
15kDa
Mock
HIV-2 Tat
HIV-1 Tat
B
α-histag
Mock
HIV-2 Tat
HIV-1 Tat
C
α-Tat-2
Mock
HIV-1 Tat
HIV-2 Tat

2.3.2.3 Biological Activity of Tat Proteins

Once the identity of the wild type and mutant Tat proteins were confirmed, the biological activity of these proteins was verified. Earlier work has shown that Tat-1 is capable of activating the HIV-2 LTR, but not vice versa [361]. This allowed for the use of the HIV-2 LTR to test the transcriptional activity of both Tat6xHis and Tat2-6xHis. A CEM line expressing luciferase under the control of the HIV-2 LTR (CEM.NKR-CCR5-Luc) was treated with 10 μg/mL of Tat6xHis, Tat2-6xHis, or a mock control and luciferase activity was measured after 24 hours. To determine the basal level of HIV-2 LTR activity prior to the addition of Tat proteins, the CEM.NKR-CCR5-Luc cell line and the CEM parent line lacking the HIV-2 LTR-luc sequence were also analyzed for luciferase activity. As shown in figure 19, the CEM.NKR-CCR5-Luc cell line produced a significant amount of luciferase without the addition of Tat-1 or Tat-2 protein. This suggests the HIV-2 LTR is relatively active prior to Tat transactivation in this cell line. Nevertheless, both Tat6xHis and Tat2-6xHis protein strongly promoted luciferase activity from the LTR-2 promoter. This confirms that both Tat6xHis and Tat2-6xHis are biologically active.

2.3.2.4 Recombinant Tat-1 Downregulates CD127 Expression

To check the biological stability and activity of Tat6xHis with regards to CD127 downregulation, Tat-1 protein was used to treat primary human CD8 T cells isolated from healthy donors. CD8 T cells were isolated from consenting volunteers and incubated in medium with purified Tat-1 protein (10 μg/mL) or a volume-matched mock control. Surface CD127expression was measured via flow cytometry at 24 hour intervals. As shown in

Figure 19: Tat6xHis and Tat2-6xHis are biologically active with regards to LTR trans-activation. A CEM cell line altered to express luciferase from the HIV-2 LTR was treated with either 10 μg/mL of Tat6xHis (CEM LTR Tat-1), Tat2-6xHis (CEM LTR Tat-2), a mock eluate (CEMLTR Mock), or left untreated (CEM LTR) (n=4). As negative controls, the CEM parental line without the integrated LTR was also analyzed for luciferase expression after no treatment (CEM), treatment with mock eluate (CEM Mock) or treatment with Tat2-6xHis (CEM Tat-2). After 24 hours, cells were lysed and luciferase activity was measured. Relative light units are shown normalized to auto luminescence from untreated CEM lysates (without a LTR-luciferase gene). Error bars represent standard error of the mean (±). * denotes p-values≤0.05.

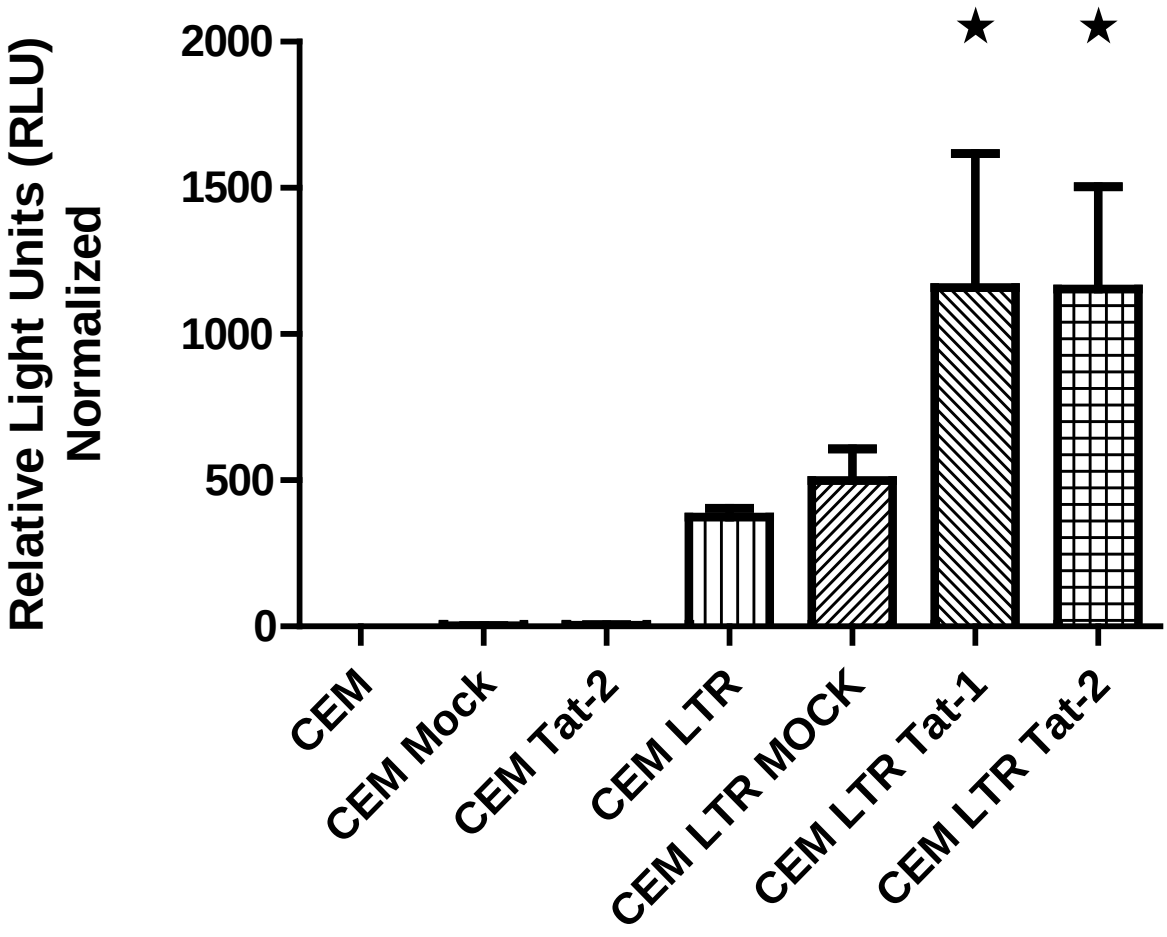

Relative Light Units (RLU)
Normalized
2000
1500
1000
500
0
CEM
CEM Mock
CEM Tat-2
CEM LTR
CEM LTR MOCK
CEM LTR Tat-1
CEM LTR Tat-2

figure 20, Tat6xHis protein treatment led to a 40% decrease in surface CD127 expression. This decrease is comparable to that observed with commercially purchased Tat-1 protein, showing similar potency and kinetics [352]. This work further confirms the biological activity of Tat6xHis protein.

2.3.2.5 Recombinant Tat-2 does not Downregulate CD127 Expression

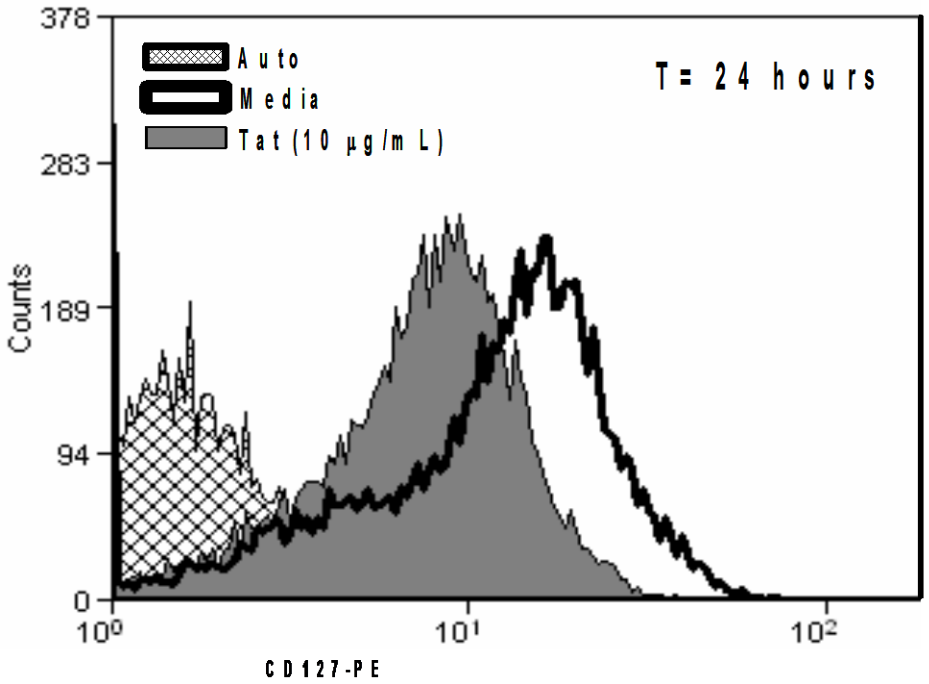
If wild type Tat-2 is able to downregulate CD127 in a manner similar to Tat-1, exchanging the first 10 aa of Tat-1for those of Tat-2 (i.e. HIV-2N10) may not produce a chimeric protein that is an acceptable substitute for an N-terminal Tat-1 deletion mutant. The effect of wild type Tat-2 on surface CD127 expression on human CD8 T cells was therefore investigated. CD8 T cells were incubated in RPMI-20 with 10 μg/mL of purified Tat2-6xHis protein or a volume-matched mock eluate and surface expression of CD127 was measured at 24 hours by flow cytometry. As shown in figure 21, Tat2-6xHis Tat did not downregulate the surface expression of CD127. This experiment suggests that Tat-2 does not interact with CD127, and therefore suggests that substitution of the first 10 aa of Tat-1 with those from Tat-2 will result in a chimeric protein which will function as a substitute for the unstable ΔAcidic protein.

2.3.3 Production of Recombinant Mutant Tat Proteins

Once the identity of wild type Tat-1 had been confirmed and the protein was shown to be biologically active, a series of mutant Tat-1 proteins was produced from the plasmids generated above: HIV-2N10, Δ17-21, Δ22-26, ΔCore, ΔBasic, ΔGln, and ΔC-term. As

Figure 20: Recombinant Tat6xHis produced in *E. coli* downregulates CD127 from the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative donors and incubated in media containing either 10 μg/mL of wild type Tat6xHis or a volume-matched mock control. Surface CD127 expression was measured at 24 hour intervals via flow cytometry. **A)** Representative flow histogram showing the effect of Tat6xHis on surface expression of CD127 on primary human CD8 T cells isolated from healthy donors 48 hours post-treatment. **B)** Percent change in CD127 expression relative to mock control over 72 hours (n=3). Error bars represent standard error of the mean (±).* denotes p-values≤0.05.

A


Auto
Media
Tat (10 µg/mL)
T = 24 hours
Counts
378
283
189
94
0
10⁰
10¹
10²
CD127-PE

B

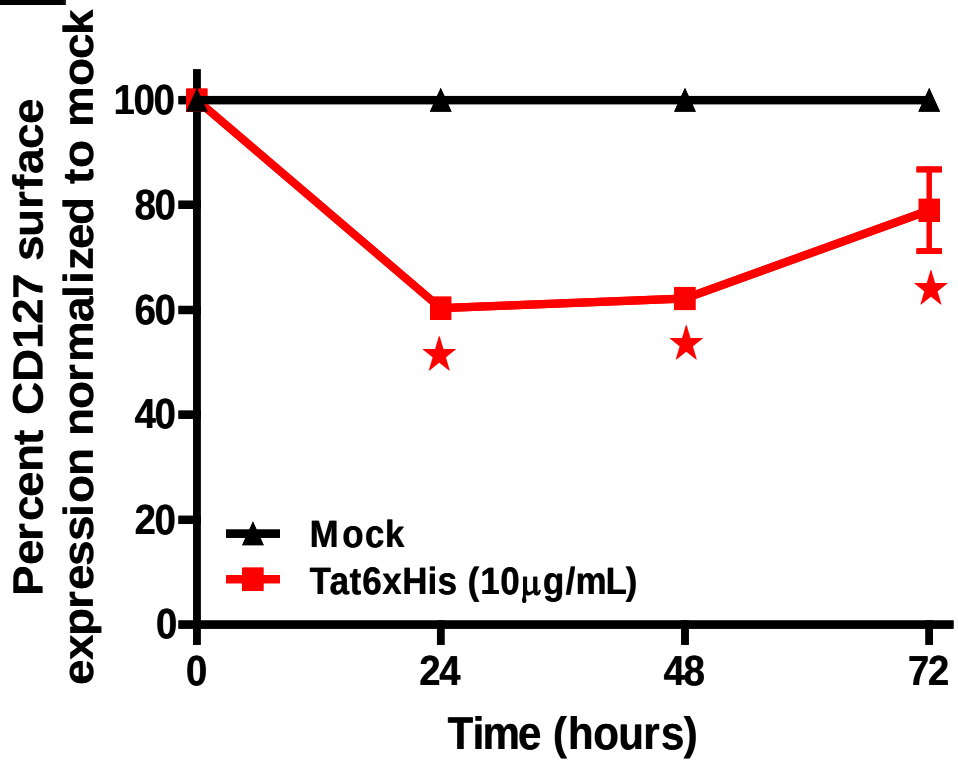
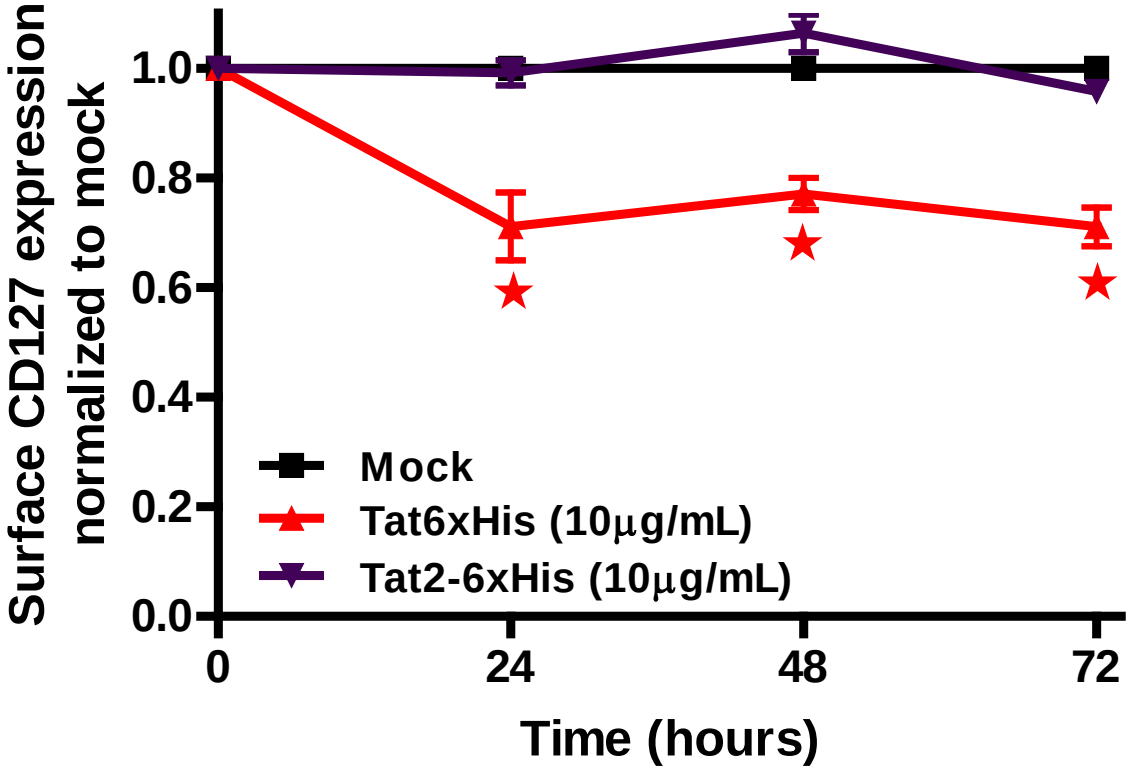

Percent CD127 surface expression normalized to mock
100
80
60
40
20
0
Mock
Tat6xHis (10µg/mL)
0
24
48
72
Time (hours)

Figure 21: HIV-2 Tat does not downregulate CD127 from the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative donors and incubated in media containing either 10 μg/mL of Tat6xHis, Tat2-6xHis, or a volume-matched mock control. Surface CD127 expression was measured at 24 hour intervals by flow cytometry. Percent change in CD127 expression relative to mock control is shown (n=4). Error bars represent standard error of the mean (±).* denotes p-values≤0.05.


Surface CD127 expression normalized to mock
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
Time (hours)
Mock
Tat6xHis (10μg/mL)
Tat2-6xHis (10μg/mL)

mutations in the first 40 aa of Tat-1 are known to prevent binding of the monoclonal anti-Tat antibody used for Western blots, the identities of these mutant proteins were confirmed by Western blot analysis using anti-polyhistidine antibodies (see figure 22). These proteins could be produced at comparable yields to wild type Tat-1 and were stored under identical conditions until use.

2.3.4 Expression of Tat-1 in Eukaryotic Cells

In order to allow for the expression of Tat-1 proteins within a eukaryotic system, all 8 coding sequences were transferred from the pTatC6H-1 backbone to the pcDNA3.1 vector, which expresses Tat6xHis from the cytomegalovirus (CMV) promoter. To ensure proper eukaryotic translation, a eukaryotic Kozak sequence was preserved between the CMV promoter and the translational start ATG coding sequence.

To confirm that these plasmids are capable of expressing Tat-1 within human lymphocytic cells, they were transfected into the Jurkat cell line and endogenous wild type or mutant Tat-1 production was measured by Western blot using anti-polyhistidine antibodies. As shown in figure 23, Tat-1 mutants and Tat-1 could be produced at comparative levels in Jurkat cells. This work shows that these plasmids could be used to produce a consistent amount of Tat-1 within eukaryotic cells, regardless of the region deleted. This also ensures that any variations in CD127 surface downregulation resulting from endogenous Tat-1 mutants are not the result of variations in protein levels.

Figure 22: Recombinant mutant Tat-1 proteins can be isolated from *E. coli*. The pTatC6H-1 plasmid encoding wild type or mutant Tat-1 linked to a 6 histidine track (Tat6xHis) was transformed into *E. coli* and purified over Ni-NTA columns. Purified proteins were analyzed by Western blot using anti-polyhistidine antibodies.

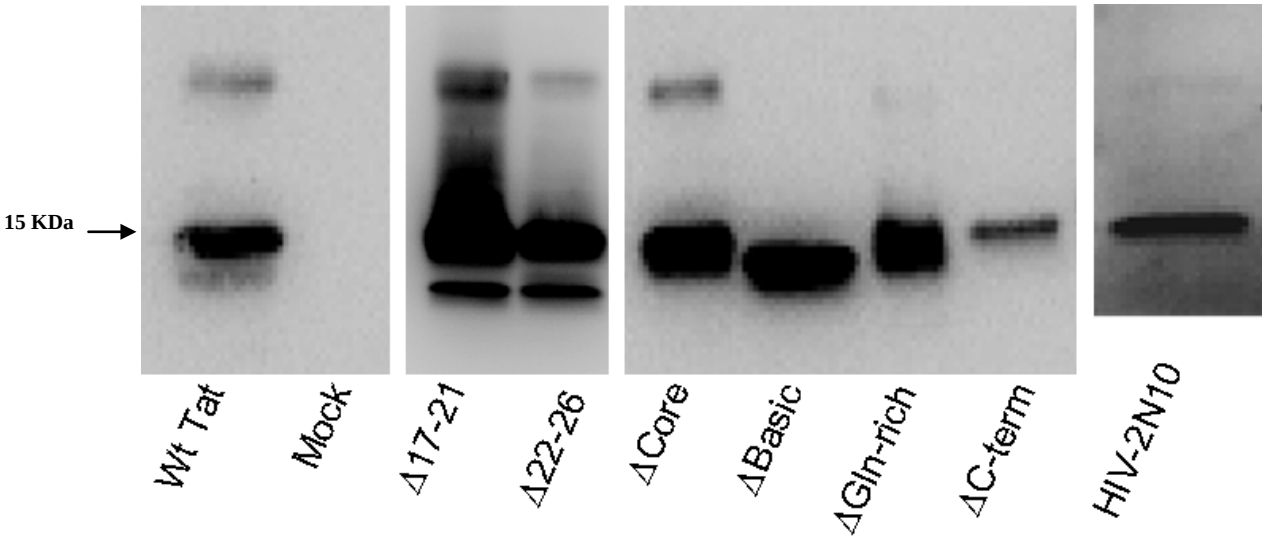
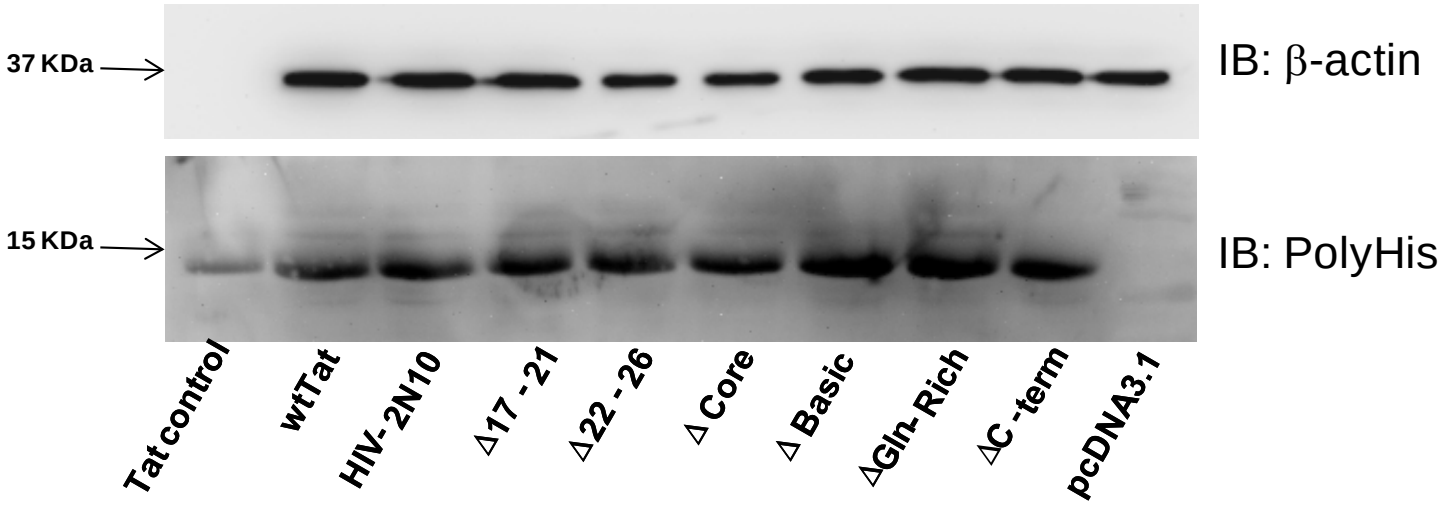

15 KDa
Wt Tat
Mock
Δ17-21
Δ22-26
ΔCore
ΔBasic
ΔGln-rich
ΔC-term
HIV-2N10

Figure 23: Tat mutants are expressed at comparable levels in Jurkat cells. Jurkat cells were transfected with empty pcDNA3.1$^{(-)}$, pcDNA3.1$^{(-)}$ encoding wild type Tat-1, or pcDNA3.1$^{(-)}$ encoding for mutant Tat-1 under control of the CMV promoter. Forty-eight hours post-transfection, cells were lysed. Proteins were separated via 10% SDS-PAGE and analyzed via Western blot using a polyclonal anti-histidine antibodies. Representative blot is shown above.


37 KDa
IB: β-actin
15 KDa
IB: PolyHis
Tat control
wtTat
HIV-2N10
Δ17 - 21
Δ22 - 26
Δ Core
Δ Basic
ΔGln- Rich
ΔC -term
pcDNA3.1

2.4 Discussion

In order to investigate Tat/CD127 interactions, it was first essential to develop a methodology to produce large quantities of concentrated, highly purified Tat-1 protein. Many different methodologies were attempted including HPLC, heparin affinity columns, cation exchange columns and cobalt affinity resin. Eventually a Ni-NTA based column system was designed, optimized and employed.

In this chapter, I have shown that a Ni-NTA column system could be optimized to isolate histidine-tagged HIV-1 and HIV-2 Tat at a high yield and purity from *E. coli*. These proteins can be stored stably at -80^{o}C and are biologically active with regards to HIV LTR transactivity. I further show that, in stark contrast to Tat-1, Tat-2 does not downregulate CD127 from the surface of human CD8 T cells.

In addition, a series of mutant Tat-1 proteins was produced. Molecular cloning techniques were used to generate two sets of plasmids encoding Tat-1 deletion mutants. One set of plasmids was designed for expression in prokaryotic cells and the optimized Ni-NTA column technique was used to produce and purify the series of Tat-1 mutant proteins from *E. coli*. The second set of plasmids allowed for expression of wild type and mutant Tat in eukaryotic cells.

Wild type Tat-1 protein is comprised of six well defined regions [304]. Originally I attempted to create six deletion mutants, each sequentially lacking a single region of the protein. Although deletions of the core region, the basic region, the glutamine-rich region and the C-terminal region did not destroy protein stability, deletions within the first 40 aa (Δ2-11, Δ22-39) resulted in a complete loss of protein production. In an attempt to generate stable deletion mutants within the first 40 aa of Tat-1, a new series of plasmids encoding

more refined Tat-1 deletion mutants (Δ2-6, Δ7-11, Δ12-16, Δ17-21, Δ22-26, Δ27-31, Δ32-36) was generated. It was hoped that these refined deletions would contain less alterations to protein structure, resulting in greater protein stability. Of these 8 plasmids, only Δ17-21 and Δ22-26 proteins could be produced.

From these results, it appears that the N-terminus of wild type Tat-1 is essential to the protein's stability. It is possible that the N-terminal domain adapts a specific conformation which protects the start methionine from exopeptidase digestion. Once removed, Tat's new N-terminus is no longer protected, and the protein is quickly destroyed by protease enzymes. This region contains the PxxPxxxPxxxPxxxP proline-rich motif, suggesting perhaps a more rigid structure within this region which may be required for Tat-1 stability. Although Tat is believed to exsist without established conformation [304], the extreme N-terminus of HIV-1 Tat may require a certain rigidity which cannot easily be altered without destroying the protein.

The cysteine rich region of Tat-1 (aa 22-39) was also essential for Tat-1 production from *E. coli*. The cysteines in this region are highly conserved and have been shown to be essential for the transactivating function of Tat [362]. Furthermore, oxidation of these cysteines results in a loss of Tat's ability to transduce the cell membrane [363], possibly through the induction of intermolecular disulphide bond formation and subsequent protein aggregation [364]. Tat-Tat homodimers can also be formed via cysteine/metal ion co-ordinate bond formation [357, 364], and newer evidence suggest an important role for intracellular disulphide bond formation in proper Tat-1 function [363].

In an attempt to produce stable Tat-1 protein lacking the protein's seven cysteine residues, a series of Tat-1 mutants containing point mutations which converted these

cysteines to serines was produced (data not shown). Perhaps not surprisingly, when these plasmids were expressed within *E. coli*, no stable protein could be isolated. As the seven cysteines of Tat are highly conserved and have been linked to the structural integrity of the protein through either divalent metal co-ordinate bond formation or intramolecular disulphide bond formation, it is possible that the removal of these cysteines exposes the protein to digestion by bacterial proteases.

In order to generate a stable protein lacking the N-terminus of Tat-1, it was necessary to replace the wild type sequence with that from a similar protein. Tat-2 was chosen because of the similar role that Tat-2 has in its respective virus, while still having a distinct primary amino acid sequence between aa 1-10 (see Figure 15).

I show here that HIV-2 Tat does not downregulate CD127 expression. This observation is extremely interesting in light of the observation that HIV-2 is less pathogenic than HIV-1, having a longer disease course on average [365]. Indeed, only approximately 20% of HIV-2 infected persons will progress to AIDS without treatment [366]. Recent work has further shown that CD127 levels on CD8 T cells are not reduced to the same degree in HIV-2 infection as compared to HIV-1 [367, 368]. We hypothesize that secreted Tat-1 is responsible for CD127 downregulation from the surface of CD8 T cells during HIV-1 infection and CD127 downregulation is an important contributor to HIV-1 pathogenesis. Indeed, the inability of HIV-2 Tat to interfere with the IL-7 signalling axis may help explain the slower progression and reduced morbidity often noted in HIV-2 infection compared to HIV-1.

In this chapter, a series of plasmids designed for the production of recombinant Tat-1 proteins from within *E. coli* cells was generated. All told, seven mutant Tat proteins were

generated: HIV-2N10, Δ17-21, Δ22-26, ΔCore, ΔBasic, ΔGln, ΔC-term. These plasmids were used to produce a series of corresponding proteins which were purified to homogeneity. Lastly, a second series of plasmids was produced encoding for these same Tat-1 mutants yet designed to express Tat-1 from within eukaryotic cells. Eukaryotic Tat-1 production from these plasmids was confirmed and proved to be relatively consistent across the panel of Tat-1 variants. These constructs were used in subsequent chapters to characterize the nature of Tat/CD127 interactions in primary human CD8 T cells.

3. Chapter 3: Effects of Exogenous and Endogenous Tat on Surface CD127 Expression on CD8 T cells.

3.1 Introduction

CD8 T cells are critical for the control and containment of numerous infectious agents. In HIV-1 infection, CD8 T cells have been identified as a major contributor in the control of infection. Without proper medical intervention however, CD8 T cells become anergic over the course of infection, displaying aberrant surface marker phenotypes and failing to destroy infected cells. We have shown that the surface expression of CD127, the specificity providing component of the IL-7 receptor, is lower on CD8 T cells isolated from HIV^+ persons when compared to cells isolated from healthy controls [193]. This downregulation is recovered, although incompletely, with successful antiretroviral therapy (ART). As CD127 greatly promotes survival and clonal expansion of CD8 T cells, as well as upregulates perforin production, lowered CD127 expression on CD8 T cells in the context of HIV-1 infect may represent a critical contributor to immunopathogenesis.

Our lab has shown previously that HIV-1 Tat downregulates the expression of CD127 from the surface of CD8 T cells [352]. Tat binds to HSPG moieties expressed on the surface of CD8 T cells via its basic region and enters host cell endosomes via clathrin-mediated endocytosis [288]. As the endosome acidifies, Tat transduces the endosomal membrane using a mechanism which requires interaction between the basic region of Tat and the cell membrane, and is facilitated by Hsp90 [288]. At the lower pH of the late endosome, this interaction allows Tat's Trp11 to insert into the endosomes membrane to begin the transduction process. Once free in the cytosol, Tat binds CD127 at the cell membrane and induces receptor clustering. After clustering, CD127 is removed from the cell surface via

accelerated internalization and is finally destroyed by the proteasome. Importantly, Tat-mediated CD127 downregulation *in vitro* results in lowered perforin production and proliferative capacity in response to IL-7 stimulation coupled with TCR engagement [352].

As CD127 is required for proper immune function, a more thorough understanding of Tat/CD127 interactions may contribute to the understanding of HIV-1 pathogenesis. To determine which regions of Tat are required for CD127 downregulation, CD8 T cells isolated from healthy volunteers were incubated with a panel of wild type or mutant Tat proteins and surface CD127 expression was measured by flow cytometry.

Identification of the regions of Tat required for the downregulation of CD127 is the first step in characterizing the underlying mechanisms by which Tat is able to accelerate the internalization of CD127 from the cell surface and target it for proteasomal degradation. A more thorough understanding of Tat/CD127 interactions may help to narrow the choice of possible mechanisms involved in these processes. Furthermore, an identification of the regions of Tat involved in this facet of HIV-1 pathogenesis may identify novel therapeutic targets.

3.2 Methods

3.2.1 Incubation of CD8 T Cells with Exogenous Tat Protein

After isolation, CD8 T cells were allowed to recover overnight in fresh RPMI-20. The following day, media was supplemented with either wild type or mutant Tat6xHis proteins at 10 μg/mL, or a volume-matched mock control. Surface CD127 expression was measured by flow cytometry at 24 hour intervals using anti-CD127-PE (Beckman Coulture IO Test; Prod. No. IM1980U). One hundred thousand cells (100 μL) were incubated with 2

μL of antibodies in the dark for 20 minutes to allow binding. Flow cytometry was performed using the cytomic FC500 flow cytometer (Beckman Coulter). Ten thousand events were acquired per condition. Cells were gated using forward and side scatter to identify the live population. $CD127^+$ gating was set against the auto-florescence of unstained CD8 T cells.

3.2.2 Jurkat-CD127 Cell Transfection and Flow Cytometry

A Jurkat cell line expressing CD127 and enhanced green florescent protein (GFP) from a bicistronic mRNA molecule driven from a CMV promoter was provided as a generous gift from Dr. Rene van Lier (Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands). The bicistronic expression of both proteins ensured that GFP^+ cells expressed CD127 mRNA. Jurkat-CD127 cells were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.2 M L-glutamine (RPMI-10) and maintained at a density between $5x10^5$ and $2x10^6$ cells/mL. Jurkat-CD127 cells were kept at 37°C and 5% CO_2 in a humidified incubator. Jurkat-CD127 cells were suspended at $0.9x10^6$ cells/mL in RPMI-10 to promote log-phase growth. The following day, pcDNA3.1$^{(-)}$ plasmids encoding for either wild type Tat or mutant Tats were transfected into Jurkat-CD127 cells using nucleofection technology (Lonza). Per condition, $2.5x10^6$ Jurkat-CD127 cells were resuspended in 100 μL of cell line V nucleofection solution containing 1.5 μg of wild type or mutant Tat-expressing pcDNA3.1$^{(-)}$, or empty vector control, and 0.25 μg of the pDsREDExpress2 plasmid (Clontech). Samples were then transfected with the Nucleofector II device using protocol X-005. Following nucleofection, Jurkat-CD127 cells were placed in serum free RPMI-1640 for 4 hours at a concentration of $2.5x10^6$ cells/mL. Cells were then transferred to RPMI-10 at a

concentration of $1x10^6$ cells/mL. Surface CD127 expression was measured on Jurkat-CD127 cells using anti-CD127- phycoerythrin-cyanine 7 (PE-PC7) (BD Bioscience; Clone HIL-7R-M21) at 4, 24 and 48 hours after nucleofection. Per condition, $3x10^5$ Jurkat-CD127 cells (300 μL) were stained with 6 μL of antibodies in the dark for 20 minutes. Cells were gated on GFP expression to assure CD127 mRNA production. Cells were also gated on DsRed florescence as an internal control for transfection efficiency. Auto-florescence from unstained Jurkat-CD127 cells was used to set the cut-off gate for $CD127^+$ staining. Twenty thousand events were acquired per condition.

3.2.3 Primary CD8 T Cell Transfection

Primary CD8 T cells were isolated as described above and cultured overnight in RPMI-20. The next day $5x10^6$ CD8 T cells were treated with anti-CD3/CD28 antibodies conjugated to magnetic beads using a T cell activation/expansion kit (Miltenyi) as per the manufacturer's instructions. After 24 hours, beads were removed using magnetic separation and CD8 T cells were nucleofected according to the manufacturer's instructions. Briefly, $5x10^6$ activated T cells per condition were resuspended into 100 μL of human T cell nucleofection solution containing 1.5 μg of wild type or mutant Tat-expressing $pcDNA3.1^{(-)}$, or empty vector control, and 0.25 μg of pGFPmax plasmid (Lonza) and transfected on the nucleofector II machine using protocol U-014. Post-nucleofection, cells were allowed to recover at a concentration of 2.5 $x10^6$ cells/mL for 4 hours in serum-free RPMI-1640 before being transferred into RPMI-20 at a concentration of $1x10^6$ cells/mL. Surface CD127 expression was monitored at 24 hour intervals via flow cytometry. Per condition, $1x10^5$ CD8 T cells (100 μL) were incubated with 2 μL of anti-CD127-PE antibodies in the dark for 20

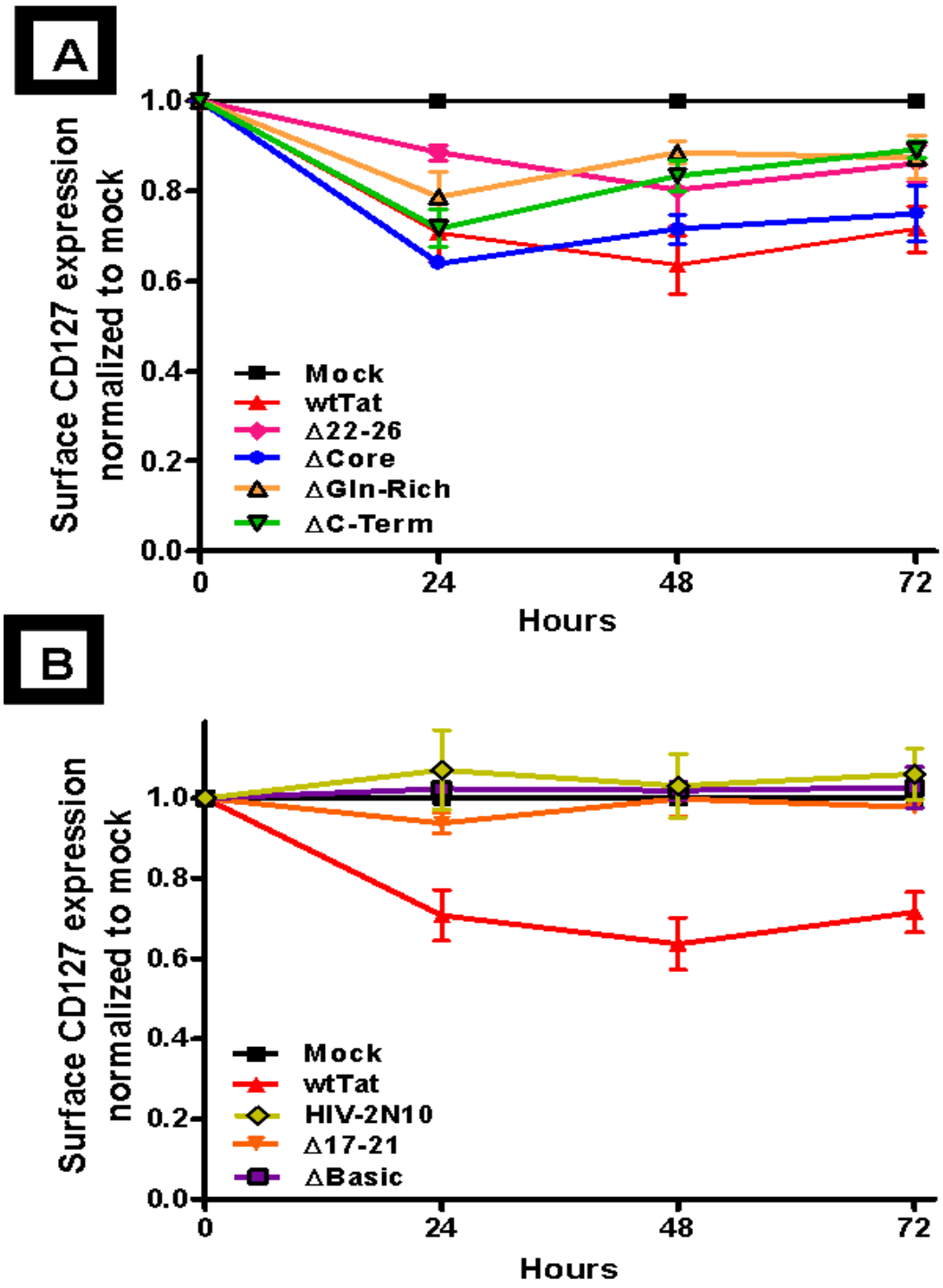
minutes to allow binding. Surface CD127 expression was measured by flow cytometry at 24 hour intervals. GFP florescence was used as an internal control for transfection efficiency. Auto-florescence from unstained CD8 T cells was used to set the cut-off gate for $CD127^+$ staining. Twenty thousand events were acquired per condition.

3.3 Results

3.3.1 Exogenous Tat Requires its N-terminal and Basic Regions to Downregulate CD127 from the Surface of CD8 T Cells

In this chapter, the regions of Tat required to downregulate CD127 were investigated. To accomplish this, the panel of mutant Tat proteins generated in chapter 2 was incubated with primary human CD8 T cells and the effect on surface CD127 expression was investigated by flow cytometry. As shown in figure 24A, aa 22-26, aa 37-50 (the core region), aa 63-78 (the glutamine rich region) or aa 73-86 (the C-terminal region) could be deleted without affecting Tat-mediated CD127 downregulation. When the N-terminal region (aa. 1-10), or aa 17-21, or the basic region (aa 48-57) were removed, Tat was no longer able to downregulate surface CD127 expression (see figure 24B). Hence, extracellular Tat requires its N-terminal and basic regions to downregulate CD127 from the surface of primary human CD8 T cells.

Figure 24: Exogenous Tat requires its N-terminal and basic regions to downregulate CD127 from the surface of CD8 T cells. CD8 T cells were isolated from healthy HIV-negative donors and incubated in media containing either 10 μg/mL of wild type Tat6xHis, a mutant variant thereof, or a volume matched mock eluate. Surface CD127 expression was measured at 24 hour intervals via flow cytometry. Percent change in CD127 expression relative to mock control is shown ($n \geq 4$). Error bars represent standard error of the mean (±). **A)** Tat mutants that retain the ability to downregulate CD127. **B)** Tat mutants that do not downregulate CD127 expression.


A
Surface CD127 expression normalized to mock
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
Hours
Mock
wtTat
Δ22-26
ΔCore
ΔGln-Rich
ΔC-Term
B
Surface CD127 expression normalized to mock
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
Hours
Mock
wtTat
HIV-2N10
Δ17-21
ΔBasic

3.3.2 Endogenous Tat Requires its N-terminal and Basic Regions to Downregulate CD127 from the Surface of Jurkat-CD127 Cells.

The basic region of Tat is required for the extracellular protein to be taken into host endosomes by interacting with surface HSPG. Furthermore, a combination of the basic region and Trp11 is required for the transduction of Tat across the cellular membrane [369]. The basic region of Tat binds the head group of the membrane phospholipids allowing Trp11 to insert itself between the hydrophobic tails of the lipid bilayer. Although both the HIV-2N10, Δ17-21 Tat deletion mutants preserve the critical Trp11, alterations in proximal residues could result in conformation changes that prevent Trp11 from properly interacting with its membrane binding partner. As Tat must first reach the cytosol to affect CD127, the observed loss of function for HIV-2N10, Δ17-21 and ΔBasic Tat may potentially be explained by a loss of cellular transduction, and not a loss of the ability to interact with CD127.

To address this possibility, wild type and mutant Tat proteins were expressed endogenously from within lymphocytic cells using the series of Tat expressing pcDNA3.1$^{(-)}$ plasmids described in the previous chapter. Expressing these proteins directly within lymphocytes removes the cellular transduction of Tat as a confounding factor.

For ease of transfection, these experiments were first carried out in the Jurkat-CD127 cell line. The wild type and mutant Tat coding sequences expressed from the CMV promoter on a pcDNA3.1$^{(-)}$ plasmid backbone were transfected using nucleofection into the Jurkat-CD127 line. As shown in chapter 2, Tat mutants are produced in relatively equal quantities in Jurkat-CD127 cells. Therefore, any variation in the ability of endogenously produced Tat mutant proteins to downregulate CD127 is not the result of variations in protein quantities

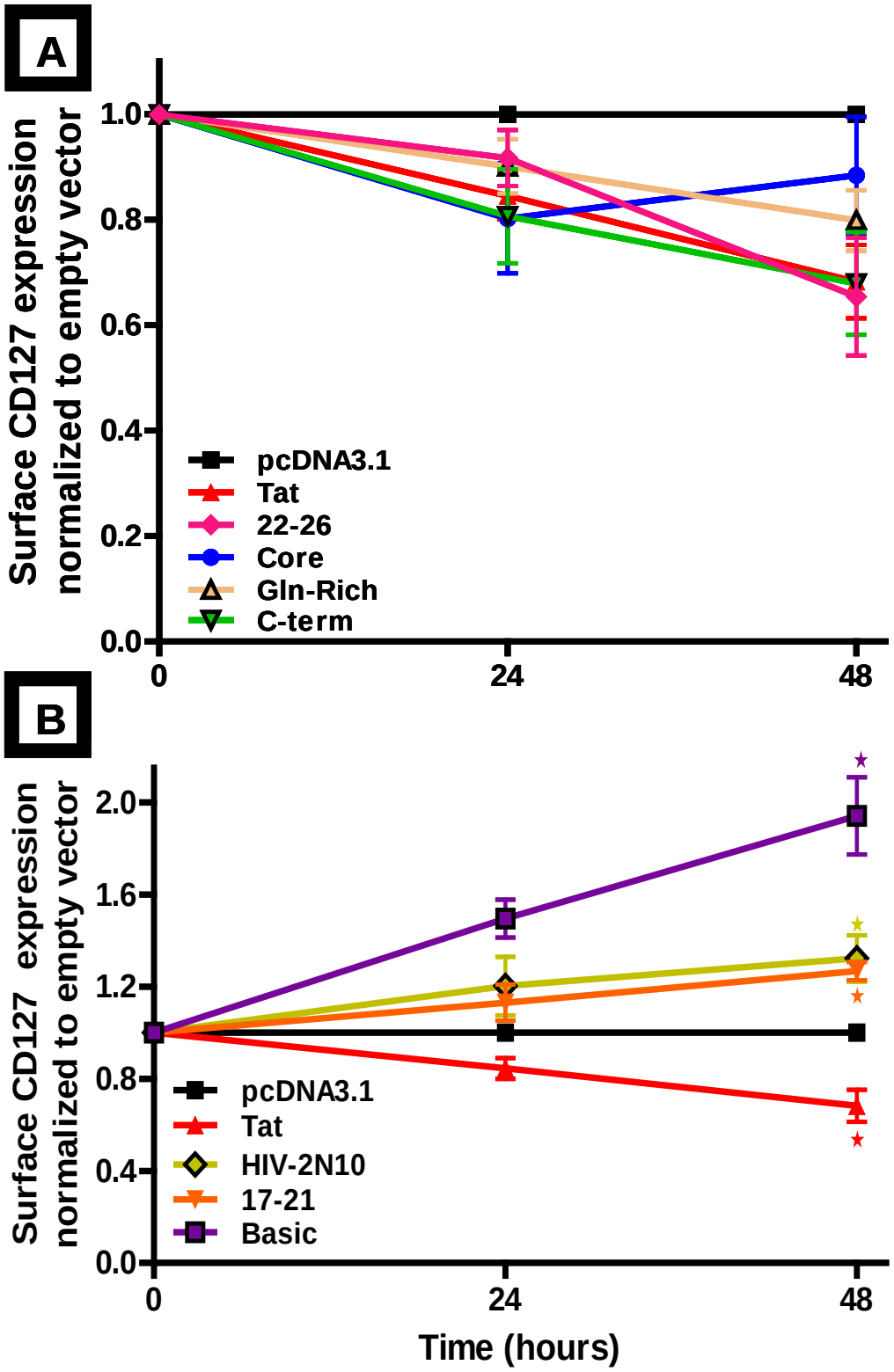
within the cells. As shown in figure 25A, endogenous expression of wild type Tat, Δ22-26, ΔCore, ΔGln or ΔC-term resulted in the downregulation of CD127 from the surface of Jurkat-CD127 cells. In contrast, endogenous expression of either HIV-N10, or Δ17-21 Tat, did not significantly alter expression of CD127 on the cell surface (figure 25B). Thus, the N-terminal region of Tat is required to downregulate CD127 expression independent of its requirement for cell membrane transduction.

Interestingly, whereas exogenous ΔBasic Tat did not alter expression of surface CD127, endogenous ΔBasic protein caused an accumulation of CD127 to two fold higher surface levels compared to empty pcDNA3.1$^{(-)}$ alone. As CD127 expression is constantly recycled at the cell membrane [229], a blockage in constitutive CD127 internalization from the cell membrane could result in an accumulation of CD127 at the cell surface. Endogenous ΔBasic Tat protein thus appears to act in a dominant negative manner and may disrupt constitutive CD127 recycling, causing an accumulation of CD127 at the cell surface.

3.3.3 Tat's N-terminal and Basic Regions are Required to Suppress CD127 Recovery from the Surface of CD8 T Cells after TCR Stimulation.

The effects of endogenously expressed Tat deletion mutants were confirmed in primary CD8 T cells. Since resting primary human lymphocytes are difficult to transfect, direct transfection of these cells could not be accomplished. In contrast, stimulated cells readily take up DNA and express foreign genes. CD8 T cell stimulation downregulates CD127 expression however, and we therefore sought to determine if the Tat deletion mutants would prevent CD127 recovery on the surface of stimulated CD8 T cells.

Figure 25: Endogenously produced Tat requires its N-terminal and basic regions to downregulate CD127 from the surface of Jurkat-CD127 cells. Jurkat-CD127 cells were transfected with either pcDNA3.1$^{(-)}$, or pcDNA3.1$^{(-)}$ encoding for wild type Tat or mutant Tat. Surface CD127 expression on Jurkat-CD127 cells was measured at 24 hour intervals by flow cytometry. Percent change in CD127 expression relative to empty pcDNA3.1$^{(-)}$ control is shown ($n \geq 4$). Error bars represent standard error of the mean (±). **A)** Tat mutants that retain the ability to downregulate CD127. **B)** Tat mutants that do not downregulate CD127 expression.


A
Surface CD127 expression normalized to empty vector
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
pcDNA3.1
Tat
22-26
Core
Gln-Rich
C-term
B
Surface CD127 expression normalized to empty vector
2.0
1.6
1.2
0.8
0.4
0.0
0
24
48
Time (hours)
pcDNA3.1
Tat
HIV-2N10
17-21
Basic

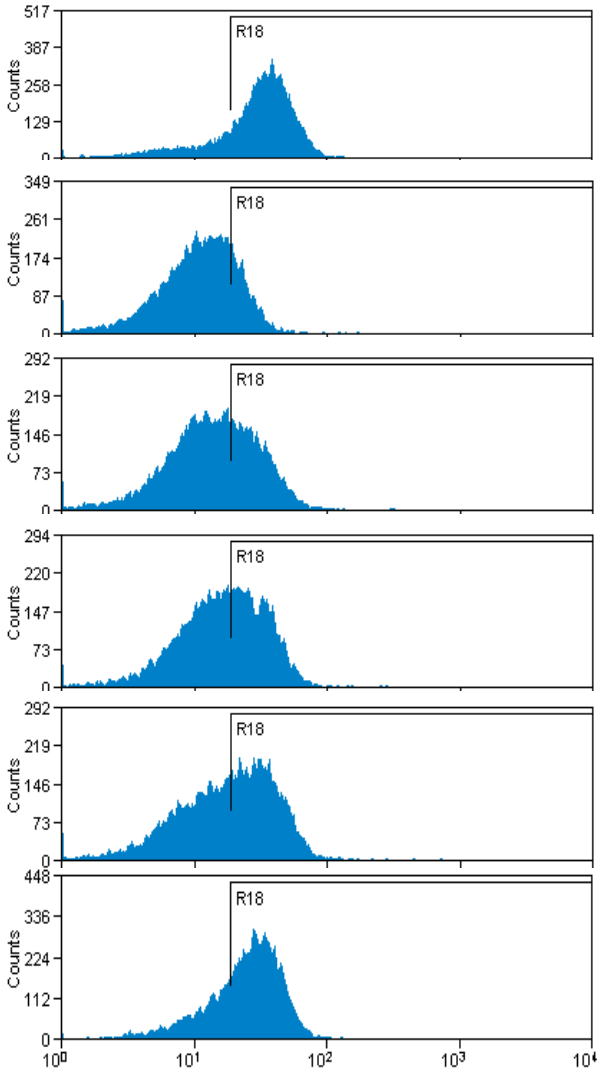
In vitro, CD8 T cells can be treated using anti-CD3 and anti-CD28 antibodies to simulate TCR (CD3) and second signal (CD28) engagement respectively. This stimulation results in downregulation of CD127 to approximately 20% of pre-stimulation levels. When anti-CD3/CD28 stimulation is removed, CD127 levels recover completely over the following 72 hours (see figure 26).

To determine if Tat could prevent the recovery of CD127, purified CD8 T cells were isolated from healthy volunteers and stimulated using anti-CD3 and anti-CD28 antibodies conjugated to magnetic beads. After 24 hours, beads were removed and CD8 T cells were transfected via nucleofection with pcDNA3.1$^{(-)}$ vectors encoding for either wild type or mutant Tat proteins, or pcDNA3.1$^{(-)}$ only control. At 24 hour intervals, surface CD127 was measured by flow cytometry. Wild type Tat expressed endogenously from within CD8 T cells, suppressed the recovery of CD127. In agreement with previous data, in this system HIV-2N10, Δ17-21 and ΔBasic Tat were unable to suppress recovery of CD127 (figure 27B). As also seen previously with exogenous Tat and Jurkat-CD127 experiments, Δ22-26, ΔCore, ΔGln, and ΔC-term Tat were all able to suppress CD127 recovery (figure 27A). This experiment again shows that the N-terminal and the basic regions of Tat are required to downregulate CD127 expression at the CD8 T cell surface.

3.4 Discussion

Our lab has shown that purified HIV-1 Tat protein will downregulate the expression of CD127 from the surface of human CD8 T cells *in vitro*, resulting in the loss of effector functions upon IL-7 and TCR co-stimulation. Therefore, Tat-mediated CD127

Figure 26: CD127 expression recovers on the surface of CD8 T cells after TCR stimulation. Isolated CD8 T cells were stimulated with α-CD3/α-CD28 beads for 24 hours before beads were removed and cells were allowed to recover. Representative flow cytometry histograms showing recovery of CD127 surface expression after removal of TCR stimulation.



Pre-stimulation

Post-stimulation

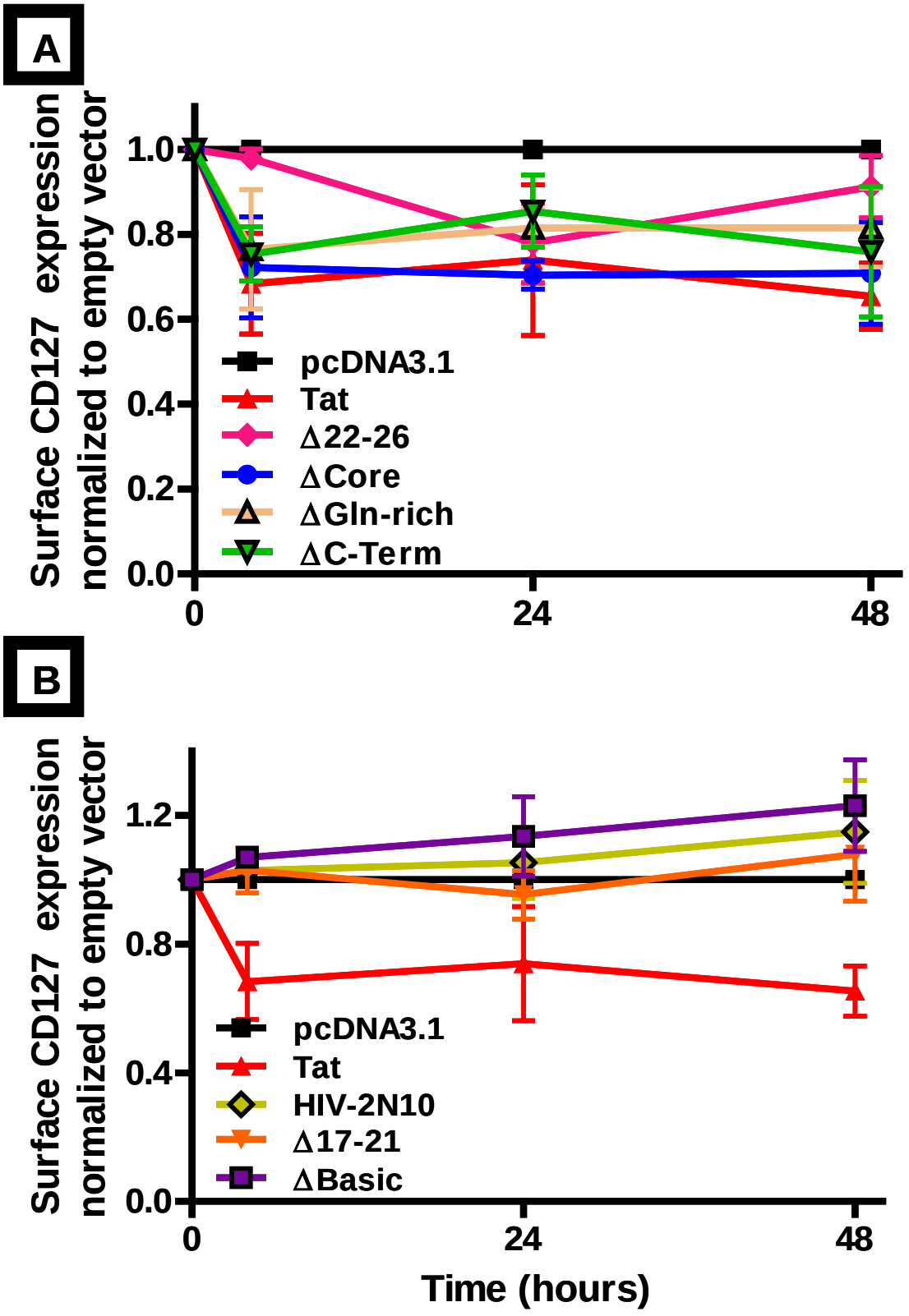
4 hours post-stimulation

24 hours post-stimulation

48 hours post-stimulation

72 hours post-stimulation

Figure 27: Endogenous Tat prevents recovery of CD127 on CD8 T cells after TCR stimulation via its N-terminal and basic regions. CD8 T cells were stimulated with α-CD3/α-CD28 beads for 24 hours before beads were removed and cells were transfected with either empty pcDNA3.1$^{(-)}$, or pcDNA3.1$^{(-)}$ encoding for wild type Tat or mutant Tat. Surface CD127 on CD8 T cells was measured at 24 hour intervals via flow cytometry. Percent change in CD127 expression relative to empty pcDNA3.1$^{(-)}$ control is shown (n=4). Error bars represent standard error of the mean (±). **A)** Tat mutants that prevent CD127 recovery. **B)** Tat mutants that do not prevent CD127 recovery.


A
Surface CD127 expression normalized to empty vector
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
pcDNA3.1
Tat
Δ22-26
ΔCore
ΔGln-rich
ΔC-Term
B
Surface CD127 expression normalized to empty vector
1.2
0.8
0.4
0.0
0
24
48
Time (hours)
pcDNA3.1
Tat
HIV-2N10
Δ17-21
ΔBasic

downregulation may in part explain the loss of cell-mediated immunity seen in HIV-1 infection. A better understanding of the mechanism of Tat/CD127 interactions could lead to new avenues of treatment and research. I sought here to further characterize the Tat/CD127 interactions and describe the regions of Tat protein required to affect receptor surface expression on primary human CD8 T cells.

I show here that Tat's N-terminal and basic regions are required for Tat-1 to downregulate CD127 from the surface of CD8 T cells. The lack of effect observed when CD8 T cells were treated with exogenous ΔBasic Tat can be explained by simple loss of the ability of Tat to transduce the cell membrane as it is well established that the basic region is required to interact with negatively charged HSPG moieties on the surface of T cells [297]. Furthermore, once in the endosome, the basic region residues 49-51 form an essential interaction with phospholipids which allows for insertion to Trp11 into the membrane [333, 370]. This insertion subsequently results in the transduction of Tat into the cytosol [333]. Moreover, it is a possibility that the HIV-2N10 and 17-21 have lost their ability to transduce the cell membrane. Although these mutations preserve the essential Trp11 needed to interact with membranes, deletions within the N-terminal regions may induce conformation changes that "hide" Trp11 and prevent Tat from interacting with membranes, causing loss of membrane transduction.

To circumvent the potential disruption of membrane transduction caused by removal of the basic and N-terminal regions, the readily transfectable Jurkat-CD127 cells were transfected to express wild type and mutant Tat endogenously. As seen with exogenous Tat treatment, intracellularly-produced Tat required the N-terminal and basic regions to downregulate CD127. Notably, in this system, internal production of ΔBasic Tat led to a

doubling of surface CD127 expression over the following 48 hours. I hypothesize that endogenously expressed ΔBasic Tat acts in a dominant negative manner by binding CD127 via its N-terminus and blocking constitutively active host machinery from recycling CD127 as a part of a normal homeostatic process. Indeed, CD127 continually recycles on and off the membrane by traveling into the early endosomal system and back to the surface [133] and we have shown that CD127 has a relatively long half-life of approximately 55 hours in human CD8 T cells [229]. Homeostatic recycling in this manner has been observed for other common γ-chain receptor family members, namely IL-2Rα [371] and IL-15Rα [372]. Given CD127s longer half-life, a halt in CD127 removal from the cell surface would be expected to result in an increase in surface CD127 over the course of a few days as observed with endogenous ΔBasic treatment. This suggests that ΔBasic Tat may still be able to interact with the receptor in some fashion, yet cannot remove the receptor from the cell surface. ΔBasic Tat may function as a competitive inhibitor of CD127 recycling. For example, Tat may be displacing the class III PI-3K Vps34, which has been shown recently to mediate CD127's return from the cytosol to the cell surface [373].

To extend the results observed using Jurkat-CD127 cells to primary CD8 T cells, a system was designed whereby TCR-stimulated CD8 T cells were transfected to endogenously express wild type or mutant Tat proteins and surface CD127 recovery was observed. This system was chosen because primary human CD8 T cells are impervious to transfection without activation. Cell stimulation, although allowing for transfection, results in dramatically decreased CD127 surface expression, making it only possible to observe the effects of endogenous Tat on the recovery of CD127 post-stimulation in human CD8 T cells.

In this system, as in Jurkat-CD127 cells, the N-terminal and basic regions of Tat were required for Tat's effect on CD127. Interestingly, in this model, ΔBasic Tat did not cause an increase in CD127 surface expression. This is likely due to the low CD127 surface expression following TCR stimulation. Hence, ΔBasic Tat can retain very little CD127 at the cell surface in this model. Given that the same regions required for exogenous Tat to downregulate CD127 were also required for endogenous Tat's suppression of CD127 recovery after TCR stimulation, I conclude that the basic (aa 48-57) and N-terminal (aa 2-10 and aa 17-21) regions of Tat are required to downregulate CD127 at the cell membrane.

Tat may be promoting CD127 downregulation by several mechanisms. For example Tat may alter CD127 endosomal trafficking to encourage shunting of the receptor towards the proteasome and away from the cell surface. Tat may alter CD127 post-translation modifications to encourage degradation, or Tat itself may represent the modification, acting as a tugboat to pull CD127 towards the proteasome. Another possibility is that Tat may interact with CD127 to act as a scaffold, inducing CD127 clumping or recruiting clathrin machinery to the cytosolic tail of the receptor. This list of possible interactions is certainly not exhaustive.

In the next chapter, the regions of Tat requiremed for CD127 physical interactions are investigated along with the molecular mechanisms of Tat-induced CD127 removal from the cell surface.

4. Chapter 4: Tat Downregulates CD127 at the CD8 T Cell Surface by Recruiting CIS and an E3 Ubiquitin Ligase to the Receptor.

4.1 Introduction

In the previous chapter I showed that the N-terminal and basic regions of Tat-1 are required to downregulate CD127 from the surface of CD8 T cells. Furthermore ΔBasic Tat will function as a dominant negative mutant to increase surface CD127 when expressed endogenously. In this chapter, I set out to define the molecular mechanisms by which Tat-1 removes CD127 from the cell surface. Based on the literature, several candidate mechanisms can be proposed.

As Tat-1 is known to affect the phosphorylation, ubiquitination, methylation and/or acetylation states of numerous host proteins [304], it may affect CD127 by promoting post-translational modifications. For example, Tat-1 may induce CD127 downregulation through recruitment of a kinase to the cytosolic tail CD127 to promote CD127 phosphorylation. Although Tat-1 does not seem to induce CD127 signalling [352], I have observed recently that CD127 phosphorylation at Y449, although dispensable for initial IL-7 mediated CD127 endocytosis, is required for degradation of CD127 protein upon IL-7 treatment (see figure 10). Tat-1 promotes the activity of signal transducing kinases including mitogen-activated protein kinase (MAPK) [374], ERK1 [375], ERK2 [375], protein kinase R (PKR) [376], PCK [377], and PI3K [378]. Tat-1 induces PKR function through an interaction that involves the basic region of Tat [379]. More interestingly, the basic region of Tat has recently been shown to bind the membrane associated protein kinases A and C (PKA; PKC)

and the Tat peptide aa 48-60, corresponding to the basic region, inhibits PKC activity competitively by binding the kinase's active site [380].

Aside from inducing CD127 phosphorylation, Tat-1 may promote the ubiquitination of the receptor. Surface expression of several members of the common γ-chain family of receptors is now known to be regulated by ubiquitin modification [381, 382]. Indeed, our lab has recently demonstrated that IL-7 binding to its receptor targets CD127 for proteasomal degradation through recruitment of members of the SOCS family (unpublished observation, Feras Ghazawi). We have shown that IL-7 signalling upregulates SOCS2 and CIS mRNA and protein in CD8 T cells. By both confocal microscopy and co-immunoprecipitation, we have shown that SOCS2 and CIS associate with CD127 at early endosomes and co-traffic with the receptor to the proteasome for degradation. This process is dependent on cellular E3 ubiquitin ligase machinery, strongly implicating ubiquitination in this process. CIS appears to play the lead role in this process, being more strongly induced than SOCS2, and CIS knockdown has been shown to prevent CD127 downregulation in response to IL-7. We have also shown that inhibition of proteasome function blocks Tat-mediated CD127 degradation [229]. As Tat-1 is known to recruit E3 ligases such as Mdm-2 to induce the ubiquitination and subsequent degradation of several proteins [383, 384], it is possible that Tat's basic region is required to recruit ubiquitin machinery to CD127. Notably, Tat-1 has been shown to induce the internalization and proteasomal degradation of the recepteur d'origine nantais (RON) tyrosine kinase in monocytes and macrophages by inducing its ubiquitination [384]. It is also possible that ubiquitnation of Tat-1 allows it to ferry CD127 to the proteasome for degradation.

In this chapter, I characterize the physical interaction between Tat-1 and CD127, as well as investigate the role of various post-translational modifications in Tat-mediated receptor downregulation. I show here that Tat-mediated CD127 downregulation is dependent on Tat/CD127 physical interaction. Furthermore, I show that Tat-1 does not affect CD127 phosphorylation, but rather induces ubiquitination of the receptor. Thus, recruitment of cellular ubiquitin ligase machinery by Tat-1 may result in CD127 downregulation and degradation.

4.2 Methods

4.2.1 Co-Immunoprecipitations (co-IP)

4.2.1.1 Co-IP of CD127 and Tat-1 from Primary CD8 T Cells

Five million purified CD8 T cells were incubated at $1x10^6$ cells/mL in RPMI-20 supplemented with 10 μg/mL of purified commercially purchased Tat-1 protein (Advanced Bioscience Laboratories) for 24 h at 37°C. Cells were then lysed on ice for 1 hour in 100 μL of WCL Buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 (NP-40), 10% glycerol, 2 mM DTT) containing HALT protease inhibitor (Thermo Fisher Scientific). Cell lysates were precleared by adding 100 μL of Immunopure Immobilized Protein G beads (Thermo Fisher Scientific) and incubating at 4°C for 1 hour with constant mixing. Protein G beads were removed by centrifugation at 17 000 g, and CD127 was immunoprecipitated by incubating the supernatants overnight with 2.5 μg of polyclonal goat anti-CD127 antibodies (R&D systems; cat. no. AF-306-PB). The next day, 50 μL of Immunopure Immobilized Protein G beads were added and incubation was continued at 4°C for an additional 2 hours. Antibody–protein G complexes were pelleted at 17 000 g for 2

minutes and washed four times with 1 mL of IP buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 2 mM DTT, 0.2% sodium azide) containing HALT protease inhibitor mixture. Antibody–protein G complexes were then pelleted and resuspended in 50 μL of 3x SDS sample buffer (New England Biolabs) and boiled for 10 min at 100^{o}C.

Immune complexes were separated by SDS-PAGE and probed for the presence of Tat-1 protein by Western blot. PAGE and transfer were carried out as described in chapter 2. Tat-1 was detected using monoclonal anti-Tat antibodies (Immunodiagnostics; Prod. No. 1102) diluted to 1:5 000. Secondary labelling was achieved using goat anti-mouse-HRP antibodies (R&D systems; cat. no. HAF007). Blots were developed by ECL as previously described.

4.2.1.2 Co-IP of CD127 and Tat Mutants from Jurkat-CD127 Cells

Five million Jurkat-CD127 cells were lysed on ice in 100 μL of WCL buffer. After centrifugation at 11 000 g for 10 minutes to pellet cellular debris, supernatants were pre-cleared by incubating with 50 μL of Immunopure immobilized protein G beads for 1 hour at 4^{o}C as described above. Cleared lysates were then incubated with either wild type or mutant Tat6xHis protein (10μg/mL) for 1 hour at 4^{o}C. CD127 protein was then immunoprecipitated with 2.5 μg of goat anti-CD127 antibodies (R&D systems; cat. no. AF-306-PB) overnight at 4^{o}C followed by the addition of 50 μL of Immunopure immobilized protein G beads for 1 hour at 4^{o}C. Following centrifugation, pellets were washed five times with IP buffer and boiled for ten minutes at 100^{o}C in 50 μL of 3x SDS sample buffer.

Samples were separated via SDS-PAGE and investigated for the presence of Tat6xHis protein by Western blot. PAGE and transfer were carried out as described in chapter 2. Bands corresponding to Tat protein were detected using monoclonal anti-polyhistidine antibodies (Sigma; clone HIS-1) diluted to 1:500. Secondary labelling was achieved using 1:10 000 goat anti-mouse-HRP antibodies (R&D systems; Prod. No. HAF007). Blots were developed by ECL as previously described.

4.2.1.3 Co-IP of CD127-CIS from CD8 T Cells

One and a half million primary CD8 T cells were incubated at $1x10^6$ cells/mL in RPMI-20 supplemented with or without commercially purchased Tat-1 (10 μg/mL). At 6 hours following the addition of Tat-1 the media was supplemented with the proteasome inhibitor MG132 (Sigma) at 5 μM and cells were incubated for another 8 hours. Cells were then lysed in 100 μL of WCL buffer supplemented with fresh cØmplete™ protease inhibitor (Roche) and CD127 was immunoprecipitated with 2.5 μg of goat anti-CD127 antibodies (R&D systems; cat. No. AF-306-PB) as described previously.

Immune complexes were separated by SDS-PAGE followed by transfer to PVDF membranes as described previously, then probed for the presence of human CIS protein by Western blot. CIS was detected using polyclonal mouse anti-CIS antibodies (abCAM; Prod. No. ab88383) diluted 1:500. Secondary labelling was achieved using 1:10 000 goat anti-mouse-HRP antibodies (R&Dsystems; Prod. No. HAF007). Blots were developed by ECL as previously described.

4.2.2 Western Blots to Detect CD127 Y449 Phosphorylation

Two million CD8 T cells were incubated in RPMI-20 supplemented with commercially purchased HIV-1 Tat-1 (10 μg/mL) for 12 hours. As a positive control, $2x10^6$ CD8 T cells were incubated in RPMI-20 supplemented with 10 ng/mL of recombinant IL-7 (Gibco) for 10 minutes. Cells were then lysed in 50 μL of RIPA buffer supplemented with fresh cØmplete™ protease inhibitor (Roche) and lysates were boiled for 5 minutes in 3x SDS sample buffer (New England BioLabs).

Whole cell lysates were separated on an 8% polyacrylamide gel via SDS-PAGE and transferred to PVDF membrane as described in chapter 2. The presence of CD127 phosphorylated at tyrosine 449 (pY449) was detected by Western blot. PVDF membranes were incubated overnight at 4^oC in 2% blocking solution containing a 1:1 000 dilution of rabbit anti-CD127-pY449 antibodies (ROCKLAND; Prod. No. 600-401-A49). The next day, blots were treated with mouse anti-rabbit-HRP antibodies diluted at 1:10 000 in 2% blocking solution for one hour at room temperature. Blots wer then washed and developed by ECL as previously described.

4.2.3 Ubiquitin Enrichment Assay

Half a million primary CD8 T cells were incubated at $1x10^6$ cells/mL in RPMI-20 supplemented with or without commercially purchased Tat-1 (10 μg/mL). At six hours following addition of Tat-1, the media was supplemented with the proteasome inhibitor MG132 (Sigma) at 5 μM and the cells were incubated for an additional eight hours. Cells were then lysed in 200 μL of RIPA buffer supplemented with fresh cØmplete™ protease inhibitor (Roche). Lysates were then cleared by centrifugation at 11 000 g for five minutes

and then diluted 1:1 with tris-buffered saline (TBS). Ubiquitinated proteins were isolated using a ubiquitin enrichment kit (Pierce) as per the manufacturer's instructions. In brief, cleared lysates were treated with a proprietary ubiquitin affinity resin (Pierce) for 2 hours at 4^{o}C with continuous mixing. Resin was then loaded into the kit-provided columns and washed five times with 300 μL rinse buffer (9:1 TBS to RIPA) by centrifugation at 5 000 g for 15 seconds. Resin was then boiled in 50 μL of 3x SDS buffer and ubiquitin enriched proteins were separated from the resin via centrifugation at 5 000 g for 30 seconds.

Samples were separated on an 8% polyacrylamide gel via SDS-PAGE and transferred to PVDF as described in chapter 2. CD127 was detected by Western blot using polyclonal goat anti-CD127 antibodies (R&D systems; Cat. No. AF-306-PB) diluted 1:500 in 2% skim milk overnight at 4^{o}C. The next day, blots were treated with donkey anti-goat-HRP antibodies (R&D systems cat. no. HAF109) diluted at 1:10 000 in 2% blocking solution for one hour at room temperature. Blots were developed by ECL as previously described.

4.2.4 CD127 Flow Cytometry

Per condition, 2 μL of anti-CD127-PE antibodies (Beckman Coulter IO Test; Prod. No. IM1980U) were added to 100 μL of purified primary CD8 T cells (10 000 cells) in RPMI-20 and incubated in the dark for 20 minutes to allow for antibody binding. Flow cytometry was performed using the Cytomics FC500 flow cytometer (Beckman Coulter). Ten thousand events were captured per condition. Cells were gated using forward and side scatter to identify the live population. Auto-fluorescence from unstained CD8 T cells was used to set the cut-off gate for CD127^{+} staining.

4.2.5 CD8 T Cell Smer3 Assay

Four hundred thousand primary CD8 T cells per condition were cultured at $1x10^6$ cells/mL in RPMI-20 with or without 1.25 μM of the pan-E3 uibquitin ligase inhibitor Smer3 (Tocris Bioscience). After 1 hour, media was supplemented with or without commercially purchased Tat-1 (10 μg/mL). At 12 and 24 hours following the addition of Tat-1, cells were analyzed by flow cytometry for surface CD127 expression as described above.

4.2.6 Statistical Methods

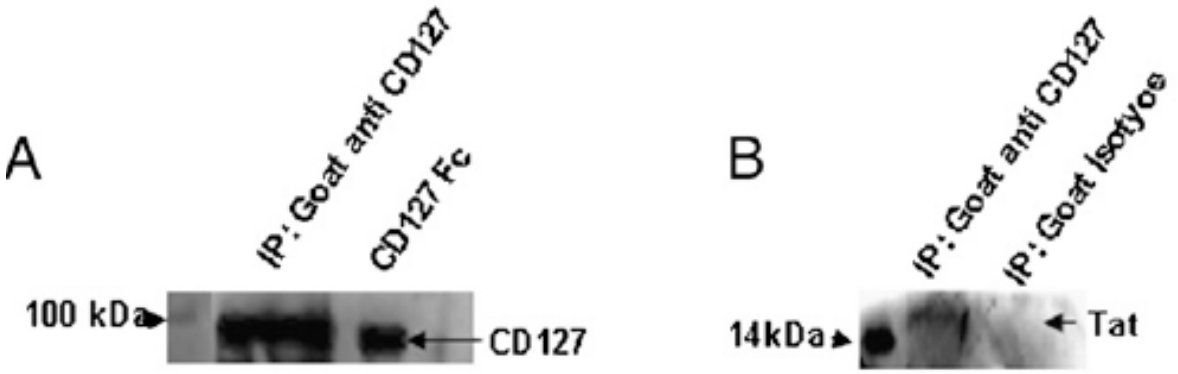
For figure 32, each treatment condition was analyzed for statistical difference against the untreated control using a paired two-tailed student t-test. Results with p-values ≤ 0.05 were deemed significantly different.

4.3 Results

4.3.1 Tat-1 Binds CD127 in Primary Human CD8 T Cells

We have shown previously by microscopy that Tat colocalizes with CD127 at the cell surface and induces receptor capping prior to receptor internalization and degradation [229]. To determine if Tat was physically bound to CD127, CD8 T cells were incubated with soluble Tat protein at 10 μg/mL for 24 hours. Cells were then lysed and CD127 was immunoprecipitated with anti-CD127 antibodies. Immune complexes were separated via SDS-PAGE and immunoblotted for Tat-1 protein. As shown in figure 28, Tat co-immunoprecipitated with CD127 but not with isotype control antibodies. This experiment confirms that Tat binds CD127 in live CD8 T cells and suggests that Tat binding to CD127 may play a direct role in Tat-induced CD127 surface downregulation.

Figure 28: Tat binds CD127 in CD8 T cells. Primary human CD8 T cells were treated with Tat protein (10 μg/mL) for 24 hours prior to CD8 T cell lysis, then immunoprecipitated with anti-CD127 antibodies or an irrelevant isotype-matched control. Immunocomplexes were separated via SDS-PAGE and analyzed by Western blot for **A)** CD127 protein or **B)** HIV-1 Tat protein. Reprinted with permission from the American Association of Immunologists. Faller, E. M., Sugden, S. M., McVey, M. J., Kakal, J. A. & MacPherson, P. A. Soluble HIV Tat protein removes the IL-7 receptor alpha-chain from the surface of resting CD8 T cells and targets it for degradation. J Immunol 185, 2854-66 (2010).

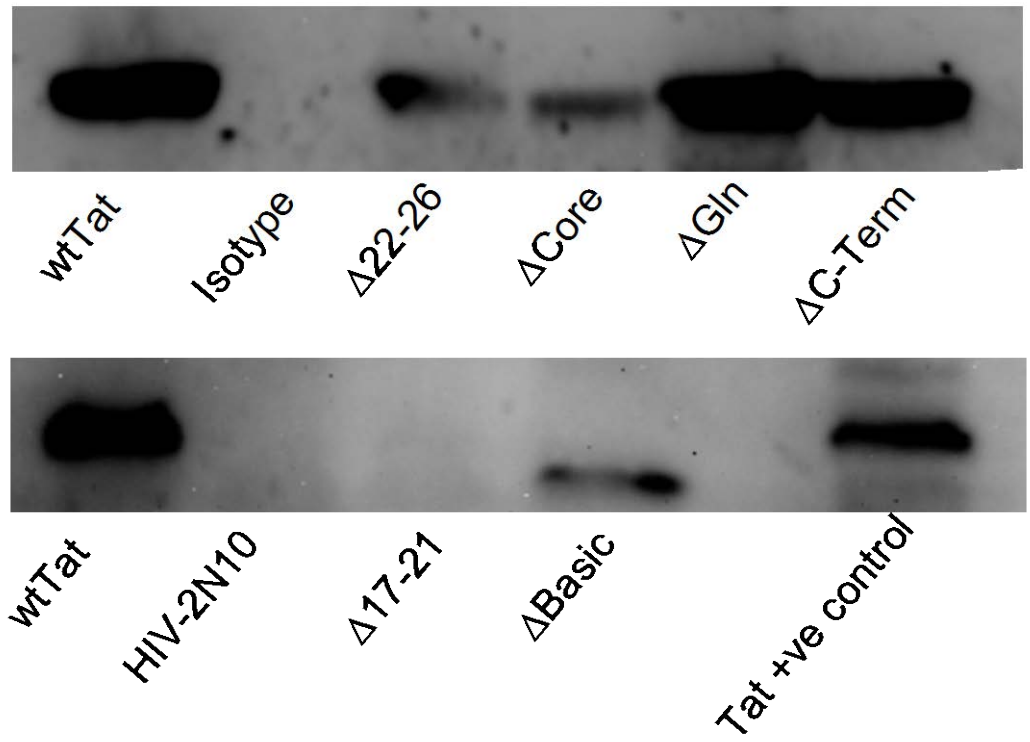

A
IP : Goat anti CD127
CD127 Fc
100 kDa
CD127
B
IP : Goat anti CD127
IP : Goat Isotype
14kDa
Tat

4.3.2 Tat-1 Binds CD127 via its N-terminal Region

Using the Tat6xHis mutant proteins, I next set out to determine which region(s) of Tat are required to interact with CD127. To accomplish this, the Jurkat-CD127 cell line was used, which has previously been shown to downregulate CD127 in response to exogenous soluble Tat (data not shown; personal observation). The panel of Tat6xHis mutant proteins were incubated with lysates from Jurkat-CD127 cells followed by anti-CD127 immunoprecipitation. Ten micrograms of Tat protein was incubated with lysates from $5x10^6$ Jurkat-CD127 cells in a 100 μL volume for 2 hours at 37°C. Anti-CD127 antibodies were added overnight and the next day immune complexes were pulled down with protein G conjugated to sepharose beads. Immunocomplexes were separated by SDS-PAGE and probed for the presence of wild type or mutant Tat6xHis protein by Western blot using monoclonal anti-polyhistidine antibodies.

As shown in figure 29, Δ22-26, ΔCore, ΔGln and ΔC-term Tat mutants, which all induce CD127 downregulation, all co-immunoprecipitated with CD127. Deletion mutants lacking aa 17-21 (Δ17-21) or the first ten aa of Tat (HIV-2N10) did not bind CD127. As shown in chapter 3, these deletion mutants also failed to downregulate CD127, suggesting that Tat must bind to CD127 to induce receptor internalization. Interestingly, the basic region, which is required for CD127 downregulation, is not required for CD127 binding. As shown in figure 29, ΔBasic Tat still co-immunoprecipitated with CD127. Based on the data to this point, I hypothesize that Tat binds CD127 via its N-terminal region and displaces host constitutive receptor recycling machinery. The basic region of Tat then acts to recruit an auxiliary factor which is required to remove CD127 from the cell surface. In the absence of the basic region, Tat is still able to bind CD127 and prevent constitutive recycling, yet is

Figure 29: Tat interacts with CD127 via its N-terminal region. Jurkat-CD127 lysates were treated with either wild type or mutant Tat6xHis then immunoprecipitated with anti-CD127 antibodies. Immune complexes were separated via SDS-PAGE and analyzed by Western blot for polyhistidine. Representative anti-polyhistidine Western blots are shown. Immunoprecipitations were carried out a minimum of three times per condition.


wtTat
Isotype
Δ22-26
ΔCore
ΔGln
ΔC-Term
wtTat
HIV-2N10
Δ17-21
ΔBasic
Tat +ve control

unable to recruit the cellular machinery required to accelerate CD127 internalization. Notably, this model explains how ΔBasic Tat may act to increase surface CD127 expression when produced endogenously from within the cell. (See figure 30.)

4.3.3 Tat Does Not Induce CD127 Y449 Phosphorylation

Since I have shown that Tat binds CD127 to directly affect surface receptor expression, I next investigated whether Tat induces post-translational modifications in CD127. IL-7 binding to its receptor results in phosphorylation of Y449 by Jak1 [203] or Jak3 [133] and subsequent CD127 downregulation. We have shown that, although initial removal of the receptor from the cell membrane can occur in the absence of Y449 phosphorylation, Y449 phosphorylation is required for subsequent CD127 degradation by the proteasome. Since Tat is known to interact with several cellular kinases [374-379], the potential for Tat to induce CD127 phosphorylation in a manner similar to IL-7 was investigated. To accomplish this, CD8 T cells were treated with Tat for 12 hours and then analyzed by Western blot for CD127 Y449 phosphorylation using anti-CD127-pY449 antibodies. Twelve hours was chosen for Tat treatment because the kinetics of Tat-mediated CD127 surface downregulation suggests that it is at this time that the maximum amount of Tat/CD127 interactions are occurring. In parallel, CD8 T cells were treated with IL-7 for ten minutes, as a positive control. Compared to Tat, the kinetics of IL-7-mediated CD127 downregulation are extremely quick and IL-7-induced CD127 Y449 phosphorylation would be expected to occur within minutes of IL-7 treatment. As shown in figure 31, while IL-7 treatment, as expected, readily caused receptor phosphorylation, Tat treatment did not induce any detectable phosphorylation of CD127 at Y449. Although Tat could potentially still

Figure 30: Tat reduces surface CD127 expression on CD8 T cells by binding to the cytosolic tail of the receptor via it's N-terminus, and targets CD127 to the proteasome for degradation via it's basic region. In the absence of Tat's basic region, Tat will bind the receptor and disrupt constitutive recycling machinery, but will not target CD127 to the proteasome. This results in an accumulation of CD127 at the cell surface.

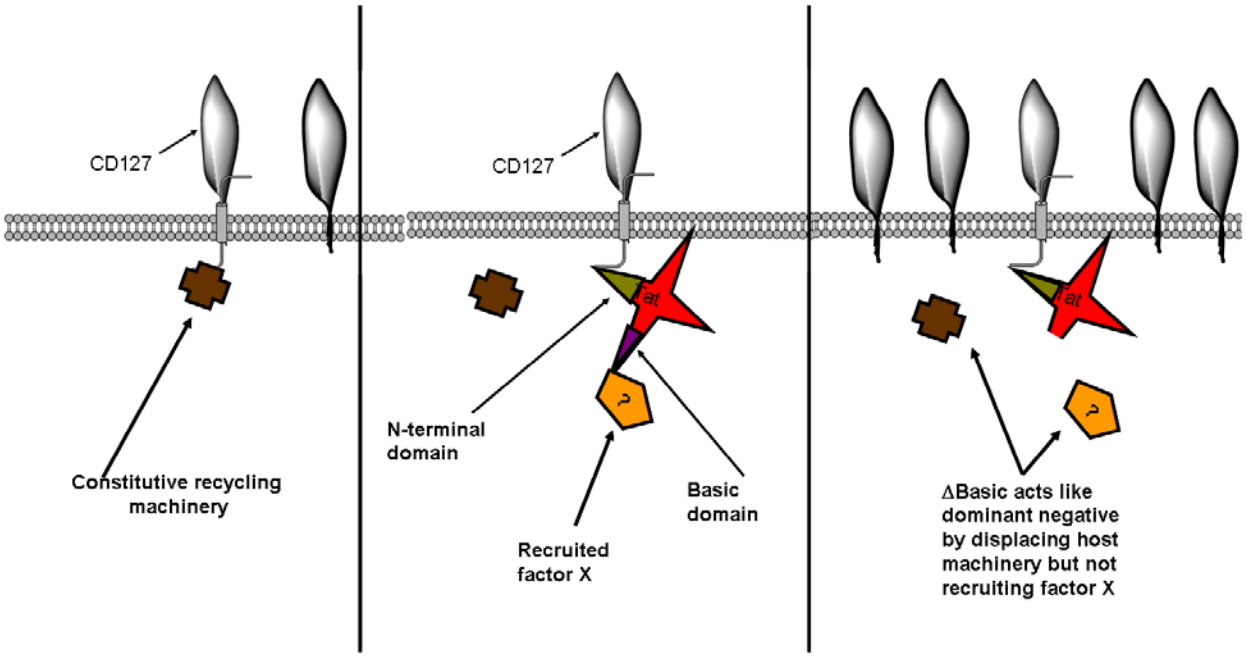
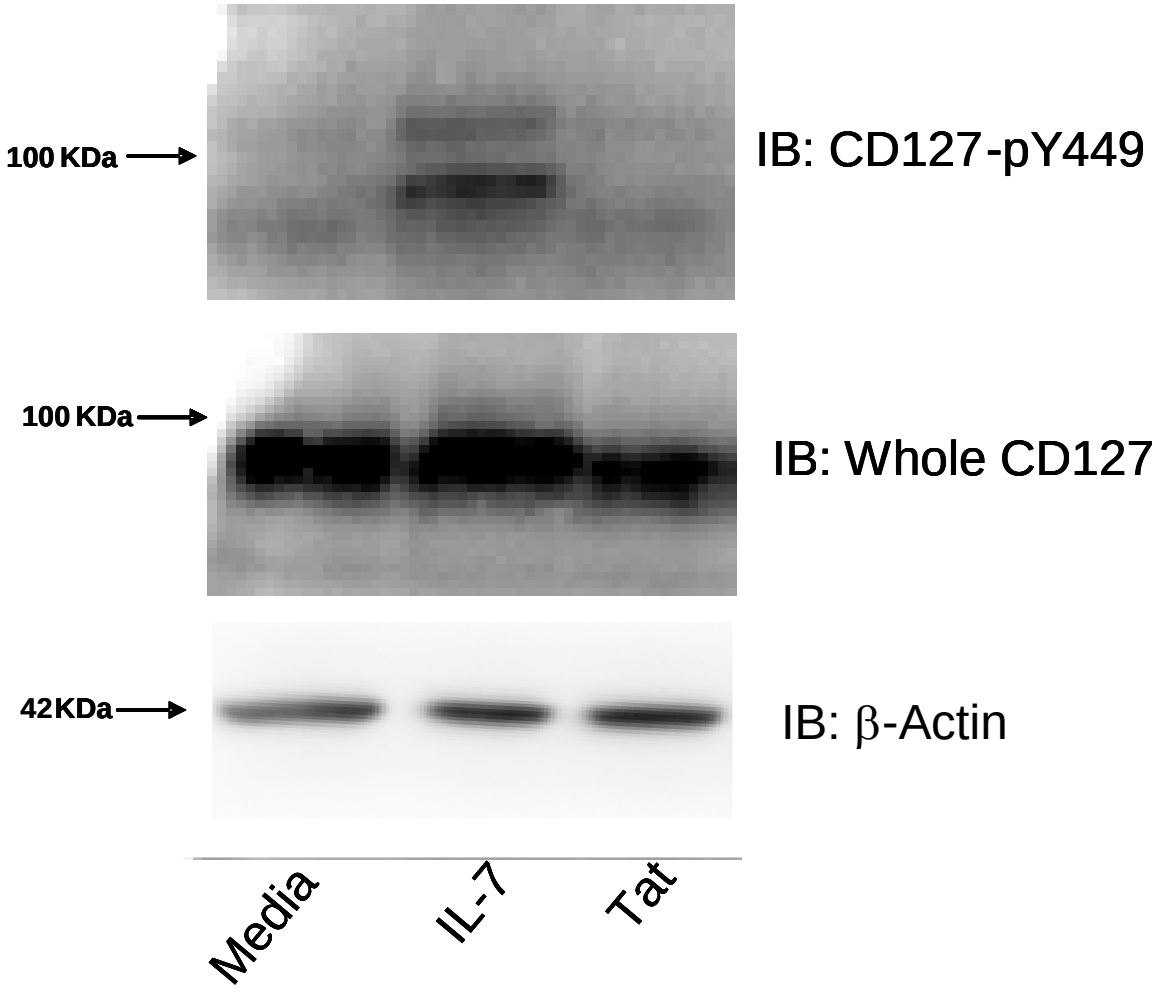

CD127
Constitutive recycling machinery
CD127
N-terminal domain
Recruited factor X
Basic domain
?
ΔBasic acts like dominant negative by displacing host machinery but not recruiting factor X
?

Figure 31: Tat does not induce phosphorylation of CD127 at Y449. Primary human CD8 T cells were treated with either Tat (10 μg/mL) for 12 hours or IL-7 (10 ng/mL) for 10 minutes. CD8 T cells were then lysed. Proteins were separated by SDS-PAGE and analyzed for CD127 phosphorylation of tyrosine at position 449 using goat anti-CD127-pY449 antibodies or CD127 using goat anti-CD127 antibodies. A representative blot is shown. Experiments were run six times per condition.


100 KDa
IB: CD127-pY449
100 KDa
IB: Whole CD127
42KDa
IB: β-Actin
Media
IL-7
Tat

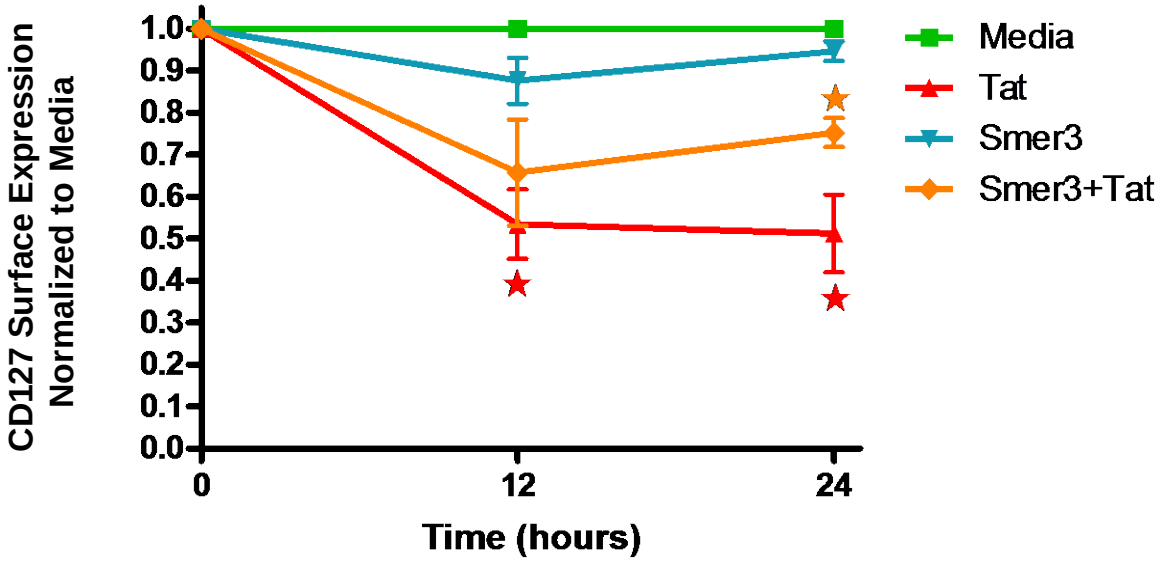
target other Thr, Ser or Tyr residues for phosphorylation, these data indicate that Tat is not targeting CD127 for proteasomal degradation by inducing Y449 phosphorylation in a manner similar to IL-7.

4.3.4 Tat-Mediated CD127 Downregulation Requires Ubiquitin Ligase Machinery

The expression of numerous receptors is known to be regulated by ubiquitination [385, 386]. Tat has been shown to recruit numerous E3 ligases, and Tat is known to be ubiquitinated itself within its basic region [338, 387]. Furthermore, we have shown previously that Tat-induced loss of CD127 is dependent on the proteasome [229]. Notably, we have shown recently that degradation of CD127 in response to IL-7 may depend on the host cell ubiquitination machinery (unpublished observation, Feras Ghazawi). Therefore, the potential role of ubiquitination in Tat-induced CD127 downregulation was investigated.

Purified human CD8 T cells were pretreated with the pan E3 ubiquitin ligase inhibitor Smer3 (1.25 μM) for 1 hour, then incubated with or without Tat protein (10 μg/mL) for 24 hours. Surface CD127 expression was then measured by flow cytometry. Owing to Smer3 cytotoxicity, this inhibitor could only be used at sub-optimal dosages for extended periods of time. As shown in figure 32, whereas Tat treatment alone reduced surface CD127 expression as expected, Smer3 pretreatment prior to Tat resulted in a 50% reduction in Tat-mediated CD127 downregulation when compared to Tat treatment alone at 24 hours. Smer3 treatment alone did not significantly alter CD127 surface expression. As Smer3 pretreatment prior to Tat results in inhibition of the cellular ubiquitination machinery, these data suggest that ubiquitination may play a role in Tat-induced downregulation of CD127 from the surface of CD8 T cells.

Figure 32: Tat-mediated CD127 downregulation is dependent on cellular E3 ubiquitin ligases. Primary human CD8 T cells were pretreated for 1 hour with or without the pan E3 ubiquitin ligase inhibitor Smer3 (1.25 μM). Cells were then treated with or without Tat (10 μg/mL) for an additional 24 hours (n=3). At 12 and 24 hours post-treatment, surface CD127 expression was measured via flow cytometry. Percent change in CD127 expression relative to untreated cells is shown. Error bars represent standard error of the mean (±). * denotes p-values≤0.05.


CD127 Surface Expression
Normalized to Media
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
Time (hours)
Media
Tat
Smer3
Smer3+Tat

4.3.5 Tat Enhances CD127 Ubiquitination

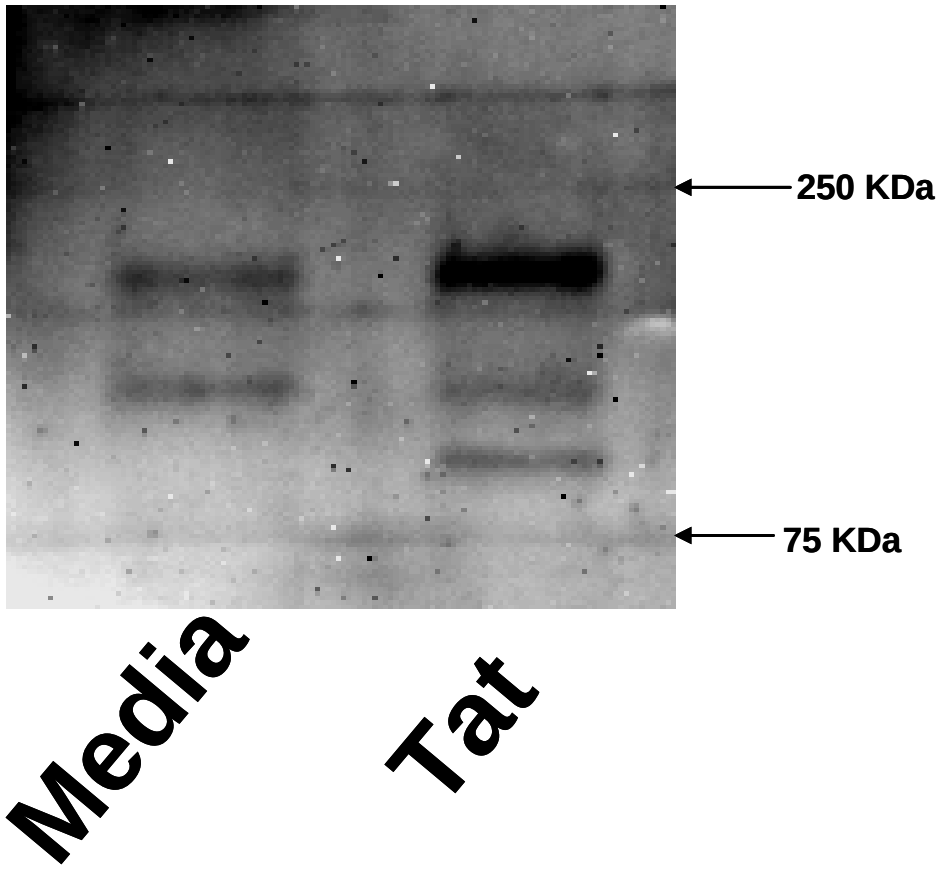
Given the potential requirement of E3 ligases for Tat-mediated CD127 downregulation, I next investigated whether CD127 was ubiquitinated in the presence of Tat in CD8 T cells. To do this, CD8 T cells were treated with Tat (10 μg/mL) for six hours to allow Tat the time required for endocytosis, then MG132 was added to prevent degradation of ubiquitin-tagged proteins, and cells were incubated for and additional eight hours. Cells were then lysed and ubiquitinated proteins were isolated using a ubiquitin affinity resin column. Eluates were analyzed for the presence of CD127 by Western blot. As shown in figure 33, addition of Tat to CD8 T cells resulted in a significant increase in the the intensity and number of bands detected using anti-CD127 antibodies. Given the high molecular weight of these bands, they likely represent poly-ubiquitinated forms of CD127.

4.3.6 Tat Recruits CIS to CD127

Our lab has shown recently that CD127 expression in human CD8 T cells is regulated primarily by the suppressor of cytokine signalling (SOCS) protein family member CIS, which binds CD127 following IL-7 stimulation, likely by associating with phosphorylated Y449 on the cytosolic tail of the receptor [388-392]. CIS may then recruit ubiquitin ligase machinery to induce receptor internalization and degradation [242, 393-395].

Since Tat has been shown to increase CD127 ubiquitination, I questioned whether Tat recruits SOCS proteins to CD127. CD8 T cells were left untreated or treated with Tat at 10 μg/mL for 14 hours, followed by the addition of the proteasome inhibitor MG132 for the last 8 hours to prevent degradation of ubiquitin-tagged molecules. CD127 was then

Figure 33: Tat promotes ubiquitination of CD127. Human CD8 T cells were treated with or without Tat (10 μg/mL) for six hours prior to the addition of the proteasome inhibitor MG132 (5 μM). At 14 hours post Tat treatment, cells were lysed and ubiquitinated proteins were captured on a proprietary ubiquitin affinity resin. Ubiquitin-enriched samples were eluted and boiled in 3xSDS buffer. Samples were separated via SDS-PAGE and analyzed by Western blot for CD127. Experiment was carried out three times per condition.


250 KDa
75 KDa
Media
Tat

immunoprecipitated using anti-CD127 antibodies, and the association of CIS was investigated by Western blot. As shown in figure 34, Tat increased the association of CIS with CD127 in human CD8 T cells. Therefore, it is possible that Tat increases CD127 ubiquitination by recruiting CIS to the cytosolic tail of the receptor.

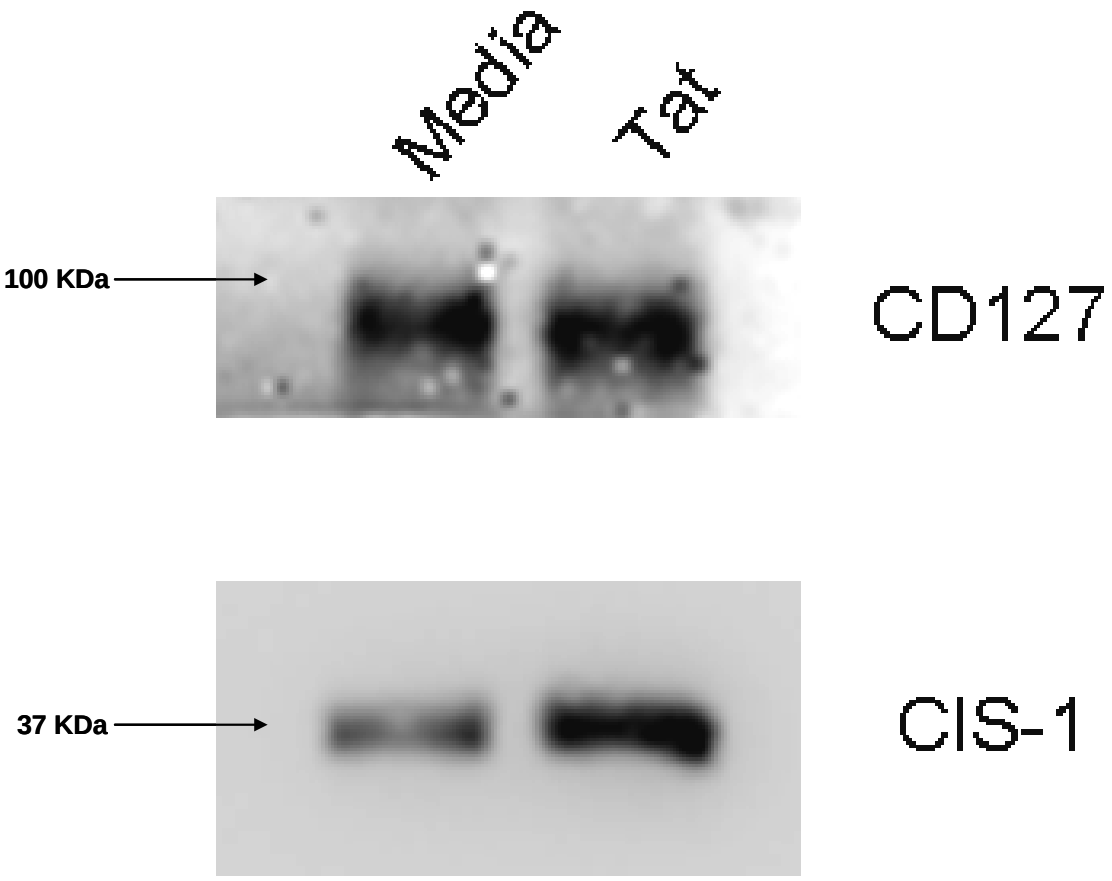
4.4 Discussion

In this chapter, I elucidate several important components of the mechanism by which Tat may downregulate CD127 expression from the surface of primary human CD8 T cells. In the previous chapter, I demonstrated a requirement for both the N-terminal region and basic region of Tat in CD127 downregulation. This requirement was independent of the cellular transduction of Tat, as endogenously produced Tat also required both the N-terminus and basic region to downregulate the receptor.

We have shown previously that Tat will directly induce degradation of CD127. Tat does not affect CD127 *de novo* synthesis or the trafficking of the receptor from the ER/Golgi to the cell membrane [229]. Furthermore, Tat co-localizes with CD127 at the plasma membrane as seen via florescent microscopy [229]. Taken together, these data suggest that Tat acts directly on the CD127 receptor to remove it from the cell surface.

In this chapter (and published elsewhere [229]) I use co-IPs to confirm that Tat binds CD127 in live human CD8 T cells. Human CD8 T cells were treated with wild type Tat protein, then CD127 was immunoprecipitated and immune complexes were investigated for the presence of Tat by Western blot analysis. This work confirms that Tat will bind CD127 in living CD8 T cells. Using co-IP experiments from Jurkat-CD127 lysates, I further demonstrate in this chapter that Tat requires its N-terminus to bind CD127. Jurkat-CD127

Figure 34: Tat recruits CIS to CD127. Human CD8 T cells were treated with or without Tat (10 μg/mL) for 6 hours prior to the addition of the proteasome inhibitor MG132 (5 μM) to prevent proteasomal degradation. At 14 hours post Tat treatment, CD127 was immunoprecipitated using anti-CD127 antibodies. Immune complexes were separated via SDS-PAGE and analyzed by Western blot for the presence of CIS protein. Representative anti-CIS Western blot is shown. Immunoprecipitations were carried out two times per condition.


Media
Tat
100 KDa
CD127
37 KDa
CIS-1

cells were lysed, then incubated with either wild type or mutant Tat proteins. CD127 was then immunoprecipitated and immune complexes were investigated for the presence of wild type or mutant Tat by Western blot analysis. As shown in figure 29, whereas deletion mutants lacking the core, basic, glutamine-rich or C-terminal region of Tat still bound CD127, removal of the N-terminus of Tat prevented Tat from co-immunoprecipitating with CD127. Performing this experiment using whole cell lysates instead of Tat-treated live cells excludes the possibility that loss of ΔN-terminal Tat binding is simply a result of protein mistrafficking.

The region of the cytosolic tail of CD127 bound by the N-terminal region of Tat is currently under investigation in our laboratory. As the N-terminus of Tat contains a PxxPxxxPxxxPxxxP motif, it is likely a semi rigid structure. The first 10 aa of Tat also contain three acidic residues which are negatively charged. Given this, an attractive target for Tat binding is the positively charged region (KKRIK) of CD127 found directly adjacent to the transmembrane domain on the cytosolic side of the protein. A second positively charged sequence is found 18 aa further into the tail of the receptor.

Comparing the N-terminus of Tat-1 (aa 1-10) to that of Tat-2 may offer important clues as to the nature of CD127/Tat interactions. Neither our HIV-2N10 Tat chimera nor wild type Tat-2 caused a downregulation of CD127 from the surface of purified CD8 T cells, again supporting the necessity of the N-terminus of Tat-1 in CD127 binding. This is interesting in light of recent work from Dr A.P. Sousa and colleagues who have shown that individuals infected with HIV-2, a less virulent strain of HIV [396], retain higher CD127 expression on most CD8 T cell populations when compared to HIV-1 infected, age and sex

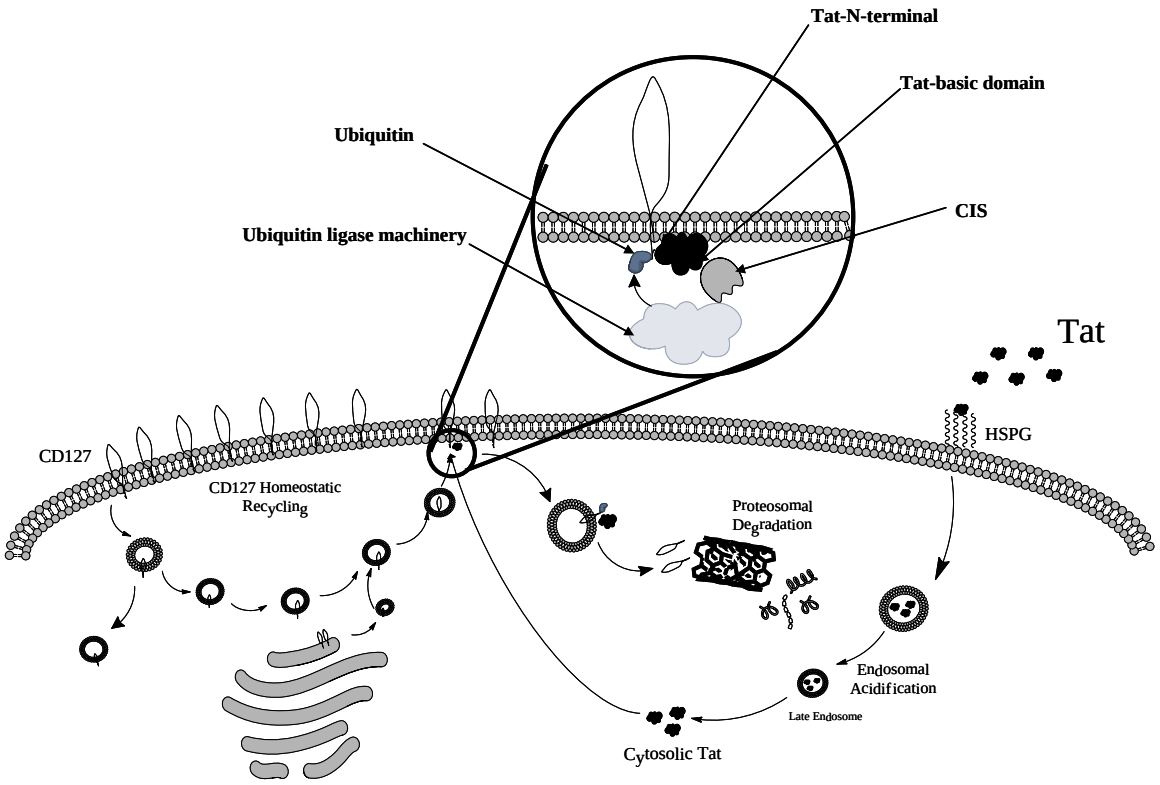
matched counterparts [367]. Taken together, these results further support the role of Tat in HIV pathogenesis.

Although essential for surface CD127 downregulation, the basic region of Tat can be removed without affecting CD127 binding. This observation coupled with the dominant negative behaviour of endogenously expressed ΔBasic Tat shown in chapter 3, I propose a model where Tat binds the cytosolic tail of CD127 via its N-terminus then recruits effector molecules to the receptor via its basic region (see figure 35). In the absence of the basic region, Tat displaces CD127's constitutive homeostatic recycling machinery, yet is unable to remove the receptor from the cell surface, resulting in an accumulation of CD127 on the membrane.

Since IL-7 is known to induce phosphorylation of CD127 at Y449, and since we have shown that this phosphorylation is required for IL-7-induced CD127 degradation, I questioned whether Tat induced phosphorylation of the receptor at the same position. Tat is known to interact with numerous cellular kinases [316, 397, 398], and it was therefore possible that Tat promotes CD127 phosphorylation to signal the subsequent degradation of the receptor. I show here that Tat does not induce the phosphorylation of the canonical signalling residue Y449. Although the CD127 cytosolic tail contains two additional tyrosine residues (Tyr 401 and Tyr 456) which are conserved between mice and humans [203, 211], these residues are not known to support signalling through their phosphorylation.

We have shown recently that IL-7 signalling induces the production of the SOCS family members SOCS2 and CIS. This results in an increased association of these molecules, particularly CIS, with CD127 followed by CD127's removal from the cell

Figure 35: Cytosolic Tat binds CD127 and targets it for proteasomal degradation through CIS-mediated receptor ubiquitin tagging. HIV-1 Tat interacts with heparan sulphate proteoglycans at the surface of CD8 T cells and is then internalized via clathrin coated pits. As the endosomes acidify, Tat escapes to the cytosol in a pH-dependent process. Once in the cytosol, Tat binds CD127's cytosolic tail at the cell membrane via its N-terminal region. Tat then recruits CIS via its basic region. CIS in turn recruits cellular ubiquitin ligase machinery resulting in CD127 ubiquitination. Ubiquitinated receptor is targeted to the proteasome for degradation.


Tat-N-terminal
Tat-basic domain
Ubiquitin
CIS
Ubiquitin ligase machinery
Tat
HSPG
CD127
CD127 Homeostatic Recycling
Proteosomal Degradation
Endosomal Acidification
Late Endosome
Cytosolic Tat

surface and proteasomal degradation. IL-7 induced CD127 downregulation can be blocked by inhibiting the host E3 ubiquitin ligase machinery, suggesting an important role for ubiquitination. Tat is known to recruit the E3 ligase Mdm-2 to induce the ubiquitination and subsequent degradation of several proteins, including microtubule-associated protein 2 (MAP-2) [383, 384] and RON [384]. I therefore investigated whether inhibition of the host E3 ubiquitin ligase machinery prevented Tat-mediated downregulation of CD127. Indeed, suboptimal concentrations of Smer3 did in fact reduce the effect of Tat on CD127 downregulation. I then asked if Tat may be recruiting the same factor, namely CIS to the receptor. I show here that indeed Tat induces the recruitment of the ubiquitination-promoting CIS protein to CD127. I go further to show that Tat also induces the ubiquitination of CD127, although the exact ligase responsible for CD127 ubiquitination remains to be determined.

Tat has been shown to bind and downregulate the receptor RON in monocytes. This mechanism mimics nearly exactly the one elucidated here. Although Tat and RON were not shown to associate physically, Tat induces the ubiquitination of RON, resulting in its surface downregulation and proteasomal degradation [384]. Like the effect of Tat on CD127, Tat's affect on RON is specific for the receptor, as well as independent of transcription and *de novo* protein synthesis. Most interestingly, a Tat mutant containing a glycine and a phenylalanine substitution in the N-terminus between aa 18 and 19 completely prevented Tat-induced RON degradation. I show here that aa 17-21 of Tat are required for CD127 binding and downregulation. Together, these data suggest a possible common mechanism whereby the N-terminus of Tat binds CD127 or RON while the basic region of Tat recruits SOCS family proteins such as CIS which subsequently recruit cellular ubiquitin ligase

machinery to target the receptors for proteasomal degradation. Interestingly both RON [399] and CD127 [400], as well as the common γ-chain [381] have been shown to be ubiquitinated by the Casitas B-lineage Lymphoma (c-Cbl) E3 ubiquitin ligase. It is tempting to speculate that this is the E3 ubiquitin ligase recruited to the receptor complex by CIS.

The effect of Tat may extend beyond CD127 degradation alone. Vranjkovic *et. al.* and others have shown that IL-7 signalling in CD8 T cells from HIV^+ people is inhibited downstream of CD127 binding. CD8 T cells from HIV^+ people displayed impaired CD127-STAT5 signal transduction [253, 401], and STAT5 nuclear localization [402], independent of CD127 surface expression. Similar impairment of downstream signalling has also been observed recently in HIV^+ thymocytes [403]. Furthermore although HIV-1 infection does not downregulate the surface expression of any components of the IL-2 receptor, STAT5 signalling in response to IL-2 stimulation has also been shown to be impaired in HIV-1 infection [404, 405].

In light of the Tat-CD127 interaction demonstrated here, it is a distinct possibility that the effect of Tat on CD127 may go beyond simple proteasomal targeting of the receptor and extend to cytosolic CD127-STAT5 disruption via CIS recruitment. CIS functions both to target receptors for degradation and to suppress Jak/STAT5 signalling [391, 392, 406]. Tat-mediated CIS recruitment may therefore have two distinct effects. Recruitment of CIS by Tat may both target CD127 for proteasomal degradation, as well as prevent IL-7 signalling through the Jak1/STAT5 axis. Indeed, we have previously shown that Tat treatment will render cells completely unresponsive to IL-7 with regards to proliferation and perforin production [352]. In light of the fact that Tat only lowers CD127 surface expression on CD8 T cells by approximately 40%, the ability of Tat to completely shut off IL-7

signalling may result in part from receptor downregulation, and in part from disruption of STAT5 signalling, both via CIS recruitment.

Tat-mediated recruitment of CIS may further explain our observation that Tat in combination with IL-7 will lead to a more sustained downregulation of the receptor than IL-7 treatment alone [181]. This sustained effect is synergistic, and cannot be explained as a result of a simple additive effect of Tat and IL-7 acting separately. Since we have shown previously that IL-7 binding to its receptor results in an increase in *de novo* CIS production, and it is shown here that Tat increases the recruitment of CIS to CD127, Tat/IL-7 synergy may be explained by convergence of both mechanisms on the CIS molecule: IL-7 treatment increases the pool of CIS protein which Tat then recruits to the cytosolic tail of the receptor.

5. Chapter 5: Conclusion

HIV-1 is a major cause of morbidity and mortality across the world. Although effective antiretroviral treatment has helped infected individuals live longer and healthier lives, no significant change in overall infection rates have been noted in the past decade. Furthermore a viable vaccine or cure has yet to be discovered.

Several major obstacles to an effective permanent solution to HIV-1 infection are apparent. A major barrier to HIV-1 eradication is the early establishment of treatment-impervious viral reservoir in resting memory CD4 T cells. A small population of activated CD4 T cells revert to a quiescent memory phenotype [76, 77]. These cells do not produce viral proteins and are hence not identified by CTLs as targets for destruction, nor is viral replication inhibited by ART treatment [79, 80]. Another major hurdle to HIV iradication is the efficiency with which it avoids and evades immunosurveillance. HIV-1 is an RNA virus that codes for its own error-prone reverse trasnscriptase. As such, the HIV-1 genome has an extremely high rate of mutation, allowing the virus to escape elimination by the adaptive immune response. HIV-1 also achieves immune dysregulation by various mechanisms, including altered surface expression of numerous receptors critical for proper immune system function, resulting in disorders in immune signalling.

Many HIV-1 proteins downregulate cell surface molecules. For example HIV-1 Vpu has recently been shown to bind CD317 (tethrin) [407] in the Golgi network, preventing antegrade transport of *de novo* CD317 and homeostatic recycling of CD317 from endosomes to the cell surface [408]. Instead, Vpu shunts the protein towards proteasomal degradation. This allows HIV-1 to bud properly from the cell membrane. HIV-1 Nef has been shown to interact with surface molecules and downregulate the surface expression of CD4, CD28,

MHCI and Fc gamma receptors [409-411]. As discussed previously, HIV-1 Tat itself is already known to downregulate the expression of numerous receptors critical for proper immune function from the surface of PBMCs including MHCI [312, 318, 412] β2-microglobulin [311], CD25 [319], and the mannose receptor [413], amongst others [414].

Our lab has shown previously that CD127 is downregulated from the surface of CD8 T cells during HIV-1 infection [193]. This observation has since been corroborated by others [194, 196, 236-238]. Since this initial observation, we have shown that HIV-1 Tat protein downregulates CD127 from the surface of CD8 T cells isolated from healthy individuals *in vitro* [352]. Tat enters the cell by endocytosis then co-localizes with CD127, leading to receptor internalization and proteasomal degradation [229]. The affect of Tat is specific for CD127. Tat does not alter the surface expression of numerous other surface receptors including the common γ-chain (CD132), CD3, CD2, and CD28 [352]. Furthermore, the effect of Tat is direct and independent of *de novo* protein transcription or translation. Tat does not affect CD127 mRNA levels nor alter the trafficking of *de novo* CD127 from the endoplasmic reticulum to the cell membrane [229]. Tat also does not require Jak1-mediated signalling to downregulate CD127 [181].

The goal of my thesis research was to better understand Tat/CD127 interactions and further elucidate the mechanism by which Tat removes CD127 from the cell surface to target it for proteasomal degradation. Here, I define the regions of Tat necessary for downregulation of CD127 from the surface of CD8 T cells and suggest that Tat recruits an E3 ubiquitin ligase to the receptor to target CD127 to the proteasome for degradation.

In chapter 2, a series of plasmids encoding for Tat deletion mutants linked to a C-terminal polyhistidine tag were created (see figures 11-14, 22). These plasmids were used to

express and purify a panel of Tat mutant proteins from *E. coli*. To the best of my knowledge, this is the first comprehensive series of Tat mutant proteins to be generated. In addition, the same series of mutant Tat coding sequences was transferred into a eukaryotic expression system to allow for endogenous production of Tat within primary human CD8 T cells and the Jurkat lymphoblastic cell line at relatively equivalent levels (see figure 23).

Using the series of Tat deletion mutants generated in chapter 2, chapter 3 of this thesis shows that aa 22-26, the core, glutamine-rich and C-terminal regions of Tat could be removed without loss of effect, whereas Tat lacking either the N-terminal regions (i.e. aa 1-10 or 17-21) or the basic region was unable to downregulate CD127 (see figure 24). Since these regions are also required for exogenous Tat to transduce the cell membrane, cells were transfected to express Tat endogenously. In this experiment, the N-terminal and basic regions were still required for CD127 downregulation (see figure 25). Hence, these regions are absolutely essential for Tat-mediated downregulation of surface CD127 expression from the surface of primary human CD8 T cells independently of their role in the transduction of Tat through the cell membrane. At this point it is interesting to note that in natural infection the predominant anti-Tat antibody responses [415-417] as well as major CD4 [418] and CD8 [125, 419-421] T cell epitopes of Tat have been mapped to aa 1-20, and 36-50 (i.e. the N-terminal and basic regions). These regions are also highly conserved across clades [417], consistant with their absolute requirement for productive HIV-1 infection [303]. Not surprisingly, Tat specific antibodies targeting these regions will neutralize cross-clade Tat variants [244].

Given that Tat and CD127 co-localize at the cell membrane [229], I next asked whether Tat/CD127 physical interactions were required for Tat-mediated receptor

internalization. CD127 was immunoprecipitated from Tat-treated CD8 T cells and Tat co-immunoprecipitation was investigated by Western blot. As shown in figure 28, Tat did indeed associate physically with the receptor in primary human CD8 T cells, confirming previous florescent microscopy experiments [229]. I next asked what regions of Tat are required to bind the receptor. To address this, lysates from Jurkat-CD127 cells were treated with wild type or mutant Tat and CD127/Tat co-immunoprecipitation was investigated as done for CD8 T cells. As seen in figure 29, all the regions that were dispensable for CD127 surface downregulation (aa 22-26, core region, glutamine-rich region, C-terminal region) were also dispensable for CD127 binding. This observation lends further support for the requirement of Tat/CD127 interactions for Tat-mediated CD127 downregulation. I show here that the N-terminal, but not basic region of Tat is required for Tat to bind CD127. Given the requirement of both these regions for CD127 surface downregulation, I propose a model whereby Tat binds CD127's cytosolic tail via its N-terminal region, and recruits a cellular factor to the receptor using its basic region (see figure 30).

Interestingly, whereas removal of the N-terminal region completely abrogated the effect of endogenously produced Tat, removal of the basic region resulted in a two fold increase in surface CD127 expression over 48 hours. Our lab has previously observed that CD127 constitutively cycles on and off the cell surface with a relatively long protein half-life of approximately 55 hours. In the absence of the basic region, I propose that Tat binds the receptor and disrupts homeostatic recycling processes, yet is unable to target CD127 for accelerated removal from the cell membrane and proteasomal degradation. This results in an accumulation of CD127 on the surface of the cell (see figure 30).

Also in chapter 4, the cellular factors recruited to the cytosolic tail of CD127 by Tat are investigated. I showed that Tat-mediated CD127 downregulation is affected by cellular E3 ubiquitin ligases. Partial inhibition of E3 ubiquitin ligase activity resulted in a partial block to CD127 downregulation (see figure 32). I also demonstrated that Tat recruits CIS to CD127 (see figure 34). CIS is a protein that is known to bind the E3 ligase complex composed of elongin B/C, Cullin5 and Rbx to downregulate receptors and target them for proteasomal degradation [241-244]. In view of this, it is perhaps not surprising that Tat treatment results in the ubiquitination of CD127 (figure 33). Consistent with this, we have shown previously that inhibition of the proteasome will abrogate the effect of Tat on CD127 [352].

Given these observations, I propose a mechanism by which Tat downregulates CD127. Tat binds HSPGs on the surface of CD8 T cells and enters the cell via endocytosis [288]. Tat is able to escape the endosome as it acidifies in a mechanism that is dependent on interactions between Tat's Trp11, Tat's basic region and the cell membrane [333], and is facilitated by Hsp90 [288]. Cytosolic Tat then traffics to the inner leaflet of the cell membrane where it binds the cytosolic tail of CD127 via its N-terminal region. Once bound, Tat both disrupts constitutive homeostatic recycling of CD127 and recruits CIS protein to the receptor's cytosolic tail. CIS in turn recruits host ubiquitin ligase machinery which ubiquitinates CD127, tagging it for internalization and subsequent proteasomal degradation. See figure 35 for the proposed model.

We have previously shown that Tat localizes with CD127 at or near the cell membrane and in punctuate structures within CD8 T cells [229]. The exact location of initial CD127/Tat interaction however, has yet to be completely determined. Visualizing

endosomal structures within CD8 T cells remains a difficult challenge, as these cells contain a relatively small volume of cytosolic space. It is difficult to distinguish the cell membrane from endosomal structures, even using florescent co-localization markers. Tat may be acting on the cytosolic tails of CD127 molecules at numerous locations, perhaps binding the receptor at the cell surface and/or early endosomes to alter its trafficking, shunting the receptor towards proteasomal degradation.

Also, whether Tat acts as a stable bridge between CIS and CD127 or acts only transiently to bring CIS into close proximity to CD127, allowing CIS to bind directly to the receptor has yet to be determined. We have shown previously that CIS will associate with CD127 in response to IL-7, and that this interaction is dependent on CD127 Y449 phosphorylation and Jak1/STAT5 signalling. CIS contains an SH2 domain and many members of the SOCS family are known to bind phosphorylated tyrosine residues [422]. Although definitive proof is still required, it is therefore our hypothesis that in response to IL-7 treatment, CIS transcription is upregulated, and *de novo* CIS protein binds directly to CD127 by recognizing pY449 via its SH2 region. I establish in this work that Tat does not induce Y449 phosphorylation (figure 31) and Tat is therefore most likely not transferring CIS to CD127 in a pY449 dependent manner. I demonstrate here that Tat itself binds directly to the CD127 receptor via its N-terminal region, and show that Tat binds CIS. We have further shown that ΔBasic Tat will act in a domainate negative manner to increase surface CD127 expression, suggesting that ΔBasic Tat is able to bind the receptor to disrupt constituative endosomal cycling, yet is unable to recruit the effector molecule(s) required to accelerated CD127 internalization. We propose therefore that Tat acts as a stable bridge

between CD127 and CIS, allowing for CIS-mediated ubiquitination of the receptor in the absence of IL-7 signalling.

Although we show here a definitive role for the ubiquitin tagging of CD127 in Tat-mediated downregulation of the receptor, the exact nature of the ubiquitin linkages have yet to be determined. Ubiquitin forms covalent bonds by attaching to lysine residues of target proteins. Ubiquitin itself has seven lysine residues at positions 6, 11, 27, 29, 33, 48, and 63, offering a large variety of possible polyubiquitin linkages. Monoubiquitination and polyubiquitination represent different molecular messages. Monoubiquitination is known to control the trafficking of membrane proteins [423, 424]. While Lys63 linkages are are found predominantly on mammalian membrane protein which are known to traffic to lysosomes [425, 426], Lys48 linkages are well known to be a prerequisite for recognition by the proteasome prior to degradation [427, 428]. The ubiquitin affinity resin used to perform the experiment depicted in figure 33 has a much greater affinity for polyubiquitin, suggesting that these bands represent multi-ubiquitinated forms of the receptor. Indeed, the high molecular mass of the bands detected with anti-CD127 antibodies suggests that these bands represent highly ubiquitinated forms of the receptor. Given the multiple roles of ubiquitin in both receptor trafficking and proteasomal degradation, it is possible that monoubiquitination occurs first altering trafficking followed by poly-ubiquitination to direct CD127 to the proteasome. These possibilities remain to be investigated.

This is far from the first example of a viral protein targeting a cellular protein for degradation. In fact, many viruses from diverse taxonomic origins manipulate cellular E3 ubiquitin ligases, and many viruses encode for their own E3 ligase homologues [429]. HIV-1 itself exploits the host cellular ubiquitin machinery to target various anti-viral molecules to

the proteasome. Vpr has also been shown to induce the ubiquitination and proteasomal degradation of uracil DNA glycosylase [430], while Vpu recruits the E3 ubiquitin ligase complex SCF-βTrCP to CD4 to induce its degradation by triggering the endoplasmic reticulum associated degradation (ERAD) pathway [200]. Vif induces the polyubiquitination and subsequent proteasomal degradation of APOBEC3G by acting as an adaptor protein linking APOBEC3G to the Cul5-elongin B/C complex [431].

As well as interacting directly with the proteasome [305, 432], Tat protein has previously been shown to recruit cellular ubiquitination machinery. Tat induces the ubiquitination and subsequent proteasomal degradation of proteins such as Tip60 [383], MAP2 [433] and RON [384]. Indeed, the similarity of the effect of Tat on RON and CD127 is striking and suggests a similar mechanism. The N-terminal region of Tat may bind the cytoplasmic tails of both RON and CD127, and then Tat may recruit CIS via its basic region. CIS in turn may recruit cellular ubiquitination machinery to ubiquitinate the receptor and target it for degradation. Interestingly, sequence alignment reveals that both RON and CD127 share a "LPPP" motif within their cytosolic tails. Although no functions have been ascribed to this motif, the fact that it is common to both these Tat-targeted receptors identifies it as a putative Tat binding site, or a ubiquitin acceptor lysine. These possibilities remain to be investigated.

Furthermore, as SOCS family members are known to both downregulate receptor expression as well as attenuate JAK1/STAT signalling [394], Tat may operate to disrupt IL-7 signalling downstream of surface receptor expression via recruitment of CIS. In fact, CD8 T cells from HIV^+ people display impaired CD127-STAT5 signal transduction [253, 401, 405], and STAT5 nuclear localization [402]. As CIS blocks STAT5 signalling [391, 392, 406],

Tat may both target CD127 for proteasomal degradation and prevent IL-7 signalling through STAT5 impairment, offering a second level of Tat-induced control over the IL-7 signalling axis.

Studies have demonstrated an inverse correlation between the degree of Tat-specific humoral [284] and cell-mediated [132, 421] immunity and HIV disease progression, with LTNPs and slow progressors having stronger anti-Tat adaptive immune responses [132, 244, 421]. Anti-Tat antibody titres are inversely correlated to serum HIV p24 levels [284], particularly in individuals with natural anti-Tat IgM production early in infection [434, 435]. CTL responses against Tat also correlate with earlier containment of the initial acute infection [405, 436-438].

Given the strong correlation between anti-Tat immunity and favourable prognosis, disrupting the function of Tat may prove to be therapeutically beneficial. Indeed, investigations into the use of Tat as a preventative or therapeutic vaccine are currently being undertaken. Preclinical [439-441] and phase I [442-445] studies using Tat as a part of vaccine regimens have been successfully completed and showed Tat to be safe for human injection. An open label phase II clinical trial using full-length active Tat to therapeutically treat ART non-responders showed that Tat is safe and immunogenic [446]. Tat vaccinations induced significant cellular immune responses against HIV-specific antigens. Tat vaccination also resulted in the production of anti-Tat antibodies [446], and an increase in $CD4^+$ and $CD8^+$ T cell responses against Env and Tat antigens. Tat vaccination also resulted in significant recovery of B cells and CD4 T cells, a decrease in $CD4^+$ regulatory T (Treg) cell counts, and a re-balancing of naive, memory and effector CD4 and CD8 T cell subsets, with immune-compromised individuals experiencing the greatest therapeutic benefits [443,

446]. Furthermore, Tat immunization resulted in the reduced expression of the activation markers HLA-DR and CD38, which are not reverted under ART treatment alone [446].

Targeting the interaction of HIV-1 Tat with CD127 in this manner may prove a fruitful avenue for novel treatment. Tat-targeting therapies could help to retain a functional IL-7 signalling axis, and in this manner be used to boost vaccines and/or supplement ART therapy. Indeed, recent investigations have shown that vaccination with Tat and gp120 protected monkeys against viral challenge better than gp120 vaccination alone, with protection correlating with CTL activity to non-Tat antigens [447]. Hence, targeting Tat strengthens the CTL-mediated immune response to non-Tat viral epitopes. It is tempting to speculate that Tat-neutralizing antibodies may strengthen CTL responses through the reconstitution of the IL-7 signalling axis. As IL-7 has been implicated in T cell lymphopoesis and CD8 T cell effector function, many of these immuno-beneficial effects may in part be due to the reconstitution of the IL-7 signalling axis.

Novel therapeutics specifically designed to target the N-terminal and basic regions of Tat may prevent CD8 T cell anergy through sustained higher CD127 expression. If Tat/CD127 interactions could be targeted and prevented, sustained CD127 expression on CD8 T cells could allow the individual's immune system to maintain strong functional CTL responses in the face of HIV-1 infection. Restoration of the IL-7 signalling axis could also help to regulate T cell homeostasis and the establishment of memory T cells. As CTL immunity is a major contributor to the control of HIV-1 infection, robust CD8 T cell mediated immune responses could potentially contain chronic HIV-1 infection or even favour immune clearance of the virus. In addition, anti-Tat immunity may contribute to a sterilizing vaccine, as vaccine-induced immunity may negate Tat-mediated CD127

interference from the onset of infection. This could allow a sustained and healthy CD8 T cell-mediated immune response to eradicate the infection prior to it establishing a beach head within the body.

Finally, as IL-7 treatment has been shown to stimulate virus production from the long lived viral CD4 T cell memory reservoirs, IL-7 therapy in combination with anti-Tat vaccination may help to purge the body of this elusive population.

6. References

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443. Bellino, S., et al., *Parallel conduction of the phase I preventive and therapeutic trials based on the Tat vaccine candidate.* Rev Recent Clin Trials, 2009. **4**(3): p. 195-204.
444. Ensoli, B., et al., *The preventive phase I trial with the HIV-1 Tat-based vaccine.* Vaccine, 2009. **28**(2): p. 371-8.
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446. Ensoli, B., et al., *Therapeutic immunization with HIV-1 Tat reduces immune activation and loss of regulatory T-cells and improves immune function in subjects on HAART.* PLoS One, 2010. **5**(11): p. e13540.
447. Ferrantelli, F., et al., *A combination HIV vaccine based on Tat and Env proteins was immunogenic and protected macaques from mucosal SHIV challenge in a pilot study.* Vaccine, 2011. **29**(16): p. 2918-32.

7. Contribution of Collaborators

Samantha Burugu—Did much of the Tat-2 work, including Western blots, protein production and flow cytometry

Feras Ghazawi—Did several of the flow cytometry time points for figure 10A, as well as credit for conceptual design.

Jessica Steinmoeller—Designed the strategy used to generate the deletion mutant coding sequences inserted into the pTatC6H-1 vectors.

8. Curriculum Vitae

Scott Sugden

Education

Doctorate of Philosophy: Immunonology	Microbiology and Immunology, University of Ottawa	May. 2007 to Present
Bachelor's of Science with Honour's in Biochemistry	University of Ottawa	Sept. 2003 to April. 2007

Academic Distinctions

2012 University of Ottawa Excellence Scholarship.
2012 University of Ottawa Department of Biochemistry, Microbiology, and Immunology Poster Day Competition, Most attractive poster.
2012 University of Ottawa Department of Biochemistry, Microbiology, and Immunology Poster Day Competition, awarded 2nd place.
2011 University of Ottawa Excellence Scholarship.
2011 Ontario HIV Treatment Network (OHTN) Research Conference Travel Grant.
2010 University of Ottawa Excellence Scholarship.
2010 OHTN Research Conference Travel Grant.
2010 CROI Young investigator award.
2009 OHTN Research Conference Travel Grant.
2009 Canadian Institute of Health Research (CIHR) Doctoral Research Scholarship 2009-2012.
2009 University of Ottawa Dean's Scholarship.
2009 University of Ottawa Excellence Scholarship.
2008 OHTN 2008 Research Conference Travel Scholarship.
2008 Canadian Institute of Health Research Master's Scholarship.
2008 University of Ottawa Excellence Scholarship.
2007 OHRI Research Day Graduate Student Poster Competition, awarded 3rd place.
2007 OHTN 2007 Research Conference Travel Grant.
2007 Ontario Graduate Scholarship in Science and Technology.
2007 University of Ottawa Excellence Scholarship.
2007 University of Ottawa Merit Scholarship for Outstanding Academic Achievement.
2007 University of Ottawa Dean's Honour's List.
2006 Member of the Chemical Institute of Canada.
2006 University of Ottawa Merit Scholarship for Outstanding Academic Achievement.
2006 Janssen-Ortho Studentship Award, Association of Medical Microbiology and Infectious Diseases (AMMI) Canada
2006 University of Ottawa Dean's Honour's List.

Research Experience

Sept.2007 - Present	*Molecular characterization of the HIV Tat protein and its interaction with cellular proteins in downregulating CD127 on CD8 T-cells.* **P.I. Dr. Paul MacPherson.** University of Ottawa, Department of Biochemistry, Microbiology and Immunology.
May 2007-Aug. 2007	*CD8 T-cells from Long Term Nonprogressors are resistant to interleukin-7 receptor downregulation by HIV Tat protein.* **P.I. Dr. Paul MacPherson.** University of Ottawa, Department of Biochemistry, Microbiology and Immunology.
Sept. 2006 – May 2007	*Characterization of the CD127 gene promoter.* **P.I. Dr. Paul MacPherson.** University of Ottawa, Department of Biochemistry, Microbiology and Immunology.

Leadership Roles--Trainees Under Direct Tutelage

Honour's Students	4
Master's Students	1
Medical Students	1
Volunteers	2

Other Teaching Experience and Tutoring

2011	University of Ottawa Faculty of Medical Problem Based Learning Study Group Leader: HIV Unit.
2011	Physiology and Biology Tutor
2007-2012	Summer/Honour's Student Supervisor. (8 Students Total).
2006	Biomechanics Tutor.
2005	Grade 11/12 Mathematics Tutor.
2005	Molecular Biology Laboratory Mentor.
2004-2005	Physics Tutor.

Knowledge Transfer and Community Outreach Activities

2011	Encounters with Canada volunteer: HIV/infectious disease.
2010	Oral Presentation on sexually transmitted infections at Talitha House Talk female detention center.
2009	Oral Presentation on Novel HIV vaccines at AIDS Committee of Ottawa.
2009	Dignitas International Youth Race for Dignity.
2008	H.O.P.E Research Fundraiser.
2008	Introductory Speaker-*Chemical Condoms: Fact of Fiction* Forum.
2008	Canadian Institute of Health Research funded *Café Scientifique* volunteer.
2007	Reviewer of *A Practical Guide to Nutrition* for the Canadian AIDS Treatment Information Exchange (CATIE).
2007	H.O.P.E Research Fundraiser.
2007	Plan Canada child sponsor.
2007	International Health Promotions and Kilimanjaro Black Students' Association World AIDS Day Fundraiser.
2007	The Farha Foundation's 15th Annual AIDS Walk.
2006	AIDS Walk for Life.
2006	H.O.P.E Research Fundraiser.
2005	Food donation volunteer for St. Joseph's church.
2002	Canadian Cancer Society Relay for Life.

1997-1999 March of Dimes volunteer.
1997-1999 St. John's Ambulance volunteer.

Oral Presentations

1. **Sugden, S.** and P. MacPherson (2012). *Molecular Characterization of the HIV Tat Protein and its Ability to Downregulate CD127 on CD8 T-Cells*. Annual Ottawa Hospital Research Institute Research Conference. Ottawa, Ontario, Canada.
2. **Sugden, S.**, F. Al-Ghazawi and P. MacPherson (2011). *HIV Tat Protein Binds the Cytoplasmic Tail of CD127 via Tat's Amino-Terminal Domain, Resulting in Downregulation of CD127 from the Surface of CD8 T cells.* Ontario HIV Treatment Network Annual Research Conference. Toronto, Ontario, Canada.
3. **Sugden, S.** and P. MacPherson (2011). *Molecular Characterization of the HIV Tat Protein and its Ability to Downregulate CD127 on CD8 T-Cells*. 20th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.
4. **Sugden, S.** and P. MacPherson (2010). *Molecular Characterization of the HIV Tat Protein and Its Ability to Downregulate CD127 on CD8 T-Cells*. Ontario HIV Treatment Network Annual Research Conference. Toronto, Ontario, Canada.
5. **Sugden, S.** (2010) *CD127 is Downregulated in HIV Infection*. First Annual Symposium on Interleukin-7 in HIV-1 Infection. Montreal, Quebec, Canada.
6. **Sugden, S.**, I. E, J. Steinmöeller, P. MacPherson (2009). *Mutational Analysis of the HIV Tat Protein and Its Ability to Downregulate CD127 from the Surface CD8 T cells.* Ottawa Hospital Research Instititute Annual Conference. Ottawa, Ontario, Canada.
7. **Sugden, S.**, I. E, J. Steinmöeller, P. MacPherson (2009). *Mutational Analysis of the HIV Tat Protein and Its Ability to Downregulate CD127 on CD8 T cells.* Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.

Published Abstracts

1. **Sugden, S.** and P. MacPherson (2012). *HIV Tat Protein binds the cytoplasmic tail of CD127 via Tat's N-terminal domain resulting in downregulation of CD127 from the surface of CD8 T cells*. International AIDS Society AIDS 2012 Conference, Washington, DC, USA.
2. Al-Ghazawi, F., P. Parmar, E. Faller, **S. Sugden**, N. Sant and P. MacPherson (2012). *Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and the Interactions with the IL-7 Receptor Alpha Chain (CD127) may Play a Role in Regulating CD127 Expression in Human CD8 T Cells.* International AIDS Society AIDS 2012 Conference, Washington, DC, USA.
3. **Sugden, S.** and P. MacPherson (2012). *HIV Tat Protein Binds the Cytoplasmic Ttail of CD127 via Tat's N-terminal Domain Resulting in Downregulation of CD127 from the Surface of CD8 T cells*. 21th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.
4. Al-Ghazawi, F., P. Parmar, **S. Sugden** and P. MacPherson (2012). *Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and May Play a Role in Regulating CD127 Expression*. Ontario HIV Treatment Network Annual Research Conference. Toronto, Ontario, Canada. 21th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.
5. Al-Ghazawi, F., P. Parmar, **S. Sugden** and P. MacPherson (2012). *Suppressor of Cytokine Signalling (SOCS) Proteins are Induced by IL-7 and May Play a Role in Regulating CD127 Expression*. Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.
6. Faller, E., F. Al-Ghazawi, **S. Sugden**, J. Kakal and P. MacPherson (2011). *HIV Tat and IL-7 Downregulate Surface Expression of the IL-7 Receptor Alpha-Chain Through Overlapping*

Pathways. 20th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.

7. Faller, E., J. Kakal, F. Al-Ghazawi. **S. Sugden** and P. MacPherson (2011). *HIV Tat and IL-7 Downregulate Surface Expression of the IL-7 Receptor Alpha-Chain Through Overlapping Pathways*. Keystone Symposia: HIV Evolution, Genomics and Pathogenesis. Whistler, British Columbia, Canada.
8. Faller, E., **S. Sugden** and P. MacPherson (2010). *Therapeutics Designed to Neutralize Soluble HIV Tat Protein Could Preserve IL-7 Signaling and CD8 T-Cell Function in HIV+ Patients*. The Tenth International Congress on HIV and Drug Therapy in HIV Infection. Glasgow, UK.
9. Faller, E., F. Ghazawi, **S. Sugden** and P. MacPherson (2010). *IL-7 induces clathrin mediated internalization and proteasomal degradation of the IL-7R alpha-chain in CD8 T-cells*. Ontario HIV Treatment Network Annual Research Conference. Toronto, Ontario, Canada.
10. Faller, E., **S. Sugden**, M. McVey and P. MacPherson (2010). *HIV Tat-induced degradation of CD127 protein is dependent on endosomal trafficking.* International Symposium on Vesicular Trafficking and Cellular Signaling. Montreal, Quebec, Canada.
11. **Sugden, S.**, I. E., J. Steinmoeller and P. MacPherson (2010). *Mutational Analysis of the HIV Tat Protein and Its Ability to Downregulate CD127 on CD8 T-Cells*. 17th Conference on Retroviruses and Opportunistic Infections. San Francisco, CA, USA.
12. Kakal, J., **S. Sugden** and P. MacPherson (2009). *Regulation of the IL-7 Receptor Alpha-Chain in Primary Human Cytotoxic T-Cells.* Ontario HIV Treatment Network Annual Research Conference. Toronto, Ontario, Canada.
13. **Sugden, S.** and P. MacPherson (2009). *Analysis of the HIV Tat Protein and its Function in Downregulating CD127 on CD8 T-Cells.* 18th Annual Canadian Conference on HIV/AIDS Research. Vancouver, British Columbia, Canada.
14. **Sugden S.**, J. Steinmöeller, P. MacPherson (2008). *Characterization of the HIV Tat Protein and Its Interactions with Cellular Proteins in Downregulating CD127 on CD8 T cells.* 17th Annual Canadian Conference on HIV/AIDS Research. Montreal, Quebec, Canada.
15. **Sugden, S.**, J. Steinmöeller and P. MacPherson (2008). *Characterization of the HIV Tat Protein and Its Interactions with Cellular Proteins in Downregulating CD127 on CD8 T cells.* Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.
16. Kakal, J., **S. Sugden**, E. Faller and P. MacPherson (2008). *Transcriptional Regulation of the CD127 Gene in CD8 T-cells*. Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.
17. Faller, E., J. Kakal, M. McVey, **S. Sugden** and P MacPherson (2008). *HIV Impairs CD8 T-cell function by removing the IL-7 Receptor alpha-chain from the cell surface and targeting it for degradation.* Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.
18. **Sugden, S.**, J. Steinmöeller and P. MacPherson (2008). *Characterization of the HIV Tat Protein and Its Interactions with Cellular Proteins in Downregulating CD127 on CD8 T cells.* Ottawa Health Research Institute Research Day. Ottawa, Ontario, Canada.
19. Kakal, J., **S. Sugden**, E. Faller and P. MacPherson (2008). *Transcriptional Regulation of the CD127 Gene in CD8 T-cells*. Ottawa Health Research Institute Research Day. Ottawa, Ontario, Canada.
20. **Sugden, S**. and P. MacPherson (2007). *Mutations in the HIV Tat Protein Correlate with Normal Interleukin-7 Receptor Alpha-Chain Expression on CD8 T-cells in a Long Term Nonprogressor.* 17th Annual Canadian Conference on HIV/AIDS Research. Montreal, Quebec, Canada.

21. Kakal, J., **S. Sugden**, E. Faller, R. Kumar and P. MacPherson (2007). *Regulation of CD127 Gene Expression in CD8 T-cells.* 17th Annual Canadian Conference on HIV/AIDS Research. Montreal, Quebec, Canada.
22. Kakal, J., **S. Sugden**, E. Faller, R. Kumar and P. MacPherson (2007). *Regulation of CD127 Gene Expression in CD8 T-cells.* Keystone Symposia: HIV Pathogenesis. Banff, Alberta, Canada.
23. Faller, E., J. Kakal, M. McVey, **S. Sugden** and P MacPherson (2007). *HIV Impairs CD8 T-cell Function by Removing the IL-7 Receptor Alpha-Chain from the Cell Surface and Targeting it for Degradation.* Keystone Symposia: HIV Pathogenesis. Banff, Alberta, Canada.
24. Faller, E., M. McVey, J. Kakal, **S. Sugden** and P. MacPherson (2007). *HIV impairs CD8 T-cell Function by Removing the IL-7 Receptor Alpha-Chain from the Cell Surface and Targeting it for Degradation.* Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.
25. Kakal, J., **S. Sugden**, E. Faller, R. Kumar and P. MacPherson (2007). *Regulation of CD127 Gene Expression in CD8 T-cells.* Ontario HIV Treatment Network, Annual Research Conference. Toronto, Ontario, Canada.
26. Faller, E., M. McVey, J. Kakal, **S. Sugden** and P. MacPherson (2007). *HIV Impairs CD8 T-Cell Function by Removing the IL-7 Receptor Alpha-Chain from the Cell Surface and Targeting it for Degradation*. Ottawa Health Research Institute, Annual Research Day. Ottawa, Ontario, Canada.
27. Kakal, J., **S. Sugden**, E. Faller, R. Kumar and P. MacPherson (2007). *Regulation of CD127 Gene Expression in CD8 T-Cells*. Ottawa Health Research Institute, Annual Research Day. Ottawa, Ontario, Canada.
28. **Sugden, S**. and P. MacPherson (2007). *Regulation of CD127 Expression on CD8 T-Cells by HIV's Tat Protein in Long Term Nonprogressors*. Ottawa Health Research Institute, Annual Research Day. Ottawa, Ontario, Canada.
29. Kakal, J., **S. Sugden**, R. Kumar, E. Faller, and P. MacPherson (2007). *Transcription of the CD127 Gene is Downregulated by IL-7 in CD8 T-cells.* 16th Annual Canadian Conference on HIV/AIDS Research. Toronto, Ontario, Canada.
30. **Sugden, S**., J. Kakal, E. Faller and P. MacPherson (2007). *Regulation of the Human CD127 Gene Promoter by Interleukin-7*. Association of Medical Microbiology and Infectious Disease, Canada, Annual Conference. Halifax, Nova Scotia, Canada.

Manuscripts

1. Faller, E., **S. Sugden**, M. McVey, J. Kakal and P. MacPherson. *Soluble HIV Tat Protein Removes the IL-7 Receptor Alpha-Chain from the Surface of Resting CD8 T cells and Targets it for Degradation.* **J Immunol.** 2010 Sep 1;185(5):2854-66
   - All "protein" work: Western blots, immunoprecipitations. **40% contribution.**
2. Al-Ghazawi F., E. Faller, **S. Sugden**, J. Kakal, and P. MacPherson. *IL-7 Downregulates IL-7Rα Expression in Human CD8 T Cells by Two Independent Mechanisms*. Accepted to **Immunology and Cell Biology** Oct 2012.
   - Jak inhibitor flow cytometry. **20% contribution.**
3. **Sugden, S.** and P. MacPherson. *HIV Tat Protein Binds the Cytoplasmic Tail of CD127 via Tat's Amino-Terminal Domain and Targets CD127 for Proteasomal Degradation via Tat's Basic Domain.* Completed. To be submitted to **J Retrovirology** in October 2012.
   - **100% contribution.**

4. McLaughlin, D., E. Faller, **S. Sugden**, J. Kakal, and P. MacPherson. *HIV-1 Tat Protein Downregulates the Expression of CD127 from the Surface of Primary Human CD4 T cells*. Completed. To be submitted Fall 2012.
    - Primary CD4 T cell transfection to express CD127, Western blots. **30% contribution.**
5. Al-Ghazawi F., P. Parmar, E. Faller, **S. Sugden,** H. Cherid, and P. MacPherson. *IL-7 Suppresses CD127 Gene Transcription in Human CD8 T-Cells via Inducing Expression of the Transcription Factor c-MYB.* Manuscript in preparation. Fall 2012.
    - Primary CD4 T cell transfection to express Myb. **20% contribution.**
6. **Sugden, S.** and P. MacPherson. *HIV Tat Protein Recruits CIS to the Cytoplasmic Tail of CD127 Resulting in Receptor Ubiquitination and Proteasomal Degradation.* Manuscript in preparation. December 2012.
    - **100% contribution.**

Research Funding Details: Major Exterior Scholarships Held

Grant/Award Name	Project Title	Funding Agency	Year(s) of Award	Total Budget ($)
Doctoral Research Award	Molecular Characterization of the HIV Tat Protein and its Ability to Downregulate CD127 on CD8 T-Cells.	Canadian Institutes of Health Research (CIHR)	2009-2012	63 000
Banting and Best Master's Scholarship	Molecular characterization of the HIV Tat protein and its interactions with cellular proteins involved in the downregulation CD127 on CD8 T-cells	Canadian Institutes of Health Research (CIHR)	2008-2009	17 500
Ontario Graduate Scholarship in Science and Technology	CD8 T-cells from Long Term Nonprogressors are resistant to interleukin-7 receptor downregulation by the HIV Tat protein.	Government of Ontario	2007-2008	15 000
Janssen-Ortho Studentship Award	Characterization of the CD127 Promoter	Association of Medical Microbiology and Infectious Diseases (AMMI) Canada	2007	5 000