Human Immunodeficiency Virus Type I Tat-Mediated Gene Regulation

by

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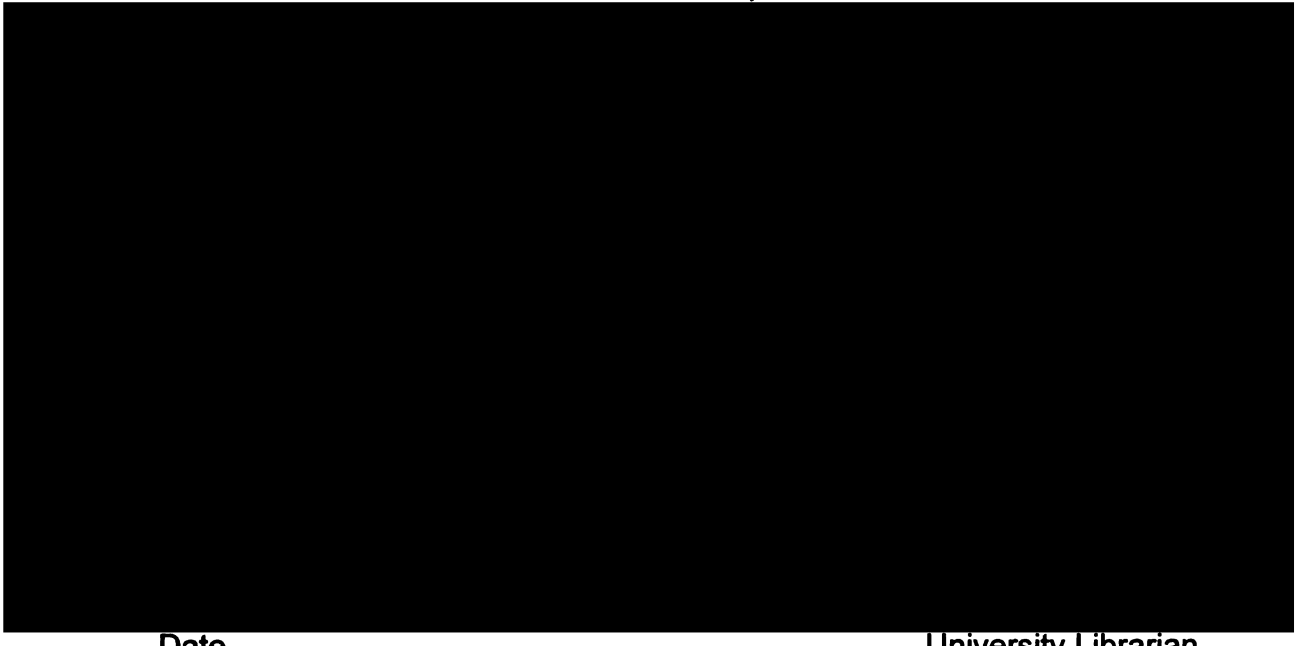

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Dedication

To my husband, for his long commutes and great pep talks, and my family, for their enthusiasm and patience

Acknowledgments

I have learned that a PhD is not simply a long process, but an experience of learning, and for that, I must thank my graduate mentors: Dr. Mark Goldsmith, who allowed me to believe in myself, and Dr. Eric Verdin, who took me on as a challenge and took pains to mold me into a true scientist. Dr. Doug Nixon, as my committee chair, provided organization, understanding, and enthusiasm, and Dr. David Erle, along with the Sandler Center for Functional Genomics, lent his critical eye and support. Dr. Melanie Ott and Dr. Warner Greene also lent me their ears and gave me advice when I most needed it. I am grateful to UCSF and to the University-wide AIDS Research Program for crucial funding.

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Abstract

HIV infection is characterized by progressive loss of CD4+ T cells and activation of the immune system. This immune activation is mediated by a number of factors, including viral proteins like Tat, as well as cytokines and chemokines. We investigated the role of Tat in activating gene expression as well as the role of a chemokine receptor, CXCR4, which is important for entry of HIV and cellular chemotaxis. We have found that CXCR4-mediated entry can occur efficiently in tissue macrophages, despite the view that macrophages are usually only targeted via CCR5. However, CXCR4-mediated entry into either T-cells or macrophages is inefficient in transgenic rats, compared with CCR5-mediated entry. In humans, pathogenic infection via CXCR4 evolves even in the absence of the accessory Nef protein. A splice variant of the ligand of CXCR4, SDF-1γ, can inhibit HIV Tat-mediated transcription. In contrast, HIV-1 Tat activates the expression of several genes including CXCR4 and Tsg101 by a transcriptional mechanism, leading to increased chemotaxis to SDF-1 and potential dissemination of infected T cells. Our work describes a complex interplay of HIV proteins, chemokines, and chemokine receptors, in which activation of chemokine-mediated pathways facilitates the pathogenesis of HIV.

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Chapter 1: Introduction

1A. Introduction to HIV

Human Immunodeficiency Virus type I (HIV-1) is the etiological agent of AIDS (Barre-Sinoussi 1983, Gallo 1984, Popovic 1984, Levy 1984). Over 40 million people are infected (UNAIDS 2005), and although drugs targeting several stages of the life cycle are available or in production (Yin 2006), a vaccine or curative treatment remains elusive.

HIV-1 is a plus-stranded RNA retrovirus of the lentivirus genus (Coffin 1979). The virus is an enveloped virus surrounding a protein coat which encloses two strands of genomic RNA of 9.2 kilobases (Terwilliger 1989). The genome contains two long-terminal repeats that are necessary for viral integration and act as transcriptional regulatory elements, including the promoter (Sanchez-Pescador 1980). The genome (Figure 1.2) encodes essential genes *gag*, which encodes the structural capsid proteins Gag (capsid CA [p24], matrix MA) and nucleocapsid (NC), *pol*, which encodes the reverse transcriptase (RT) and integrase (IN), *pro*, which encodes the viral protease (PR), *env*, which encodes the viral Env envelope protein. In addition, the genome encodes *tat*, the transcriptional activator, rev, necessary for RNA nuclear export, and accessory non-essential proteins *nef*, an infectivity factor, *vif*, which counteracts cellular restriction factors, *vpr*, viral protein R which is necessary for nuclear entry, and *vpu*, viral protein U which is involved in viral egress. These nine genes (Figure 1.2) encode a total of 15 proteins that participate throughout the viral life cycle (Figure 1.1) (reviewed in Greene 2002).

The mature HIV virion is coated with the envelope protein. Envelope is produced as a precursor protein called gp160, which is then cleaved into (SU, surface) gp120 and (TM, transmembrane) gp41 forms. Envelope is arranged as trimers on the virion surface (Doms 1991), with the gp120 protein making direct contacts with the cell. The gp41 molecule is a transmembrane protein, revealed only upon gp120 binding to the cellular receptors (CD4 and a coreceptor) and its subsequent conformational change. When gp41 is exposed, its fusion peptide makes contact with the cellular membrane and initiates fusion of the viral membrane with the cellular membrane (Sattentau 1993). This process leaves the viral membrane proteins on the cell surface (Dimitrov 1991), and releases the virion core, matrix, and some molecules of protease into the cytoplasm.

Inside the cell, the released virion core is coated with the capsid protein p24 and p6, processed forms of Gag. This pyramid-shaped core also contains some molecules of reverse-transcriptase and integrase, Vpr, and the nucleocapsid-coated RNA (Accola 2000). The virion RNA is uncoated by means of a process known only to be inhibited by the cellular protein TRIM5α (Stremlau 2004).

The uncoated RNA is reverse transcribed into DNA by the viral reverse transcriptase using cellular dNTPs (Barre-Sinoussi 1983). The process of reverse transcription leads to the full reconstitution of the HIV LTRs and the formation of ends suitable for integration.

These viral DNAs are imported into the nucleus by a viral protein complex called the pre-integration complex (PIC), which contains Vpr, IN, MA, and other proteins (Farnet 1991). The complex enters the nucleus through the nuclear pore and

integrase causes the integration of the DNA into the host chromosomes (Kulkosky 1990). Preferential integration is thought to occur within transcribed units (Schroder 2002).

After integration, expression of the viral genome requires cellular factors for initiation (Zhou 2000; Alcami 1995), followed by expression of Tat, which allows efficient elongation of transcription by RNA polymerase II (Kao 1987). Viral RNAs that are produced are initially spliced by the cellular splicing machinery. In the presence of the viral protein Rev, unspliced and partially spliced RNAs are also exported to the cytoplasm via CREM1-mediated nuclear export, and these unspliced messages can be incorporated into new viral particles (Fischer 1994).

Spliced and partially spliced messages are translated in the cytoplasm to produce viral proteins (Fischer 1994). Proteins such as Gag-Pol (p160) must be cleaved by protease to produce functional proteins. Gag p55 is cleaved to produce p24 (CA), p17 (MA), and NC. It also produces p6, p1, and p2. Gag-Pol is cleaved to produce protease, two subunits of RT, and integrase (reviewed in Dunn 2002).

After protease cleavage, viral proteins pass through the normal cellular transport pathways for modification. Gag, bound to viral unspliced genomic RNA, as well as Env, assemble at the plasma membrane (Vincent 1999). Other cellular proteins, such as APOBEC3G (Zennou 2004) and Cyclophilin A (Franke 1994), also may be found at the site of assembly. Proteins of the vesicular transport pathway, including Tsg101 (Martin-Serrano 2001), assist in the creation of membrane curvature. The viral particle produced is once again enveloped, but does not contain a core, but only a Gag precursor. This immature particle is cleaved by protease to produce

mature particles (Navia 1990). Protease cleaves Gag to MA and CA, p6 and NC, and these form the outer and inner compartments, respectively, of the mature viral particle that is competent for infection (Figure 1.2)

Studies have shown that several cellular factors are necessary for productive viral infection (reviewed in Sorin 2006). However, many host factors necessary for infection remain unknown. Furthermore, while it is known that HIV can infect CD4+ T lymphocytes (Barre-Sinoussi 1983), macrophages (Gartner 1986), and dendritic cells (Cimarelli 1994), and that the virus can kill infected lymphocytes (Levy 1994), the mechanism of viral action on uninfected cells remains elusive. The observation that several HIV proteins are soluble and may exert effects on neighboring uninfected cells highlights the importance of studying these proteins in isolation.

1B. HIV Pathogenesis

HIV infects cells by means of a receptor CD4 and coreceptor (Kwong 1998). The progressive loss of CD4+ cells by HIV infection or by bystander apoptosis of cells leads to a weakening of the immune system, which in turn provides the conditions necessary for opportunistic infections and clinical AIDS (Finkel 1995).

Infection of T cells in vivo usually requires CD4 and also a coreceptor which is a chemokine receptor (Doms 2000). One of two major alternate coreceptors may be used, CCR5 or CXCR4. Typically, viruses using CCR5 for entry are less pathogenic, infect macrophages, are found in early infections, are non-syncytium-inducing, and are more often transmitted. CXCR4-using viruses, instead, are often cytopathic, primarily infect T cells, evolve later in infection, induce syncytia, and are rarely transmitted (Table 1.1) (Scarlatti 1997).

CCR5 viruses predominate earlier in infection (Scarlatti 1997), and several potential causes result in the shift of the *in vivo* population towards CXCR4-using population. Several theories exist to explain the evolution of CXCR4-using viruses, which occurs in approximately 50% of AIDS patients (Koot 1993). R5 viruses are thought to be more easily transmitted because of their infection of dendritic cells which line the genital mucosa (Berlier 2005). Even in patients infected with both R5 and X4 viruses, the R5 viruses' ability to infect many long-lived macrophages, may contribute to their survival (Gorry 2004). Also, as chemokines that bind CCR5, such as MIP1 and RANTES, are produced in response to R5 infection, X4 variants may emerge (Kinter 2000). Viral intrinsic properties such as envelope shedding, relative burst size, and their ability to infect more activated cells may result in greater viral

loads with R5 than X4 viruses (Roy 2005). In addition, antiviral immunity may play a role. Studies suggest that X4 viruses may be selectively inhibited by antibodies (Bunnik 2006), in part due to lower glycosylation (LI 2001), and by cellular responses (Gorry 2004). As immune function wanes in later stages of disease, X4 viruses may emerge. Rates of viral mutation may also play a role in the transition from X4 to R5 (Gorry 2004).

Aside from viral properties, the cytokine milieu plays a role in controlling and exacerbating infection. Cytokines such as the interleukins IL-2, IL-7, and IL-15 are known to increase viral infection of resting T cells (Kreisberg 2006). However, inflammatory cytokines such as the interferons and tumor necrosis factor may act in an antiviral fashion as they respond to viral RNA (Preble 1993; Wong 1998). Activation of these cytokine genes is also regulated by viral proteins (Ott 1996; Buonaguro 1992; Kim 2004; Kinter 2000; Robichaud 2000).

As mentioned above, chemokines also play a role in infection. Beta chemokines like Macrophage Inflammatory Protein-1 (MIP1) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) counteract R5 infection by binding to and signaling through CCR5, while SDF-1 counteracts X4 infection by binding to and signaling through CXCR4 (reviewed in Kinter 2000). In addition, other chemokine receptors such as CCR7, CX3CR1, CCR2, CCR3, and CCR4 play a role in HIV pathogenesis (Kinter 2000). CCR7 is downregulated upon infection, while CCR2 and CX3CR1 may be used as alternative coreceptors (Kinter 2000). CCR3 and CCR4 upregulation leads to increased T cell permissivity for infection (Ancuta 2006).

The cell type infected by the virus also affects viral pathogenesis. The majority of cells infected and targeted by HIV are CD4+ T cells, which are found in blood and lymphoid organs. Among T cell subsets, memory and activated cells produce more virus than naïve cells (Eckstein 2001a, Zhang 1999). Furthermore, because CCR5 is expressed more highly on memory cells and CXCR4 more highly on naive cells (Lacey 1997), these expression patterns may play a role in the evolution of viral tropism (Davenport 2002, Blaak 2000). Cytokines like IL-7 which cause proliferation of naïve T cells may also affect viral evolution (Llano 2001). The long survival of some memory cells further contributes to prolonged retention of viruses in the host.

In addition to T cells, HIV targets macrophages. Macrophages are relatively long-lived, and unlike T cells, are not susceptible to activation- or infection-induced apoptosis. Studies have shown that infection of macrophages can contribute up to 50% of the infectious viral load (Johnson 1993; Eckstein 2001; Sherman 2003). Macrophages are typically infected by R5 viruses. However, recent studies have shown that macrophages are also susceptible to infection by viruses that solely use CXCR4 both in vitro and in vivo, despite their lower expression of CXCR4 (Jayakumar 2005; Yi 2005). Because macrophages reside in tissue, they have the potential to spread virus by close contact with T cells. Macrophages residing in the lung (alveolar macrophages), the gut, and brain (microglia) have all been shown to be infected with HIV-1, and may contribute to high viral loads. They are also potential reservoirs of infection after treatment.

1C. Roles of CXCR4

CXCR4, also called fusin or CD184, is a CXC-chemokine receptor expressed on a wide variety of cells. These receptors are seven-transmembrane proteins with a C-terminal region coupled to a Gαi protein, upstream of phosphoinositol-3 kinase and the mitogen-activated protein kinase (MAPK) pathways (Figure 1.3). CXCR4 and other CXC-receptors are sensitive to pertussis toxin, and when bound by their cognate ligand, induce Ca++ signaling. Active CXCR4 has been found in lipid raft regions of the cell membrane (Wysoczynski 2005).

CXCR4 is essential for early heart development, outward migration of mesenchymal stem cells (CD34+ cells), and differentiation of osteoclasts. Mice lacking both copies of CXCR4 die in embryogenesis. Furthermore, CXCR4 is necessary for the maturation of B cells into plasma cells, including the migration of these cells from the bone marrow. Expression of this receptor is also associated with neuronal cell migration (Peng 2004).

The exclusive ligand for CXCR4 is the chemokine stromal-derived factor, or SDF-1 (α or β). SDF-1 is an alpha chemokine and expressed in a variety of tissues. SDF-1 is also necessary for heart development and plasma cell development, and mice lacking SDF-1 also die during development.

CXCR4 and SDF-1 are implicated in a variety of cancers. Upregulation of CXCR4 is common among metastatic cancers, and migration of cells towards SDF-1 is thought to play a role in dissemination of transformed cells. An additional role for CXCR4 in cancers is in cooperation with integrins to promote cell adhesion and survival (Hartmann 2005). Furthermore, CXCR4 and SDF-1 have roles in heart development

and are crucial for angiogenesis, which is necessary for tumor maintenance (Gupta 1998). CXCR4 mutations are associated with WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome (Hernandez 2003).

The CXCR4 gene is known to be regulated by a number of cellular transcription factors (Figure 1.4). NFκB, Sp1, and USF-1 positively regulate CXCR4, whereas the transcription factor YY1 downregulates CXCR4. The gene is upregulated upon stimulation of T cells with PHA/IL-2 or CD3 and CD28 (Secchiero 2000), where 62-64kD species of CXCR4 have been shown to be ubiquitinated and to associate with CD4 (Zaitseva 2005). CXCR4 expression is also induced by NFkB (Hebig 2003), vascular-endothelial growth factor (VEGF) (Bachelder 2002), IL-6 (Odemis 2002), and insulin-like growth factor receptor (IGF1R) (Akekawatchai 2005). Follicular dendritic cells have also been demonstrated to increase CXCR4 on the T cell surface (Estes 2002).

In the context of HIV-1, SDF-1 and the HIV envelope protein gp120 bind to an overlapping site on CXCR4 and induce signaling. SDF-1 is a potent inhibitor of HIV binding to CXCR4. The binding of SDF-1 to CXCR4 is improved by tyrosine sulfation of CXCR4 (Farzan 2002). Mutations in the 3' untranslated region of *SDF1* have been associated with slower progression to AIDS or with later onset of evolution to X4 viruses (Marechal 1999; Winkler 1998). One study has shown that overexpression of CXCR4 may be associated with the emergence of X4 strains (Lin 2005).

In HIV pathogenesis, CXCR4 expression on M cells of the gut allows HIV transport across the gut monolayer to gain access to target T cells (Fotopoulos 2002).

Several HIV proteins can regulate CXCR4 expression. Nef downregulates CXCR4 protein levels from the cell surface by direct binding. Envelope binding to CXCR4 is also thought to downregulate CXCR4 expression. Extracellular forms of recombinant Tat have been demonstrated to upregulate CXCR4 expression on the cell surface by an unknown mechanism.

The V3 region of HIV-1 gp120 is the coreceptor binding site and associates with CXCR4. Binding to CXCR4 is dependent on as little as one amino acid within the V3 region of the envelope protein, and may be regulated by V3 glycosylation. In most strains, binding to CXCR4 is dependent on initial envelope binding to CD4 and a subsequent conformational change. Tat also binds to CXCR4 competitively at the SDF-1 or gp120 site and can downregulate CXCR4 expression by binding (Ghezzi 2000; Xiao 2000).

HIV opportunistic infections such as tuberculosis also induce CXCR4 expression, allowing infection of alveolar macrophages (Hoshino 2004). Lipopolysaccharides, however, downregulate CXCR4 expression in monocytes (Juffermans 2002).

1D. Roles of Tat

The HIV-1 *trans*activator of *t*ranscription, Tat, is an early gene in the viral life cycle. Originally known as p5, it is a 10kD protein encoded by two exons, the first 72 amino acids long and the second exon encoding an additional 29 amino acids (Jones 1993). Tat's role in infection occurs after integration, when the viral DNA must be transcribed into RNA in order to complete the cycle of infection. Early transcription, thought to be induced by immune activation of T cells, results in the expression of the Tat protein as well as the Rev protein. This early Tat is 101 amino acids long and encoded by multiply spliced mRNA. As Rev accumulates, unspliced RNAs are exported from the nucleus and a shorter 72 amino acid Tat protein is produced later in the infection cycle (Figure 1.5).

Both forms of Tat can bind to an RNA stem loop corresponding to the first 59 nucleotides of the HIV LTR, called the trans-activation responsive RNA (TAR). TAR is a stem loop structure, and mutations that disrupt the stem abolish Tat transactivation. Compensatory mutations, accordingly, restore TAR-Tat interactions. Tat binds to TAR at a small bulge at residues +22 to +24 via its arginine-rich motif (ARM) (Dingwall 1989; Cordingley 1990; Roy 1990; Gait 1993).

In the absence of Tat, RNA polymerase II cannot elongate efficiently from the HIV LTR due to hypophosphorylation of its C-terminal domain (CTD), although basal transcription occurs. The short transcripts that are produced form the stem loop structure that is TAR, to which Tat specifically binds in conjunction with the cellular protein Cyclin T1 (Fujinaga 1998). Cyclin T1 in turn recruits Cdk9 and other cellular transcription factors to form the positive transcriptional elongation factor b (PTEFb,

also known as Tat-associated kinase, TAK). CDk9 in PTEFb phosphorylates the polymerase at serine 2 and 5 of the CTD, resulting in more efficient transcription (Zhou 2000). The entire process, called transactivation (Figure 1.6), allows RNA polymerase II to transcribe the integrated virus into full-length RNA efficiently (see Figure 1.7), resulting in hundred-to-thousand-fold activation of the HIV LTR. This results in production of viral structural proteins and assembly and eventual budding of virus, completing the replication cycle. Thus, Tat is necessary at an early step for any production of virus, and viruses lacking Tat do not replicate.

The Tat protein has several domains (Figure 1.8). The N-terminal region contains an acidic domain. This is followed by the cysteine-rich region, which binds to CyclinT1 and coordinates zinc. The 16-residue hydrophobic core of the protein is followed by the arginine-rich motif (ARM), which binds to TAR, provides protein stability, and is responsible for Tat's nuclear and nucleolar subcellular distribution. The ARM overlaps with a basic region called the protein transduction domain (PTD), which is responsible for Tat's ability to enter cells by an active, but unknown, process. All of these domains are found within the first exon of Tat. The second exon is known only to contain an RGD motif which binds integrins. In this regard, it is notable that some molecular strains (LAI/BRU) of HIV-1 typically carry a shorter two-exon Tat86. However, other molecular and primary HIV-1 isolates encode full length Tat101.

Beyond simple transactivation, post-translational modifications of Tat also affect transcription. Forms of Tat acetylated at Lysine-50 cannot associate with TAR. Instead, acetylated Tat is thought to associate with the histone acetyltransferase p300/CREB-binding protein-associated factor (PCAF), which in turns remodels the

local chromatin environment to allow transcription to proceed (Kaehlcke 2003; Dorr 2002, Nakatani 2002, Bres 2002). Tat can also be de-acetylated by the histone deacetylase SIRT1 (Pagans 2005). Tat is also reported to be ubiquitylated (Bres 2003) and phosphorylated (Endo-Munoz 2005).

In addition to transcription of HIV genes, Tat has other effects on the cell. Overexpression of Tat in lymphocytes results in apoptosis (Li 1995). Full-length Tat (Tat 101) can activate T cells and induce the production of IL-2 (Ott 1997), while both forms of Tat stimulate IL-8 production by T cells (Ott 1998). The protein transduction domain (PTD) of Tat allows it to enter cells, likely through endosomal or clathrin-mediated pathways (Vendeville 2004), activate the HIV LTR, and induce cytokine expression. Addition of Tat to macrophages and dendritic cells induces production of inflammatory molecules such as TNFα (Chen 1997) and interferon-responsive genes (Izmailova 2003). These immune activation effects have garnered interest in Tat as an immunomodulatory molecule in vaccines. Furthermore, Tat induces post-transcriptional changes through the upregulation of Tat-associated kinases (such as T-cell derived kinase, TTK) which in turn phosphorylate components of the basal transcriptional machinery (Nekhai 2002).

Previous experiments have shown that both Tat and Nef regulate gene expression in different cell types. HIV-1 Nef initiates a program of gene expression that mimics T cell activation by CD3 and CD28 antibodies (Simmons 2001). Tat also regulates cellular activation and can induce the expression of interferon-responsive genes, including inflammatory chemokines and chemoattractants in dendritic cells, an effect

recapitulated in HIV-1 infection (Izmailova 2003). These results make Tat a likely candidate for many viral effects on uninfected cells.

The mechanism of these changes is thought to be through the upregulation of key transcription factors such as NFκB at the transcriptional level, possibly by binding to enhancer sequences (Yeung 2004; Dandekar 2004). Only in the case of the tumor necrosis factor beta gene (TNFβ) is Tat known to act through a TAR-like element (Buonaguro 1994; Brother 1996).

Tat also represses the activity of many genes. Repression of some genes may be due to sequestration of transcription factors by Tat at the HIV promoter. However, for genes such as the mannose receptor (Caldwell 2000), β2 microglobulin (Carroll 1998), and MHC class I (Matsui 1996; Weissman 1998), repression is enhanced by the presence of TAR and is targeted to the basal promoter via known Tat-associated factors (TAFs), potentially indicating a direct effect on transcription by Tat.

Tat interacts with a variety of proteins. Among the proteins identified to interact with Tat are transcription factors like NFAT, Sp1, TFIID, CREB-binding protein, and TFIIH, as well as histones and histone-associated proteins.

Several mutants of Tat have been identified. Mutants of the Cysteine-22 residue no longer bind to Cyclin T1 and cannot transactivate. Mutants in the ARM region also fail to transactivate due to lack of interaction with TAR. Mutants of acetylation sites, like Lysine-41, fail to transactivate for unknown reason, but other acetylation mutants, like Lysine-50, are defective only under certain conditions. Various mutants of Tat, including mutants of the second exon, have no known function.

1E. Figure Legends

Figure 1.1 – Human immunodeficiency virus type 1 (HIV-1) viral life cycle. Adapted from Warner Greene.

Figure 1.2 – Schematic representation of HIV-1 genome organization. Open reading frames are shown as boxes, with alternative and shifted reading frames shown above or below. LTR: long terminal repeat.

Figure 1.3 – Pathways activated by CXCR4 ligation with SDF-1. From ttp://www.biocarta.com/pathfiles/cxcr4Pathway.gif

Figure 1.4 – CXCR4 promoter organization. Predicted transcription factor binding sites are shown, along with relative positions. Asterisks indicate published binding sites verified by experimental methods.

Figure 1.5 – Tat72 and Tat101 are produced by alternate splicing. Tat101 is produced in the absence of Rev-dependent nuclear export by splicing of an intron, and can be found both early and late in the infection cycle. Tat72 is produced only from transcripts exported to the cytoplasm after the accumulation of Rev and encodes exon 1 only, terminated by a stop codon in the intron.

Figure 1.6 – Tat-mediated transcription of the HIV-1 promoter. In the absence of Tat, transcription does not proceed efficiently. Tat recruits cellular cofactors Cyclin T1 and Cdk9 to phosphorylate RNA polymerase II and promote elongation. Similar mechanisms of activation may occur at cellular promoters.

Figure 1.7 – Tat mediated transcription occurs in two steps. In the early step in the absence of Tat, short transcripts accumulate. In the late step, Tat binds to TAR and activates transcription.

Figure 1.8 – Domains of Tat. Exon 1 contains the acidic domain, cysteine-rich region, the core, and the arginine-rich motif. Exon 2 contains an RGD motif. Critical residues for transactivation (Cys-22 and Lys-41) as well as the protein-transduction domain (PTD) are indicated.

Chapter 2: Collaborations

2A. CXCR4 and Nef

Paper: Coreceptor Phenotype of Natural Nef-deleted HIV-1 Evolves *in Vivo* Leading to Increased Virulence

2A.1 Abstract

The Sydney Blood Bank Cohort is a group of patients with slowly progressive infection by a human immunodeficiency virus (HIV) strain containing spontaneous deletions within the *nef*-LTR region. In 1999, 18 years after the initial infection, one of the members (D36) developed AIDS. In this work, we used an *ex vivo* human lymphoid cell culture system to analyze two viral isolates obtained from this patient prior to the onset of AIDS in 1995, and after disease progression in 1999. Both D36 isolates were less potent in depleting $CD4^+$ T-cells compared to a reference dual-tropic, *nef*-bearing viral isolate. However, the 1999 isolate was measurably more cytotoxic to $CD4^+$ T-cells than the 1995 isolate. Interestingly, although both isolates were nearly equally potent in depleting $CCR5^+$ $CD4^+$ T-cells, the cytotoxic effect of the 1999 isolate towards $CCR5^-$ $CD4^+$ T-cells was significantly higher. Furthermore, GHOST cell infection assays and blocking experiments with the CXCR4 inhibitor AMD3100 showed that the later D36/99 isolate could infect both $CCR5^+$ and $CCR5^-$ $CXCR4^+$ cells efficiently while infection by the 1995 isolate was restricted to $CCR5^+$ cells. In conclusion, these data show that an *in vivo* evolution of the tropism of this *nef*-deleted strain towards an X4 phenotype was associated with a higher cytopathic potential and progression to AIDS.

2A.2 Introduction

In human immunodeficiency virus (HIV) -infected individuals, both viral and host factors regulate viral replication, depletion of $CD4^+$ T-cells, and disease progression. Host factors include genetic determinants such as the expression of coreceptors (Dean et al 1996, Fauci 1996, Stewart 1997) and the ability to establish an efficient immune response (reviewed in McMichael 2001 and Rowland-Jones 2001). Furthermore, mutations in the HIV envelope protein Env that cause a change in coreceptor usage have been shown to influence disease progression (Connor 1997; Kreisberg 2001; Schramm 2000a; Schuitemaker 1992; Xiao 1998). In many, but not all, patients disease progression coincides with broadened coreceptor usage. HIV strains isolated early after infection encode Env proteins that utilize CCR5 as a coreceptor to enter host cells (R5 viruses), whereas viruses isolated at later disease stages often represent Env-variants that can utilize CXCR4 (X4 viruses) or both CCR5 and CXCR4 (R5X4 viruses) (reviewed in Berger 1999). In addition, viral accessory genes such as *rev*, *tat*, *vif*, *vpr*, *vpu* (Michael 1995; Yamada 2000; Zhang 1997) and *nef* (Deacon 1995; Mariani 1996) have been implicated in disease development. In particular, Nef is a multifunctional protein (Peter 1998) that enhances viral replication (Miller 1994; Spina 1994) and infectivity (Chowers 1994), modulates apoptosis (Geleziunas 2001; Robichaud 2000; Yoon 2001), and decreases the expression of CD4 (Aiken 1994), the major histocompatibility (MHC) class I (Schwartz 1996), and CD28 (Swigut 2001). Long-term studies have shown that disease progression is markedly delayed in humans or rhesus macaques infected with HIV or SIV strains, respectively, that carry deletions in the *nef* gene

(Daniel 1992; Deacon 1995; Kirchhoff 1995). Interestingly, spontaneous partial repair of the *nef* gene *in vivo* have been correlated with enhanced infectivity and disease progression (Carl 2000; Sawai 2000).

One of the best examined patient groups of long-term nonprogressors (LTNP) and long-term survivors (LTS) is the Sydney Blood Bank Cohort (SBBC) (Deacon 1995), which consists of one blood donor (D36) and eight transfusion recipients who were infected with an HIV-1 strain containing multiple spontaneous alterations in the viral genome. These alterations include deletions in the *nef* open reading frame and a part of the long terminal repeat (LTR) region as well as duplications and rearrangements within the LTR. Three of the cohort members remained asymptomatic for at least 14 years and are classified as LTNP (Birch 2001), two died of causes unrelated to HIV, and one member with systemic lupus erythematosus died of causes possibly related to HIV (Learmont 1999). Three members had declining CD4 counts and detectable viral loads and, therefore, are grouped as LTS (Birch 2001). One of these LTS, the original blood donor D36, developed AIDS 18 years after infection with this *nef*-deleted HIV-1 strain and started highly active antiretroviral therapy (HAART) in 1999 (Learmont 1999).

In this study, we sought to determine whether the clinical progression of patient D36 corresponds to changes in the virulence of HIV isolates from this patient, and to identify possible mechanisms underlying such changes. Using a modified version of *ex vivo* human lymphoid histoculture, we compared virus isolated from patient D36 prior to disease progression in 1995 (D36/95) and after progression but before therapy was initiated in 1999 (D36/99). We found that the later viral isolate was more

cytotoxic than the earlier isolate and that this increased cytotoxicity was caused by a more efficient CXCR4 usage and expanded target cell range.

2A.3 Materials and Methods

Preparation of viral stocks.

NL4-3 was a gift from Malcolm Martin via the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Diseases, National Institutes of Health. The molecular clone 49-5 was a gift from Bruce Chesebro. Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as described previously (Atchison 1996). The primary isolates D36/95, D36/99, 7/86, and 1/85 were expanded by infection of heterologous peripheral blood mononuclear cells (PBMC). 7/86 and 1/85 were gifts from Ruth Connor (Connor 1997). The p24 Gag concentrations of viral stocks were assessed by enzyme linked immunosorbent assay (ELISA) (NEN, Life Sciences, Boston, MA).

Culture and infection of human lymphoid tissues ex vivo.

Human noninflammatory tonsil tissue removed during tonsillectomy (provided by the National Disease Research Interchange (NDRI, Philadelphia, PA) the Kaiser Hospitals in San Francisco, South San Francisco and San Rafael) was prepared for lymphoid aggregate culture as previously described (Eckstein 2001; Kreisberg 2001). In brief, tonsil tissue was mechanically dispersed and isolated cells were transferred to 96-well U-bottom plates at a concentration of $1x10^7$ cells per ml, 200 µl per well. Cells were allowed to aggregate at the bottom of the well and were not dispersed for the remainder of the culture period. *Ex vivo* human lymphoid cell cultures were inoculated within 24 h of preparation with HIV-1 at 80 50%-tissue culture infective doses ($TCID_{50}$), as determined by terminal dilution of the virus

stocks in quadruplicate on heterologous phytohemagglutin-activated PBMC as described previously (Penn 1999).

Assessment of $CD4^+$ T-cell depletion by FACS analysis.

At the indicated time points, cells from infected and uninfected lymphoid cell cultures were stained for cell surface markers CD3, CD4, CD8, and CCR5 as described previously (Penn 1999; Schramm 2000a), by using the following monoclonal antibodies: anti-CD3 (clone SK7, phycoerythrin conjugated), anti-CD4 (clone SK3, fluorescein isothiocyanate conjugated), anti-CD8 (clone SK1, PerCP conjugated) (Becton Dickinson) and anti-CCR5 (clone 2D7, allophycocyanin conjugated) (Pharmingen). Then, 10,000 lymphocytes positive for CD3 surface marker were counted and the data were analyzed with CELLQUEST software (Becton Dickinson). To facilitate comparison among experiments, $CD4^+$ T-cell depletion was assessed by measuring the ratio of $CD4^+$ to $CD8^+$ T-cells. This value was normalized to the CD4/CD8 ratio of control (uninfected) samples.

Measurement of apoptosis.

At the indicated time points, cells from infected and uninfected lymphoid cell cultures were washed with phosphate-buffered saline, 2% fetal bovine serum, 2.5 mM $CaCl_2$, stained for cell surface markers CD3, CD4, and either AnnexinV-PE (Alexis) or 200 nM tetramethylrhodamine-methyl ester (TMRM, Molecular Probes) for 30 min at room temperature, washed again, and subjected to flow cytometric analysis. To determine activation of caspase-3, cells were incubated with 10 μM PhiPhiLux-G_1D_2

(Alexis) for 1 h at 37°C, washed, stained for CD4 and CD3, and subjected to FACS analysis.

GHOST cell and HeLa infection assays.

GHOST cell assays were performed as reported previously (Cecilia 1998). Briefly, 20,000 $CXCR4^+$ $CD4^+$ (GHOST-X4) or $CCR5^+$ $CD4^+$ GHOST (GHOST-R5) cells were plated in 12-well plates and infected at a multiplicity of infection (MOI) =0.1. 72 h after infection, infected cells were identified by flow cytometric analysis. $CXCR4^+$or $CCR5^+$ HeLa-CD4 cells were pretreated for at least 12 h with the indicated concentrations of AMD3100 and inoculated with HIV at a MOI=0.01. 72 h after infection, the concentration of p24 Gag in the culture supernatant was assessed by anti-p24-ELISA.

2A.4 Results

Characterization of the virulence of D36/95 and D36/99.

First, the clinical isolates from patient D36 were tested for their potential to deplete $CD4^+$ T-cells in *ex vivo* human lymphoid cultures. The kinetics of HIV-induced $CD4^+$ T-lymphocytes depletion was measured by staining cultures with antibodies to CD3, CD4 and CD8, and was displayed as a ratio of $CD4^+$ to $CD8^+$ T-cells as described previously (Eckstein 2001; Kreisberg 2001; Penn 1999; Schramm 2000; Schramm 2000a). We compared the two D36 isolates with two other primary isolates from a different cohort. These isolates called 7/86, an R5X4 strain and 1/85, an R5 strain (Connor 1997) are wildtype (WT)-*nef* strains. As expected, the number of $CD4^+$ T-cells in 1/85-infected cultures decreased only slightly over time, since R5 strains can infect and deplete only the small subset of CCR5-expressing $CD4^+$ T-cells (Grivel 2000; Penn 1999; Schramm 2000; Schramm 2000a). In contrast, the dual-tropic virus 7/86 depleted $CD4^+$ T-cells markedly due to its expanded target cell range (Figure 2.1A) (Schramm 2000a). Overall CD4 depletion was less severe in cultures infected with either of the two D36 isolates than in cultures infected with the R5X4 reference strain 7/86 (Figure 2.1A). However, the later D36/99 isolate was measurably more cytopathic than the earlier D36/95 isolate with more severe $CD4^+$ T-cell depletion despite comparable inoculum size (Figure 2.1A). The increased virulence of D36/99 was also reflected in faster replication kinetics. D36/99 replicated with a profile similar to the two reference strains, whereas replication of D36/95 was significantly delayed (Figure 2.1B).

The elevated potential of D36/99 to deplete $CD4^+$ T-lymphocytes was paralleled by its ability to induce apoptosis in $CD4^+$ T-cells. Apoptosis was measured independently by several assays: (1) AnnexinV-binding to phosphatidylserine as a marker for the loss of cell membrane asymmetry (Figure 2.2A); (2) activation of caspase-3, a key enzyme of the apoptotic signal transduction pathway (Figure 2.2B); and (3) depolarization of the mitochondrial membrane as a marker of the mitochondrial branch of apoptotic signaling (Figure 2.2C). To this end, cells were stained with antibodies to CD4 and CD3 along with AnnexinV-FITC, the membrane-permeable caspase-3-substrate PhiPhiLux G_1D_2 or tetramethylrhodamine-methyl ester (TMRM), and subjected to FACS analysis (Bernardi 1999; Hirata 1998). Loss of TMRM binding to mitochondria is an indicator for the breakdown of the mitochondrial membrane potential (Bernardi 1999). Examination by all three methods showed that cultures infected with D36/99 had much higher levels of apoptosis in $CD4^+$ T-cells compared to those infected with the earlier D36/95 isolate (Figure 2.2). However, apoptosis in D36/99-infected cultures was still slightly lower than that observed in cultures infected with the WT strain 7/86.

Depletion and apoptosis of $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cells.

Disease progression in HIV-infected patients has been shown to correlate with a change in coreceptor usage in many, though not all, individuals (Connor 1997; Schuitemaker 1992; Xiao 1998). Therefore, we addressed the question whether the increased potential of D36/99 to induce apoptosis and hence deplete $CD4^+$ T-cells was caused by a broadened coreceptor usage. We used flow-cytometry to distinguish $CD4^+$ T-cells into $CCR5^+$ and $CCR5^-$ subsets as described previously

(Grivel 2000; Kreisberg 2001; Penn 1999; Schramm 2000a). Interestingly, D36/95 depleted the $CCR5^+$ subset of $CD4^+$ T-cells to an extent comparable to that of both D36/99 and the R5X4 reference strain 7/86 (Figure 2.3A). In contrast, while D36/99 and 7/86 caused marked depletion of the $CCR5^-$ subset of $CD4^+$ T-cells, the earlier D36/95 isolate and the R5 reference strain 1/85 did not affect this subset significantly (Figure 2.3B). Similarly, D36/95 and 1/85 induced apoptosis exclusively in $CCR5^+$ $CD4^+$ T-cells, whereas D36/99 and 7/86 promoted apoptosis equally in both the $CCR5^+$ and $CCR5^-$ cellular subsets (Figure 2.3C and D). Thus, the effects of D36/95 appeared to be restricted to $CCR5^+$ $CD4^+$ T-cells, while cytopathic effects of the later D36/99 isolate include both $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cells.

Coreceptor phenotype of the D36 isolates.

To define the coreceptor usage by the two D36 isolates more directly and quantitatively, GHOST cell infection assays were performed. CCR5-expressing GHOST (GHOST-R5) cells and CXCR4-expressing GHOST (GHOST-X4) cells were infected with D36/95 and D36/99 at a MOI of 0.1 (Cecilia 1998). The prototypical strains 49-5 (R5) and NL4-3 (X4) were used as positive controls for CCR5 and CXCR4 usage, respectively (Toohey 1995). As expected, 49-5 infected exclusively GHOST-R5 cells as indicated by LTR-mediated expression of the fluorescent marker, whereas NL4-3 infected GHOST-X4 cells (Figure 2.4A). The residual infection of GHOST-R5 cells by the X4 strain NL4-3 was due to the endogenous low-level expression of CXCR4 in parental GHOST cells. Importantly, both D36/95 and D36/99 infected GHOST-R5 cells to an extent comparable to 49-5. In contrast, infection of GHOST-X4 cells by D36/95 was very limited in comparison to D36/99.

These results indicate that D36/99 has a significantly higher potential to infect CXCR4-expressing cells compared to D36/95 (Figure 2.4A).

We tested the differential CXCR4 utilization by these viruses further by analyzing their sensitivity to the CXCR4 antagonist AMD3100 (Donzella 1998; Schols 1997). Dose-response curves were determined using AMD3100 to inhibit infection of $CXCR4^+$ $CCR5^+$ HeLa-CD4 cells (Bestwick 1988; Kabat 1994) by D36/95, D36/99 and the two reference viruses 49-5 (R5) and NL4-3 (X4). Infection by either 49-5 or D36/95 was not significantly inhibited by AMD3100, thereby confirming their independence from CXCR4 (Figure 2.4B). In contrast, infection by NL4-3 or D36/99 was inhibited effectively by AMD3100 with a 50%-inhibitory concentration (IC_{50}) of 38 nM and 85 nM, respectively (Figure 2.4B). Notably, even at AMD3100 concentrations as high as 2500 nM, infection with D36/99 could not be inhibited completely while that by NL4-3 was nearly fully abolished (Figure 2.4B). This suggests that D36/99 can use both CCR5 and CXCR4 as a coreceptor. Taken together, these results demonstrate that both D36 isolates can utilize CCR5 as a coreceptor, and that D36/99 can also use CXCR4 efficiently.

Finally, we analyzed the coreceptor usage of the D36 isolates within the biologically relevant *ex vivo* lymphoid cell culture system. Lymphoid cultures were pretreated with AMD3100 (250 nM) and infected with equal doses of D36/95 or D36/99. Depletion and apoptosis of $CD4^+$ T-cells overall as well as specific depletion of the $CCR5^-$ and $CCR5^+$ subsets of $CD4^+$ T-cells were determined 12 days after the infection. As expected, the modest depletion (Figure 2.5A) and apoptosis (Figure 2.5B) of $CD4^+$ T-cells induced by D36/95 were not significantly affected by

AMD3100. In contrast, both CD4 depletion (Figure 5A) and apoptosis (Figure 2.5B) induced by D36/99 were strongly abrogated by AMD3100 (Figure 2.5A and 2.5B). Furthermore, analysis of the $CCR5^-$ and $CCR5^+$ subsets showed that AMD3100 prevented depletion of $CCR5^-$ $CD4^+$ T-cells by D36/99, but not depletion of $CCR5^+$ $CD4^+$ T-cells (Figure 2.5C). Again, depletion of the $CCR5^+$ subset by D36/95 was unaffected by AMD3100 (Figure 2.5C). These results confirm that D36/99 has a broadened coreceptor usage that allows it to infect both $CCR5^+$ cells and $CCR5^-$ $CXCR4^+$ $CD4^+$ T-cells.

2A.5 Discussion

Evidence from *in vivo* observations and *ex vivo* experiments has strongly pointed to a switch in coreceptor usage as an important factor in HIV disease progression. Although the precise mechanism remains unclear, such phenotypic and genotypic changes are thought to be the result of an evolutionary selection process (Connor 1997; Schuitemaker 1992; Xiao 1998). The present study reveals that the low level of replication *in vivo* of an HIV-1 strain with spontaneous deletions in the *nef*-LTR region (Figure 2.1B and Deacon 1995) was sufficient to allow evolution of the viral *env* gene from a predominantly R5 phenotype toward a predominantly X4 phenotype. Moreover, such viral evolution seems to have been dictated by the same evolutionary processes that cause changes in typical *nef*-WT HIV strains. Importantly, the change in coreceptor usage was manifested by an increased potential of the virus to infect $CCR5^{-}$ $CXCR4^{+}$ lymphocytes (Figure 2.4A and B) and to deplete and induce apoptosis in this T-cell subset of human lymphoid tissue (Figure 2.1A, Figure 2.3 and Figure 2.5). Earlier studies had implicated a more promiscuous coreceptor usage and a wider target cell range as a key pathological mechanism underlying disease acceleration following emergence of X4 strains in patients infected with typical *nef*-WT strains (Connor 1997; Schuitemaker 1992; Xiao 1998). The increased cytotoxicity of the D36 virus observed in this study strongly suggests that a similar evolution of the viral tropism is an important factor in the disease progression of patient D36 despite the absence of Nef. However, additional mechanisms not delineated here may be involved as well.

Recently, Kirchhoff and co-workers reported a case of a long-term nonprogressor infected with an HIV-1 strain with deletions in the *nef* gene that were found to be partially repaired (Carl 2000). Moreover, the virus with this "repaired" Nef protein displayed augmented infectivity and increased downregulation of CD4 and of MHC class I molecule expression (Carl 2000). Similarly, rhesus macaques infected with SIV strains with mutations of the *nef* start codon, as well as deletions and insertions in the coding sequence of *nef,* were able to express a truncated version of Nef with a reverted start codon after several months of infection (Sawai 2000). Virus isolates with these truncated Nef proteins were much more pathogenic than their parental strains and caused an AIDS-like disease in the macaques (Sawai 2000). In contrast, the increased cytopathicity of the D36/99 isolate reported here was not caused by a reversion or partial repair of the *nef* gene. Sequence analysis showed that the deletions in the *nef* gene of the D36/99 isolate are even more extensive compared to that of D36/95 (Birch 2001). Furthermore, immunoblot analysis of PBMC infected with the D36/95 and D36/99, respectively, revealed that the two D36 isolates do not express a Nef protein that can be recognized by a polyclonal anti-Nef antibody (data not shown). However, a contribution of further genetic rearrangements in the *nef*-LTR region to the enhanced cytotoxic potential of the later D36/99 isolate cannot be entirely excluded (Birch 2001).

Live attenuated HIV strains like the one seen in the SBBC have been proposed as a vaccine to prevent infection with wildtype HIV (Desrosiers 1992). A key challenge in developing a safe, effective attenuated virus vaccine is that a strong immune response seems to depend on the degree of viral replication (Ruprecht 1999).

However, even at a very low level, replication harbors the risk of evolution towards higher virulence. Several reports of spontaneous repair of deletions in the *nef* gene *in vivo* resulting in increased virulence and the onset of an AIDS-like disease (Dittmer 1995; Sawai 2000; Whatmore 1995) have challenged the safety of this vaccination concept. Therefore, vaccine candidates have been developed with multiple mutations in accessory genes (Desrosiers 1998; Gibbs 1995; Mills 2000). These second-generation vaccines are thought to be much safer but may still be able to elicit an effective immune protection (Johnson 1998; Mills 2000). However, the present study shows that the attenuation of a virus with deletions in the *nef* gene can be overcome not only by a repair of the *nef* gene, but also by compensatory changes in other genes. In particular, the deletions in the D36 virus did not prevent it from evolving to higher virulence by broadening its coreceptor usage. Indeed, in contrast to *nef* and other accessory genes, *env* is an essential HIV gene that cannot be deleted to provide a higher level of protection without completely abrogating replication. The high risk of using a live attenuated virus as a vaccine is further supported by the fact that two more members of the SBBC now have declining CD4 counts and detectable viral loads (Birch 2001), although the underlying mechanisms in these two cases remain unknown.

In conclusion, we have shown that an HIV-1 strain with multiple deletions in the *nef*-LTR region can still mutate to a higher level of virulence *in vivo* by widening its coreceptor usage and target cell range. This implies that the low level of replication of an attenuated virus is sufficient to allow substantial viral evolution and shows that the concept of live attenuated *nef*-deleted viruses as a vaccine is not safe.

2A.6 Acknowledgments

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2A.7 Figure Legends

Figure. 2.1. The D36/99 isolate depletes $CD4^+$ T-cells more efficiently and replicates faster than D36/95 in *ex vivo* human lymphoid cell cultures. (A) CD4 depletion induced by infection with viral strains D36/99, D36/95, 7/86, and 1/85 was assessed by FACS analysis at the indicated time points. Shown are the mean relative CD4/CD8 ratios (n=3) with SEM of a representative experiment from among experiments with 6 different donor tissues. (B) Viral replication was monitored in the same infections by assessing the accumulation of p24 in the culture medium at days 3, 6, 9 and 12 after infection using an anti-p24 ELISA. Shown are the mean values (n=3) with SEM of a representative experiment from among experiments with 6 different donor tissues.

Figure. 2.2. D36/99 induces high levels of apoptosis among $CD4^+$ T-cells in human lymphoid cell cultures. Apoptosis in $CD4^+$ T-cells induced by infection with viral strains D36/99, D36/95, 7/86, and 1/85 was measured at the indicated time points independently by AnnexinV-binding (A), activation of caspase-3 (B), and depolarization of the mitochondrial membrane potential (C) using FACS analysis. Shown are the mean values (n=3) with SEM of a representative experiment from among experiments with 6 different donor tissues.

Figure. 2.3. D36/99 induces depletion and apoptosis of both $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cells in human *ex vivo* lymphoid cell cultures. CD4 depletion induced by infection with viral strains D36/99, D36/95, 7/86 and 1/85 within the $CCR5^+$ (A) and $CCR5^-$ (B) subsets of $CD4^+$ T-cells was analyzed at the indicated time points by multiparameter FACS analysis. Shown are the mean relative CD4/CD8 ratios (n=3)

with SEM of a representative experiment from among experiments with 6 different donor tissues. In the same infections, apoptosis was measured in the $CCR5^+$(C) and $CCR5^-$ (D) $CD4^+$ T-cell subsets by AnnexinV-binding. Shown are the mean values (n=3) with SEM of a representative experiment from among experiments with 6 different donor tissues.

Figure. 2.4. D36/99 can use CXCR4 as a coreceptor more efficiently than D36/95. (A) GHOST-R5 (open bars) and GHOST-X4 cells (solid bars) were infected with D36/99, D36/95, NL4-3, and 49-5 at a MOI=0.1 and cultured for 72 h. Infected cells were identified by FACS analysis. Shown are the mean values (n=3) with SEM of a representative experiment from among 3 independent experiments. (B) $CXCR4^+$ $CCR5^+$ HeLa-CD4 cells were pretreated for 12 h with the indicated concentrations of AMD3100 and infected with D36/99, D36/95, NL4-3, and 49-5 at a MOI=0.01. Viral replication was measured 72 h after infection by assessing the concentration of p24 in the culture medium. Shown are the mean values (n=3) with SEM of a representative experiment from among 3 independent experiments.

Figure. 2.5. D36/99 depletes and induces apoptosis in both the $CCR5^+$ and $CCR5^-$ subsets of $CD4^+$ T-cells in human *ex vivo* lymphoid cultures. Human *ex vivo* lymphoid cell cultures were pretreated with AMD3100 (250 nM) for 12 h and infected with D36/95 and D36/99. AMD3100 was replenished to the cultures at each media change. Depletion of total $CD4^+$ T-cells (A) and of the $CCR5^+$ and $CCR5^-$ subsets of $CD4^+$ T-cells (C) as well apoptosis of total $CD4^+$ T-cells (B) was measured by FACS analysis 12 days after infection. Shown are the mean values (n=3) with SEM of a representative experiment from among experiments with 4 different donor tissues.

2B. CXCR4 and Rats

hCXCR4/hCD4 Transgenic Rat Tissues Partially Support HIV-1 Entry and Early Gene Expression *ex Vivo*

2B.1 Abstract

Small animal models of human immunodeficiency virus type 1 (HIV-1) infection have so far proved elusive, despite efforts to introduce a variety of human genes into mice by transgenesis. Despite the introduction of human CD4, coreceptors, and CyclinT1, mice sustained a late block to HIV-1 replication. We have employed the outbred Sprague Dawley rats (*Rattus norvegicus*) as a potential small animal model of HIV-1 replication. Upon introduction of cell type-specific transgenic vectors encoding for human CD4 and CXCR4, human gene expression was noted in relevant HIV-1 target cells, including CD4 lymphocytes and macrophages. Lymphocytes from these animals supported entry and early gene expression at low levels with X4, but not R5 HIV-1 strains. However, macrophages from these double-transgenic animals remained refractory to infection with T cell line-adapted X4 HIV-1 viruses. Instead, they were partially susceptible to infection by primary X4 viruses, previously shown to productively infect human macrophages. These studies indicate in rats expressing the appropriate HIV receptor complex, X4 HIV-1 infection is not as efficient as R5 HIV-1 infection, and that infection of hCD4/hCXCR4-transgenic rat macrophages with X4 HIV-1 viruses may in part resemble characteristics of the infection in human macrophages.

2B.2 Introduction

Small animal models of disease are necessary in addition to in vitro studies, especially in cases of HIV-1, where human studies cannot be performed. In addition, a small animal model of HIV will provide a testing ground for vaccines, drugs, and immunological studies. Current models include the SCID-huThy-Liv mice, in which human tissue is implanted into mice, as well as transgenic mice expressing HIV genes or receptors. However, none of these support systemic infection of HIV, nor are they susceptible to infection leading to AIDS.

These limitations indicate a need for a small animal model of HIV that supports replication and which can be easily replicated. Rat cells support productive replication in vitro when infected with viruses pseudotyped with the VSV-G envelope protein (Keppler 2001). Recent studies have shown that transgenic hCCR5 expression in laboratory rats along with hCD4 renders macrophages from these animals susceptible to a moderate degree of productive infection (Keppler 2002). These hCD4(hCCR5-transgenic animals suppored entry and early gene expression in both CD4 T cells and macrophages. In T cells, a yet to be identified block at the later stages of replication apparently prevented productive replication. macrophages.

Given the promise of animals that co-express hCCR5 and hCD4, we tested the ability of CD4 T cells and macrophages from hCD4/hCXCR4-transgenic rats to support HIV replication. We found that these rats support significant levels of viral entry in lymphocytes, but only minimal entry in macrophages.

2B.3 Materials and Methods

Construction of transgenic rats

Rats transgenic for human CD4 (hCD4) were created as described previously (Keppler 2002). Rats transgenic for hCXCR4 were created as described for hCCR5, with the exception of the use of a hCXCR4 cDNA cloned into the identical transgene construct. Founders were generated by pronuclear microinjection of the transgene vector and identified by transgene-specific PCR. Transgenic founders were mated with nontransgenic Sprague-Dawley rats and F1 progeny were screened by flow cytometry for expression of human proteins in peripheral blood samples. Transgene-expressing F1 rats were interbred with previously established hCD4-transgenic animals.

Flow cytometry

FACS® analyses were performed as described previously (Keppler 2002), using FITC-, PE-, or APC-conjugated mAbs (BD PharMingen): anti-hCD4 (mAb Leu-3a); anti-hCXCR4 (mAb 12G5); anti–rat (r)CD3 (mAb G4.18); anti-rCD4 (mAb OX-35); anti-rCD8a (mAb OX-8); anti–rat macrophage subset marker (ED2-like antigen, mAb HIS36); anti-rCD11b (mAb WT.5); anti-rCD11b/c (mAb OX-42); and anti-rCD45RA (mAb OX-33).

Primary cell culture

Ex vivo cultures of primary rat lymphocytes, macrophages, or microglia, and cultures of PBMCs or human monocyte–derived macrophages were prepared and propagated as described previously (Keppler 2001).

Preparation of Viral Stocks

The molecular clone pNL4–3 was obtained from Malcom Martin (National Institutes of Health, Bethesda, MD), via the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. The molecular clone p49–5 (Chesebro 1992) was a gift from Bruce Chesebro (Rocky Mountain Laboratories, Hami Hou, MT). Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as described previously (Atchison 1996). Primary isolates UG021 and UG024 were obtained via the NIH AIDS Research and Reference Reagent Program. The primary isolates J130 and J34, and tybe were gifts from Gabriella Scarlatti (San Raffaele Scientific Institute, Milan, Italy) and Ron Collman (University of Pennsylvania, Philadelphia, PA), respectively. Viral stocks were expanded by infection of heterologous human PBMCs. For concentrated infections, viral stocks were concentrated using Centricon® Plus-150 columns (Millipore) following the manufacturer's protocol. The infectivity of replication-competent viral stocks was determined by terminal dilution in quadruplicate on heterologous phytohemagglutinin-activated PBMCs. The p24 CA concentration was assessed by ELISA (NEN Life Sciences). The molecular clone pNL-4–3 Luc $E^{-}R^{-}$ (Connor 1995), a replication-incompetent NL4–3 provirus (along with mutations in *env*, *nef*, and *vpr*), carrying a luciferase gene within the *nef* locus driven by the 5' LTR, was a gift of Nathaniel Landau (Salk Institute for Biological Studies, La Jolla, CA) via the NIH AIDS Research and Reference Reagent Program. pVSV-G, the mammalian expression vector for VSV-G protein (Emi 1991), was a gift from Jane Burns (University of California, San Diego, CA). The

preparation of NL4–3 Luc E^-R^- pseudotype viruses with autologous or heterologous envelopes (Env) has been described previously (Chan 1999).

Viral Infections

HIV-1 infections of ex vivo cultures were performed in 24-well (lymphocytes, macrophages) or 96-well plates (microglia, macrophages) with the indicated multiplicity of infection (MOI) or p24 CA concentrations. In infection studies on transgenic rat macrophages, the following reagents were used: anti-hCXCR4 mAb 12G5 (BD PharMingen) at 50 µg/ml; zidovudine (AZT) (3'-azido-3'deoxythymidine; Sigma-Aldrich) at 100 µM; Ritonavir (RTV) (Abbott Laboratories, Abbott Park, IL) at 1 µM; LPS (*Escherichia coli* serotype 0128:B12; Sigma-Aldrich) at 100 ng/ml; or a formalin-fixed *Staphylococcus aureus* crude cell suspension (Sigma-Aldrich) at 0.01%. All rats were housed under SPF conditions with food and water ad libitum. Rats were killed with CO_2 and bilateral thoracotomy and organs were removed aseptically. Coded splenocyte, thymocyte, and PBMC samples were analyzed for the presence of human markers by flow cytometry as described above.

2B.4 Results

CXCR4 and CD4 expression in transgenic rats

Rats transgenic for hCD4 and the human chemokine receptor hCXCR4 were generated that express the transgenes in CD4(+) T lymphocytes, macrophages, and microglia. First, we could demonstrate hCXCR4 and hCD4 expression on these specific target cells (Figure 2.6). We also analyzed rat CD3, CD4, and CD8

T cell subsets, and found a slight increase in the relative frequency of CD8+ T cells in hCXCR4/hCD4 homozygous transgenic animals (Figure 2.6C). No other alterations in lymphocytes were detected. We found strong expression of hCD4 in transgenic lymphocytes, and moderate to low expression of hCXCR4 (Figure 2.6A). Similar results were found in macrophages (Figure 2.6B).

Entry and early gene expression in double-transgenic lymphocytes

Lymphocytes were extracted from the spleens and thymus of hCD4/hCXCR4-transgenic animals and dispersed, followed by culture in Concavalin A and IL-2. Using a virion-based fusion assay (Cavrois 2002), we found that the X4 virus NL4-3 could fuse with hCD4/hCXCR4 transgenic but not hCD4 transgenic rat lymphocytes (Figure 2.7A) at a rate comparable to that of human PBMCs. Entry was completely inhibited by the CXCR4 antagonist AMD3100. A control VSVg-pseudotyped virus also entered rat cells efficiently (Figure 2.7A). Using a virus construct that expresses luciferase in the position of *nef* and contains a frameshift in *env*, we pseudotyped these viruses with envelopes from different R5 or X4 HIV-1 viruses. We infected cells with laboratory strains of isogenic luciferase viruses – NL4-3, LAI.2 and Ada-M. As expected, the R5 virus Ada-M was unable to enter rat hCD4+ hCXCR4+ lymphocytes. However, the X4 strains NL4-3 and LAI.2 were able to enter and drive luciferase expression to some extent in these cells (Figure 2.7B). We also found similar results in human lymphocytes isolated from peripheral blood and activated for 3 days. However, when we infected lymphocytes with infectious HIV-1, we found no productive infection in transgenic lymphocytes, as measured by p24-Gag (Figure 2.7C).

Entry and early gene expression in hCD4/hCXCR4-transgenic macrophages

We then tested whether macrophages from these transgenic animals would be susceptible to infection by X4 viruses. We isolated macrophages from freshly prepared splenocyte suspensions by adhesion to flasks for 5-7 days, washed away lymphocytes, leaving a majority of cells with macrophage-like morphology. We found that neither 49-5 nor NL4-3 were able to infect hCD4+ hCXCR4+ macrophages. This was not entirely unexpected, since these cells do not express human CCR5, which is required for 49-5 entry, and NL4-3 has been reported not to infect human macrophages efficienctly. In order to test whether hCXCR4 on macrophages was functional, we obtained several primary X4 HIV-1 viruses of subtype D that have been reported to infect human monocyte-derived macrophages, and we used their envelopes to create pseudotyped luciferase virus stocks to infect macrophages from hCD4/hCXCR4-transgenic rats. We found that only some of the macrophage-tropic X4 viruses were able to infect rat macrophages, as determined by a low level luciferase signal 3 days post infection, reflecting early viral gene expression (Figure 2.8). Addition of human serum, M-CSF, or administration of higher doses of viral inoculum did not augment infection (data not shown). These viruses were, however, able to infect human monocyte-derived macrophages isolated in a similar manner, and they were also able to infect macrophages in human lymphoid histoculture (Jayakumar 2005, and Chapter 3A).

Infection of hCD4/hCXCR4-transgenic microglia

We next measured the susceptibility of rat microglia to infection by X4 viruses. In one double-transgenic animal, we found some evidence for infection and early gene expression in microglia, albeit at a very low level. The virus was unable to infect non-transgenic microglia in an infection conducted in parallel (Figure 2.9).

2B.5 Discussion

We have found that lymphocytes, macrophages, and microglia from hCD4/hCXCR4 transgenic rats were partly susceptible to infection by HIV-1 X4 viruses leading to expression of low levels of early HIV-1 gene products. These levels were lower than those found in rats transgenic for hCCR5 and hCD4 (Keppler 2002 and Goffinet 2007). Primary rat lymphocytes and microglia, but not macrophages, from double-transgenic rats support entry and early gene expression but not productive infection by X4 strains of HIV-1.

2B.6 Acknowledgments

We thank the members of the Gladstone Animal Care Facility (Gladstone Institutes) for their expert assistance with transgenic animals. We acknowledge the assistance of Marty Bigos and Valerie Stepps for flow cytometry, and Andreas Jekle, Jason Kreisberg, Marielle Cavrois, and Becky Schweighardt for viral preparation and valuable discussions.

2B.7 Figure Legends

Figure 2.6 – Transgenic tissues express human CD4 and CXCR4. (A) hCD4 and hCXCR4 expression on nontransgenic and transgenic homozygous rat lymphocytes from spleen, after culture with Concavalin A and IL-2 are shown. (B)

hCD4/hCXCR4 on transgenic macrophages, isolated by adherence after 5 days. (C) Rat CD4 and CD8 and hCD4/hCXCR4 expression on nontransgenic and transgenic homozygous rat lymphocytes from thymus, as in A. (D) hCD4/hCXCR4 expression in nontransgenic and transgenic rat blood. Representative results from flow cytometry after culture are shown.

Figure 2.7 – Transgenic lymphocytes support entry and early gene expression but not productive infection. (A) Virion-based fusion assay of activated rat lymphocytes. (B) Luciferase-reporter virus infection of rat lymphocytes. Activated lymphocytes were infected with pseudotyped luciferase viruses and luciferase activity was measured after 48 hours. Results are the mean of triplicate experiments and normalized to protein expression. (C) Infection of rat lymphocytes with replication-competent virus. p24-Gag was measured by ELISA at the indicated timepoints.

Figure 2.8 – Macrophages from transgenic rats support early gene expression with some X4 viruses. Luciferase expression in rat macrophages (as in Figure 2.7). Results are the mean of triplicate experiments and normalized to protein expression.

Figure 2.9 – Microglia from transgenic rats support early infection with X4 viruses. Luciferase activity was detected in microglia after culture for 14 days and infection for 2 days. Results are the mean of triplicate experiments from a single animal.

2C. SDF-1γ

Paper: Inhibition of X4 and R5 HIV-1 by human SDF-1γ, a novel chemokine that interferes with HIV transcription

Christian Callebaut, Prerana Jayakumar, Brian J. North, Marielle Cavrois, Khaoula Bourara and Eric Verdin

2C.1 Abstract

Human *SDF1* encodes two known splice variants, SDF-1α and SDF-1β, which are the unique ligands of CXCR4. We have identified a new human splice variant of *SDF1*, SDF-1γ. This isoform is expressed in several tissues and exhibits strong chemotactic activity on Peripheral Blood Lymphocytes (PBL). SDF-1γ encompasses the full SDF-1α protein and an additional 30 amino acid at the C-terminus. This extra domain shows significant homology to the arginine-rich motif (ARM) of the HIV Tat protein and acts as a nuclear localization sequence. Accordingly, SDF-1γ binds to the target of Tat, the TAR RNA *in vitro*. Overexpression of SDF-1γ, but not of SDF-1α, inhibits HIV LTR transactivation by Tat and production of both X4 and R5 viruses. These observations demonstrate that SDF-1γ is a novel HIV restriction factor that interferes with the entry of X4 viruses and with the transcription of both X4 and R5 viruses.

2C.2 Introduction

Chemokines were initially linked to HIV biology by the purification of three soluble factors able to inhibit HIV infection (Cocchi 1995). This discovery was immediately followed by the functional cloning of CXCR4, a chemokine receptor, as an HIV coreceptor (Feng 1996). Subsequently, the ligand for CXCR4 was identified as SDF-1α, a chemokine capable of blocking HIV entry (Oberlin 1996; Bleul 1996). Since then, other chemokines and chemokine receptors, notably CCR5, have been characterized to play a significant role in HIV biology (Berger 1999). This has lead to the current nomenclature of viruses as X4 isolates, those using CXCR4 as a coreceptor, and R5 isolates, those viruses using the CCR5 family of receptors. A series of genetic mutations in human CCR5 restrict HIV infection, suggesting the importance of host factors to limit HIV replication. Other host factors have been shown to restrict HIV replication at various stages, including budding, reverse transcription, and packaging. However, there are no currently characterized host factors that inhibit HIV transcription.

HIV transcription is characterized by two temporally distinct phases. The early phase occurs after integration and relies only on cellular transcription factors. The late phase, characterized by a much higher rate of transcription, is Tat-dependent (for review, see Karn 1999). This second phase of transcription involves a positive regulatory loop, since increased transcriptional elongation results in more Tat synthesis, leading to a further increase in transcriptional elongation (Bieniasz 1998). The Tat protein of HIV is a unique viral transactivator that binds to the Tat-responsive element (TAR), an RNA stem–loop structure that

forms at the 5' extremity of all viral transcripts. In the absence of Tat, HIV transcription is highly inefficient because the assembled RNA polymerase II complex cannot elongate efficiently on the viral DNA template (Garber 1999). The binding of Tat to TAR stimulates the production of full-length HIV transcripts, and the integrity of the Tat/TAR axis critically determines the dynamics of viral replication in infected cells.

The role of Tat in HIV transcription has also recently been defined as an adaptor that coordinates the recruitment of critical co-factors at the HIV promoter. Distinct domains of Tat are involved in the recruitment of different cellular co-factors. The cysteine-rich region interacts with the cyclinT1/CDK9 complex (Wei 1998). Recruitment of CDK9 is thought to lead to hyper-phosphorylation of the C-terminal domain of RNA polymerase II and increased elongation efficacy. The arginine-rich motif (ARM) in Tat is essential for RNA binding to TAR and nuclear localization. Additionally, lysine residues in the ARM motif become acetylated by acetyl-transferases (Ott 1999) and regulate Tat-TAR-Cyclin interactions (Kaehlcke 2003).

We were interested in proteins sharing sequence similarity with Tat ARM. We found a high similarity to a novel splice variant of rat SDF-1, SDF-1γ (Gleichmann 2000). This SDF-1 splice variant is identical in sequence to SDF-1α but incorporates an additional 30 amino acids at the C-terminus. Interestingly, the additional domain located at the C-terminus of SDF-1γ contains a region displaying strong homology to the RNA-binding motif of Tat (ARM). Notably, the two basic stretches and a central arginine residue (R) both essential for the

binding of the ARM domain to TAR are conserved (Calnan 1991). The high degree of conservation of the C-terminal domain of SDF-1γ and the ARM domain of Tat suggested that SDF-1γ might be able to bind to the TAR RNA element. Competition between Tat and SDF-1γ for binding to TAR would inhibit Tat-mediated transactivation and Tat-dependent HIV transcription. We hypothesized that SDF-1γ inhibits Tat-dependent HIV transcription and suppresses HIV replication by binding to TAR or mimicking other Tat functions. We propose a series of experiments that will further test this model and define the potential role of SDF-1γ in HIV infection.

2C.3 Materials and methods

SDF-1γ Cloning (GenBank Accession numbers)

Human SDF-1γ was cloned from human heart polyA+ RNA (Clontech, Mountain View, CA) by standard RT-PCR-based strategies. Oligonucleotides were designed according to the genomic sequence of the *SDF1* gene. We used primer EV1276 (5'-TGAACTGTGGTCCATCTCGAGGTG) for the RT reaction (Superscript, Life Technologies), and EV1278 (5'-TCCGGCCCCCGACCCGCGCTCGTC) and EV1279 (5'-GTCCATCTCGAGGTGGCAGATAAC) primers for the PCR amplification. The 434 bp fragment obtained containing the SDF-1γ ORF was subsequently cloned into pCR-Blunt (Invitrogen).

Alignments and deduced protein structure

After *in silico* translation of the deduced cDNA sequence, comparisons and

alignments were carried out with MacVector 6.5.3 software (Oxford Molecular plc, UK). Deduced amino-acid sequences were analyzed by 'MOTIF: Searching Protein and Nucleic Acid Sequence Motif' (http://motif.genome.ad.jp/). Predicted nuclear localization sequences were identified using PSORT II software (http://psort.nibb.ac.jp/form2.html).

Tissue-blot

Human multi-tissue-expression array membrane (MTE, Clontech Palo Alto, CA) was hybridized with a SDF-1γ specific oligonucleotide probe (EV1298) (Genset, La Jolla, CA), radiolabeled with 32P-g-ATP (Amersham) and polynucleotide kinase (NEB).

Northern-blot

Human multi-tissue-northern membrane was obtained from Clontech (12-Lane MTN Blot, Clontech Palo Alto, CA). 32P labeled probes corresponding to SDF-1γ (NEB, Beverly, MA). The blots were prehybridized and hybridized with ExpressHyb hybridization solution (Clontech) and washed under high stringency conditions. Autoradiographs were analyzed with a FUJIX BAS1000 phosphorus imaging system (Fuji, Tokyo, Japan).

Plasmids and Mutagenesis

The SDF-1γ ORF was subcloned into pcDNA3.1(+) vector (Invitrogen) for expression. The SDF-1γ ORF was also subcloned to generate C-terminal FLAG-tagged fusion in a derivative of the pcDNA3.1(+) backbone (FLAG vector) by

standard PCR-based strategies and confirmed by sequencing. The C-terminal tail of SDF-1γ was cloned into pEGFP-C1 vector (Clontech).

Site-directed mutagenesis for SDF-1γ constructs was performed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Rabbit anti- SDF-1γ peptide KLH

We raised an antiserum against SDF-1γ by immunizing rabbits with a peptide corresponding to the C-terminus (indicate peptide sequence or residues) of SDF-1γ coupled to keyhole limpet hemocyanin (KLH).

SDF-1γ was cloned into a vector containing the T7 promoter and a 13.5-kDa protein was expressed by in vitro translation. We raised an antiserum against SDF-1γ by immunizing rabbits with a peptide corresponding to the C-terminus of SDF-1γ coupled to keyhole limpet hemocyanin (KLH) (repeated from above). In western blotting analyses, this polyclonal antiserum in unpurified form specifically recognized SDF-1γ, but not SDF-1α, in extracts of transfected 293T cells.

Soluble recombinant SDF-1α is was purchased from R&D Systems (Minneapolis, MN) and used at the indicated concentrations.

Cells

HeLa, HEK-293T, and Jurkat cells were obtained from the ATCC. MAGI-C5 and GHOST X4R5 cells were obtained from the NIH AIDS Reagent Program. Cells were cultivated in DMEM or RPMI-1640 complemented with 10% fetal calf serum and 1% penicillin-streptomycin (Invitrogen).

Western blotting

Samples (Cellular lysates) were separated on 15% SDS-polyacrylamide gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Inc.). Membranes were blocked with 5% blocking reagent (Bio-Rad, Richmond CA) in TBS-Tween (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and were probed with anti-FLAG M2 (Sigma), diluted 1:2000; anti-HA, or anti-lamin A (Cell Signaling Technology, Inc., Beverly, MA), each diluted 1:1000. Secondary detection was performed using HRP-coupled sheep anti-mouse IgG (Amersham Pharmacia Biotech, Inc.) or goat anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) diluted 1:5000 and the ECL Western blotting detection system (Amersham Pharmacia Biotech, Inc.).

Chemotaxis

The chemotaxis assay, using a modified Boyden chamber method, was performed in order to evaluate the biological activity of rhSDF-1 (R&D systems, Minneapolis, MN) on PBMC (10^6 5-days-PHA-activated) or Jurkat cells. Control, SDF-1α, or SDF-1γ containing supernatants were produced by transfection of HEK-293T cells with pcDNA3.1, pcDNA3.1-SDF-1α or pcDNA3.1-SDF-1γ respectively. Supernatants were harvested 48hrs after transfection, passed through a 0.22 μm filter, aliquoted and stored at –20°C. Cells were suspended at a concentration of 10^6 cells/ml in RPMI 1640 medium containing 0.25% inactivated fetal calf serum (FCS). The cell suspension (100 μl) was transferred into the top chamber of a Transwell (Costar #3415; Corning, NY). Media containing rhSDF-1 or SDF-1 containing supernatant were put into the bottom

chamber of the Transwell (600 μL). After incubation at 37°C for 5 h the transmigrated cells to the bottom chamber were manually counted.

Transfections and luciferase assays

Transfections with Tat-, and SDF-1-expressing plasmids were performed in 293T cells with the calcium phosphate precipitation method. Cells were harvested 48 hrs after transfection of plasmids DNA and analyzed for luciferase activity. Transient transfections into GHOSTX4R5 cells (NIH AIDS reagent program) were performed with FuGene 6 (Roche Diagnostics, Indianapolis, IN). Cells and supernatant were harvested after 48 h, and luciferase activity was measured.

Protein assay Biorad

Protein concentration was measured in total cell lysates by the *Dc* Protein Assay Kit (BioRad, Hercules, CA)

Virus infections

X4 and R5 HIV isolates were produced from transfection of HEK-293 T cells with pNL4.3 (Adachi 1986) and p49-5 (Toohey 1995) DNA plasmids respectively.

Viral Replication

At the indicated time points, sample of culture media was taken from infected cells, mixed with Triton-X-100 (?) and stored at –20°C. The concentration of HIV-1 p24 in the media was measured by ELISA (NEN Life Science).

TAR binding assay

The TAR binding assay was performed as previously described (Dorr et al, 2002). Briefly, streptavidin-sepharose beads (Amersham Biosciences, Piscataway, NJ) were blocked for 1 hr at 4°C in binding buffer containing 3% nuclease-free BSA (NEB, Beverly, MA). After equilibration in binding buffer, 30 µl beads were incubated with 0.5 µg biotinylated TAR element (biotin-AATTCCAGATCTGAGCCTGGGAGCTCTCTGGA; Xeragon, Zurich, Switzerland) and incubated for 1 hr at 4°C. TAR bound to beads was mixed with 2 µL lysates from control, SDF-1 or Tat expression plasmids *in vitro* translated in the presence of 20 µCi [^{35}S]-L-methionine (Amersham Biosciences, Piscataway, NJ) using the TNT T7 coupled Reticulocyte Lysate System (Promega, Madison, WI). Reactions were incubated for 10 min at 30°C and washed twice with binding buffer prior the addition of loading buffer. Proteins were separated by SDS–PAGE analysis, fixed and amplified (Amplify; Amersham Pharmacia Biotech) for 30 min, dried and exposed to a BioMax MR film (Kodak, Rochester, NY).

Immunofluorescence Microscopy

HeLa cells grown on coverslips were transfected with Fugene 6 (Roche Diagnostics, Indianapolis, IN.) according to the manufacturer's instructions. Cells on coverslips were washed twice in PBS for 10 min, fixed in 4% paraformaldehyde (EMS, Ft. Washington, PA) for 10 min, followed by permeabilization in 0.5% Triton-X-100 in PBS for 10 min. After three washes for 10 min in PBS, cells were incubated in 10% BSA for 10 min and then incubated

for 1 hr with anti-Flag-M2 1:1000 in PBS + 0.1% Tween-20. Cells were washed three times 10 min in PBS + 0.1% Tween-20, followed by incubation with goat anti-mouse IgG (Fc specific) TRITC-conjugated secondary antibody (Sigma) diluted 1:100 in PBS + 0.1% Tween-20. Cells were then incubated in 20 ug/ml DAPI for 5 min, washed three times for 10 min each in PBS and once briefly in ddH_2O, and mounted on slides with Gel Mount (Biomeda Corp., Foster City, CA). Slides were visualized on a Nikon E600 microscope system equipped with a SPOT 2 Digital Camera. Confocal images were acquired by laser-scanning confocal microscopy with an Olympus BX60 microscope equipped with a Radiance 2000 confocal setup (Bio-Rad).

GFP Fluorescence

293T cells grown on coverslips were transfected with either GFP (pEGFP) or GFP-gamma-tail (pEGFP-SDF1-γ (69-98)). Cells were visualized after 48 hours.

2C.4 Results

Rat SDF-1γ cDNA was recently cloned from a cDNA library of injured nerves (Gleichmann 2000). Preliminary experiments using RT-PCR indicated that human SDF-1γ mRNA was expressed in several tissues (data not shown). Using the information from the rat cDNA cloning, the human *SDF1* gene organization (Bleul 1996) and the human SDF-1 locus sequence (http://www.ncbi.nlm.nih.gov/, LOCUS AL137026), we designed primers to clone the putative human SDF-1γ by standard RT-PCR methods. The open reading frame of the sequence amplified is shown in Figure 2.10A. Compared to SDF-1α, SDF-1γ contains an extra 30 amino-acid tail, encoded by a single additional exon, generating a novel protein with a predicted size of 98 amino acids (Figure 2.10A). The human protein is 94% identical to the rat isoform (97% for the mature form without the signal peptide) (Figure 2.10B). Both species of SDF-1γ contain a highly basic region (103-115) that is similar to the Tat ARM. *SDF1* gene organization is showing in Figure 2.10C. The gene contains one cryptic donor site in exon 3 that can be used with a lower efficiency to produce SDF-1β and SDF-1γ, both of which are generated by alternative splicing. Using a probe against exon I-III, a region common to all three variants, we observe that both SDF-1β and SDF-1γ result in mRNAs larger than that of SDF-1α (Figure 2.11A). Overall, SDF-1α can be seen as the main transcript, whereas SDF-1β and SDF-1γ are expressed at lower levels. In some tissues, like spleen or kidney, SDF-1β

is the predominant alternative splice variant, whereas in tissues like heart or lung, SDF-1γ is more highly expressed.

The expression of SDF-1γ in different tissues was examined by Northern blot using a probe specific for exon IVb (Figure 2.11B). A major transcript of 6.5 kb was detected in most tissues, with a lower expression in thymus, placenta and unstimulated PBLs. A weaker band corresponding to 9.0 kb might represent an additional differentially spliced isoform of the SDF-1 mRNA, or an alternative poly-adenylation site. The size of 6.5kb is in good agreement with the length of ESTs previously cloned (data not shown). Additionally, a dot-blot human multi-tissue-expression array membrane hybridized using the same probe showed a ubiquitous expression of SDF-1γ mRNA (data not shown).

We first cloned SDF-1γ into a vector under the control of a T7 promoter and showed that a 13.5-kDa protein was expressed by in vitro translation (not shown). We raised an antiserum against SDF-1γ by immunizing rabbits with a peptide corresponding to the C-terminus of SDF-1γ coupled to keyhole limpet hemocyanin (KLH). In western blotting analyses, this polyclonal antiserum in unpurified form specifically recognized SDF-1γ, but not SDF-1α, in extracts of transfected HEK-293T cells (Figure 2.11C). As previously shown, SDF-1α is a strong chemoattractant for PBMCs (Aiuti 1999). To test whether SDF-1γ has similar activity, supernatants from transfected cells were used in a chemotaxis assay using a modified Boyden chamber system. Both SDF-1α and SDF-1γ were able to attract PBMCs to a similar level (Figure 2.11D).

We next wanted to determine the intracellular localization of SDF-1γ. Using indirect immunofluorescence, we observed that tagged SDF-1γ was predominantly cytoplasmic (a typical cell is shown in Fig 2.12a). Concurrent DAPI staining of nuclei showed that the discrete SDF-1γ staining in the nucleus was nucleolar. The nucleolar expression seems limited to the "crown" of the nucleoli, where the snRNP complexes are mainly formed (references) (Fig 2.12a). This unexpected nuclear expression for a chemokine suggested that the additional residues in SDF-1γ contain a nuclear localization signal (NLS). Computer analysis of the sequence showed the presence of four putative NLS's, two monopartite and two bipartite (Fig 2.12b). To confirm the function of these sequences, we created a fusion protein between the C-terminal tail of SDF-1γ and the Green Fluorescent Protein (GFP). In 293T cells, GFP alone produces diffuse, whole-cell staining. In contrast, the fusion protein was only localized to the nucleus. We could also see more intense GFP signal in nucleolar-like structures within the nucleus (Fig 2.12c). We conclude that this C-terminal domain specific to SDF-1γ contains at least one functional NLS responsible for its localization to the nucleus, and that this domain could be further useful for addressing heterologous proteins to this cellular compartment. Interestingly, Tat nuclear localization gives a similar pattern of expression (data not shown).

The high degree of homology between Tat consensus sequences (from Los Alamos National Laboratory) and a region of SDF-1γ tail is shown in Fig2.13a. This degree of homology leads us to suggest that, like Tat, SDF-1γ would be able to interact with TAR. To investigate this hypothesis, we used a very specific TAR-

RNA pull down assay, where TAR RNA is linked to biotin at its 5' extremity, incubated with *in vitro* translated radiolabeled Tat or SDF-1, bound to avidin-agarose beads and pulled-down by centrifugation (Fig 2.13b). After extensive washing, Tat or SDF-1 was released from the beads by nuclease treatment. The eluted material was analyzed by SDS–PAGE, followed by autoradiography. Figure 2.12C shows that Tat binding to the synthetic TAR is saturable. Using the same assay, we showed that SDF-1γ was binding to TAR. In contrast, SDF-1α lacking this additional basic region showed no TAR binding (Fig 2.13d), suggesting that this domain was responsible for the specific binding of SDF-1γ to the TAR element.

The presence of SDF-1γ in the nucleus and its binding to TAR fulfill two pre-requisites for potential inhibition of the HIV LTR by SDF-1γ. To test whether SDF-1γ can interfere with HIV Tat-mediated transcription we co-transfected an LTR-luciferase reporter construct and Tat expression vectors with expression vectors for SDF-1α or SDF-1γ, and measured their effect on the activity of the HIV promoter. The HIV promoter was activated 10-fold in response to Tat (not shown). As we introduced and increased the SDF-1γ concentration, we saw a dose-dependent inhibition of the luciferase activity (Fig 2.14a). SDF-1α, in contrast, did not significantly inhibit Tat-mediated LTR activation. This implies that LTR inhibition is specific for the SDF-1γ tail. This effect was also specific for the HIV promoter and was not observed with the promoters HSV–TK, CMV-IE, LTR stimulated with PMA and 4xNF-κB activated with TNFα (Fig 2.14b).

Because SDF-1γ was able to alter HIV transactivation, we postulated that it could also inhibit HIV infection independent of coreceptor usage. To test this hypothesis, we co-transfected HIV encoding vectors (pNL4.3 or p49-5) with expression vectors for SDF-1 in GHOST cells. HIV p24 gag antigen production in the supernatant was monitored by ELISA at day 3 (Fig 2.15a). As previously shown, the addition of SDF-1α inhibited p24 production of the X4 virus NL4-3. SDF-1γ also reduced the p24 output of NL4-3 to a similar extent. This inhibitory effect can be explained by the fact that the CXCR4 binding site in SDF-1α is located in the first 17 amino acids of the mature protein and is identical in both isoforms. The same experiment was done with 49-5, an R5 isolate (isogenic of NL4-3). SDF-1α increased virus production with the R5 isolate, similar to what has been reported (Marechal 1999). This effect is believed to be mediated through NFkB activation. In contrast, virus production was inhibited when the cells were transfected with SDF-1γ (Fig 2.15b). The level of SDF-1γ inhibition was comparable with both NL4-3 and 49-5 isolates. Similar results were obtained in MagiC5 cells, in which virus production was monitored by LacZ activity (not shown). Taken together, these experiments demonstrate that SDF-1γ is the first chemokine that functions at multiple steps of the HIV life cycle. In contrast to previously characterized anti-HIV chemokines, SDF-1γ acts to inhibit not only entry of X4 viruses but also transcription of both R5 and X4 isolates.

2C.5 Discussion

The rate of progression to AIDS correlates directly with the level of plasma viremia. Therefore, changes in viral transcriptional rates directly affect disease pathogenesis. Pro-inflammatory cytokines that activate the transcription factor NF-κB are likely to activate HIV transcription and contribute to an increase in HIV replication. Other cytokines could exert opposing activities on the HIV promoter and inhibit HIV transcription and replication. Such cytokines could contribute significantly to the establishment of a latent infection or to a partial inhibition of HIV replication, thereby modulating the rate of disease progression. We have identified one such possible cytokine, the human ortholog of SDF-1γ, the third splice variant of the *SDF1* gene. The open reading frame for this variant contains an additional domain of 30 amino acids, of which 66% are basic lysine or arginine residues. As previously shown for rat SDF-1γ, human SDF-1γ is ubiquitously expressed, with higher levels in heart.

Interestingly, one of the prime causes of lethality in CXCR4 and SDF1 knockouts in mice is heart defects. These defects are thought to be caused mainly by homing defects in these mice due to the lack of SDF-1α, but may in fact be due to deficiencies in the non-chemotactic properties of SDF-1γ due to its presence in the nucleus. We also report here that human SDF-1γ exhibits equivalent chemotactic activity to SDF-1α. Cai et al recently reported that the C-terminal alpha helix plays a role in the stability of SDF-1α binding to its receptor (Cai 2004). Computer modeling of SDF-1γ structure suggest that the additional 30

amino acids extends the alpha helix, suggesting a possible enhancement of binding to CXCR4 (data not shown). However, we showed here that neither the chemotactic activity nor the inhibition of X4 viruses through CXCR4 engagement is significantly different between SDF-1α and SDF-1γ. This suggests that the C-terminal domain may not stabilize SDF-1γ binding to CXCR4.

As previously shown for the Tat basic domain, the C-terminal tail of SDF-1γ confers nucleolar localization. In Tat, this region is also responsible for protein transduction. Preliminary results indicate that SDF-1γ has protein transduction properties (CC, unpublished observations). We have also shown here that SDF-1γ was able to bind TAR very efficiently, similar to Tat. In addition, Tat is thought to bind other RNAs. Consequently, we would like to evaluate SDF-1γ binding to other RNAs/DNAs, including highly structured regions in the HIV genome.

The observation that SDF-1γ inhibits both X4 and R5 isolates is a small soluble protein, and acts at the transcriptional level, suggests that it may be a good candidate as part of the CAF activity previously described (Levy 1994). Dr. Levy's laboratory identified several years ago a cellular antiviral factor (CAF), secreted by CD8+ T-cells (Mackewicz 1995), whose levels correlate inversely with the rate of progression to AIDS (Mackewicz, 1991). CAF was also shown to block HIV replication *in vitro,* at least partially by interfering with HIV transcription in $CD4^+$ T cells (Chen 1993; Mackewicz 1995). We and others have recently shown that CAF specifically inhibits HIV transcription (Le Borgne 2000; Mackewicz 2000; Tomaras, 2000). Possible identity between CAF and SDF-1γ is

under investigation. However, SDF-1γ obviously does not fulfill all the requirements for CAF, e.g. it is not exclusively expressed in CD8+ T cells.

Despite this apparent lack of identity with CAF, SDF-1γ may nonetheless play a role in the reported delay of AIDS progression associated with the SDF1 3'A genotype (Winkler 1998). This mutation present in the 3' UTR of SDF-1β transcripts was shown to have little influence on SDF-1β production, and the techniques used to evaluate the effect of the mutation were specific for SDF-1β. However, the 3'A mutation is present in SDF-1γ mRNA as well, and it may modify SDF-1γ abundance and then be associated with a delay in HIV infection. Finally, this mutation may modulate alternative splicing in favor of SDF-1γ. The role of this mutation in SDF-1γ abundance will be investigated.

Recent reports showed that Tat was able to bind CXCR4 and act as a chemokine-like molecule. It has also been speculated that Tat is structurally related to chemokines. We have shown here that SDF-1γ also recapitulates many of the properties of Tat, including (Xiao 2000) cysteine organization, basic domain, size, NLS, NOS, PTD, chemotaxis, CXCR4 binding, binding to GAGs, and TAR binding. Therefore, because SDF-1 is believed to be the ancestor of all known chemokines, very conserved among species, and has given rise to other chemokines by gene duplication, it is possible that HIV Tat also evolved from the same gene.

2C.6 Acknowledgments

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2C.7 Figure Legends

Figure 2.10 - Cloning of human SDF-1γ. (A) The nucleotide sequence and the deduced amino acid sequence corresponding to the SDF-1γ open reading frame are shown. The additional residues specific to SDF-1γ are highlighted in black. (B) Alignment of the human and rat SDF-1γ proteins. Proteins were aligned with Clustal. Identical residues are boxed in *black*; conserved residues are boxed in *gray*. Alignment of the Tat-ARM is shown. (C) Schematic representation of *SDF1* and its splicing variants. Exons I-IV are shown in black, and the new exon (Ivb) is shown in white. Solid lines indicate exclusive splicing; dotted lines show alternative splicing. SA, splice acceptor site; SD, splice donor site.

Figure 2.11 - Tissue-specific expression and function of SDF-1γ. (A) Northern blot analysis of SDF-1 isoforms showing tissue distributions. (B) Western blot analysis of SDF-1γ expression in transfected 293T cells. (C) Chemotactic activity of SDF-1α and SDF-1γ on PHA-treated PBMCs. A representative experiment of three is shown.

Figure 2.12 - SDF-1γ is expressed in the cytoplasm and in the nucleolus. (A) HeLa cells were transfected with an expression vector encoding FLAG-tagged SDF-1γ. Left panel shows staining with anti-FLAG antibody. Center panel shows staining of DNA with DAPI. Right panel shows a merged image in which DAPI is blue and SDF-1γ-FLAG is red. (B) HeLa cells were transfected with an expression vector encoding a fibrillarin-GFP fusion protein and FLAG-tagged SDF-1γ. Left panel shows GFP fluorescence of the fusion protein. Center panel shows staining with the anti-FLAG antibody. Right panel shows a merged image in which GFP is green and SDF-1γ-FLAG is red. (C) Amino acid sequence of the specific SDF-1γ C-terminal tail is shown. Bipartite nuclear localization signals (NLS) are underlined (1 and 2); monopartite NLS are boxed (3 and 4). (D) HeLa cells were transfected with expression vectors encoding GFP or GFP fused to amino acids 90–119 of SDF-1γ (GFP-γ-Tail).

Figure 2.13 - SDF-1γ binds specifically to TAR RNA in vitro. (A) Alignment of SDF-1γ basic domain with Tat Arginine Rich Motif consensus sequences (from http://www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-INDEX.html). Capital letters indicate conserved residues in Tat for each consensus sequence. Boxed letters show alignment with SDF-1γ (103-115). (B) Cartoon showing the TAR RNA pull-down assay. ^{35}S-labeled proteins were mixed with biotin-TAR. Complexes were pulled down with avidin-agarose before loading on SDS-PAGE gel. (C) Binding of Tat to TAR. ^{35}S-labeled Tat protein (0, 1, 3, and 9 uL of TnT extracts) was used as shown in panel B. Gels were dried and exposed overnight

in a PhosphorImager. Band intensities were plotted for each corresponding lane. (D) Binding of SDF-1γ to TAR. ^{35}S-labeled SDF-1γ proteins (3 uL of TnT extracts) was used as shown in panel B. Gels were treated as in C. Lanes 1 and 2: input (20%); lanes 3 and 4: pull-down.

Figure 2.14 - SDF-1γ blocks Tat-dependent LTR transactivation. (A) 293T cells were co-transfected with fixed quantities of LTR-Luciferase and CMV-Tat plasmids, and EF-1a-renilla plasmid as an internal control. The co-transfection contained increasing quantities of control SDF-1α or SDF-1γ plasmids as indicated, complemented with control plasmids. (B) 293T cells were transfected with fixed quantities of different promoters linked to luciferase (LTR/PMA, 4xNF-kB/TNFa, HSV-TK or CMV-IE). As in A, cells were cotransfected with increasing quantities of SDF1γ.

Figure 2.15 - SDF-1γ blocks both X4 and R5 virus infection in GHOST cells. GHOST cells expressing CXCR4 and CCR5 were co-transfected with 100 ng of (A) pNL4.3 or (B) p49-5, and 0, 100, or 300 ng of control, SDF-1α or SDF-1γ encoding plasmids, complemented with empty plasmid. HIV infection was monitored by p24 released in the supernatant at day 3. p24 values are the average of duplicate samples. Values were normalized to control plasmid transfection.

2D. Tat and SIRT1

Paper: Human Immunodeficiency Virus Type 1 Tat Protein Inhibits the NAD^+-Dependent Deacetylase SIRT1 and Induces T-Cell Hyperactivation

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2D.1 Abstract

Symptoms of generalized immune hyperactivation shape the course and outcome of HIV-1 infection, but underlying causes of immune activation are not well understood. We find that the viral transactivator Tat hyperactivates T cells by blocking the nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase SIRT1. Tat directly interacts with the deacetylase domain of SIRT1 and blocks the ability of SIRT1 to deacetylate lysine 310 in the p65 subunit of NFκB. As a consequence, Tat hyperactivates the expression of NFκB response genes, a function lost in SIRT1-/- cells. These results support a model, in which SIRT1 serves as a negative regulator of T-cell activation, which is inactivated by Tat during HIV infection.

2D.2 Introduction

Immune activation is a hallmark of HIV-1 infection and a significant factor in continuous viral replication and CD4+ T cell depletion (Douek 2003). In HIV-infected individuals, levels of circulating activation markers correlate with accelerated disease progression and shortened survival (Lawn 2001). HIV infection is critically dependent on the activated state of $CD4^+$ T cells since the virus cannot replicate efficiently in resting T cells (Stevenson 1990). Quiescent T cells are refractory to infection because of blocks at the level of reverse transcription and proviral integration (Bukrinsky 1991; Chiu 2005). In addition, T-cell activation enhances viral transcription through activation of transcription factors, such as nuclear factor κB (NFκB) (Liu 2005).

HIV-1 infection itself manipulates the activation status of infected T cells through the expression of viral proteins, including Env, Nef and Tat (Chirmule 1996; Fackler 2002; Peruzzi 2006; Schindler 2006). The viral transactivator Tat potently activates HIV transcription and binds to an RNA stem-loop structure called TAR that spontaneously forms at the 5′ extremities of all viral transcripts (Barboric 2005). Tat also influences the expression of cellular genes in infected T cells. For example, Tat synergizes with signals mediated via the T-cell receptor (TCR) and the CD28 coreceptor to superactivate interleukin (IL)-2 gene expression (Fortin 2004; Ott 1997; Westendorp 1994; Wu 2001).

IL-2 is a T-cell growth factor with critical functions in lymphocyte proliferation, survival and immune functions (Smith 1988). IL-2 expression is dependent on coordinated signals delivered via the TCR and coreceptors and is tightly

regulated at the transcriptional level. IL-2 is important during HIV infection: it primes nonactivated bystander cells for infection in the absence of antigenic stimulation (Kinter 1995; Polacino 1995; Unutmaz 1994). This important mechanism increases the pool of target cells permissive for HIV infection, specifically in the confines of the lymphoid organs (Kreisberg 2006).

While the function of Tat in viral transcription is well studied, the molecular mechanism of its immunomodulatory functions is less clear. Many previous reports linked Tat with the transcription factor NFκB (Buonaguro 1994; Cota-Gomez 2002; Dhawan 1997; Hofman 1999; Lee 2004; Liu 2002; Lusic 2003; Ott 1997; Ott 1998; Scala 1994; Westendorp 1994; Westendorp 1995). Beside its central role in the regulation of the innate and adaptive immune response, NFκB is a critical regulator of HIV transcription. The HIV promoter contains two tandem NFκB binding sites (Nabel 1987). The activity of the prototypical NFκB complex (a heterodimer of p50 and p65 subunits) is regulated through association with inhibitory IκB molecules and posttranslational modifications. IκB molecules complexed to the NFκB dimer in the cytoplasm are rapidly phosphorylated after T-cell activation via the TCR, allowing nuclear translocation of NFκB (Ghosh 2002). After translocation, the p65 subunit undergoes posttranslational modifications, including reversible acetylation by the histone acetyltransferase p300 (Chen 2002; Kiernan 2003). The acetylation of lysines 218 and 220 enhances DNA binding and prevents assembly with IκB and nuclear export of p65, while acetylation of lysine 310 (K310) is critical for the transcriptional activity of NFκB (Chen 2001; Chen 2002). The transcriptional activity of NFκB is

suppressed by the deacetylase activities of histone deacetylase 3 and SIRT1, two proteins that deacetylate p65 (Chen 2001; Yeung 2004). SIRT1 specifically deacetylates K310 in p65 and inhibits the antiapoptotic function of NFκB (Yeung 2004).

SIRT1 is a homologue of the yeast transcriptional repressor silent information regulator 2p (Sir2p), an important regulator of longevity in yeast (Gasser 2001). Like Sir2p, SIRT1 requires nicotinamide adenine dinucleotide (NAD^+) as a cofactor, which links its activity to the metabolic state of the cell. Despite its enzymatic activity on histone substrates *in vitro*, recent experimental evidence suggests that SIRT1 predominantly targets nonhistone proteins for deacetylation (Sauve 2006). We recently reported that Tat is a substrate for the deacetylase activity of SIRT1 (Pagans 2005). Acetylation of lysine 50 (K50) in Tat is mediated by the histone acetyltransferase activity of p300 (Kiernan 1999; Ott 1999) and human GCN5 (Col 2001) and generates binding sites for the bromodomains of PCAF (Dorr 2002; Mujtaba 2002) and Brg1 (Mahmoudi 2006). SIRT1 binds and deacetylates Tat at K50, a process necessary to recycle nonacetylated Tat protein for binding to TAR RNA and the cofactor pTefb (Kaehlcke 2003; Kiernan 1999).

Because SIRT1 is a regulator of NFκB and interacts with the HIV Tat protein, we examined the effects of Tat on the deacetylase activity of SIRT1. We propose a molecular mechanism for the longstanding observation that Tat participates in the hyperactivation of immune cells infected with HIV.

2D.3 Materials and Methods

Cells and Plasmids

HeLa, 293 and Jurkat cells were cultured under standard tissue culture conditions. MEF cells derived from SIRT-/- cells (McBurney 2003)were obtained from M. McBurney (Ottawa Regional Cancer Center) and were grown as described (Pagans 2005). Expression constructs for human Myc-tagged SIRT1 (Langley 2002) were provided by T. Kouzarides (University of Cambridge), E-Selectin (De Luca 1994) and IκB luciferase reporter constructs (Luecke 2005) by J. Pober (Yale University) and K. Yamamoto (University of California, San Francisco), respectively. Murine Sir2 deletion mutants (Fulco 2003) were gifts from V. Sartorelli (NIH) and the expression vector for p53 (Baker 1990) from B. Vogelstein (Johns Hopkins Oncology Center). Mutant constructs for SIRT1 HDAC domain were prepared by site-directed mutagenesis using FLAG-tagged SIRT1 as a template. Three copies of the NFκB binding site present in the α subunit of the high-affinity IL-2 receptor promoter were cloned upstream of the thymidine kinase minimal promoter in pGL-2 basic vector.

Infection with LTR-GFP and LTR-Tat-GFP Lentiviral Vectors

LTR-GFP and LTR-Tat-GFP are HIV-based vectors derived from the pHR' series (provided by D. Trono, Swiss Institute of Technology Lausanne), in which GFP or Tat101 (corresponding to the two-exon form of the HIV-1 Tat gene) and GFP are under the control of the HIV-1 LTR through the use of an internal ribosome entry site (Jordan 2001). Each vector was cotransfected into 293T cells together with a

packaging construct (pCMV-R8.91) that provides all HIV genes required for production of infective particles and a plasmid encoding the vesicular stomatitis virus envelope G protein (VSV-G) to produce pseudotyped viral particles with broad host range and high infectivity. The next day, viral particles were harvested from the supernatant of transfected cells and quantified with a p24 ELISA assay. p24 (1500 ng) were used to infect 18×10^6 Jurkat T cells. Infections were carried out in 6-well plates at 2400 rpm in a Beckman-Coulter centrifuge (Allegra 6R, rotor GH3.8A) for 2 h at 32°C in the presence of polybrene (1 μg/ml, Sigma). After infection, cells were washed repeatedly in phosphate-buffered saline and plated at a concentration of 5×10^5 cells/ml in RPMI complete medium. Jurkat cells (10^6) were stimulated with α-CD3 (3 μg/ml) and α-CD28 (1 μg/ml) antibodies 36 h after infection as described (Ott et al., 1998) or were preincubated with nicotinamide (10 mM, Sigma) for 1 h before treatment with α-CD3/28 antibodies. Flow cytometry analysis of GFP expression was performed (FACSCalibur, BD Bioscience). Infection efficiencies ranged from 70–98% GFP+ cells in individual experiments. Tat expression was visualized by western blot with polyclonal α-FLAG and α-actin (Sigma) antibodies.

Generation of SIRT1-Negative and -Positive MEF Cell Lines

Open reading frames corresponding to Myc-tagged human SIRT1 and FLAG-tagged HIV-1 Tat (101 amino acids) cDNAs were inserted into murine stem cell virus (MSCV)-based retroviral vectors, MSCV-puromycin and MSCV-zeocin, respectively (Clontech). To obtain recombinant virus, 10 μg of each constructs for SIRT1 and Tat expression along with control vectors were transfected into

BOSC23 cells, a retroviral packaging cell line derived from the 293 human embryonic kidney cell line (Pear 1993), grown in a 10-cm dish until 80% confluence with Lipofectamine (Invitrogen). The supernatants were collected 48 h after transfection and filtered through a 0.45-μm membrane. SIRT1-/- MEF cells (2×10^5) in a 6-cm dish were incubated with 2 ml of the supernatant containing SIRT1 retrovirus and control virus together with polybrene (8 μg/ml). Cells were selected after 48 h with puromycin (2.5 μg/ml, Invitrogen) for 5 days. Polyclonal puromycin resistant MEF cells were then reinfected with Tat and its control retroviruses as the same condition above. Cells were reselected in the presence of both puromycin (2.5 μg/ml) and zeocin (100 μg/ml, Invitrogen). Four polyclonal populations of MEF cells grown in a 6-cm dish at 90% confluence were treated with recombinant human TNFα (20 ng/ml, Invitrogen) in the presence of both puromycin and zeocin for 2 and 8 h. Cells were processed for RNA isolation.

RNA Purification, cDNA Synthesis, and Real-Time RT-PCR

Total RNA was extracted using RNA STAT-60 reagent (Tel-Test) according to the manufacturer's instruction. The first strand cDNA was generated using 2 μg of total RNA and SuperScript reverse transcriptase (Invitrogen). Real-time RT-PCR was performed in duplicate on the ABI PRISM 7700 thermocycler (Applied Biosystems). Human IL-2 and murine E-selectin mRNAs were quantified by QuantiTect gene expression assays (Qiagen). Murine IκBα and murine/human GAPDH mRNA concentrations were determined with SYBR Green I master mix (MCLab). Primers were designed using Primer Express software (Applied

Biosystems). Mouse IκBα (NM_010907); Forward: 367 TGGCCTTCCTCAACTTCCAG, Reverse: 487 TCTCGGAGCTCAGGATCACA. Mouse GAPDH (BC095932); Forward: 204 ACTCCACTCACGGCAAATTCA, Reverse: 324 GCCTCACCCCATTTGATGTT. Human GAPDH (BC001601); Forward: 344 AGTCCACTGGCGTCTTCACC, Reverse: 464 TGGTTCACACCCATGACGAA.

Gene expression was analyzed quantitatively using a comparative *C*t method (Pfaffl 2001) (Applied Biosystems). The expression of each gene was first normalized to GAPDH expression by subtraction of the GAPDH *C*t value to the one obtained for the gene at any condition tested (Δ*C*t values). The ΔΔ*C*t was then calculated as the difference between the Δ*C*t values from stimulated (α-CD3/28 antibodies or TNFα) and nonstimulated control samples. Relative expression ratios between stimulated and nonstimulated conditions were calculated by the equation, ratio = $2^{-\Delta\Delta Ct}$. All real time RT-PCR were performed from at least three independent samples.

Transfections and Coimmunoprecipitations

Luciferase reporter constructs and expression vectors for various proteins were cotransfected into HeLa cells with 12-well plates and Lipofectamine. Twenty-four h after transfection, cells were lysed and processed for luciferase assays (Promega). Transfection efficiency was normalized by protein concentration. P values (paired t-test) were used for statistical analysis.

In coimmunoprecipitation experiments, 293 cells grown in 6-well plates at 70% confluence were cotransfected in triplicate using Lipofectamine and expression

vectors as indicated in the figure legends. Twenty-four hours after transfection, cells were lysed in P300 lysis buffer (250 mM NaCl, 0.1% NP40, 20 mM NaH_2PO_4 (pH 7.5), 5 mM EDTA, 30 mM sodium pyrophosphate, 10 mM NaF) containing protease inhibitors (Roche Molecular Biochemicals). Triplicates were pooled, and 2 mg of cell lysate was immunoprecipitated with either α-FLAG M2 agarose (Sigma) or α-T7-agarose (EMD Biosciences) at 4°C over night. Beads were washed three times in P300 lysis buffer, boiled in SDS loading buffer, and analyzed by western blotting with polyclonal α-FLAG (Sigma), α-Myc (sc-40, Santa Cruz Biotechnology), or monoclonal α-T7 (EMD Biosciences) antibodies.

For detection of acetylated p65 and p53 in cells, 293 cells were transfected using Lipofectamine and p65, wildtype p53, and various expression vectors as indicated in the figure legends. Transfection and lysis of cells were carried out with the same procedures used in coimmunoprecipitation experiment. Cell lysate (2 mg) was immunoprecipitated with either α-T7-agarose for p65 or with α-goat p53 antibody (sc-1311, Santa Cruz Biotechnology) together with protein G-Sepharose (Amersham Biosciences) at 4°C over night. Immunoprecipitated material was analyzed by western blotting with α-AcK310 p65 (Chen et al., 2005), α-AcK382 p53 (Cell Signaling Technology), monoclonal α-T7, and mouse α-p53 (sc-126, Santa Cruz Biotechnology).

In Vitro *HDAC Assays.*

FLAG-tagged SIRT1 expression plasmids (1 μg) were transfected in 293 cells with lipofectamine reagent (Invitrogen). Transfection and lysis of cells were

carried out with the same procedures used in coimmunoprecipitation experiment. Equal amounts of total cell extracts (2 mg) were immunoprecipitated with α-FLAG M2 agarose at 4°C over night. In vitro HDAC assays were performed as previously (Pagans 2005).

Recombinant p65 protein prepared from baculovirus-infected Sf9 insect cells (BD biosciences) was acetylated by immunoprecipitated full-length p300 expressed in 293 cells as described (Chen 2005). Acetylated p65 protein was incubated with recombinant SIRT1 (1 μg/5U) in SIRT1 deacetylase buffer (50 mM Tris-HCl pH 9, 4 mM $MgCl_2$, 0.2 mM DTT) in the presence of NAD^+ for 3 h at 37°C. Reactions containing synthetic Tat (0.1 μg) or nicotinamide (10 mM) were preincubated for 15 min at room temperature. Reactions were stopped by the addition of SDS loading buffer, and the mixture was boiled, and after brief centrifugation, analyzed by western blotting with rabbit α-AcK310 (Chen 2005) or α-p65 antibodies (sc-8008, Santa Cruz Biotechnology).

2D.4 Results

Tat and Nicotinamide Hyperactivate T Cells Via the Same Cellular Pathway

To recapitulate the Tat effect on immune activation in a manner as close to the natural infection as possible, we used an HIV-based retroviral vector containing the full-length Tat (101 amino acids) and the GFP open reading frames under the control of the HIV promoter in the 5' long terminal repeat (LTR). We infected Jurkat T cells with viral particles containing this vector or a control vector expressing GFP alone. Infected cultures were stimulated with antibodies specific for the CD3 and CD28 receptors to mimic physiological T-cell activation.

Tat expression was visualized by western blot analysis (Figure 2.16A). Treatment with α-CD3/28 slightly increased the levels of Tat because of the stimulatory effect of these antibodies on the viral LTR. In cells expressing Tat, a ~1200-fold induction of IL-2 mRNA was measured in response to α-CD3/28 treatment, whereas IL-2 expression in cells expressing GFP was only induced ~250 fold (Figure 2.16B). A similar difference was observed when IL-2 protein levels were measured in the culture supernatant (Figure 2.16C). No effect of Tat was observed in nonactivated cultures. These results reproduced data previously obtained in purified $CD4^+$ T cells (Wu 2001), peripheral blood lymphocytes (Ott 1997), and Jurkat T cells (Fortin 2004) infected with infectious HIV and support the model that Tat synergizes with T-cell activation to hyperactivate IL-2 production.

To examine the role of SIRT1 in IL-2 production, we treated Jurkat cells with nicotinamide, a natural byproduct of the deacetylase reaction of sirtuins and a feedback inhibitor of these enzymes (Bitterman 2002). Treatment with nicotinamide superinduced IL-2 mRNAs to similar levels as Tat in response to α-CD3/28 stimulation (Figure 2.16D). To test whether Tat and nicotinamide synergize in the hyperactivation of IL-2 expression, we treated Tat- or control-infected Jurkat cultures with nicotinamide before activation. This experiment showed no additive effect of Tat and nicotinamide on IL-2 expression (Figure 2.16E). Tat superinduced IL-2 mRNA in the absence of nicotinamide, but no additional superinduction was measured in nicotinamide-treated cultures. Levels of Tat protein were equal in infected cultures treated with nicotinamide and control-treated cultures excluding the possibility that nicotinamide suppressed the expression of Tat (Figure 2.16F). These results demonstrate that Tat and nicotinamide target related cellular pathways and support the hypothesis that Tat hyperactivates T cells via SIRT1.

SIRT1 Is Required for the Hyperactivation of NFκB Target Genes by Tat

T-cell activation is coupled to the activation of several transcription factors that regulate the transcriptional activation of the IL-2 gene (Liu 2005). We focused our efforts on NFκB since its activity is regulated by SIRT1 (Yeung 2004). We and others previously reported that Tat enhanced IL-2 promoter activity via NFκB binding sites within the IL-2 promoter (Fortin 2004; Ott 1997; Ott 1998; Westendorp 1994). We speculated that Tat, like nicotinamide, targets the SIRT1 deacetylase activity to hyperactivate NFκB-responsive genes including IL-2.

We tested this hypothesis in cells derived from SIRT1-/- mice. Mouse embryonic fibroblasts (MEFs) were infected with a retroviral vector expressing SIRT1 or the empty vector alone. Polyclonal populations of SIRT1-expressing or SIRT1-negative cells were each infected with retroviral vectors expressing Tat or with the empty vector alone. This protocol generated four polyclonal MEF cultures, allowing the analysis of the Tat effect in the presence or absence of SIRT1 (Figure 2.17A). MEF cell lines were treated with TNFα to activate NFκB. Expression of IκBα, an endogenous NFκB response gene, was examined by real-time RT-PCR. We found that Tat expression increased endogenous levels of IκBα mRNA 3- to 4-fold over control-infected cells in response to TNFα (Figure 2.17B). Remarkably, this effect was only observed in cultures where SIRT1 expression had been reconstituted. No difference between Tat- and vector-infected cells was detected in SIRT1-negative cells, indicating that Tat-mediated superinduction of NFκB response genes was dependent on SIRT1.

A similar result was observed with E-selectin, another NFκB response gene that is also superinduced by Tat in endothelial cells (Cota-Gomez 2002; Dhawan 1997; Lee 2004). After TNFα treatment, endogenous E-selectin gene expression was enhanced 6-fold by Tat in SIRT1-expressing, but not in SIRT1-negative cells (Figure 2.17C). Here, the Tat effect was only visible at 2 h after TNFα treatment. At 8 h, we did not measure any induction of E-selectin gene expression, consistent with the finding that E-selectin mRNA accumulation peaks 2 h after TNFα treatment and rapidly declines (Edelstein 2005). Tat had no effect on the

expression of the endogenous glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), which was used as a control gene (Figure 2.17D).

Tat Neutralizes the Negative Effect of SIRT1 on NFκB Function

Next, we studied the effect of Tat on the IκBα and E-selectin gene promoters. We transfected an IκBα promoter luciferase construct together with expression vectors for p65, SIRT1, and Tat in HeLa cells (Figure 3A). SIRT1 suppressed the activation of the IκBα promoter by p65, confirming that deacetylation by SIRT1 inactivates p65 activity (Yeung 2004). Coexpression of Tat restored p65 activity, indicating that Tat interfered with the deacetylation of p65 by SIRT1 (Figure 2.18A). No change in expression levels of p65 was observed by western blotting in the presence of SIRT1 or Tat (data not shown; Figure 2.20C). The suppressive activity of SIRT1 was dependent on the intact deacetylase activity of SIRT1, since a catalytically inactive SIRT1 mutant (P447E; described in Figure 2.19C) did not suppress the activity of p65 and was unresponsive to the action of Tat. Expression of Tat did not affect basal or p65-mediated activity of the IκBα promoter in the absence of SIRT1 overexpression (Figure 2.18A).

The same results were observed using an E-selectin promoter luciferase construct. Again, SIRT1 reduced p65-activated E-selectin promoter activity, and Tat reversed the negative effect of SIRT1 (Figure 2.18B). No reduction was observed with the mutant SIRT1, which was also unresponsive to Tat (Figure 2.18B).

To verify that Tat and SIRT1 modulated the activities of the E-selectin and IκBα promoters through NFκB, we transfected p65-, Tat-, and SIRT1-expressing

constructs together with a NFκB luciferase reporter. Expression of SIRT1 reduced the p65-mediated activation of the NFκB reporter, while coexpression of Tat reversed this negative effect (Figure 2.18C). Again, expression of the catalytically inactive SIRT1 mutant did not interfere with the activity of p65, and additional Tat expression had no effect in this background (Figure 2.18C). Likewise, overexpression of a p65 mutant (K310R) which lacked the deacetylation site for SIRT1 and reduced activation of the NFκB luciferase construct was unaffected by the SIRT1 and Tat (Figure 2.18C). These results demonstrate that Tat targets the SIRT1 deacetylase activity to hyperactivate NFκB function.

Binding of Tat to the Deacetylase Domain of SIRT1

We showed previously that Tat and SIRT1 interact in cells (Pagans 2005). To map the Tat-interacting domain in SIRT1, we performed coimmunoprecipitation assays with SIRT1 mutants and Tat using deletion constructs of the murine SIRT1 protein as described (Fulco 2003). Gradual N-terminal deletions at amino acids 120, 236, or 341 did not affect coimmunoprecipitation with Tat (Figure 2.19A, lanes 1–3). However, deletion of the first 513 amino acids abrogated binding to Tat, indicating that the Tat-interaction domain lies between amino acids 342 and 512 of SIRT1 (Figure 2.19A, lane 4). Accordingly, expression of amino acids 236 to 510 was sufficient for the interaction between Tat and SIRT1 protein (Figure 2.19A, lane 5). The results of the coimmunoprecipitation studies are summarized in Figure 2.19B.

The region spanning amino acids 342–512 harbors the catalytic domain of SIRT1, which is shared among all sirtuins (Sauve 2006). It is 98% conserved in murine and human SIRT1 proteins and consists of two distinct domains that bind NAD^+ and the acetyl-lysine substrate, respectively (Avalos 2002). To determine whether Tat interacts with one of these subdomains, point mutations were introduced into human SIRT1 expression construct to disrupt NAD^+-binding (R274A, N346A) or acetyl-lysine binding (F414D, E416A, V445E, P447E) (Figure 2.19C). In addition, we mutated two conserved cysteines (C371/374G) located in a zinc-finger like structure predicted to serve as a protein-protein interaction domain (Min 2001).

The catalytic activities of these mutants were measured by *in vitro* histone deacetylase (HDAC) assay using a radioactive acetylated histone peptide as a substrate. We transfected wildtype and mutant SIRT1 proteins into 293 cells and immunoprecipitated the FLAG-tagged proteins (Figure 2.19D). The immunoprecipitated material was tested for enzymatic activity. Incubation with wildtype SIRT1 or the R274A mutant resulted in deacetylation of the histone peptide in the presence of NAD^+ (Figure 2.19D). The deacetylase activity of the F414D, E416A, and V445E mutants was severely impaired, while no activity was measured for N346A, C371/374G, and P447E mutants.

Next, we cotransfected wildtype and SIRT1 mutants together with T7-tagged Tat. Wildtype and mutant SIRT1 proteins were expressed at similar levels (Figure 2.19E, upper panel). After pull down with T7-agarose, immunoprecipitated complexes were analyzed for the presence of SIRT1 (Figure 2.19D, lower

panels). While binding to Tat was preserved in SIRT1 R274A, N346A and C371/374G, no binding to Tat was observed with any of the SIRT1 mutants carrying a mutation within the acetyl-lysine-binding domain (F414D, E416A, V445E, P447E). The same results were obtained when SIRT1 proteins were immunoprecipitated, and coimmunoprecipitation of Tat was determined (data not shown). These data demonstrate that Tat interacts with the substrate-binding domain of SIRT1.

Inhibition of the SIRT1 Deacetylase Activity by Tat

Since an intact acetyl-lysine binding function is critical for the deacetylase activity of SIRT1, we tested whether Tat binding affected the enzymatic activity of SIRT1. We performed *in vitro* HDAC assays with recombinant SIRT1 enzyme in the presence of synthetic Tat peptides (amino acids 1-72) (Kaehlcke 2003). Incubation of SIRT1 with Tat inhibited deacetylation of the histone peptide in a dose-dependent manner (Figure 2.20A). At equimolar concentrations of Tat and SIRT1, a 70% reduction in deacetylation was observed, while a complete suppression occurred when a 10-fold molar excess of Tat versus SIRT1 was included in the reaction.

Tat is itself acetylated and a substrate for the deacetylase activity of SIRT1 (Pagans 2005). To test the effect of acetylated Tat on the deacetylase activity of SIRT1, we incubated synthetic Tat carrying a single acetyl group at K50 (Ac-Tat) with SIRT1. Acetylated Tat inhibited deacetylation of the histone peptide with the same efficiency as nonacetylated Tat (Figure 2.20A). These results agree with

our previous data showing that Tat binds to SIRT1 regardless of the acetylation status of K50 (Pagans 2005).

Next, we examined the effect of Tat on deacetylation of p65 by SIRT1. We incubated *in vitro* acetylated recombinant p65 protein with recombinant SIRT1 and synthetic Tat proteins. The extent of deacetylation was determined by western blotting with antibody specific for acetylated K310 in p65 (α-AcK310 p65). SIRT1 deacetylated p65 in the absence of Tat (Figure 2.20B, lane 2). In the presence of Tat, deacetylation of p65 by SIRT1 was completely suppressed, confirming that direct interaction between Tat and SIRT1 inhibits the deacetylase activity of SIRT1 (Figure 2.20B, lane 3). The same inhibition of p65 deacetylation by SIRT1 was observed with nicotinamide (Figure 2.20B, lane 4). These results demonstrate that Tat functions as an inhibitor of the SIRT1 deacetylase activity and prevents deacetylation of p65 *in vitro*.

Hyperacetylation of Cellular Targets of SIRT1 in the Presence of Tat

To test the effect of Tat on p65 acetylation in cells, we transfected expression vectors for p65, SIRT1 and Tat into 293 cells and immunoprecipitated the p65 protein. To induce efficient p65 acetylation, we overexpressed the acetyltransferase p300 (Chen 2002). Overexpression of SIRT1 reduced acetylation of p65 at K310 caused by p300 (Figure 2.20C, lanes 2, 3). Coexpression of Tat partially restored p65 acetylation in the presence of SIRT1 (Figure 2.20C, lane 4). Interestingly, expression of a Tat mutant containing two glutamines at position 50 and 51 (Tat K50/51Q) and mimicking an acetylated Tat

protein in cells completely restored acetylation of p65 in the presence of SIRT1 (Figure 2.20C, lane 5).

Neither Tat nor SIRT1 proteins affected the expression of total p65 protein (Figure 2.20C). These results demonstrate that expression of Tat causes hyperacetylation of cellular p65 in the presence of SIRT1. They further suggest that acetylated Tat functions as a better SIRT1 inhibitor than nonacetylated Tat in cells, although both proteins bind to SIRT1 and inhibit the SIRT1 deacetylase activity with the same efficiency *in vitro* ((Pagans 2005), Figure 2.20A).

To further test the concept that Tat modulates acetylation levels of SIRT1 substrates, we analyzed the acetylation status of p53. p53 is deacetylated by SIRT1 at K382 (Vaziri 2001). We transfected an expression vector of p53 together with constructs for p300, SIRT1 and Tat in 293 cells and immunoprecipitated p53 with α-p53 antibody. Coexpression of p53 with p300 induced acetylation of K382 in p53 as determined by western blotting with antibodies specific for acetylated K382 (α-AcK382 p53). Overexpression of SIRT1 suppressed acetylation of K382 in p53 (Figure 2.20D, lane 2). The suppression was reversed when increasing amounts of Tat or Tat K50/51Q were coexpressed with SIRT1. Again, mutant Tat reversed the action of SIRT1 on p53 more efficiently than wildtype Tat, supporting the concept that acetylated Tat might act as a better inhibitor of SIRT1 in cells. These results demonstrate that Tat inhibits SIRT1 function in cells leading to hyperacetylation of SIRT1 substrates including p65 and p53.

2D.5 Discussion

Our finding that Tat inactivates cellular SIRT1 provides the first molecular mechanism for the Tat-induced hyperactivation of T cells. It further uncovers a novel role of SIRT1 in T cell activation.

We find that Tat binds to the SIRT1 catalytic site and impairs the ability of SIRT1 to deacetylate K310 in NFκB/p65. Consistent with previous data that deacetylation by SIRT1 inactivates NFκB function we find NFκB activity "superactivated" in the presence of Tat. Based on these results, we propose a model in which Tat hyperactivates T cells through inhibition of SIRT1 and derepression of NFκB activity. In the absence of Tat, a balance of p300 and SIRT1 activities regulates p65 acetylation. This balance tightly controls the activation of cellular genes involved in the immune response of T cells (Figure 6A). During HIV infection, Tat expression induces a dysequilibrium and increases levels of acetylated p65 through inhibition of SIRT1 (Figure 2.21B). This increase hyperactivates the function of NFκB and the expression of NFκB responsive genes including IL-2.

One of the most studied NFκB response genes is HIV itself. The two NFκB-binding sites in the HIV enhancer link HIV transcription to the activation status of the infected T cell. This is particularly important at the beginning of the infectious process and during reactivation from latency when Tat levels are limited. During these times, the action of cellular transcription factors, most importantly NFκB, is essential for the production of full-length viral transcripts necessary for the synthesis of Tat. Once Tat accumulates to sufficient levels, it binds to TAR RNA

and dramatically increases the elongation competence of the RNA polymerase II complex through recruitment of the PTEFb complex. At that time, Tat may also enhance NFκB function through inhibition of SIRT1 (Figure 2.21B). Our preliminary studies show that the two Tat functions (via TAR or via SIRT1) are genetically distinct. Tat mutants such as TatC22G and TatK41A, which are deficient in binding to PTEFb, are unaffected in their affinity for SIRT1 (H.-S. Kwon and M. Ott, unpublished observations).

Tat itself is deacetylated by SIRT1. Although SIRT1 is a negative regulator of NFκB, it functions as a coactivator of Tat transactivation through deacetylation of K50 (Pagans 2005). We favor the model that deacetylation of Tat recycles acetylated Tat to the unacetylated form, which is the only Tat form able to engage in the formation of the Tat/TAR/PTEFb complex. Interestingly, binding of Tat to the SIRT1 deacetylase domain occurs independently from the acetylation status of K50 (Pagans 2005). However, we show here that a Tat K50/51Q mutant, which mimics an acetylated Tat protein in cells, restores p65 acetylation in the presence of SIRT1 more efficiently than wild type Tat. This raises the possibility that Tat might act as a "supersubstrate" of the SIRT1 enzymatic activity (Borgstrom 1975). Acetylated Tat is efficiently deacetylated by SIRT1, but stays bound to SIRT1 and blocks access of other substrates to the catalytically active site.

SIRT1 targets many transcriptional activators, including p65, p53, Forkhead transcription factors, as well as transcriptional coactivators PGC-1alpha and p300 (Sauve 2006). The observation that Tat inhibits a central regulator of the

function of many transcription factors and transcriptional coactivators provides an explanation of Tat's reported pleiotropism. The finding that p53 is hyperacetylated in the presence of Tat supports the concept that Tat inhibits deacetylation of many SIRT1 targets. Whether the hyperacetylation of p53 is associated with increased apoptosis in Tat-expressing cells remains to be determined. Interestingly, p53 is hyperacetylated in SIRT-/- mice, but whether this hyperacetylation is associated with an increase in the apoptosis-inducing function of p53 is a matter of controversy (Cheng 2003; Kamel 2006). However, we show that the functional "knockout" of SIRT1 by Tat leads to an increase in NFκB transcriptional activity. Tat specifically counterbalances the suppressive effect of SIRT1 on the function of p65, but not of a p65 mutant that cannot be acetylated on K310. These data support the concept that K310 acetylation is important for NFκB activity and link the Tat effect to the hyperactivation of the T cell response during HIV infection.

T-cell activation is a highly controlled process and involves positive and negative regulatory mechanisms. Negative control prevents inappropriate activation or terminates ongoing activation in a timely manner. Our results showing that inhibition of SIRT1 by Tat or nicotinamide leads to T cell hyperactivation identify SIRT1 as a negative regulator of T-cell activation. This concept is supported by our preliminary findings that SIRT1 protein levels are strongly upregulated after activation of primary $CD4^+$ T cells (H.-S. Kwon and M. Ott, unpublished data). A similar upregulation of SIRT1 protein levels has been described during fasting

and supports the model that SIRT1 is involved in life span extension induced by caloric restriction (Cohen 2004; Rodgers 2005).

The finding that Tat induces T-cell hyperactivation through inhibition of SIRT1 opens the possibility for therapeutic intervention. The role of yeast Sir2p and possibly human SIRT1 in lifespan extension has fueled the search for sirtuin activators (Grubisha 2005). One of the compounds that activates SIRT1 is resveratrol found in red wine (Howitz 2003). Interestingly, resveratrol was shown to lower the *K*m for the acetylated substrate, which might specifically counterbalance the blockade by Tat. Because of the many health benefits associated with red wine and resveratrol, the prospect of resveratrol-mediated restoration of SIRT1 function is an intriguing approach to the treatment of HIV-induced immune hyperactivation.

2D.6 Acknowledgments

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2D.7 Figure Legends

Figure 2.16. - Tat and nicotinamide cause T-cell hyperactivation (A) Western blot of Tat protein in Jurkat cells infected with lentiviral vectors expressing LTR-GFP and LTR-Tat-GFP. Infected cultures were activated for 2 h and 8 h with α-CD3/28 antibodies. (B) Real-time RT-PCR analysis of IL-2 mRNA levels in infected cultures 2 h after activation with α-CD3/28. Results are expressed as fold induction by α-CD3/28 treatment. (C) IL-2 protein levels measured by ELISA in the culture supernatant of infected cells 8 h after α-CD3/28 treatment. (D) Real-time RT-PCR analysis of IL-2 mRNA levels in Jurkat cells after treatment with nicotinamide and activation with α-CD3/28. (E) Real time RT-PCR analysis of IL-2 mRNA levels in Jurkat cells infected with lentiviral vectors expressing LTR-GFP or LTR-Tat-GFP after treatment with nicotinamide for 1 h and activation with α-CD3/28 antibodies for 2 h. (F) Western blot of Tat protein in Jurkat cells infected with lentiviral vectors after treatment with nicotinamide and

activation with α-CD3/28. In B–E averages of three independent experiments (±SEM) are shown.

Figure 2.17. - Tat-mediated superinduction of NFκB response genes requires SIRT1 (A) Western blot of SIRT1-/- MEF cells infected with MSCV-based retroviral particles expressing SIRT1 or Tat. (B-D) Real-time RT-PCR analysis of endogenous IκBα (B), E-selectin (C), and GAPDH (D) gene expression in the four MEF populations after incubation with TNFα (20 ng/ml) for 2 h and 8 h. Data are presented relative to values obtained in cells lacking Tat (100%) in a SIRT1-negative (vector) or SIRT1-positive (SIRT1) background. The mean of three independent experiments (±SEM) is shown.

Figure 2.18. - Tat neutralizes SIRT1 inhibition on NFκB promoter activity

Promoter reporter assays using (A) the IκBα, (B) the E-Selectin, and (C) NFκB reporter constructs. Luciferase constructs (0.2 μg) were transiently cotransfected with expression vectors for p65 or p65 K310R (1 ng), SIRT1 or SIRT1 P447E (0.2 μg), and Tat (50 ng) into HeLa cells. The mean of three independent experiments (±SEM) is shown.

Figure 2.19. Tat binds to the acetyl lysine-binding site in SIRT1 (A) Coimmunoprecipitation assay of Tat and SIRT1 deletion mutants. Myc-tagged murine SIRT1 deletion mutants (1 μg) were coexpressed with FLAG-tagged Tat (1 μg) in 293 cells. Immunoprecipitation was performed with α-FLAG agarose and western blotting with α-Myc and α-FLAG antibodies. (B) Schematic summary of Tat binding to SIRT1 deletion mutants. The relative localization of the human

SIRT1 HDAC domain is shown at the bottom. (C) Schematic representation of the SIRT1 HDAC domain and point mutations. (D) *In vitro* HDAC assays of immunoprecipitated SIRT1 mutants with and without NAD^+. (E) coimmunoprecipitation of SIRT1 mutants and Tat in 293 cells. Cell extracts were immunoprecipitated with α-T7 agarose followed by western blotting with α-FLAG and α-T7 and antibodies.

Figure 2.20. - Tat inhibits the SIRT1 deacetylase activity (A) *In vitro* HDAC assay of recombinant human SIRT1. SIRT1 was preincubated for 15 min with increasing amounts of synthetic Tat or AcTat proteins. (B) *In vitro* HDAC assay of recombinant SIRT1 and recombinant acetylated p65 protein in the presence of nonacetylated Tat or nicotinamide. Western blotting was performed with α-AcK310 p65 and α-p65 antibodies. (C) T7-tagged p65 (0.5 μg), HA-tagged p300 (2 μg), Myc-tagged SIRT1 (0.1 μg), Tat and Tat K50/51Q mutant (50 ng) were coexpressed in 293 cells as indicated. Immunoprecipitations were performed with α-T7 agarose followed by western blotting with α-AcK310 p65 antibody. Expression of p300 and SIRT1 was detected in total cell extract using α-HA and α-Myc antibodies. (D) Full-length, untagged p53 (0.5 μg) was overexpressed in 293 cells, immunoprecipitated with α-goat p53 antibody followed by western blotting with α-AcK382 p53 and α-mouse p53 antibodies.

Figure 2.21. - Tat blocks the SIRT1 deacetylase activity and superinduces T-cell activation and HIV transcription via NFκB. See text for details.

Chapter 3: CXCR4 and Macrophages

3A. Macrophage infection

Paper: Tissue-Resident Macrophages Are Productively Infected *ex vivo* by Primary X4 Isolates of Human Immunodeficiency Virus Type 1

3A.1 Abstract

The infection of macrophages has been implicated as a critical event in the transmission and persistence of human immunodeficiency virus type 1 (HIV-1), and the development of the AIDS dementia complex. Here, we explore whether primary X4 HIV-1 isolates can productively infect tissue macrophages that have terminally differentiated *in vivo*. Using immunohistochemistry, HIV-1 RNA hybridization, and confocal immunofluorescence microscopy, we demonstrate that macrophages residing in human tonsil blocks can be productively infected *ex vivo* by primary X4 HIV-1 isolates. This challenges the model in which macrophage tropism is a key determinant of the selective transmission of R5 HIV-1 strains. The infection of tissue macrophages by X4 HIV-1 viruses may be of high relevance *in vivo* and contribute to key events in HIV-1 pathogenesis.

3A.2 Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) into cells is mediated by binding of the gp120 subunit of the envelope (Env) glycoprotein to its main receptor, CD4, on the cell surface. Binding initiates a conformational change in gp120 that allows subsequent interaction with cellular coreceptors, most notably the human chemokine receptors CCR5 and CXCR4 (Kinter 2000). In human cell lines and primary T cells, the expression of CD4 in conjunction with CXCR4 and/or CCR5 typically correlates well with an HIV isolate's ability to productively infect these cells. Most of our current knowledge about HIV-1 infection of macrophages has been inferred from experiments conducted with monocyte-derived macrophages (MDMs). Cultured MDMs express CD4, CCR5, and CXCR4 on the cell surface, albeit at relatively low levels (Collman 1999). Despite the presence of CXCR4, a number of reports demonstrated that MDMs could be productively infected only by R5 and R5X4 strains, but not by X4 strains (Adachi 1986; Fisher 1985; Gendelman 1988; Schmidtmayerova 1998; Schuitemaker 1991). Based on these observations, it was generally believed that the tropism of an HIV-1 strain for macrophages was directly linked to its ability to utilize CCR5 as a coreceptor. On the basis of an "R5 only" susceptibility of tissue macrophages a model of HIV transmission was proposed in which a strong selection for HIV-1 isolates with CCR5 coreceptor use may occur *in vivo* during transmission (van't Wout 1994).

In addition to their postulated role as a selective portal of entry, macrophages constitute an important long-term reservoir for HIV-1 in infected individuals

(Orenstein 1997) and may be critical for pathogenesis in the central nervous system (Kaul 2001). Remarkably, in rhesus macaques infected with a simian immunodeficiency virus (SIV)/HIV-1 chimera (SHIV), macrophages sustain a high level of plasma viremia for several months in the virtual absence of CD4 T lymphocytes (Igarashi 2002; Igarashi 2001; Igarashi 2003). In this model, the SHIV DH12R strain, which exclusively uses CXCR4, was shown to infect macrophages *in vivo*.

Recently, the dogma of an absolute restriction of X4 infection in macrophages has been challenged by the identification of primary X4 isolates that can replicate to high levels in MDM cultures (Naif 2002; Verani 1998; Yi 1999). Earlier studies analyzed the susceptibility of MDMs to X4 infection by using prototypic T cell line-adapted (TCLA) strains, including NL4-3, LAI.2, HXB2, and IIIB (Naif 2002; Verani 1998; Yi 1998). In contrast to these TCLA-X4 viruses, primary X4 isolates are not adapted to growth on transformed T cells. These primary isolates have an absolute requirement for CXCR4 as a coreceptor. This was demonstrated by a complete inhibition of infection by CXCR4 receptor antagonists as well as by their ability to replicate efficiently in CCR5-deficient MDMs from patients homozygous for the CCR5 delta 32 allele (Scarlatti 1995; Verani 1998; Yi 1998). To obtain MDM cultures, monocytes are typically purified from human peripheral mononuclear blood cells (PBMC) and differentiated *in vitro* using a variety of techniques (Collman 1989; Stent 1997; Yi 1999). It is unclear to what extent *in vitro* cultures of MDMs reflect the characteristics

of tissue macrophages in general terms and, in particular, in the context of an HIV-1 infection.

In the current study, we investigated whether primary X4 viruses are capable of productively infecting macrophages that are residing within their native tissue. To address this, we conducted HIV-1 infections of explants of human tonsils. These tonsil blocks can be infected independent of exogenous stimuli and harbor a wide variety of cell types including T and B lymphocytes, dendritic cells, stromal cells, as well as macrophages that, importantly, have terminally differentiated *in vivo* (Glushakova 1995; Penn 1999).

3A.3 Materials and Methods

Virus stocks

Virus stocks were prepared as described in Chapter 2D.

Cell preparation

Human tonsil were obtained from Kaiser Hospitals and prepared as described (Schramm 2000).

Virus infections

Virus infections were performed as described in Chapter 2D.

CD3 Immunohistochemistry

Costaining for the CD68 and the T cell marker CD3 (polyclonal rabbit antiserum; 1:20; Diagnostic Biosystems) were performed as for HIV. As secondary and tertiary reagents, sheep anti-mouse IgG3 (1:100; Serotec Ltd.), Cy5-conjugated donkey anti-rabbit (1:100, Jackson Immunotherapy), and Cy2-conjugated donkey anti-sheep antibodies (1:50; Dianova) were used.

HIV Double Immunohistochemistry

After paraffin-embedding, sections from infected tonsil blocks were de-paraffinized, hydrated through graded ethanols and briefly incubated in 3% hydrogen peroxide. Following epitope retrieval, sections were stained with an anti-p24 antibody solution (1:10; Kal-1, DAKO) and detected with the Envision (+) DAB Kit (DAKO). Subsequently, anti-CD68 mAb PG-M1 (1:100; DAKO) was applied and detected with the Envision Alkaline Phosphatase (DAKO) using the Vector Red Alkaline Phosphatase Substrate Kit (Vector). Slides were counterstained with hematoxylin. Analyses were performed as described (Autschbach 1999). As the primary antibody reagent, a mixture of anti-p24 mAb Kal-1 and anti-human CD68 mAb PG-M1 was used. Subsequently, sections were incubated with biotinylated goat anti-mouse IgG1 (1:100; Southern Biotech) and sheep anti-mouse IgG3 (1:100; Serotec Ltd.). A final incubation step included Cy3-conjugated streptavidin (1:1000; Dianova) and Cy2-conjugated donkey anti-sheep antibodies (1:50; Dianova).

3A.4 Results

Primary HIV-1 isolates J130 and UG021 use CXCR4, but not CCR5, as a coreceptor.

The primary isolates J130 and UG021 were reported to replicate in MDM cultures and have been shown to exclusively utilize CXCR4 as a coreceptor for cellular entry (Verani 1998; Yi 1999). To confirm the published coreceptor usage before infection of human tonsil blocks, we performed infection studies with expanded viral stocks on HeLa-H11 cells, which stably express CD4 and CCR5, besides

expression of endogenous CXCR4 (J.F. Kreisberg, personal communication). As controls, the prototypical TCLA-X4 NL4-3, which exclusively utilizes CXCR4 as a coreceptor, and the R5 molecular clone 49-5, which differs from NL4-3 only in the coreceptor-determining V3 loop region (C2-C3) (Toohey 1995), were used. The R5X4 HIV-1 primary isolate 7/86 served as reference isolate with MDM-tropism (Connor 1997). To selectively manipulate coreceptor accessibility, we used two coreceptor antagonists, TAK-779 and AMD3100, which specifically block the infection of R5 and X4 viruses, respectively. HeLa-H11 cells were infected for two days in the presence or absence of one of these coreceptor antagonists. Subsequently, the percentage of p24-positive cells (mAb KC57-RD1, Coulter) was scored by flow cytometry, and results were plotted relative to untreated infections (Figure 3.1).

As expected, infection with the primary X4 isolates J130 and UG021 was completely inhibited in the presence of the CXCR4 antagonist AMD3100, but not TAK779. Confirming the specificity of the inhibitors, this phenotype mirrored that of the prototypic X4 strain, NL4-3. Conversely, the relative percentage of cells infected by the isogenic R5 HIV-1 virus 49-5 was drastically reduced by TAK-779, but not by the CXCR4 antagonist. Neither coreceptor antagonist alone abrogated infection of the dual-tropic primary isolate 7/86 (Figure 3.1). The observation that TAK-779 treatment alone did not have a significant effect likely reflects the ability of this isolate to use CXCR4 more efficiently than CCR5 either in general or, specifically, when CCR5 is blocked. The addition of both coreceptor antagonists completely inhibited infection by 7/86 (data not shown). These results

demonstrated that the expanded viral stocks of the primary isolates J130 and UG021 utilize CXCR4, but not CCR5, as a functional coreceptor.

Primary X4 viruses efficiently replicate in ex vivo human tonsil histoculture. To assess the ability of the primary X4 HIV-1 isolates to replicate in human tonsil histocultures *ex vivo*, tissue blocks were infected and maintained in culture for 11 days under standard conditions as described (Eckstein 2001; Jekle 2003; Jekle 2002; Penn 1999; Schramm 2000a). The kinetics of viral replication were followed by determining the p24 concentration in culture supernatants (Figure 3.2) using an anti-p24 enzyme-linked immunosorbent assay. Reference viruses NL4-3 and 7/86 showed a typical replication kinetic (Jekle 2002) reaching a p24 concentration of approximately 70 ng/ml 11 days after infection. The primary X4 isolates J130 and UG021 displayed a slightly delayed replication kinetic with J130 reaching peak p24 concentrations comparable to NL4-3 and 7/86 (Figure 3.2). These results demonstrated the potential of these primary X4 isolates to efficiently replicate in human tonsil histocultures *ex vivo*. Tissue blocks infected with the individual HIV-1 strains were harvested at day 11, fixed in paraformaldehyde, paraffin-embedded and processed for further analyses.

Primary X4 viruses productively infect CD68-positive tissue macrophages. To determine whether primary X4 isolates can productively infect tissue-resident macrophages, we used immunohistochemistry or confocal immunofluorescence microscopy to identify p24 Gag antigen, which accumulates in productively infected cells during the late phase of the viral replication cycle. We also performed *in situ* hybridization for viral RNA transcripts using an antisense RNA

probe cocktail that spans 90% of the HIV-1 genome (Ward 2000). To stain macrophages, either mAb PG-M1 to the CD68 antigen (Holness 1993; Micklem 1989; Pulford 1990) or mAb HAM56, which detects an as-yet undefined molecule on macrophages (Adams 1990; Gown 1986), were employed. By using staining of native tissue sections rather than a flow cytometry-based approach, macrophages could also be identified by their typical cytomorphological appearance, including their relative size, single nuclei (to distinguish them from syncytia of CD4 T cells), and pseudopods.

The mock-infected tonsil sections showed an intense brown staining for CD68 of cells with macrophage morphology (Figure 3.3B, C). There was a complete absence of p24 staining (red) in these sections, indicating a very low background for detection of the viral capsid protein. In agreement with the replication data obtained from culture supernatants (Figure 3.2), tonsils infected with all four viruses showed a robust cellular HIV-1 infection level reflected by an intense p24 staining (Figure 3.3D, G, J, M and "p24", red). All viruses showed productive infection of cells of lymphocyte morphology, most likely representing CD4 T cells (Figure 3.3E, F, H, I, L, N), and infection of CD4 T cells was independently demonstrated by multi-color flow cytometry of dispersed tonsil blocks as described (Eckstein 2001; Jekle 2003; Jekle 2002) (data not shown). By this analysis, the percentage of p24-positive CD4 T cells ($CD3^+CD8^-$) was in a similar range for all viruses at day 5-7 ranging from 5-15%. At day 11 post-infection, UG021 and J130 had depleted CD4 T cells by up to 90%, which was comparable to the depletion seen for NL4-3 (data not shown).

For NL4-3, a clear colocalization of immunohistochemical stainings for CD68 (Figure 3.3, "M", brown) and p24 could not be detected (Figure 3.3E, F). This is in line with the absence of convincing data for the a productive NL4-3 infection of MDM cultures under standard cultivation and infection conditions in previous reports (Adachi 1986; Fisher 1985; Gendelman 1988; Schuitemaker 1991). In striking contrast, in tonsils infected with the primary R5X4 isolate 7/86 and with primary X4 HIV-1 isolates J130 and UG021, colocalization of brown staining for CD68-positive macrophages and intense red p24 staining could frequently be detected (Figure 3.3H, I, K, L, N, O; double-positive cells indicated by black arrows). These results strongly suggested the productive infection of tissue-resident macrophages in *ex vivo* tonsil histocultures by primary X4 viruses.

HIV-1 RNA in situ hybridization in conjunction with CD68 immunohistochemistry. As an independent confirmation, sections from infected tonsil blocks were analyzed by HIV-1 RNA *in situ* hybridization (Fox 1993; Fox 1993a; Ward 2000) in conjunction with an immunohistochemical identification of macrophages. In mock-infected tissues no specific hybridization signal for viral RNA transcripts could be seen, indicating a low level of nonspecific hybridization (Figure 3.4A, B). Sections from tonsils infected with NL4-3 hybridized strongly with the HIV-1 RNA probe (Figure 3.4C, D). However, there was no colocalization of the hybridization signal and CD68 staining. In contrast, sections infected with the primary R5X4 (7/86) and the two primary X4 isolates (J130, UG021) revealed intense HIV-1 RNA *in situ* hybridization signals also in CD68-positive macrophages (Figure 3.4F, G, H, I, J, double-positive cells indicated by black arrows). These results

support the results obtained by double-immunohistochemistry that, like 7/86, primary X4 isolates can productively infect tissue macrophages in human tonsils.

Detection and quantification of productively infected tissue macrophages using confocal double-immunofluorescence microscopy. We then sought to corroborate the productive infection of tissue-resident macrophages by primary X4 isolates and to quantify their abundance. We stained p24 and CD68 by indirect double-immunofluorescence, which allowed for a rapid and more unambiguous identification and quantification of productively infected tissue macrophages upon examination of individual fluorochrome images as well as merged images (Figure 3.5).

For both primary X4 isolates, a significant number of macrophages showed a strong p24 staining (Figure 3.5A-C, D-F, indicated by arrows), reflected by a yellow image in merged pictures. As specificity controls, uninfected sections showed a low background staining for p24 (data not shown), and p24-negative macrophages as well as p24-positive, CD68-negative cells of lymphocyte morphology could frequently be detected in sections from infected tonsils (Figure 3.5). For infections with J130 and UG021 in tonsils from two donors, the frequency of p24-and CD68-double positive macrophages was considerable, ranging from 9.4 to 12.1%. Remarkably, this percentage was in the same range as determined for the primary R5X4 virus 7/86 (12.2–12.5%), but higher than that previously reported for the R5 molecular clone 49-5 (6%, Grivel 2000). The NL4-3-infected tissue showed a large number of p24-positive lymphocytes, since they did not colocalize with the CD68 macrophage marker. Upon careful examination,

three macrophages in the field of view shown in Figure 3.5 were scored positive (Figure 3.5G-I, indicated by arrows and insets) on grounds of a cytoplasmic staining for p24, albeit at low intensity. In contrast to the primary X4 isolates, strongly p24-positive macrophages could only very rarely (less than 0.5%) be detected in the context of an NL4-3 infection. At this point it is unclear whether this level of p24 staining reflects endocytosed virions or, indeed, represents a low level of productive infection of tissue macrophages by NL4-3.

Based on this evaluation, the frequency of p24-positive macrophages was at least 3- to 4-fold higher for the primary X4 isolates, compared to the TCLA-X4. Although we did not address the kinetics of macrophage infection, this percentage probably reflects the cumulative number of macrophage infections over the entire time course since HIV-mediated cytopathology of this cell type is believed to be limited and the half-life of infected macrophages has been estimated to be nearly two weeks (Perelson 1996).

Macrophages in infected tonsil tissue are CD3-negative. To address whether the colocalization of HIV-1 p24 and CD68 could, in part, be due to phagocytosis of infected CD4 T cells or T cell-macrophage fusion events, we performed a costaining for the CD68 and the T cell marker.

In contrast to the p24 staining (Figure 3.5, middle row), CD3 staining showed virtually no colocalisation with CD68 staining in any of the HIV-infected sections or the uninfected control (Figure 3.6). A very rare event of a CD3 signal within a CD68-positive macrophage is highlighted in panel I, possibly reflecting the presence of T-cell membrane fragments within a phagosome. However, the

subcellular localization of this signal was quite different from p24 signals detected both by immunohistochemistry (Figure 3.3) and confocal immunofluorescence microscopy (Figure 3.5). As a specificity control, the CD3 antiserum strongly stained cells of lymphocyte morphology in the tissue surrounding the macrophages. This indicates that the considerable level (up to 12%) of strongly p24-positive macrophages in primary X4-infected tonsil histocultures are probably not a consequence of engulfment of infected T cells or fusion events with T cells, but further supports the interpretation that these macrophages are productively infected by HIV-1.

3A.5 Discussion

The current study establishes the quantitative nature and specificity of the productive infection of tissue macrophages by primary HIV-1 isolates that exclusively utilize the CXCR4 coreceptor, in *ex vivo* tonsil histocultures. This HIV-1 model system provided a convenient experimental platform for the analysis of macrophages that have differentiated *in vivo* and are embedded within a natural tissue microarchitecture, complex cellular environment, and unaltered cytokine milieu. The productive infection of macrophages was demonstrated using several independent approaches.

The ability of certain primary X4 viruses and relative inability of prototypic TCLA-X4 viruses to productively infect cells from the monocyte/macrophage lineage was previously found in *in vitro* MDM cultures (Grivel 2000; Schmidtmayerova 1998; Verani 1998; Yi 1999) and is now shown for tissue-resident macrophages. In line with the recently proposed extension of the tropism classification of HIV-1

isolates addressing both target cell permissivity and coreceptor usage (Yi 1999), the primary isolates J130 and UG021 can be classified as X4 dual-tropic based on their *in vitro* coreceptor usage and their *ex vivo* cytotropism with concurrent infection of primary CD4 T cells and tissue macrophages in tonsil histocultures.

The productive infection of macrophages may be a relatively common (Simmons 1998; Verani 1998; Yi 1999), although not a universal (Connor 1996; Rana 1997), feature of primary X4 isolates. This suggests that determinants other than macrophage tropism must be underlying the central role of R5 variants in HIV-1 transmission. According to our results, a model proposing an *in vivo* selection for R5 strains based on a postulated exclusive macrophage-tropism appears unlikely. This is also supported by studies that identified CD4 T cells as the only SIV- or HIV-infected cell type during primary infection (Schacker 2001; Zhang 1999).

Other investigators have addressed the molecular basis underlying the distinct MDM tropism of TCLA-X4 viruses as compared to primary X4 isolates. One important restriction lies at the level of cellular entry, related to the relatively low expression levels of CXCR4 and CD4 (Lapham 1999; Tokunaga 2001; Yi 1998), the lower affinity of TCLA-Env for the HIV receptor complex (Xiao 2000b), or the presence of specific forms of CXCR4 on MDM (Lapham 1999). Additional blocks appear to exist at post-entry steps in the replication cycle. In particular, signaling events mediated by the viral Env (Liu 2000), chemokines (Arthos 2000), or bacterial lipopolysaccharides (Moriuchi 1998) have been implicated.

Taken together, our data demonstrate that primary X4 isolates productively

infect a considerable fraction of macrophages residing within human lymphoid tissue. This shows that macrophage tropism is not an exclusive characteristic of R5 or R5X4 viruses and reemphasizes the question of which factors, other than coreceptor usage and macrophage tropism, determine the selective transmission of R5 strains *in vivo*.

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3D.7 Figure Legends

Figure. 3.1 - Primary X4 viruses UG021 and J130 do not use CCR5 as a coreceptor and require CXCR4 for infection. HeLa CD4/CCR5 cells (HeLa-H11) were infected with two primary X4 isolates (UG021, J130), the TCLA-X4 virus NL4-3, R5 virus 49-5, or the R5X4 primary isolate 7/86. Infected wells were either left untreated (open bars), treated with the CCR5 antagonist TAK-779 (0.5 μM, hatched bars), or with the CXCR4 antagonist AMD3100 (1 μM, closed bars) 30 min prior to viral challenge and throughout the course of infection. Two days post-infection the percentage of HIV-infected cells was scored by intracellular p24 staining and FACS analysis. Values are arithmetic means + S.D. (n=3) and are presented as relative percentages of untreated infections.

Figure. 3.2 - Primary X4 viruses replicate in *ex vivo* human tonsil histocultures. Tissue blocks were infected with 80 $TCID_{50}$ doses per tissue block and culture supernatants collected at the indicated time points. Arithmetic means of duplicate samples from a representative donor (T190) are shown.

Figure. 3.3 - Primary X4 isolates UG021 and J130, but not the TCLA-X4 strain NL4-3, productively infect tissue macrophages in human tonsil histocultures. Tonsil blocks were either mock-infected or infected with 80 $TCID_{50}$ of either NL4-3, 7/86, J130, or UG021. At day 11 post-challenge, tissue blocks (6-12 blocks per donor) were harvested. Sections were costained for the p24 antigen (red staining) and the macrophage marker CD68 (brown staining). Cells showing red staining only, representing p24 staining, are labeled "p24". Cells showing a brown staining only, representing CD68 macrophage marker staining, are labeled "M".

Double-labeled cells are highlighted by black arrows. Magnifications: Left micrograph column 20X, middle and right micrograph column 63X (b, 20X). a, H&E stain.

Figure. 3.4 - Tissue-resident macrophages in tonsils histocultures infected with primary X4 isolates are HIV-1 RNA positive. Tonsil blocks were infected and processed as described for Figure 3. Viral infection was detected by HIV-1 RNA *in situ* hybridization (Ward 2000). In parallel, CD68-positive macrophages were identified with mAb HAM56 and an immunohistochemical staining reaction (brown staining). HIV-1 RNA/CD68-double positive macrophages are highlighted by black arrows. Magnifications: Left column 20X, right column 63X.

Figure. 3.5 - Confocal double-immunofluorescence microscopy allows determination of frequency of infected tissue macrophages. Productive HIV-1 infection was detected by staining with the anti-p24 mAb Kal-1, CD68-positive macrophages were detected using mAb PG-M1. Quantitative analysis of macrophage infection was based on evaluation of individual fluorochrome images as well as the digital merge by a pathologist (I.B.). Slides were viewed with a Laserscan microscope (Leica spectral confocal microscope TCS-SL). Magnification 20X.

Figure. 3.6 - Markers for T cells and macrophages do not colocalize in infected tonsil histocultures. The CD3 antigen was detected using a rabbit polyclonal antiserum, CD68-positive macrophages using mAb PG-M1. Confocal digital images for Cy2 and Cy5 were acquired independently. Cy5 images are

presented in red to allow better assessment of merged pictures. Images were evaluated by a pathologist (F.A.). Magnification 63X.

3B. Glycosylation

Prerana Jayakumar, Oliver Keppler, Becky Schweighardt, and Mark Goldsmith

3B.1 Abstract

Glycosylation comprises half the weight of the HIV-1 envelope and is responsible in part for viral immune evasion from antibodies. However, this advantage comes at the cost of permitting less efficient access of viral envelope proteins to host proteins. This effect may be pronounced in cells where the stoichiometry of viral coreceptor or receptors is crucial for efficient entry, such as in macrophages, which express low levels of CXCR4. We noted that viruses that use CXCR4 for entry into macrophages tend to lack specific V1-V3 glycosylation sites and studied whether deglycosylation of these residues results in macrophage entry. We found that deglycosylation alone was not sufficient for macrophage entry. However, all the viruses analyzed which use solely CXCR4 for macrophage entry lack a V1-V3 glycosylation site and have an intermediate V3 charge, indicating a possible recognition motif for dual-tropic X4 viruses.

3B.2 Introduction

The HIV envelope proteins are heavily glycosylated. Nearly 50% of the weight of the 120kD envelope protein is due to glycosylation moieties, all of which are N-glycosyl groups. These mannose-rich oligosaccharide groups are co-translationally added by enzymes in the rough endoplasmic reticulum.

The process of N-glycosylation begins cotranslationally. As the approximately 850-residue protein is translated from spliced RNA (Earl 1990; Coffin 1997), the envelope binds to a signal recognition particle and is transferred to the

endoplasmic reticulum (ER). In the ER, the protein's signal peptide is cleaved, and the envelope forms a trimer (Lu 1995). The enzyme oligosaccharyl transferase adds a glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$ (a 14-sugar precursor) to the asparagine groups on the growing protein, transferring it from a dolichol lipid intermediate. The consensus site for glycosylation requires an Asparagine (N) residue, followed by a non-proline residue and a serine or threonine (NXS/T). The standard HIV envelope sequence contains 24 sites for N-linked glycosylation in gp120 (88, 136, 141, 156, 160, 186, 197, 230, 234, 241, 262, 276, 289, 295, 301, 332, 339, 356, 386, 392, 397, 406, 448, and 463). There are seven glycosylation sites in gp41 (611, 616, 624, 637, 674, 750, and 816). Of these 31 glycosylation sites, not all are always glycosylated (Alberts 2002; Coffin 1997).

The 14-sugar carbohydrates are then processed in the ER and Golgi to create the complete N-glycan. In the ER, the three glucose residues and one mannose are removed on each sugar, and the envelope oligomerizes and must be folded correctly. The envelope is exported to the Golgi, where further mannose residues may be removed, usually three. In the Golgi, further N-acetylglucosamine (GlcNac) residues, usually one, are added, and typically two more mannose residues are removed. Finally, in the trans-Golgi, two types of complete N-glycans may be produced: high mannose, which contain 5 or more mannose groups, or complex oligosaccharides, which may contain other sugar groups in addition to mannose, such as galactose, GlcNac, *N*-acetylgalactosamine, fucose and sialic acid (Figure 3.7). HIV envelope contains 11 high-mannose

oligosaccharides and 13 complex oligosaccharides. Because complex carbohydrates are more modified than high-mannose forms, complex glycosylation occurs to a greater extent on the surface of the protein, whereas high-mannose glycosylation occurs in relatively inaccessible regions of the envelope. In addition, gp120 has an immunologically "silent" face that is heavily glycosylated (and likely accessible). The other regions of the protein may be inaccessible due to their role in trimer or oligomer interactions.

Glycosylation plays several roles in HIV pathogenesis. Glycosylation of the envelope provides protection from host neutralizing antibodies, rendering them completely ineffective (reviewed in Hunter 1990). Several anti-HIV antibodies, including the b12 antibody known to be neutralizing, are ineffective due to the occlusion of their native antigens by glycosylation. It is thought that glycosylation is the primary reason for the failure of the humoral response to HIV.

Glycosylation also provides protection from cellular or extracellular proteases that might degrade the envelope and reduce infectivity. It may further stabilize the virion envelope, preventing envelope shedding. In this regard, it has been reported that X4 envelopes may be more susceptible to envelope shedding.

Glycosylation may also be necessary for proper folding and assembly of the HIV envelope. Viruses produced in the absence of glycosylation (in insect cells, for example) or in the presence of a glycosylation inhibitor like tunicamycin (which inhibits dolichol) are unable to bind CD4 and are likely incorrectly folded. In contrast, *in vitro* deglycosylation of fully folded and processed envelope proteins leads to no decrease in receptor binding (Fenouillet 1989; Li 1993).

On the contrary, glycosylation may also prevent binding of the envelope to its receptor or coreceptor. In this regard, it has been found that glycosylation of envelope may occlude the CD4 binding site. Glycosylation could also affect infectivity negatively in other ways. Saifuddin et al. have reported that the 11 high-mannose group-containing N-glycosylation sites on gp120 may bind more efficiently to cell-surface mannose binding lectins (MBLs), which play a role in complement-mediated opsonization and phagocytosis (Saifuddin 2000). These high-mannose residues are crucial for entry, as demonstrated by the fact that deoxynojirimycin, which inhibits the glucosidase I that produces high-mannose residues, is necessary for HIV syncytium formation. In contrast, deoxymannojirimycin, an inhibitor of alpha-mannosidase I and of complex oligosaccharides, had no effect (Gruters 1987; Montefiori 1988; Pal 1989).

Glycosylation is also thought to play a role in HIV coreceptor tropism. It has been shown that two N-glycosylated residues in gp120, when mutated, confer CD4-independent entry to the X4 isolate m7NDK (Dumonceaux 1998). CXCR4 viruses also tend to have a smaller number of glycosylation sites than do R5 viruses (http://www.aids.harvard.edu/conferences_events/2004/world_aids_04_present/4_Chakabva.pdf and our observations). This suggests that CXCR4 viruses may be more susceptible to antibody-mediated neutralization, and therefore less likely to be selected early in infection. Also, specific sites (along with V3 charge) in the V3 variable regions are associated with CXCR4 tropism (Clevestig 2006; Polzer 2002; reviewed in Dong 2005). In particular, a glycosylation site (g15) within the V3 region of gp120 has been shown to confer increased infectivity to X4 viruses

and in some cases, to confer CXCR4 usage on otherwise R5 viruses. Two other sites immediately outside the V3 region (here called g13 and g17) have also been implicated in coreceptor usage (Polzer 2002).

CXCR4 is traditionally associated with T cell infection, but recent studies have shown that macrophages are also susceptible to CXCR4 infection (Collman, Jayakumar). These X4 macrophage-tropic viruses (dualtropic X4) are associated with lower CD4 counts.

3B.3 Materials and Methods

Virus stocks

Viruses were obtained and prepared as in Chapter 2D. Glycosylation-deficient viruses were a gift of Michael Schreiber (Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) and were produced by calcium phosphate transfection of 293T cells.

Virus infections

Infections were performed as described in Chapter 2D.

Cells

Macrophages were isolated and prepared as described in Chapter 2D. Cells were replated into 24-well dishes for further studies and infections. HeLa CD4/CCR5 cells were a gift of Warner Greene. GHOST CD4, CD4/CCR5, and CD4/CXCR4 cells were obtained from the NIH AIDS Reagent and Reference Program.

Sequence Analysis

Sequences obtained from the HIV sequence database (http://hiv-web.lanl.gov) were aligned using Multalign. The sequence of tybe was determined by sequencing using T7 and M3 primers. Glycosylation sites were determined by the consensus NXT or NXS, where X is not P. V3 charge was determined for the V3 region only using the calculation (R+K)-(D+E) (i.e., the number of positively charged residues minus the number of negatively charged residues).

3B.4 Results

We sought to determine the genetic determinants of X4 macrophage tropism in a panel of viruses previously shown to infect monocyte-derived macrophages (Jayakumar 2005; Collman 1989). We obtained these primary virus isolates and cultured them on PBMCs for no more than a week to produce viral stocks. We quantified the viruses by p24 ELISA and TCID50 on activated human PBMCs. Viral stocks were normalized, frozen and thawed once for subsequent analysis. To ensure that these viruses were all replication competent, we infected HeLa CD4-CCR5 cells with equal 50% tissue culture infectious doses (TCID50s) of the X4-lab strain NL4-3, R5-lab strain 49-5, X4/R5-dualtropic 89.6, and our X4-dualtropic viruses. The viruses used were UG024, UG021, (subtype D), J130, J34, and tybe (subtype B).

We also used three mutants of NL4-3. These mutants are lacking in one, two, or three glycosylation sites. As shown in Figure 3.8, X4-dualtropic viruses were able to infect HeLa CD4-CCR5 cells and result in robust infection kinetics, similar to lab-adapted strains and other primary isolates.

To ensure that these viruses also were not altered in their tropism, we measured coreceptor usage on GHOST cells that were stably transduced with CD4 only (GHOST CD4), CD4 and CXCR4 (GHOST X4), or CD4 and CCR5 (GHOST R5). GHOST cells express a low level of CXCR4 on their cell surface. These cells contain an LTR-driven GFP reporter construct. Upon viral entry, reverse transcription, integration, and transcription, infected cells will express GFP.

As shown in Figure 3.9, the X4 lab-adapted strain NL4-3 was unable to infect GHOST CD4 or GHOST CCR5 (not shown) cells. In agreement with NL4-3's

CXCR4 usage, it was only able to infect GHOST X4 cells (data not shown). Similarly, 49-5 infected only GHOST CCR5 cells (data not shown). In contrast, all of the glycosylation mutants (g15, g15/17, and g13/15/17) were able to infect the low-CXCR4 GHOST-CD4 cells. Similarly, UG021, UG024, and tybe were also able to infect GHOST-CD4 cells. None of these viruses is able to infect cells that do not express CD4 (e.g. 293T cells, data not shown).

These results indicate that loss of glycosylation in the V3 and surrounding regions of NL4-3 can lead to improved sensitivity to CXCR4. Furthermore, these results suggest that like the glycosylation mutants, some viruses that use CXCR4 to enter macrophages also are more sensitive to CXCR4. This is supported by studies that show that artificial introduction of increased CXCR4 in macrophages supports NL4-3 infection of macrophages (Tokunaga 2001).

The correlation between macrophage-tropic X4 viruses and glycosylation mutants suggested that the NL4-3 mutants might be able to infect monocyte-derived macrophages. Therefore, we produced concentrated stocks of viruses expressing the envelope protein of these mutants along with a NL4-3 based replication-incompetent luciferase virus. However, none of the mutants was able to significantly infect macrophages. Concentrated stocks of replication-competent viruses also demonstrated no infection (Figure 3.10).

Therefore, we speculated that macrophage infection by X4 viruses might involve glycosylation in addition to other envelope sequence changes or V3 charge. We analyzed the sequences of a number of viruses, including our X4-dualtropic viruses, as shown in Table 3.1.

As shown, the three X4-dualtropic viruses we tested (UG021, UG024, and tybe) had lower V3 charges compared to X4 T-tropic viruses, but had higher charges than R5 viruses, indicating an intermediate charge between these virus types may be responsible for macrophage tropism of X4 viruses. These X4-dualtropic viruses also demonstrated loss of glycosylation sites g13, 15 and/or 17.

3B.5 Discussion

We have shown that dual-tropic X4 viruses replicate efficiently and can more efficiently use lower levels of CXCR4. These viruses lack several key V3 glycosylation sites. However, glycosylation alone is not sufficient for macrophage entry, as glycosylation-deficient viruses do not infect macrophages. We suggest that a combination of lower V3 charge and glycosylation changes in the V1-V3 region contribute to macrophage infection by X4 viruses.

In our sequence analysis, several other X4-dualtropic viruses also showed loss of glycosylation. However, R5X4 dualtropic viruses did not appear to follow this trend. The dualtropic virus 89.6 did not show any loss of glycosylation, and does have a slightly high V3 charge (+7), and has been shown to infect macrophages in the absence of CCR5. Furthermore, the R5X4 dualtropic virus DH12, also shown able to infect macrophages through CXCR4, has a high V3 charge, though it does lack g15. Several R5 viruses also lack glycosylation sites, though the role of these changes on R5 tropism is unknown. A few X4 viruses reported to infect only T cells also lacked glycosylation sites. The ability of some of these viruses to infect monocyte-derived macrophages is unknown, possibly explaining their X4-T cell tropic classification.

A recent study has further elucidated the determinants of macrophage tropism, suggesting that not only V3 charge and glycosylation, but also certain amino acid changes in V3, V5 and V1V2, play a role in macrophage tropism. Specifically, a methionine or leucine to isoleucine at position 326 in V3 (previous to g17) is necessary, along with cysteine or proline in V1 and charged residues in V2.

(Ghaffari 2005). In UG021, UG024, and tybe, this residue is indeed an isoleucine. These viruses were also more sensitive to the CXCR4 inhibitor AMD3100 (data not shown).

The authors found no correlation of envelope glycosylation with macrophage tropism by X4 viruses, but a study of the envelope sequences indicates that the two viruses studied contain altered glycosylation sites. D01 lacks g13 and has a shifted g17, whereas D02 lacks g15. The net V3 charge of these viruses is 5, an intermediate charge. This suggests that a combination of V3 charge and glycosylation may indeed play a role in macrophage tropism of X4 viruses.

3B.6 Acknowledgments

We thank Jason Kreisberg, Marielle Cavrois, and Wes Yonemoto for critical discussion. Technical assistance was provided by Duncan Mieklejohn.

3B.7 Figure Legends

Figure 3.7 – The mammalian N-glycosylation pathway.

Figure 3.8 – X4 dualtropic viruses NL4-3 (A) and glycosylation mutants (B) infect HeLa CD4-CCR5 cells. Cells were infected with equal p24 of virus, washed after 12 hours, and supernatant p24 was measured by ELISA at the indicated timepoints. Results are the mean of triplicate experiments.

Figure 3.9 – X4 dualtropic viruses and glycosylation mutants infect GHOST-CD4 cells. Equal titers of various viruses were used to infect GHOST-CD4 cells. The percent GFP+ cells was determined by flow cytometry and is the result of triplicate experiments.

Figure 3.10 – Glycosylation mutants fail to infect monocyte-derived macrophages. Equal p24 (MOI 0.01) of glycosylation mutants g15, g15/17, g13/15/17 was used to infect human monocyte-derived macrophages. Cell supernatants were collected after 3 or 5 days and p24 was measured by ELISA.

Chapter 4: Tat and microarrays

4A. Microarray studies

Prerana Jayakumar, Peter Henklein, Melanie Ott, and Eric Verdin

4A.1 Abstract

The HIV-1 Tat protein is a transcriptional activator of the HIV promoter. Tat also regulates the level of activation of immune T cells (Ott 1997). We have analyzed the expression of 21,000 human genes using oligonucleotide microarrays in response to Tat expression. Two splice variants of Tat, Tat72 or Tat101, and the green fluorescent protein (GFP) were expressed from a bicistronic mRNA under the control of the HIV promoter using an HIV-based retroviral vector in Jurkat cells. A total of 68 genes were significantly upregulated greater than 2-fold by Tat72 or Tat101. Among the upregulated genes was Tsg101, a protein recently implicated in the budding of HIV.

4A.2 Introduction

We have studied the effects of both endogenous and extracellular Tat on T cell activation and cellular transcription. The interaction of Tat with cellular factors has also been a major focus of study. In order to carry out studies of endogenous viral Tat expression, systems to express Tat from retroviral vectors have been established. The *tat 101* and *tat 72* open reading frames were cloned into an HIV retroviral vector under the control of the HIV LTR, followed by an internal ribosome entry site (IRES) and green fluorescent protein (GFP). These genomic vectors can be packaged using a retroviral packaging vector expressing the *gag* and *pol* genes of HIV. The virus is pseudotyped with the envelope of vesicular stomatitis virus (VSV G protein). Resulting viral particles can infect cells of many types to high levels (>90%) in a single cycle of infection and exhibit green fluorescence (GFP expression) as a marker of Tat expression. Viral titers can be quantified both by the expression of the viral gag/p24 protein as well as by the rate of GFP expression upon infection of target cells as analyzed by flow cytometry.

Such viruses are produced by transfecting 293T cells with genomic vectors, the packaging vector, and the envelope plasmid. We have titered these virus stocks on Jurkat T cells to determine the kinetics of infection and ensure the infectivity of the viruses (Figure 4.1). Infections are carried out with increasing quantities of viral supernatant. The minimal amount of p24 antigen necessary to get maximal infection within 48 hours was quantified and used for subsequent experiments.

4A.3 Materials and Methods

Extracellular Tat

Synthetic Tat protein was isolated as described (Dorr 2002). Clone A2 and A72 were cultured and created as described (Jordan 2003). Cells were cultured in complete RPMI and treated with the indicated concentrations of synthetic Tat72 for 24 hours before flow cytometry analysis.

Virus preparation

Viruses were prepared as described in Chapter 2D.

Virus infection

Cells were infected as described in Chapter 2D.

Cells

Jurkat and 293T cells were cultured under standard tissue culture conditions. A common stock of Jurkat cells was obtained, frozen once, and freshly thawed (at most one month in culture) cells were used for all experiments. Cells were cultured in RPMI (Gibco) containing 1% penicillin-streptomycin and 1% L-glutamine (both Gibco). Uninfected Jurkat and 293T cells were treated similarly to infected cells in all experiments.

Flow cytometry

Cells were suspended in phosphate-buffered saline containing 2% fetal bovine serum. Flow cytometry was performed on a FACSCalibur (BD Biosciences), acquired with CellQuest software, and analyzed using FlowJo (www.flowjo.com).

RNA preparation and isolation

RNA was prepared as described in Chapter 5.

Microarray analysis

Microarrays were performed and analyzed as described in Chapter 5.

4A.4 Results

We infected Jurkat T cells with these viruses and determined GFP expression. As shown in Figure 4.2B, Jurkat cells infected with these viruses expressed Tat. Tat101 was expressed at a greater level than Tat72, likely due to improved stability. We then measured GFP levels on infected cells. All the infections resulted in 90% GFP+ cells. Only viruses expressing Tat were able to activate viral GFP expression to a detectable level (Figure 4.2C).

Furthermore, we have used a purified preparation of synthetic Tat as a source of extracellular Tat. Added to Jurkat cells, this Tat preparation can activate the HIV LTR at concentrations ranging from 6.25-100ug/ml (Figure 4.3).

We used Jurkat cells infected for 48 hours for further microarray studies. The strategy of microarray comparison is outlined in Figure 4.4. The microarray preparation method is outlined in Figure 4.5.

Microarray studies resulted in the identification of a number of genes, shown in Table 4.1 and 4.2. Tat101 resulted in the increase of a larger number of genes than Tat72. Virus infection alone resulted in the increase of a number of genes, as expected. However, none of these genes was uniquely upregulated by the GFP virus alone.

4A.5 Discussion

We have performed microarray studies on Jurkat T cells using printed microarrays. Our microarray experiments were performed in Jurkat cells, which are a CD4+ T cell line, representative of the population of cells that is a target of HIV infections. Although these cells are not primary cell cultures, they are

untransformed (Schneider 1977). However, the non-clonal and pseudodiploid nature of these cells may make them different from primary cells in several regards. Nevertheless, Jurkat cells are well-studied, easy to infect, and provide sufficient material for microarray analysis.

Our results indicate that Tat72 and Tat101 have important differences in their ability to activate cellular genes. The fact that only a 29 amino acid difference can lead to profound changes in cellular transcriptional profiles may indicate that the heretofore relatively uncharacterized second exon of Tat has unknown functions. Mutation of the RGD (integrin-binding) motif in Tat101 will be necessary to determine whether activation of these genes requires integrin-binding or cellular adhesion. Further study is necessary to verify additional individual genes and to replicate the induction of these genes by Tat in primary lymphocytes and other cell types, including macrophages.

4A.6 Acknowledgments

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4A.7 Figure Legends

Figure 4.1 – Schematic of virus preparation. Viruses were prepared by calcium phosphate transfection of 293T cells, and supernatants were quantified for p24. Viruses were used to spin infect Jurkat T cells, which were subsequently used in flow cytometry (to determine infection kinetics) or microarray studies.

Figure 4.2 – Expression of Tat and GFP in infected Jurkat cells. (A) LTR-Tat-IRES-GFP constructs used. Constructs without Tat, with Tat72, and with Tat101 were used. (B) Tat expression by Western blot. Lysates were harvested after 48 hours and boiled in Laemmli buffer, and run on a 15% polyacrylamide gel. Rabbit anti-flag (Tat) and mouse anti-tubulin alpha primary antibodies were used. (C) GFP mean fluorescence intensity. After infection, an aliquot of cells was harvested at the indicated time points and GFP was measured by flow cytometry. Results are the average of triplicate experiments (±SEM).

Figure 4.3 – Activation of the LTR by extracellular Tat. Jurkat latent clone A72 (Jordan 2003) was treated with the indicated concentrations of synthetic Tat protein. After 24 hours, cells were analyzed by flow cytometry.

Figure 4.4 – Strategy of microarray comparison. All samples were individually compared with uninfected cells, which were mock-infected by spinning with DMEM media, HEPES, and polybrene.

Figure 4.5 – Microarray preparation. Total RNA isolated from cells is reverse transcribed into amino-allyl cDNA, followed by labeling with Cy3 or Cy5 dyes, and competitive hybridization on a 20,000 gene microarray slide.

4B. Tat and CXCR4

Paper: Tat expression in T cells leads to increased CXCR4 expression and increased chemotaxis to SDF-1

4B.1 Abstract

Using DNA microarrays, we identified the HIV coreceptor, CXCR4 as a cellular gene that is reproducibly activated by Tat72 and Tat101, both at the RNA and protein levels (by flow cytometry). At a functional level, this increase in CXCR4 expression is paralleled by an increase in the chemotaxis of cells that express Tat to SDF-1, the CXCR4 ligand. Using GPF expression as a marker for cells expressing Tat, we also observed that CXCR4 expression and increased chemotaxis only occurred in Tat-expressing cells, excluding a cell non-autonomous role for Tat. Using a CXCR4 promoter reporter construct, we also observed that Tat72 and Tat101 wild-type, but not the mutants K41A and C22G, activated the CXCR4 promoter. The part of the CXCR4 promoter necessary for Tat activation is restricted to between -600 and -357 from the transcriptional start site. The results indicate that Tat alters chemokine receptor expression and activity and could modulate the homing of infected cells in vivo.

4B.2 Introduction

The Tat protein of human immunodeficiency virus type I (HIV-1) is a potent transactivator of HIV-1 transcription. Tat binds to the Tat-responsive element TAR, an RNA stem loop structure, to recruit cellular factors that in turn activate RNA polymerase II to allow for processive transcriptional elongation.

The role of Tat is not limited to the HIV-1 promoter. Several studies have examined the role of Tat in activation of other promoters. In T-cells, Tat has been demonstrated to induce the secretion of cytokines such as IL-2 and IL-8 (Ott 1996), by activation of a CD28-responsive element (IL-2) or NF-κB (IL-8). Tat also activates NF-κB directly, potentially by binding to unknown sites in the NF-κB promoter. In basophils, Tat stimulates the release of TH2 cytokines IL-4 and IL-13 (Marone 2001), and IL-1 in endothelial cells (Lee 2004). In dendritic cells, by contrast, Tat induces a TH1 response, including secretion of TNFα and IL-12 (Fanales-Belasio 2002). In astrocytes (Kutsch 2000), Tat induces secretion of interferon-responsive genes including the chemokines IP-10, HuMIG, MCP2, and MCP3, as well as RANTES (El-Hage 2005). In astrocytes, macrophages, glia, and in Kaposi's sarcoma, Tat upregulates MCP-1 (Conant 1998, Kelly 1998, Mengozzi 1999, McManus 2000), resulting in cellular migration (Weiss 1999). This upregulation is at the transcriptional level, and Tat acts by activating the TGF-beta pathway and increasing the binding of Smad, SP1, NF-κB, and AP-1 transcription factors to the MCP-1 promoter (Lim 2000; Abraham 2003). Tat also induces VCAM-1 and ICAM-1 adhesion molecules as well as IL-6 in Kaposi's sarcoma (Kelly 1998). In epithelial cells, Tat upregulates CXCR4, CCR3, SDF-1,

RANTES, IL-6, TGFβ, BMP-1, BMP-2, L-10, IL-19, and IL-20 (Bettaccini 2005). Other studies have shown Tat induction of other signaling molecules and transcription factors.

Tat has also been shown to upregulate the expression of chemokine receptors. Recombinant Tat72 upregulates CCR5 and CCR3 on macrophages (but not lymphocytes) (Huang 1998). At the blood brain barrier, Tat upregulates CCR1 and CCR3 (Shaw 1999), and activates secretion of CCL2, CXCL8, CXCL10, CCL3, CCL4, and CCL5 in microglia (D'Aversa 2004).

Tat has further been shown to downregulate the beta chemokine MIP1 alpha, a ligand of CCR5. Other studies have shown upregulation of MIP1 alpha and beta in glia and lymphocytes (McManus 2000, Kim 2004), along with upregulation of MIP2, CXCL10, and XCL1 (Kim 2004) or other chemokines in microglia (Sheng 2000). Tat mimics several beta chemokines, such as MCP1, MCP3, and eotaxin, likely through basic residues in the protein, and thereby influences receptor levels (Albini 1998).

Studies in resting or activated CD4+ T cells, macrophages, erythrocytes, and glia have demonstrated the upregulation of CXCR4 by synthetic Tat protein. Only in macrophages, however, have physiological picomolar levels of Tat been demonstrated to induce CXCR4 (Secchiero 1999). These studies have shown that the upregulation of CXCR4 results in improved HIV infection of uninfected cells treated with Tat. CXCR4 is also inhibited by pertussis toxin, as are many Tat-mediated effects, suggesting that G proteins are involved in Tat effects, and lending support to the idea that Tat might act through CXCR4 (Rizzi 2004).

In contrast, recombinant Tat has also been demonstrated to downregulate CXCR4 by binding via its basic domain to the ligand-binding site on the chemokine receptor and result in diminished X4 HIV infection (Ghezzi 2000; Xiao 2000). Interestingly, an inhibitor of Tat transactivation, CGP64222, also inhibits CXCR4-mediated entry (Daelemans 2000). Furthermore, rhesus macaques treated with Tat toxoid, a chemically-inactivated form of Tat used as a vaccine, demonstrate reduced CXCR4 and CCR5 levels (Pauza 2000). In neurons, Tat upregulates the CXCR4-ligand SDF-1α (Langford 2002).

Most of studies of CXCR4 have used synthetic extracellular Tat or recombinant purified Tat proteins. A few studies have used adenoviral Tat or CMV-promoter driven Tat. None of these studies has addressed the role of virally-produced intracellular Tat driven by the HIV-1 LTR at endogenous levels on CXCR4 expression, nor is the mechanism of CXCR4 upregulation known.

The majority of studies also investigate Tat1-86 or Tat72 (one exon Tat), with few differences explored between Tat72 and two-exon Tat101. We chose to investigate roles of two forms of the Tat protein, Tat 1-72 and Tat 1-101, for their effects on gene expression by microarray analysis in Jurkat cells. We found CXCR4 among the upregulated genes, and investigated the mechanism of upregulation by Tat.

4B.3 Materials and methods

RNA extraction

Infected and uninfected cells were pelleted by centrifugation and resuspended in Trizol (Invitrogen) and stored at –80°C. Total RNA was harvested according to manufacturer's directions, quantified, and analyzed for purity on a 1% formaldehyde gel as well as on an Agilent Bioanalyzer.

Gene-specific RNA Quantification

CXCR4 RNA levels were determined by SYBR Green quantitative PCR (Qiagen). cDNA was made from total RNA using ImProm II reverse transcriptase (Invitrogen). cDNA was subjected to PCR with 2x HotSYBR PCR Reaction Mix (Molecular Cloning Laboratories) on an ABI Prism 7700 Sequence Detector (AME Bioscience). Human GAPDH (BC001601); Forward: 344 AGTCCACTGGCGTCTTCACC, Reverse: 464 TGGTTCACACCCATGACGAA. Human CXCR4 (NM_003467); Forward: GTTACCATGGAGGGGATCAG, Reverse: CAGATGAATGTCACCTCGC

Microarray analysis

Total RNA from Jurkat cells was prepared from three independent experiments for each condition (mock-infected, infected with control LTR-GFP vector, Tat72, or Tat101) after 48 hours using Trizol (Invitrogen). For each probe, 20ug of total RNA was reverse-transcribed using ImProm II reverse transcriptase (Promega), 500 μM dATP, dCTP, and dGTP, 350 μM aminoallyl dUTP (Sigma), and 150 μM dTTP. After RNA degradation by alkaline hydrolysis, cDNA was recovered by ethanol precipitation and coupled to NHS-Cy3 or Cy5 (Amersham). Cy3 and Cy5-

labeled probes were purified with the Qiaquick PCR purification kit (Qiagen). Probes were resuspended in Ambion hybridization buffer with 30% formamide and hybridized to glass slides printed with 20,000 human-gene specific 70mer oligonucleotides (Operon 7.0 mouse oligonucleotide set, Qiagen). Hybridizations were performed at 50°C for 40 hours in hybridization chambers (Die Tech). Slides were washed twice with 1x SSC/0.03% SDS, twice with 0.2x SSC, and twice with 0.05x SSC, and scanned on a GenePix 4000B scanner (Axon Instruments). Fluorescence data was acquired with GenePix 6.0 software. Median fluorescence ratios were subjected to LOWESS and print-tip normalization, without background subtraction, on Acuity 4.0 software. Ratio data were then analyzed for statistical significance using paired t-tests. For each condition, comparisons were made between infected (control, Tat72, or Tat101) with mock-infected samples from three independent experiments. These were compared with arrays in which mock-infected samples were self-hybridized (mock vs mock). This allowed comparisons to be performed indirectly between the GFP and Tat conditions. Each of the three arrays for each sample was performed with dye-swapped replicates, for six arrays per sample, and eighteen arrays in total. Statistically significant genes were analyzed by paired t-tests with a confidence interval of 95%.

Western blot

Infected and uninfected cells were pelleted by centrifugation, washed with PBS, and resuspended in 2x Laemmli buffer containing DTT, or in IPLS, as indicated. Lysates were normalized for protein content by the Biorad Protein Assay and

separated on 10 or 15% acrylamide gels (Biorad). Proteins were transferred to Hybond XL 0.2um membranes with transfer buffer containing 20% methanol. Membranes were blocked with 5% milk (Biorad) in TBS-Tween (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and probed with rabbit anti-FLAG M2 (Sigma), mouse anti-tubulin (Sigma), mouse anti-actin (Sigma), or rabbit anti-CXCR4 (Abcam), diluted 1:1000. Secondary detection was performed with goat anti-mouse or anti-rabbit HRP (Pierce), diluted 1:2000. Membranes were analyzed by ECL (Amersham) or Supersignal West Femto (Pierce) with 0.2 micron Hybond film.

Plasmids and Mutagenesis

Tat72 and Tat101 LTR and CMV constructs are as described (Jordan et al. 2003, see also Figure 4.2A). CXCR4 promoter luciferase constructs pGL-CXCR4 and –357, -93, -42 were a gift of Hiroyuki Moriuchi. Forward: -1000 GTAACCCGGGAAGACGCCGAGTATGGGGACC, -800 GTAACCCGGGCTG-TGATGGTAATACCCACACGG, -600 GTAACCCGGGCTCCAGAATTATGCCA-AATCCTACC and Reverse: GGAATGCCAAGCTTACTTAGATCT. PCR products were cut with SmaI and BglII, and recloned into the parent vector pGL2-basic (Invitrogen). pCMV-luciferase was obtained from Promega.

Flow cytometry

Cells were pelleted at 1500rpm in a tabletop centrifuge for 5 minutes and stained with fluorescence-conjugated antibodies in the presence of phosphate-buffered saline containing 2% fetal bovine serum. The following antibodies were used at a

1:25 dilution: CXCR4-PE, CXCR4-APC, CD4-PerCP (Leu3a), CCR7-PE (Beckton Dickinson). All flow cytometry was performed on a FACSCalibur cytometer (Beckton Dickinson), with acquisition using Cell Quest software, and analyzed by FlowJo software (Treestar).

Chemotaxis

The chemotaxis assay was performed using a modified Boyden chamber method (R&D systems, Minneapolis, MN) using peripheral blood-derived lymphocytes (activated for 3 days with PHA and IL-2, R&D Systems) or Jurkat cells. At the indicated times, cells were suspended at a concentration of 5 x 10^6 cells/ml in RPMI 1640 medium containing 0.25% inactivated fetal calf serum (FCS). 100μl of cells were placed in the upper chamber of a 3μm Transwell (Costar #3415, Corning, NY) dish. Cells were incubated for 30 minutes at 37°C in the presence or absence of 250nM AMD3100 (Sigma). After incubation, 600μl of media containing indicated concentrations of SDF-1α, RANTES, or CXCL21 (R&D Systems) were added to the bottom chamber of the Transwell dish. Plates were incubated for 3.5hrs and cells in the upper and lower chamber were analyzed by flow cytometry. Cell numbers were normalized using TruCount beads (Beckton Dickinson).

Transfections and luciferase assays

Transfections with virus expressing plasmids were performed in 293T cells with the calcium phosphate precipitation method. Cells were harvested 48 hrs after transfection of plasmids DNA and analyzed for luciferase activity. Transfections

into Jurkat cells were performed by electroporation at 250V, 900μF (Biorad electroporator). Cells were harvest 24 hrs after transfection and analyzed for luciferase activity (Promega luciferase assay).

Protein assay

Protein concentration was measured in total cell lysate by the *Dc* Protein Assay Kit (BioRad, Hercules, CA)

Virus preparation

HIV isolates were produced from calcium phosphate transfection of HEK-293 T cells with the following vectors: packaging construct CMVΔR8.2 (from D Trono, EFPL, Lausanne, Switzerland), pMD.G (CMV-VSV.G, from D Trono; Naldini 1996), and a lentiviral vector containing either LTR-GFP (pRRLGFP-W, from D Trono) or LTR-Tat101-IRES-GFP (Jordan 2001; Jordan 2003). In experiments involving HIV-1, NL4-3 GFP+ Nef- (a gift from N Landau, Salk Institute, San Diego, CA) was pseudotyped with the VSV envelope. Viruses harvested after 48 hours and filtered through a 0.2μM membrane. Various amounts of virus supernatant were titered by infection of 1 x 10^6 Jurkat cells in the presence of 4μg/ml polybrene (hexadimethrine bromide; Sigma), and virus-associated p24 (Gag) protein was measured by fluorescence-linked antigen quantification as described (Hayden 2003) and ELISA (Alliance).

Viral infections

Eighteen x 10^6 Jurkat T cells were infected by centrifugation with 1500ng p24-Gag of each virus in a total volume of 5ml, with 10mM HEPES (pH 7.5, Invitrogen) and 4μg/ml polybrene. Infections were carried out in 6-well plates (Falcon) at 2400 rpm in a Beckman-Coulter centrifuge (Allegra 6R, rotor GH3.8A) for two hours at 32°C. After infection, cells were washed with PBS and plated at 0.5 x 10^6 cells/ml in RPMI containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. GFP expression was measured after 24 hours to verify infection levels using a FACSCalibur flow cytometer (Beckton Dickinson) and CellQuest software (BD Biosciences), and data were analyzed with FlowJo (Treestar).

Sequence analysis

CXCR4 promoter region sequence from -600 to -357 (Figure 5A) was analyzed for evolutionarily conserved regions by the UCSC Genome Browser (Kent 2002; Karolchick 2003; Lander 2001) at http://genome.ucsc.edu. Analysis of this sequence for transcription factors was performed by TRANSFAC TfBlast (Altschul 1997) and PATCH at http://gene-regulation.com, and by TF-SEARCH at http://www.cbrc.jp/research/db/TFSEARCH.html with default parameters. Hits present at multiple sites or present in two out of three search programs were considered significant.

4B.4 Results

Microarray results

To study Tat in a viral context, we expressed Tat from a vector driven by the HIV-1 LTR and containing GFP with an internal ribosomal entry site (IRES). We used three alternative vectors: LTR-Tat72-IRES-GFP, LTR-Tat101-IRES-GFP, or the control vector LTR-GFP (Figure 4.2A). These vectors were packaged with a HIV-1 packaging vector expressing essential viral proteins and with a vesicular-stomatitis virus G protein envelope (VSV-G). Infection of Jurkat cells with these viruses resulted in expression of the appropriate Tat protein. As shown in Figure 4.2B, Tat72 and Tat101 were expressed at 48 hours after infection. Tat101 appeared to be expressed more highly despite equal levels of infection. To determine if this was due to increased transcription, we also measured GFP expression in Jurkat cells. All infections resulted in 90% GFP+ cells (data not shown). However, the mean fluorescence intensity (MFI) of GFP expression was low in the control LTR-GFP vector, because it lacks Tat to transactivate the LTR (Figure 4.2C). By contrast, GFP MFI was identical in Tat72 and Tat101 infected cultures and increased over time, indicating that Tat was functional in these cells. These results also indicate that Tat101 was not transcribed more highly than Tat72 and that increased protein expression is likely due to the greater stability of Tat101 protein or RNA.

We performed microarray analysis with Jurkat cells 48 hours after infection. All infected cell populations were compared with a standard uninfected cell population. Studies were performed in triplicate and resulted in a set of

significantly up- and down-regulated genes. Notably, Tat101 induced the expression of a large number of genes uniquely (Figure 4.2D and Table 4.1). No genes were upregulated by the control vector alone, and few genes were downregulated by any of the infections (Table 4.2). Several genes were found at the intersection of Tat72 and Tat101 (Figure 4.2D), indicating genes upregulated by either form of Tat (Table 4.2)

Tat upregulates CXCR4 RNA and protein

We chose to investigate one Tat-induced gene further, CXCR4. Microarray results for CXCR4 are shown in Figure 4.6A. To verify Tat's induction of CXCR4, we measured CXCR4 RNA levels by SYBR Green. Tat72 and Tat101 both increased CXCR4 RNA relative to the control vector (Figure 4.6B). We went on to measure CXCR4 protein expression by Western blotting. As shown in Figure 4.6C, Tat72 and Tat101 increased CXCR4 protein in Jurkat cells.

Cell surface CXCR4 expression

We next verified CXCR4 protein expression at the cell surface. We used flow cytometry to detect cell-surface CXCR4 mean fluorescence intensity. Uninfected Jurkat cells express CXCR4. However, infection with LTR-GFP often resulted in reduced CXCR4 expression (data not shown). Relative to LTR-GFP, Tat72 and Tat101 virus infection resulted in increased cell surface expression of CXCR4 in the infected (GFP-expressing cells) only (Figure 4.7A).

Because Tat contains a protein transduction domain and can enter uninfected cells, we also measured CXCR4 expression in uninfected cells in the same population. Increases in CXCR4 expression on both infected and uninfected cells

would indicate transfer of Tat from infected to uninfected cells. However, we found no increase in CXCR4 expression in uninfected cells with either Tat72 or Tat101 (Figure 4.7B), indicating either an inability of Tat to exit infected cells or to enter uninfected cells and activate CXCR4 expression. Similar results were found in blood-derived lymphocytes activated with PHA and IL-2 (Figure 4.7C and D). We also measured the cell surface expression of non-related proteins not demonstrated to be upregulated by Tat. Neither Tat72 nor Tat101 upregulated the expression of CCR7 (Figure 4.7E) or CD3 (not shown).

Chemotaxis

Although Tat increases CXCR4 expression only in infected cells, these cells are not likely more susceptible to HIV infection, as uninfected cells might be. Instead, we studied the sensitivity of Tat-infected cells to the CXCR4 ligand SDF-1. We performed chemotaxis assays with Jurkat cells in Transwell chambers. Uninfected cells migrated downward in response to SDF-1 in the lower chamber only (Figure 4.8A). We then went on to study migration of Tat-infected cells using increasing doses of SDF-1α. LTR-GFP, LTR-Tat72, and LTR-Tat101 expressing cells migrated downward in response to SDF-1. In agreement with greater CXCR4 expression, more uninfected cells migrated than LTR-GFP infected cells. However, infection with Tat72 or Tat101 relieved this defect in migration, as shown in Figure 4.8B. As with CXCR4 expression, increased migration was only seen in infected cells and not in uninfected cells (Figure 4.8B and C). The cell-autonomous effect of Tat on CXCR4 expression was also evident in primary lymphocytes (Figure 4.8D and E).

Migration of all infected cells was completely inhibited by the CXCR4 antagonist AMD3100 (Figure 4.8F). We also measured migration of cells to other chemokines. Jurkat cells did not migrate to the beta chemokine RANTES, as these cells do not express CCR5. Tat also had no effect on the CCR7 ligand CXCL21 (Figure 4.8F).

Tat activates the CXCR4 promoter

Upregulation of CXCR4 by Tat at the RNA level indicated a possible transcriptional activation of the CXCR4 promoter by Tat. To test this possibility, we expressed CMV-promoter driven Tat constructs to provide a steady level of Tat. We also used a 1.2kb CXCR4-promoter construct driving a luciferase reporter, pGL-CXCR4 (Moriuchi 1997). We cotransfected these constructs into Jurkat cells and measured normalized luciferase activity. CXCR4 was expressed in uninfected cells, and empty CMV vector or CMV-FLAG had no effect on CXCR4 luciferase activity (Figure 4.9 and data not shown). Tat72 increased CXCR4 promoter activity 3-fold, while Tat101 increased luciferase 5-fold (Figure 4.9). To test the specificity of Tat for this effect, we also cotransfected two mutants of Tat, C22G, which does not interact with CyclinT1, and K41A, which is unable to transactivate the HIV LTR. Neither of these mutants was able to activate CXCR4 luciferase (Figure 4.9).

Tat-responsive promoter element

To map the elements in the CXCR4 promoter, we tested the ability of truncated CXCR4 constructs to respond to Tat. Cotransfection of CXCR4 promoter constructs from –357 or shorter did not result in activation by Tat, indicating that

the responsive element was upstream of this region (Figure 4.10). We constructed upstream truncation mutants of CXCR4 at positions –1000, -800, and –600. All of the elements were responsive to Tat, but not to Tat mutant C22G (Figure 4.10). This indicates that the Tat-responsive element is located between –600 and –357 of the CXCR4 promoter (Figure 4.11).

4B.5 Discussion

We have demonstrated that Tat72 and Tat101 activate CXCR4 gene expression, increase cell surface expression, and improve chemotaxis to the CXCR4 ligand SDF-1. Tat's effects appear to be cell-autonomous; only infected cells are susceptible to increased CXCR4 and chemotaxis. These effects are also specific to CXCR4 and inhibited completely by CXCR4 antagonist AMD3100, suggesting that no other chemokine receptors are responsible for improved migration to SDF-1 in T cells.

It is interesting that uninfected cells, however, express greater levels of CXCR4 than infected cells. The inhibition of CXCR4 expression by infection with LTR-GFP virus indicates that viral infection alone may reduce CXCR4 expression, possibly through the activation of interferons, which have been shown to downregulate CXCR4 (Shirazi 1998). This may be relevant in vivo, where CXCR4 levels can be downregulated by infection and prevent superinfection. However, subsequent upregulation of CXCR4 by Tat would allow migration of infected cells to SDF-1 gradients. CXCR4 upregulation could lead to chemotaxis of lymphocytes to lymph nodes, where large numbers of uninfected cells reside. The recruitment of infected cells to lymphoid organs may be a mechanism of viral spread. In lymph nodes, these cells can also be activated by antigen-presenting cells (APCs), resulting in greater viral gene expression and viral budding. In fact, studies have shown that SDF-1 is expressed on high endothelial venules (HEVs) in the lymph node (Okada 2002) and in lymph nodes of infected patients (Derdeyn 1999).

We also find that Tat activation of CXCR4 requires a specific element in the CXCR4 promoter. We found that this element contains a distinct 33nt region that is highly conserved among mammals (Figure 4.11B). This region (Figure 4.11A) includes a C/EBP binding site (Figure 4.11C). The element also contains a predicted binding site for IL-6 responsive element binding protein (Figure 4.11C). IL-6 is known to be induced by Tat through C/EBP (Lee 2005; El-Hage 2005; Nath 1999; Zidovetzki 1998; Ambrosino 1997), and in turn, IL-6 activates CXCR4 in astroglia (Odemis 2002). The cascade of Tat effects through activation of proinflammatory cytokines such as IL-6, IL-1, and TNFα via transcription factors like C/EBP may result in downstream effects of chemokine receptor activation, interferon responses, and activation of other genes seen in Tat microarray studies. However, because Tat effects appear to be cell-autonomous, it is more likely that Tat may directly activate CXCR4 by activation of C/EBP, NF-1, and other transcription factors in the absence of cytokine activation. Interestingly, although CXCR4 can be activated by NFkB, we did not find any putative NFkB sites in this region of the promoter. However, we found that addition of NFkB did not further increase Tat-mediated activation of CXCR4 (data not shown).

The increase in CXCR4 and chemotaxis by Tat is countered by the downregulation of CXCR4 by HIV-1 Nef. Nef has been shown to downregulate cell surface CXCR4 to a great extent post-translationally (Michel 2006, Hrecka 2005). Unlike Tat, Nef requires a physical interaction with CXCR4. These studies have also shown that in replication-competent full-length HIV, CXCR4 is downregulated (Hrecka 2005 and F.Kirchhoff, personal communication),

indicating that Nef may counteract Tat's effects on CXCR4. However, in cells such as macrophages and neuronal cells where Nef has less of an effect on cells, or in Nef-deficient viruses, Tat may play a role in cell migration and virus dissemination (Figure 4.12).

For this and other genes, Tat101 appears to produce a greater increase in protein expression than does Tat72. In cell surface expression, Tat101 induces CXCR4 earlier than Tat72, and it activates the CXCR4 promoter to a greater extent. These results may be explained by the greater stability of Tat101 (Figure 4.2B and C), or they could be a result of differences between the proteins themselves. The fact that Tat72 is only slightly less active than Tat101 lends credence to the former hypothesis. Further studies are necessary to elucidate the pathway of CXCR4 transcriptional activation by transcription factors and subtle differences between the two forms of Tat.

4B.6 Acknowledgments

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4B.7 Figure legends

Figure 4.6: Tat increases CXCR4 RNA and protein expression. (A) CXCR4 RNA, as determined by microarray. Results are the average of triplicate experiments (±SEM). (B) CXCR4 RNA, as determined by SYBR Green PCR. Ct values were normalized to GAPDH. Results are the average of triplicate experiments (±SEM). (C) Western blot of CXCR4. Boiled Laemmli lysates were separated on a 10% polyacrylamide gel and blotted with rabbit anti-CXCR4 antibody (12G5).

Figure 4.7 – Cell surface expression of CXCR4 protein. (A) Mean fluorescence intensity of CXCR4 in infected GFP+ Jurkat cells. A representative time course is shown. (B) Mean fluorescence intensity of CXCR4 in uninfected GFP- Jurkat cells in the same population as A. A representative time course is shown. (C and D) Mean fluorescence intensity of CXCR4 in infected GFP+ (C) and uninfected GFP- (D) PBMCs. A representative time course is shown. (E) Mean fluorescence intensity of CCR7 in infected GFP+ Jurkat cells.

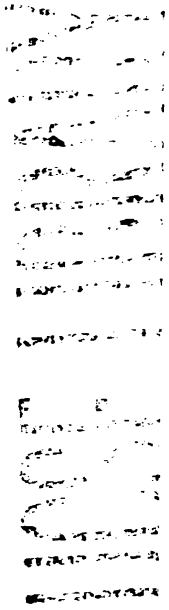
Figure 4.8 – Tat improves chemotaxis to SDF-1α. (A) Chemokinesis of uninfected cells to SDF-1α placed in different chambers of a transwell. (B and C) Chemotaxis of infected (B) and uninfected (C) Jurkat cells to increasing amounts of SDF-1α. Live migrated cells were quantified by flow cytometry and normalized to total cell number. Results are the average of quadruplicate wells (±SEM). (D and E) Chemotaxis of infected (D) and uninfected (E) PBMCs to increasing amounts of SDF-1α. Live migrated cells were quantified by flow cytometry and normalized to total cell number. Results are the average of quadruplicate wells (±SEM). (F) Tat-induced Jurkat migration to SDF-1α is CXCR4-specific. 250nM AMD3100 was added to cells in the upper chamber 30 minutes prior to chemotaxis experiments. 250nM of RANTES or CXCL21 were added to the lower chamber of transwells instead of SDF-1α.

Figure 4.9 – Tat activates the CXCR4 promoter. CMV-Tat72 and CMV-Tat101 were electroporated into Jurkat cells along with pGL-CXCR4. Luciferase activity was measured after 24 hours and normalized to cellular protein content. Mutants K41A and C22G are mutants of Tat101. Results are the average of triplicate experiments (±SEM).

Figure 4.10 – The Tat-responsive CXCR4 promoter element is between -600 and -357. (A) CXCR4 promoter truncation mutants (indicated) were elecroporated along with CMV-empty vector, CMV-Tat72, CMV-Tat101, or CMV-Tat101-C22G. Luciferase values were measured after 24 hours and normalized to cellular protein content. Results are the average of triplicate experiments (±SEM).

Figure 4.11 – A conserved region in the Tat-responsive element of CXCR4. (A) Sequence of -600 to -357. The conserved region is shown in red. (B) Conservation plot among mammalian species of the Tat-responsive element. (C) Transcription factors with predicted binding sites within the Tat-responsive element. Proteins with sites in the conserved region are shown in red.

Model of in vivo CXCR4 upregulation. Infected cells expressing Tat and having upregulated CXCR4 traffic in response to SDF-1 chemokine gradients to lymphoid organs, where many target cells reside. This may provide a means of virus dissemination and access to antigen-presenting cells.

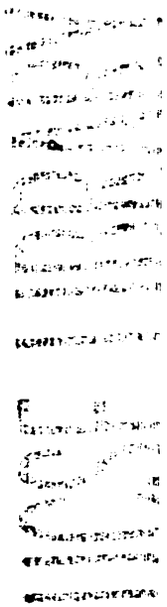


Chapter 5: Tat and other genes

5A. Tat and Tsg101

5A.1 Abstract

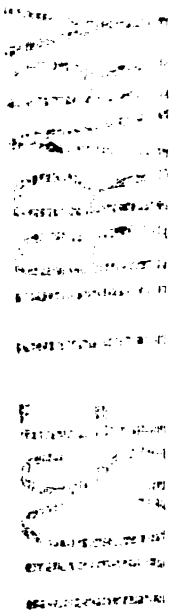
The HIV-1 Tat protein potently modulates the expression of a number of cellular genes. We asked whether two alternative splice forms of Tat, Tat72 and Tat101, could activate the expression of cellular genes. Among the genes differentially regulated by the two forms of Tat is the vesicular budding protein Tsg101, which is also necessary for HIV-1 release. We found that Tsg101 was uniquely upregulated by Tat101. The increase in Tsg101 RNA results in increased budding and viral release. Our results suggest a novel link between the early and late stages of HIV infection.



5A.2 Introduction

We recently determined a number of genes upregulated by Tat72 and Tat101. Among the genes upregulated by Tat101 but not Tat72 is Tsg101. Tsg101 is a member of the endosomal sorting complex required for transport (ESCRT) family of proteins. Tsg101 (also known as Vps23p), STAM1/2, Hrs, and Vps28 comprise the ESCRT-I complex (reviewed in Slagsvgold 2006). Proteins shown to associate with the RNA polymerase II elongation factor ELL were named the ELL associating proteins (EAP) and formed ESCRT-II (Kamura 2001). ESCRT-III consists of the charged multivesicular body proteins (CHMP) (Howard 2001). These three protein complexes are involved in vesicular sorting of proteins from the plasma membrane to the late endosome and the multivesicular body.

Tsg101 was originally identified as a tumor suppressor. Deletion of Tsg101 in mouse fibroblasts resulted in tumor formation in mice (Li 1996). Subsequent studies revealed that the viruses, including HIV, cannot exit the plasma membrane in the absence of Tsg101 (Martin-Serrano 2001). This was shown to be true for several other ESCRT components (Strack 2003; Slagsvgold 2006). Viruses as diverse as Ebola, Marburg, EIAV, HIV, and others require components of the ESCRT machinery to bud from the cell (Slagsvgold 2006). ESCRT-I components, including Tsg101, tether the Gag or structural protein of these viruses to the plasma membrane, and ESCRT-II components bridge ESCRT-I and ESCRT-III. ESCRT-III contains an ATPase (Vps4), which in turn is thought to allow release of the complex from the membrane. Furthermore, an

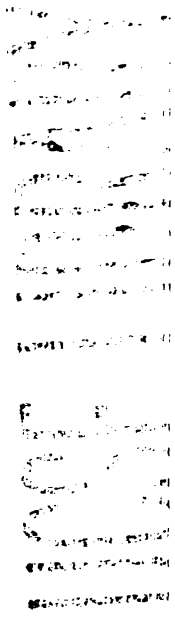


adapter, AIP1/Alix connects some Gag proteins to the CHMP/ESCRT-III complex (Figure 5.1) (Slagsvgold 2006).

Studies have shown that HIV viral particles form aberrantly in the absence of either Tsg101 or AIP1, and that the particles that are produced are not infectious (Martin-Serrano 2001; Strack 2003). Furthermore, these complexes are thought to be adapted by the virus at the plasma membrane, away from the multivesicular body.

The ESCRT complexes are also thought to play a role in the release of small 100nm particles called exosomes (de Gassart 2004). These particles are released by the maturation of the multivesicular body and its fusion with the plasma membrane. The release of these particles is thought to play a role in the transfer of antigens from antigen presenting cells to lymphocytes, as has been shown with the MHC class II molecule (Raposo 1996). One hypothesis is that viruses have used the exosomal pathway to bud into multivesicular bodies and be released into the extracellular medium directly, rather than budding at the plasma membrane. This has been shown to be the case in macrophages, but not in lymphocytes. This "Trojan horse" hypothesis surmises that viral evolution proceeded from an exosomal origin (de Gassart 2004).

ESCRT components are primarily thought to be involved in budding, with a poorly described role in transcription. Therefore, we asked whether Tat-mediated upregulation of Tsg101 would lead to an increase in Tsg101 protein, and whether this increase indicated improved viral budding.



5A.3 Materials and Methods

Plasmids

Tat vectors were prepared as described in Chapter 2D. pMDLgRRE and pRev were obtained from D.Trono. CMV-Tsg101 was a gift of Wes Sundquist (University of Utah, Salt Lake City, UT). LTR-luciferase constructs were a gift from Warner Greene.

Virus stocks and infections

Viruses were prepared and infected as described in Chapters 2D and 4A.

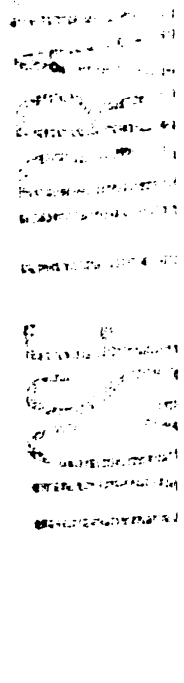
Tsg101 RNA quantification

RNA was prepared as described in Chapter 2D. Tsg101 RNA was detected by SYBR Green PCR using HotSYBR (MCLab) and with the primers Tsg101 (NM_006292); Forward: 462 TTATCTACATGAATGGAAACACCCACAG, Reverse: 798 GTCCTCGCTGATTGTGCCATCCCTACTG. Human GAPDH (BC001601); Forward: 344 AGTCCACTGGCGTCTTCACC, Reverse: 464 TGGTTCACACCCATGACGAA.

Viral budding assay

Virus budding was determined by transfection of 293T cells using Fugene reagent (Roche). Transfected cells were plated at a density of 0.05 million in 24-well plates (Falcon). At the indicated time points, supernatants and PBS-washed cells from the same well were harvested and lysed. p24-Gag was measured by ELISA (Alliance).

Exosome isolation



Exosomes were isolated by serial centrifugation as described (Fritzsching 2002). Exosomes were isolated from 293T cell media and protein was normalized to cell number.

Western blotting

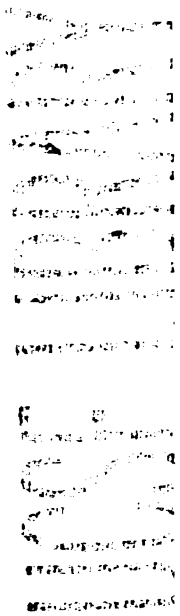
Western blots were performed as described in Chapter 2D. Mouse anti-Tsg101 antibody was obtained from eBiosciences and used at 1:1000 dilution after blotting with 1% milk (Biorad). Rabbit anti-AIP1 antiserum was a gift of Dr. Wes Sundquist (University of Utah, Salt Lake City, UT).

5A.4 Results

We infected Jurkat T cells as above and measured Tsg101 RNA in the presence and absence of Tat using SYBR-Green real-time PCR. As shown in Figure 5.2, only Tat101 increased Tsg101 RNA levels compared to uninfected or LTR-GFP infected samples. Tat72 also had no significant effect on Tsg101 RNA levels. The amount of increase was greater with Tat101 than with Tat72, reaching a five-fold increase.

We then went on to measure protein levels of Tsg101 in Jurkat cells. As shown in Figure 5.3, we saw an increase in Tsg101 protein by the expression of Tat101, but not Tat72. However, this protein level increase was minimal and was seen only at 48 hours after infection. To determine if the excess protein was being expelled from the cells by the lysosomal pathway in exosomes, we pelleted exosomes by centrifugation at increasing speeds and measured Tsg101 in various fractions in infected or uninfected samples. As shown in Figure 5.4, no increase was seen in Tsg101 protein in exosomes.

We speculated that a small increase in Tsg101 that was not detectable by Western blotting might nevertheless result in an enhancement of viral budding. Therefore, we designed an experiment to express viral proteins under the control of the CMV promoter using the pMDLgRRE vector (provided by D.Trono). This vector contains the HIV protein Gag-Pol in the absence of viral accessory proteins, as well as a Rev-responsive element (RRE), which is necessary for Rev-dependent RNA export from the nucleus and protein expression. CMV-promoter-driven Rev was expressed independently, and we also expressed



CMV-driven constructs containing either empty vector, Tat72, or Tat101. Cotransfection of these constructs in 293T cells resulted in virus. We were able to measure viral production inside 293T cells as a measure of total or intracellular virus, and compare it to extracellular viral release by measuring Gag-p24 by ELISA (Alliance/NEN Life sciences), as shown in Figure 5.5. Individual wells were harvested (intracellular) or washed (extracellular) at each time point to measure only new p24 production and not cumulative virus.

We found that production of viral p24 peaked at 36-48 hours, and that viral production was similar with empty vector, Tat72, or Tat101 intracellularly (Figure 5.5). However, a great increase in viral release was seen after 48 hours with Tat101, indicating greater viral release in the presence of Tat101 (Figure 5.5). This effect was not seen with Tat72 (Figure 5.5). Furthermore, neither Tat72 nor Tat101 increased the activity of the CMV promoter in this system (data not shown). On the contrary, Tsg101 reduced LTR activity alone (data not shown).

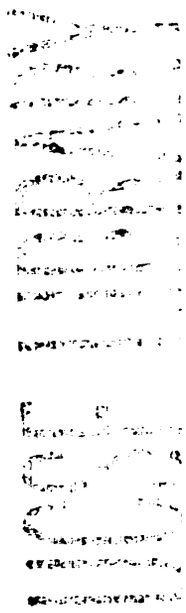
We also found an increase in AIP1 in our microarray screen. AIP1 is also necessary for HIV budding. Investigation of AIP1 protein levels indicated a possible increase in protein with Tat72, but not Tat101 (Figure 5.6).

5A.5 Discussion

We conclude that the increase in viral budding that appears in cells expressing Tat101 but not Tat72 is likely due to the increase in Tsg101 induced to a greater extent by Tat101. The increase is not likely due to increased expression of AIP1, which is only activated by Tat72.

The enhancement of budding by Tat is a novel mechanism of control between early transcriptional events in the HIV viral cycle and late events such as budding. The results indicate that viral proteins may prepare cellular factors for later stages of the viral cycle by initiating a program of transcription that is more suitable for viral assembly and release.

Tsg101, however, does appear to have a specific effect on the HIV-1 LTR. Unlike the CMV promoter, the HIV-1 LTR is strongly suppressed by Tsg101, even in the absence of Tat. This may indicate that Tsg101 regulates transcription as a form of feedback. This may be advantageous for the virus, in that as later stages of the viral cycle take place, and Tat upregulates Tsg101, the HIV LTR is suppressed to facilitate budding and the shutdown of transcription. Further study is necessary to determine whether LTR suppression is a specific process.



5A.6 Acknowledgments

We thank Wes Sundquist for reagents and advice. Björn Schwer provided technical assistance with exosome isolation. We are grateful to Melanie Ott, Katrin Kaehlcke, Angelika Pedal, and Vanessa Soros for discussions and advice.

5A.7 Figure Legends

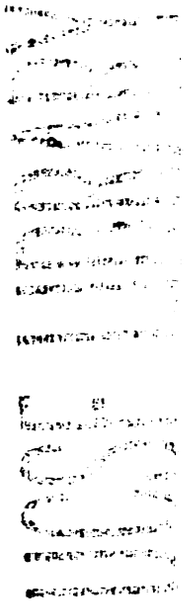
Figure 5.1 – The ESCRT complexes. ESCRT-I, containing Tsg101, is colored in light pink. ESCRT-II is colored in orange. ESCRT-III and AIP-1 are colored red. Green, HIV-1 Gag. Blue – plasma membrane.

Figure 5.2 – Tat101 increases Tsg101 RNA expression. RNA expression in infected Jurkat cells after 48 hours was determined by SYBR Green PCR. Normalized Ct values are shown. Results are the average of triplicate experiments (±SEM).

Figure 5.3 – Tsg101 protein levels are slightly increased in Jurkat cells. Western blot of Jurkat cells at 48 and 72 hours after infection. Laemmli lysates were analyzed on 10% polyacrylamide gels and blotted with anti-Tsg101 or anti-tubulin.

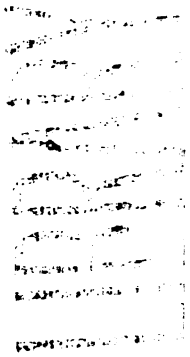
Figure 5.4 – Tsg101 protein levels are not increased by Tat in exosomes. After serial centrifugation, cell pellets and subsequent pellets were analyzed by 10% polyacrylamide gel electrophoresis and blotted with anti-Tsg101. Shown below is the Coomassie stain of the same gel as a loading control. P4, P5 – exosome containing pellets. P1 – cell pellet.

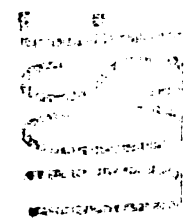
Figure 5.5 – Tat101 increases budding in the viral budding assay. (top) Viral budding assay. 293T cells were transfected, and intracellular (cell pellet) or



extracellular (supernatant) virus was quantified by ELISA. (bottom, left) Intracellular time course of viral p24 production. At the indicated time points, cells were washed with PBS, lysed, and analyzed for p24 by ELISA. p24 was normalized to cellular protein content. Results are the average of triplicate experiments (±SEM) (bottom, right) Extracellular time course of viral p24 production. At the indicated time points, 200ul aliquots of supernatant were removed and analyzed for p24 by ELISA. p24 was normalized to cellular protein content in the cell pellet. Results are the average of triplicate experiments (±SEM).

Figure 5.6 – Tat101 does not increase AIP1. Western blots of Laemmli-boiled Jurkats were performed 48 hours after infection with viruses. AIP1 antiserum was used at 1:1000 dilution.





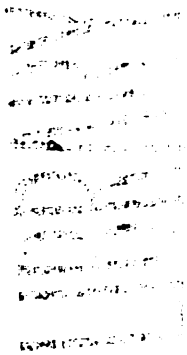
Chapter 6: Conclusions

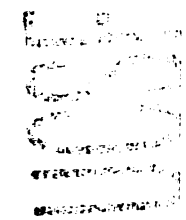
HIV-1 pathogenesis involves a complex interplay of viral proteins and host factors. The viral proteins Tat, Nef, and Env and the host proteins CD4, CXCR4, and Tsg101 play crucial roles in viral replication, viral dissemination, and disease progression.

Microarray studies provide a wealth of information that allows a global view of cellular gene expression. We find that the two alternatively spliced forms of Tat, Tat72 and Tat101, activate a distinct set of cellular genes, with Tat101 inducing the expression of a large number of unique genes. Tat72 activated a small set of genes which largely overlapped with Tat101. We observed little downregulation of specific genes. The genes modulated by Tat are involved in transcription, homeostasis, signaling, and inflammation, indicating that downstream Tat effects are not limited to transactivation. One common thread may be the modulation of Tat activity and Tat-associated transcription factors by deacetylases such as SIRT1. These studies may explain the variety of Tat-induced phenotypes reported.

Among the unique genes activated by Tat101 uniquely is Tsg101, a component of the ESCRT complexes that are essential for virus release. Expression of Tat101 improves viral egress, although whether this improvement is achieved through Tsg101 is unknown. The involvement of Tat in viral budding suggests a novel role for a transcriptional activator in modulating late steps in the viral life cycle.

Both forms of Tat also activate CXCR4, an HIV-1 coreceptor associated with later stages of infection. Tat72 and Tat101 act through an upstream transcriptional element and may activate a transcription factor that in turn induces CXCR4 expression. The change in CXCR4 expression may result in a change in cellular



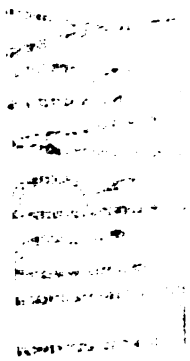


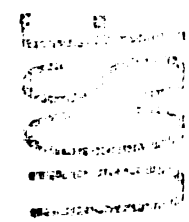
migration to SDF-1, and the subsequent relocalization of infected cells to sites of inflammation for further viral dissemination.

Two-exon Tat101 appears to have multiple novel effects, including the activation of Tsg101, the increased induction of CXCR4, and the activation of many other cellular genes. The second exon of Tat may have distinct interaction partners that lead to the activation of additional genes. The integrin-binding domain in the second exon may also play a role in cell signaling and activation of inflammatory responses.

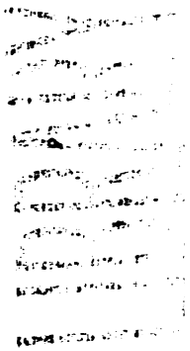
HIV-1 Nef facilitates rapid replication and increased viral infectivity, but it is not necessary for infection, as changes in Env can lead to pathogenic Nef-deficient viruses, which use CXCR4. Because Nef itself downregulates CXCR4, the absence of Nef may promote CXCR4 tropism by increasing the availability of cell surface CXCR4. This suggests that the coordinated action of Nef and envelope may lead to pathogenic viruses even when viral replication is poor.

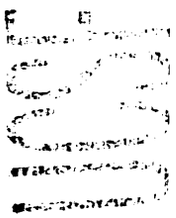
Env assists in the assembly and exit of viral particles and interacts through Gag with the ESCRT machinery. However, in entry, envelope is absolutely necessary, and when engaged with CXCR4, allows entry into not only T cells but also macrophages, when modified accordingly – by glycosylation and containing an appropriate envelope charge. Charged regions interact with CXCR4, as indicated by the binding of envelope, SDF-1, and AMD3100. SDF-1 itself has multiple roles in the HIV-1 life cycle to counteract viral effects. SDF-1 binds CXCR4 to induce signaling, to block HIV-1 entry, and to promote chemotaxis. Inside the cell, SDF-1γ can inhibit Tat-mediated transactivation.





Thus, the viral proteins Tat, Nef, and Env appear to interact at multiple levels with CXCR4, Tsg101, and a number of other host proteins at each stage of the life cycle. The interaction of these proteins demonstrates the importance of each aspect of viral pathogenesis, and highlights the need to study viral molecular mechanisms in order to create more effective therapeutic strategies.





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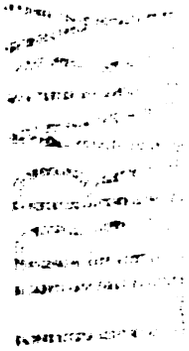

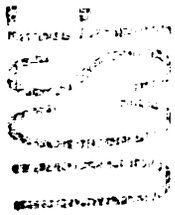

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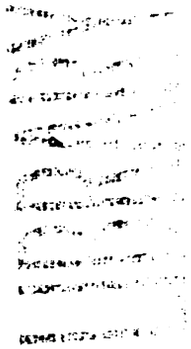

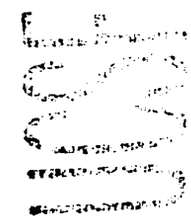

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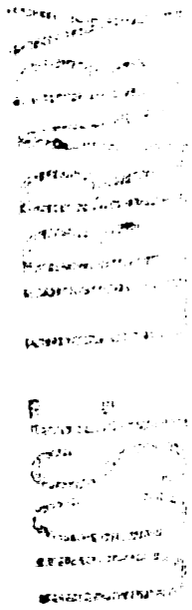

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Chapter 8: Notes and Appendices

8A. Tables

Table 1.1 – Two major viral phenotypes

Viral coreceptor	CCR5	CXCR4
Prevalence	100%	50-90%
Time of emergence	Early	Late
T cell infection	5-10%	Most T cells
T cell type	Activated, memory	Naive
Macrophage infection	Most isolates	Only some isolates
Transmission rate	High	Low
Cytopathicity	Few isolates	Most isolates (with syncytia)

[a] The percentage of p24-positive macrophages was determined from images acquired by confocal double-immunofluorescence microscopy.

Table 3.1 – V1-V3 sequences, glycosylation sites, and V3 charge of R5, R5X4, X4 lab-adapted, and X4 dualtropic viruses.

Virus	V3 sequence[a]	Coreceptor[b]	Charge[c]	MDM[d]	HLH Mϕ[e]
	g13 g14 g15 g16 g17				
NL4-3	QLNTSVKINCTRPNNNTRKSIRIQRGPGRAFVTIGKI-GNMRQAHCNISRAKWNNT	X4	+9	No	No
HXB2	QLNTSVKINCTRPNNNTRKRIRIQRGPGRAFVTIGKI-GNMRQAHCNISRAKWNNT	X4	+10	No	-
UG024	KLNKSVVINCTRPYNNTRQR-TPI-GLGQALYTTRRI-GDIRRAHCNISRAAWKKT	X4	+5	Yes	-
UG021	KLNKSVPINCTRPYDKKVSYR-TPI--GVGRASYTTRIKGDIRQAHCNISGKKWNKT	X4	+5	Yes	Yes
J130	QLNKSVQINCTRPNNTLRKRVTIH-GPGRAFYT-GKIKGTIRQAHCNLSRAQWNDT	X4	+5	Yes	Yes
J34	QLNTSVKINCTRPNNTIRR-IHI-GPGRAFYT-GKTTGNIRKAYCNISRAKWNDT	X4	+6	Yes	-
TYBE	QLNKSVQINCTRVSKNRQKRIKI-GPGRAFVATGDI-GDIRKAHCNISKAKWNKA	X4	+7	Yes	-
7/86	QLKRSVKINCIRPNNNTRKG-IHI-GPGRAWYATGKIVGDIRQAYCNISKTKWNNT	R5X4	+4	Yes	Yes
YU2	QLNKSVVINCTRPNNNTRKS-IHI-GPGRALYTTGKIIGDIRQAHCNLSKTQWENT	R5	+3	Yes	-
Ba-L	QLNKSVKINCTRPNNNTRKS-IHI-GPGRAFYTTGKIIGDIRQAHCNLSRAKWNDT	R5	+2	Yes	-

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[a]The sequence from C2 to C3 of gp120 is shown relative to NL4-3 sequence positions. *N*-glycosylation sites are indicated and highlighted in NL4-3. Underlined sequences indicate loss of potential *N*-glycosylation sites.

[b]Coreceptor tropism as determined by HeLa-H11 infection (see also Fig. 1) and as published.

[c]V3 loop charge calculated by the number of K or R residues minus the number of D or E residues ((K+R)-(D+E)).

[d]Productive infection of MDM (Yi 2005; Scarlatti 1997).

[e]Productive infection of tissue-resident macrophages in human lymphoid histocultures (this study) (-, not determined).

Table 3.2 - Quantification of HIV-1 p24-positive macrophages in tonsil histocultures from two donors.

Virus	Donor	$p24^+$ Macrophages [a]
NL4-3 (TCLA-X4)	T189	22/644 (3.4%)
7/86 (R5X4)	T189	46/376 (12.2%)
	T190	43/344 (12.5%)
UG021 (X4)	T189	51/542 (9.4%)
	T190	47/388 (12.1%)
J130 (X4)	T189	62/628 (9.9%)

[a] The percentage of p24-positive macrophages was determined from images acquired by confocal double-immunofluorescence microscopy.

Table 4.1 – Genes upregulated by GFP, Tat72, or Tat101 on microarray

Tat72 [a]	Tat101 [a]	Method of verification [b]
CXCR4 (2)	CXCR4 (2.5)	R, F, L WB
MAPK3 (2.2)	MAPK3 (2)	–
ASMTL (2.1)	ASMTL (2.1)	–
	Tsg101 (2.5)	R, WB
	BMP8 (2)	–
	CAMK1 (2)	
	Thymosin beta	

[a] Values in parentheses are fold changes, determined by statistical analysis with Acuity software.

[b] R: RNA quantification (SYBR green); W: western blot; F: flow cytometry; L: luciferase assay.

Table 4.2 – Genes downregulated by GFP, Tat72, or Tat101 on microarray.

Tat72 [a]	Tat101 [a]	Verified by
AP2B (2)	TIEG (2.1)	–
CD59 (2.5)	PCNA (2.6)	–
ASMTL (2.1)	Vimentin (2.5)	–
	PGK1 (2.4)	–
	RSP3 (2)	–
	Poly A BP (2)	–
	Thymosin beta	

[a] Values in parentheses are fold changes, determined by statistical analysis with Acuity software.

8B. Figures

Figure 1.1 – HIV life cycle

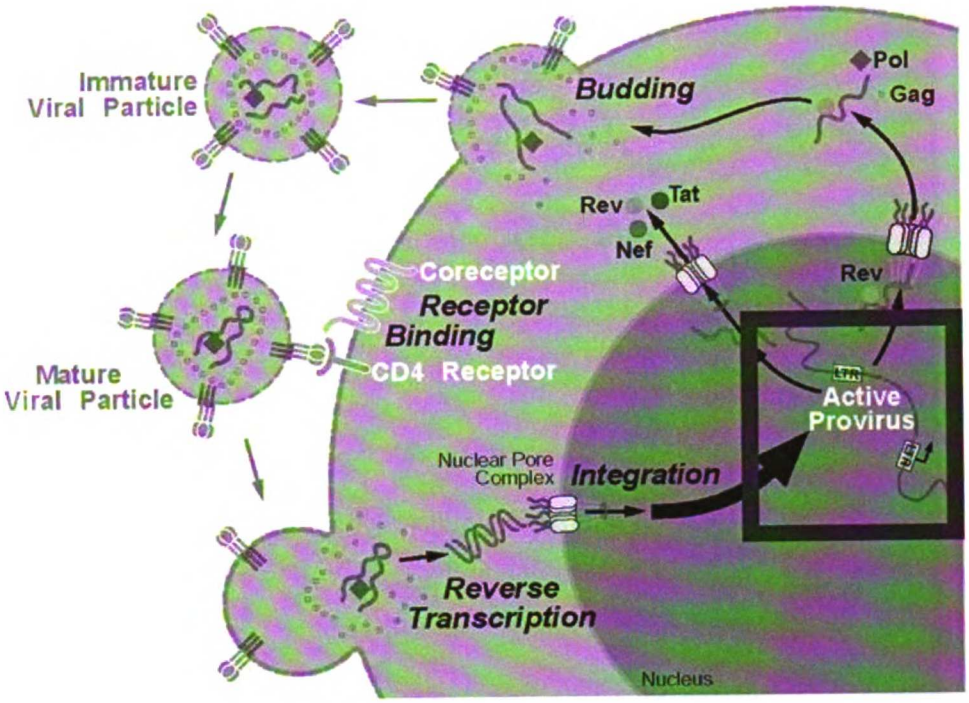


Figure 1.2 – HIV genome

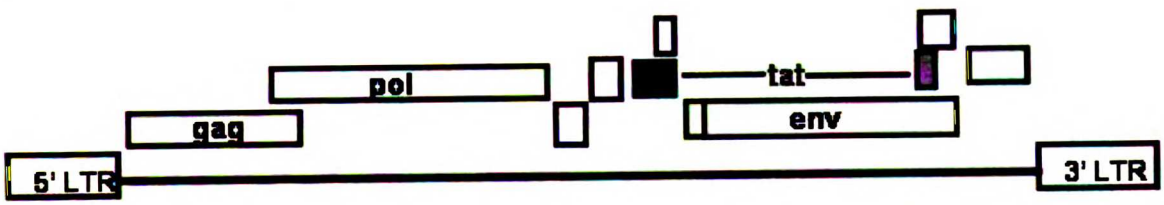
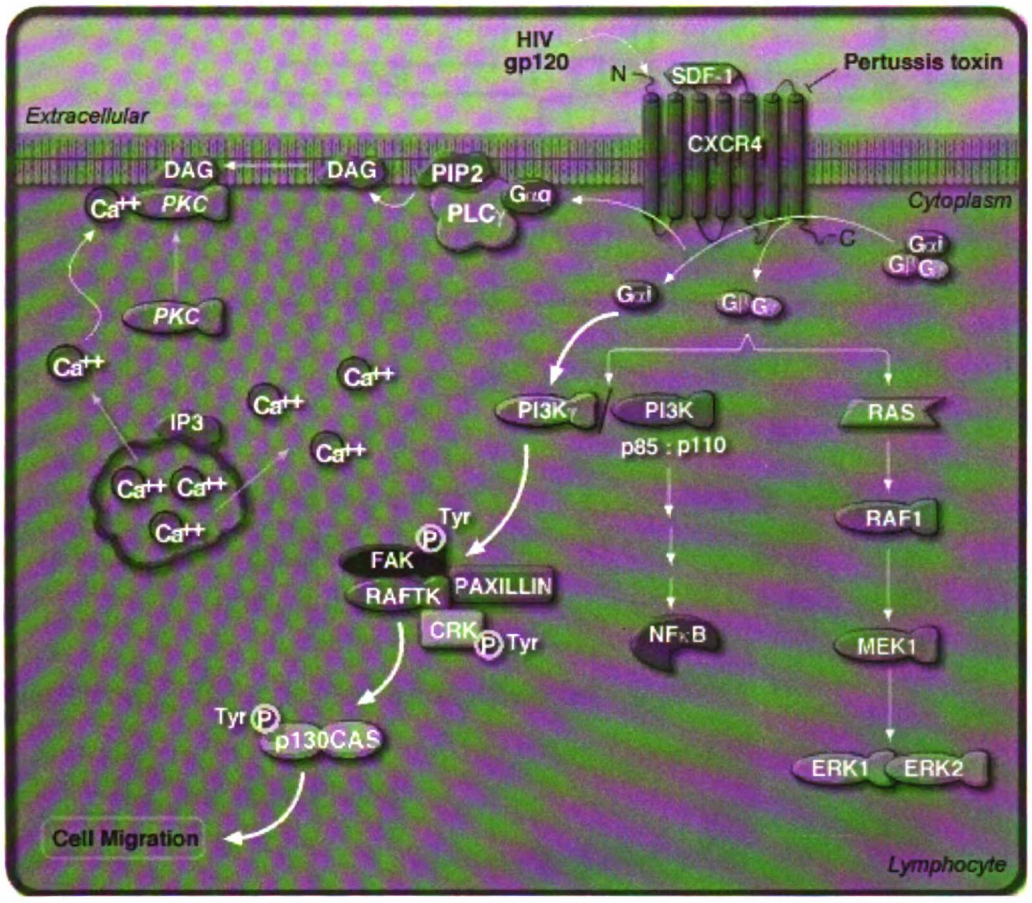


Figure 1.3 – CXCR4 signaling pathways



From http://www.biocarta.com/pathfiles/cxcr4Pathway.gif

Figure 1.4 – CXCR4 promoter

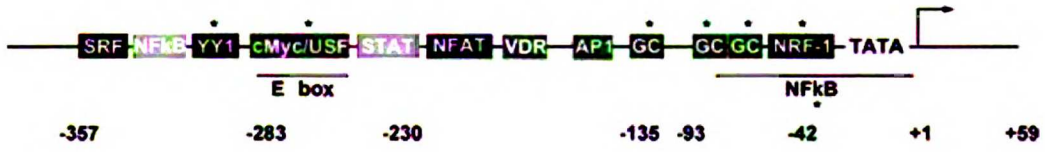


Figure 1.5 – Tat72 and Tat101

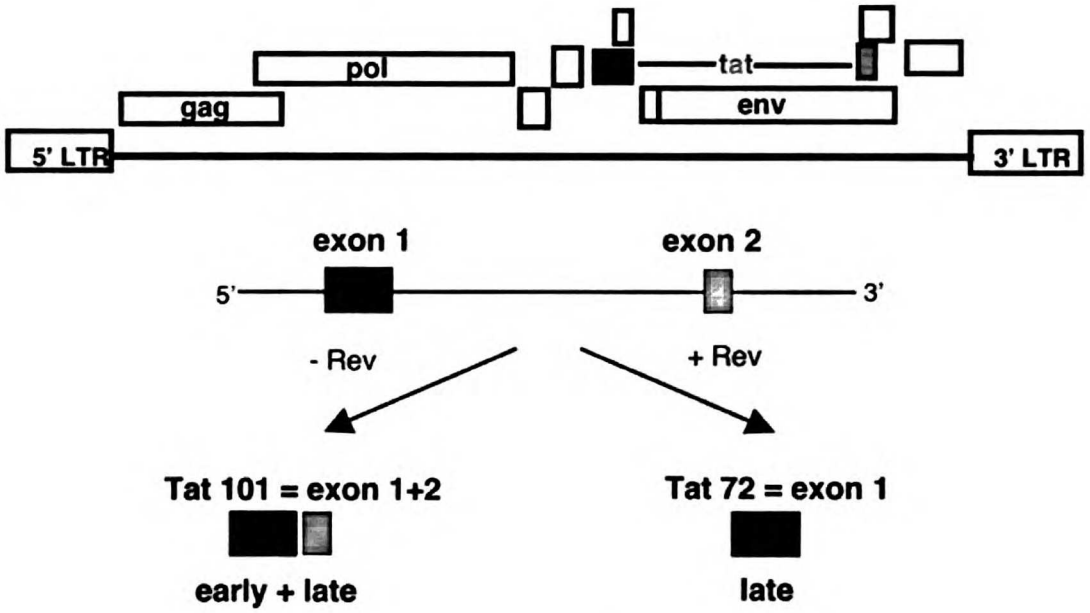


Figure 1.6 – Tat-mediated transactivation

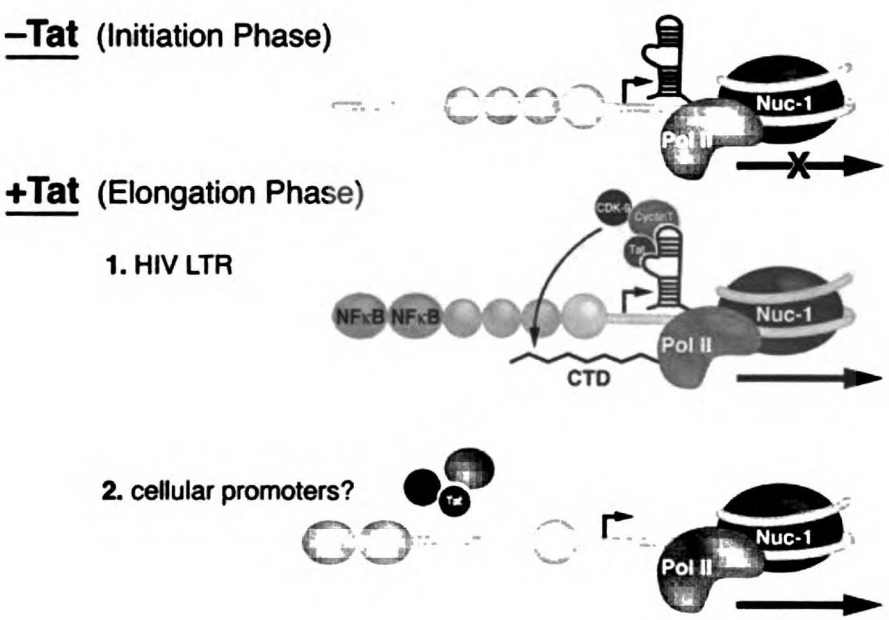


Figure 1.7 – Two steps of transactivation

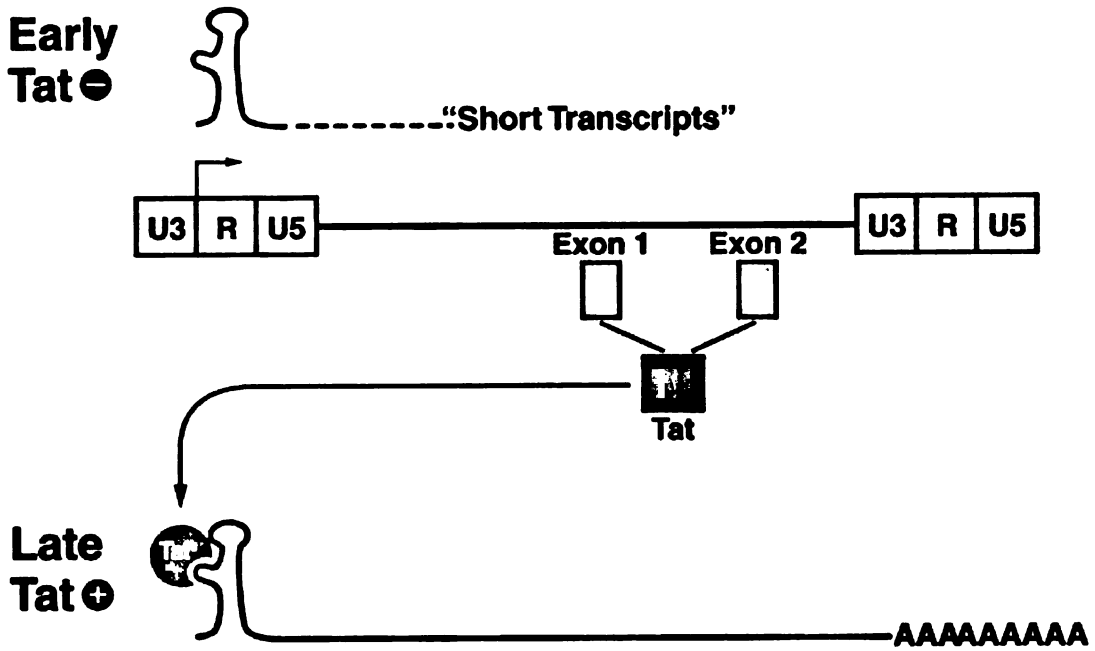


Figure 1.8 - Tat domains

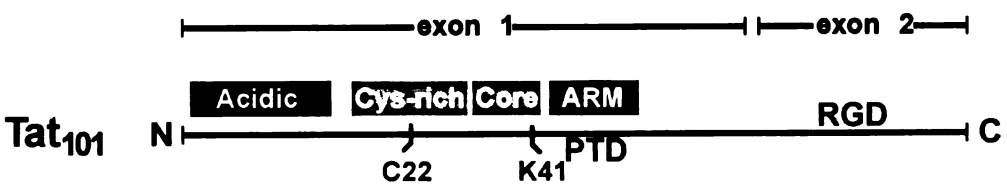


Figure 2.1 – The D36/99 isolate depletes $CD4^+$ T-cells more efficiently and replicates faster than D36/95 in *ex vivo* human lymphoid cell cultures.

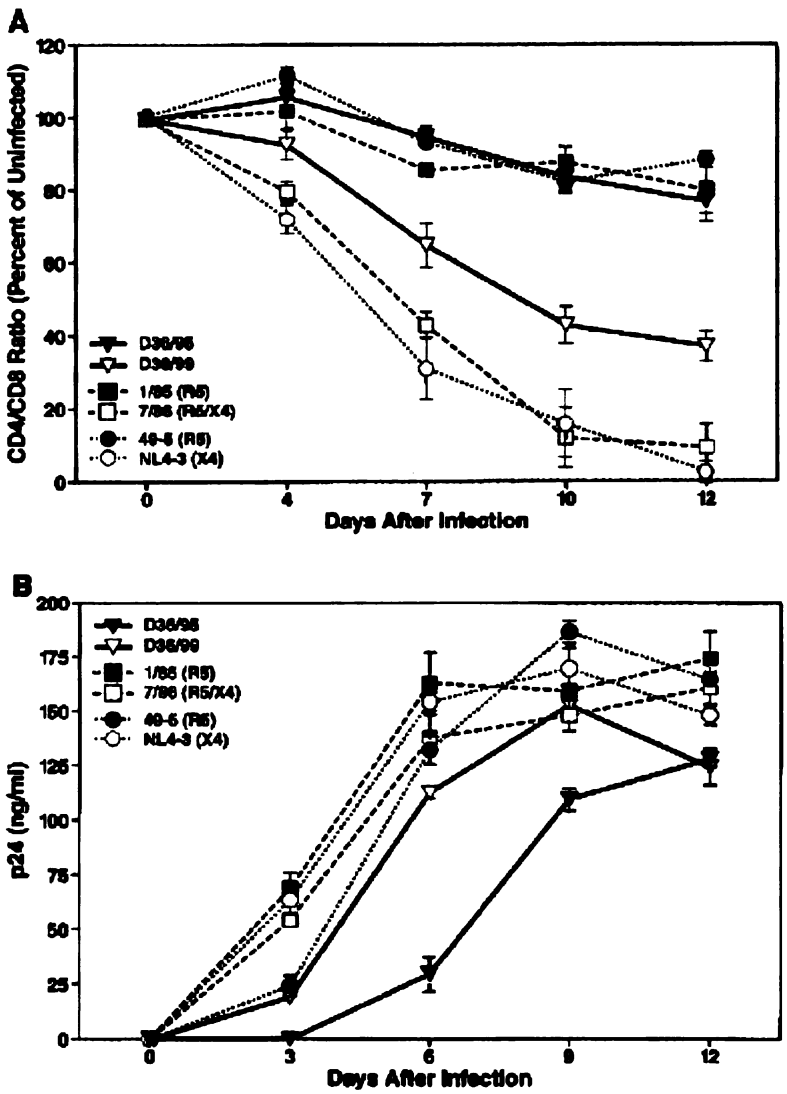


Figure 2.2 - D36/99 induces high levels of apoptosis among $CD4^+$ T-cells in human lymphoid cell cultures.

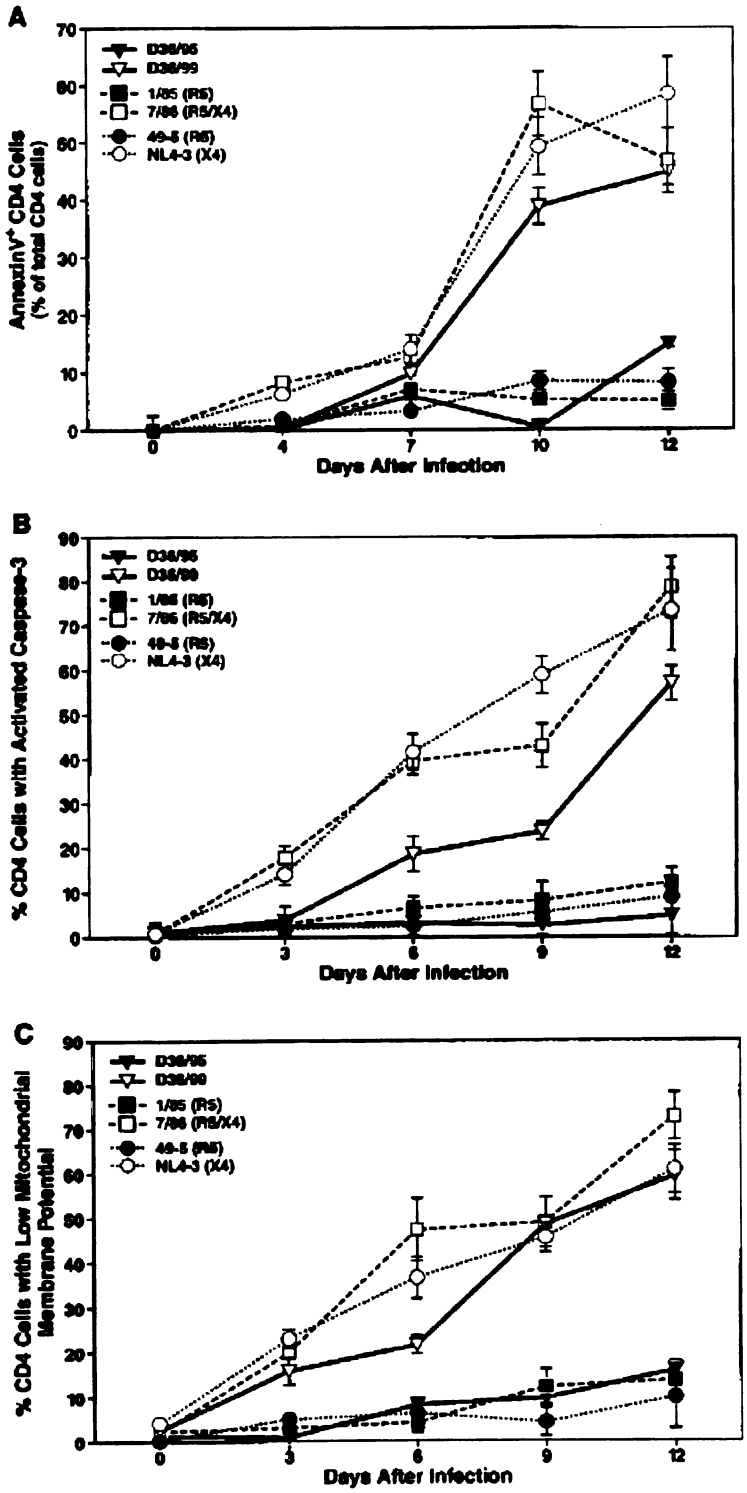


Figure 2.3 - D36/99 induces depletion and apoptosis of both $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cells in human *ex vivo* lymphoid cell cultures.

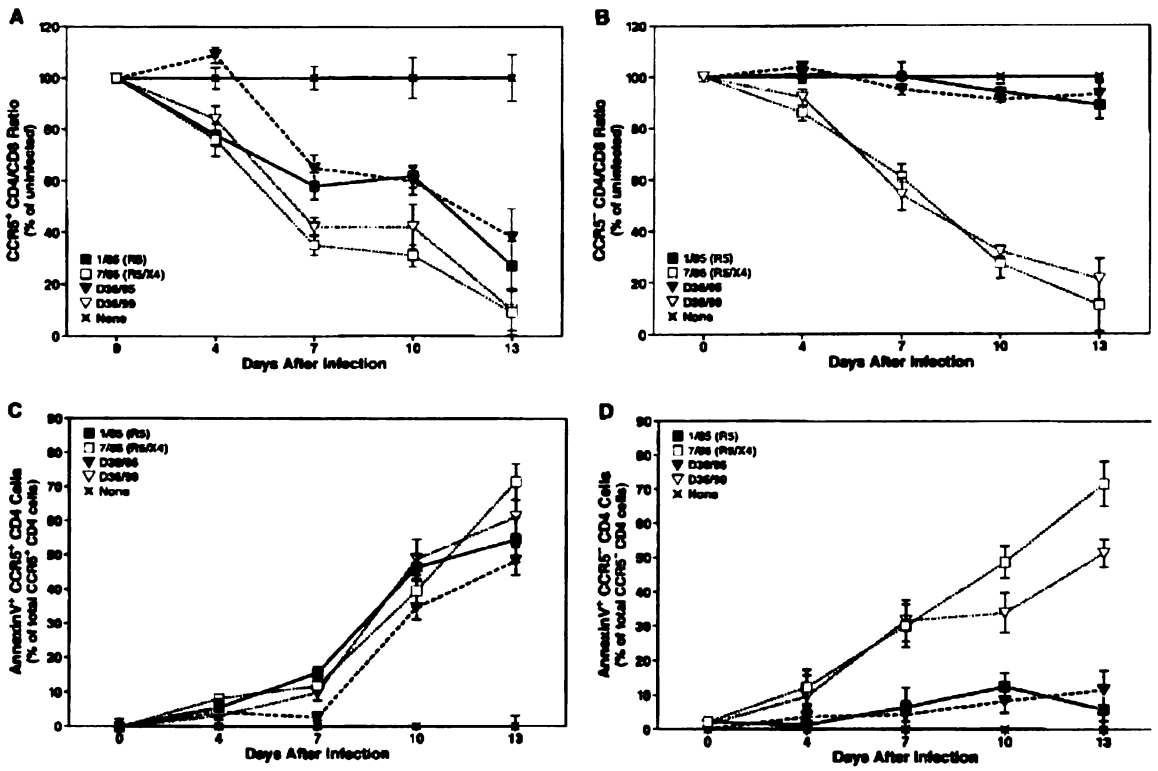


Figure 2.4 - D36/99 can use CXCR4 as a coreceptor more efficiently than D36/95.

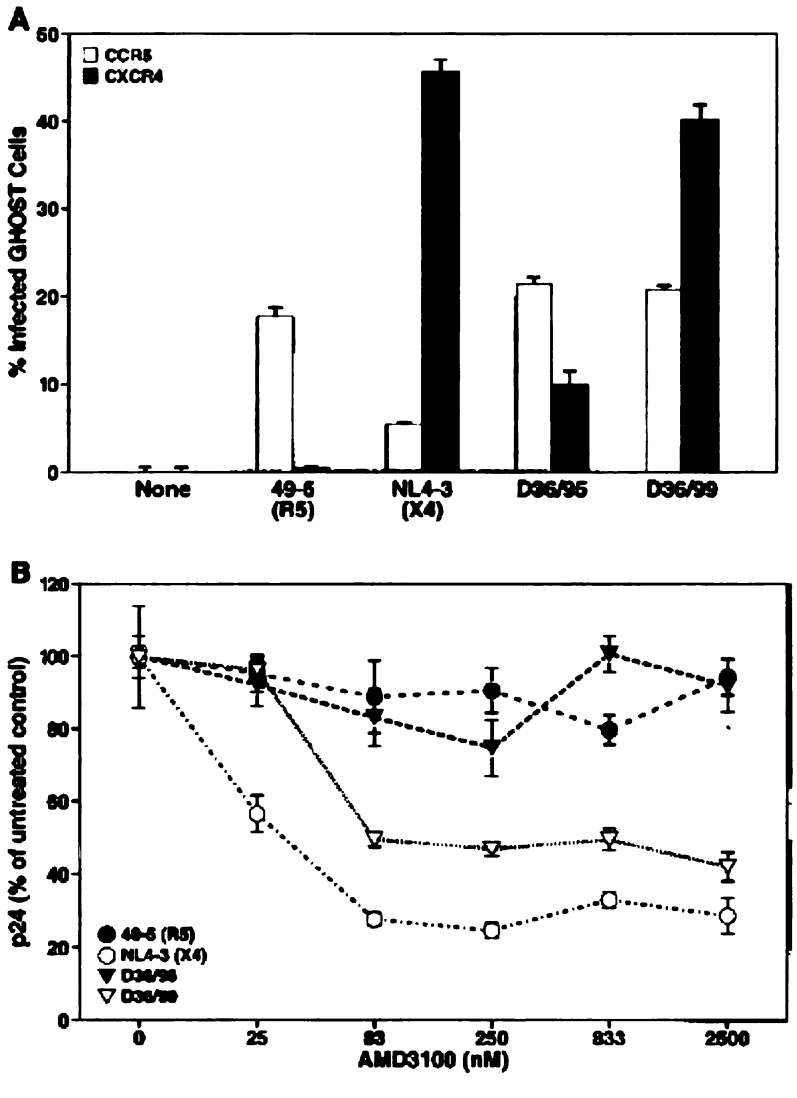


Figure 2.5 - D36/99 depletes and induces apoptosis in both the CCR5$^+$ and CCR5$^-$ subsets of CD4$^+$ T-cells in human *ex vivo* lymphoid cultures.

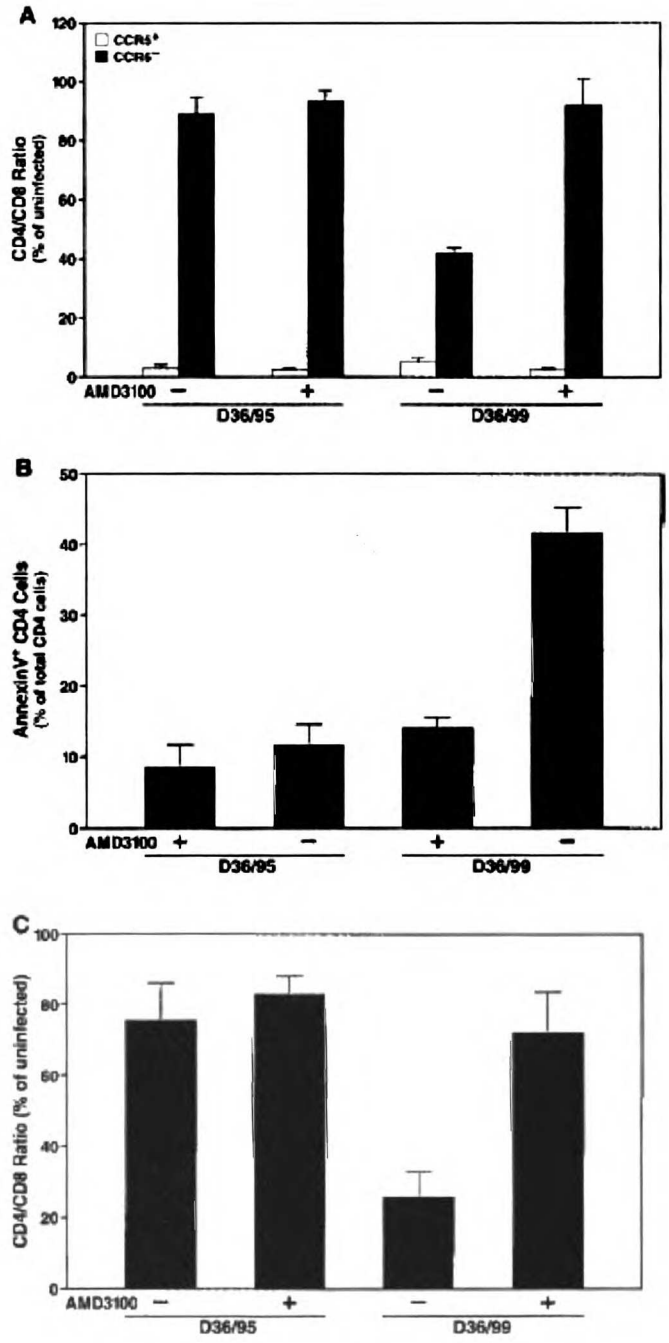


Figure 2.6 – Human CD4 and CXCR4 are expressed on rat lymphocytes and macrophages.

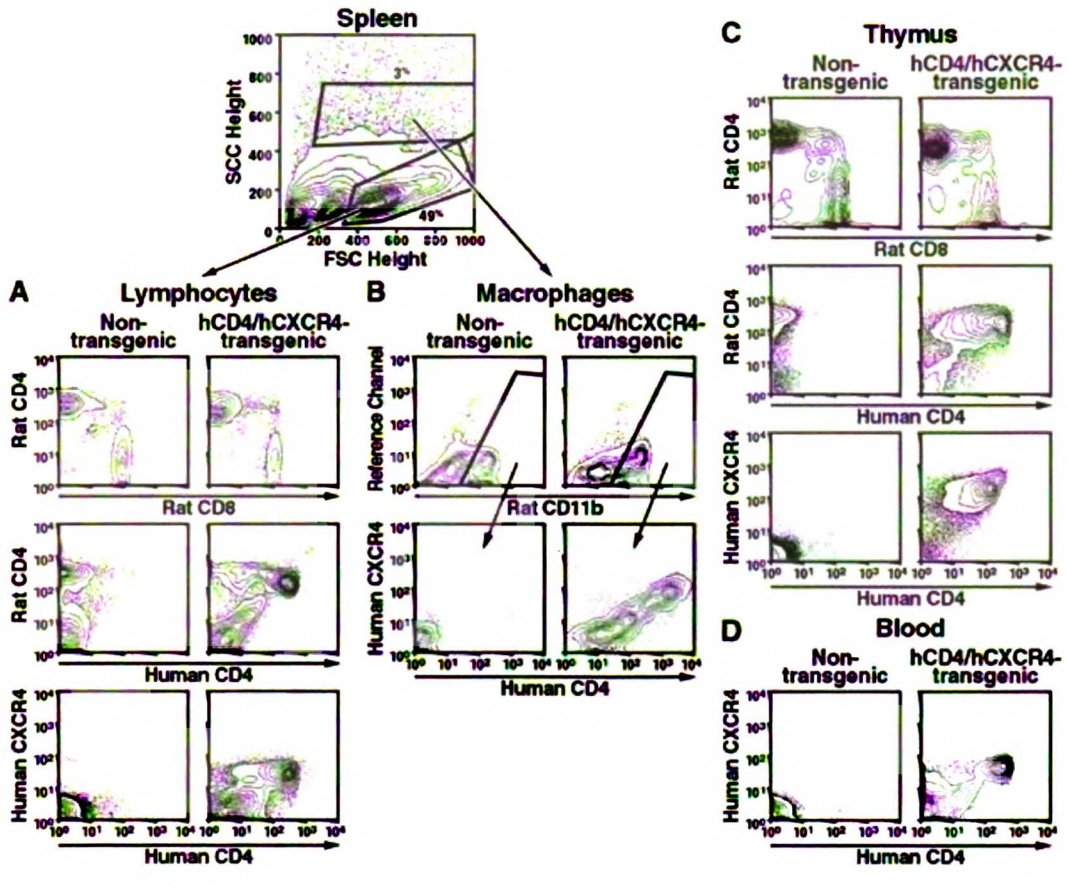
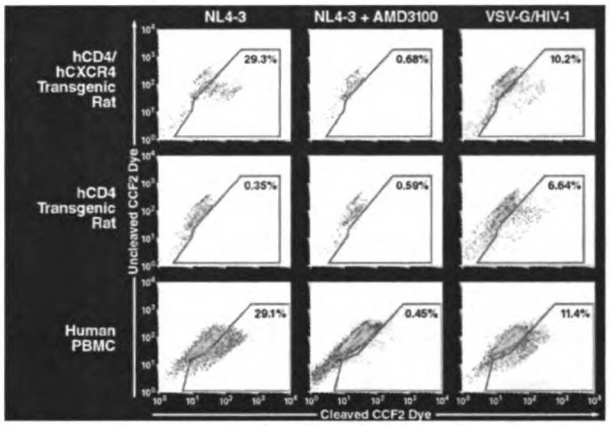


Figure 2.7 – Entry and early gene expression in transgenic rat lymphocytes.

A.



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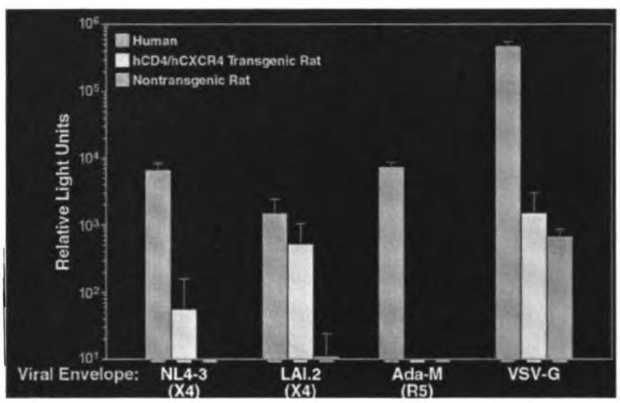


C.

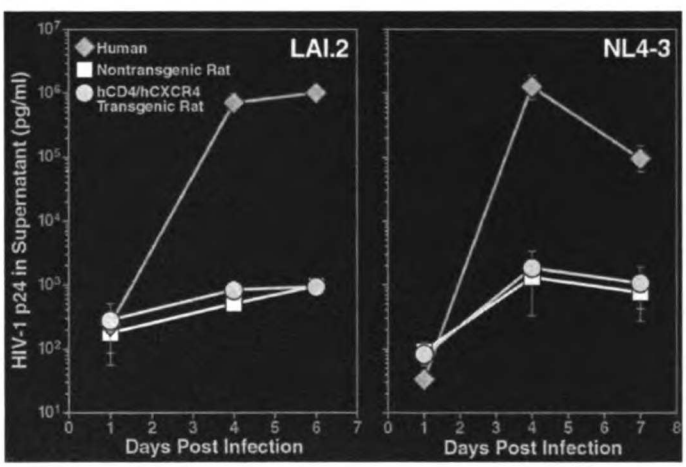


Figure 2.8 – Limited entry and early gene expression in transgenic rat macrophages.

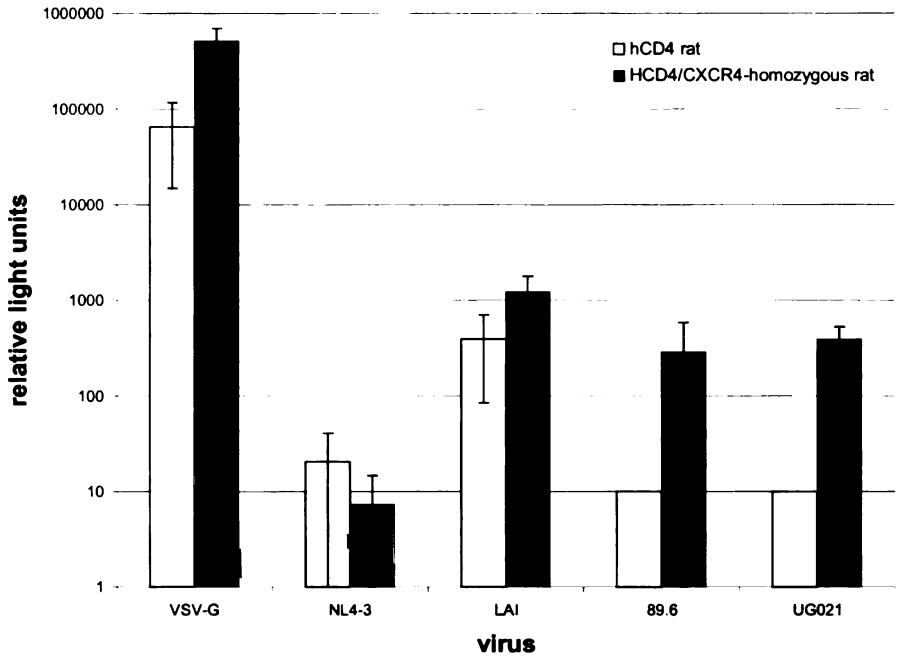


Figure 2.9 – Entry and early gene expression in transgenic rat microglia.

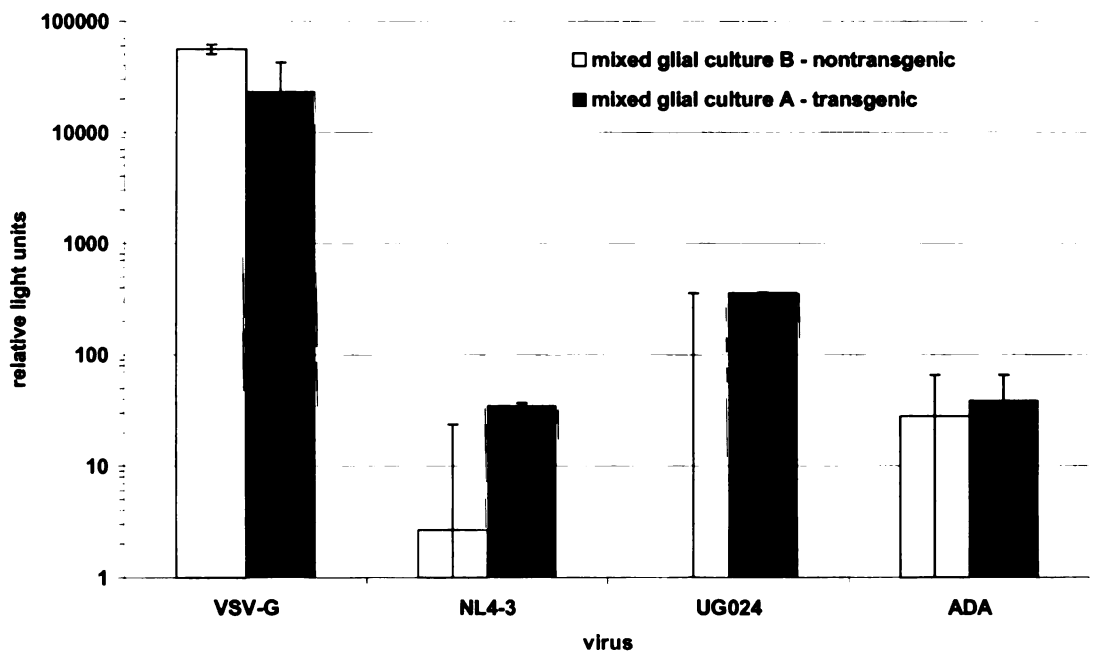


Figure 2.10 – Cloning of human SDF-1gamma.

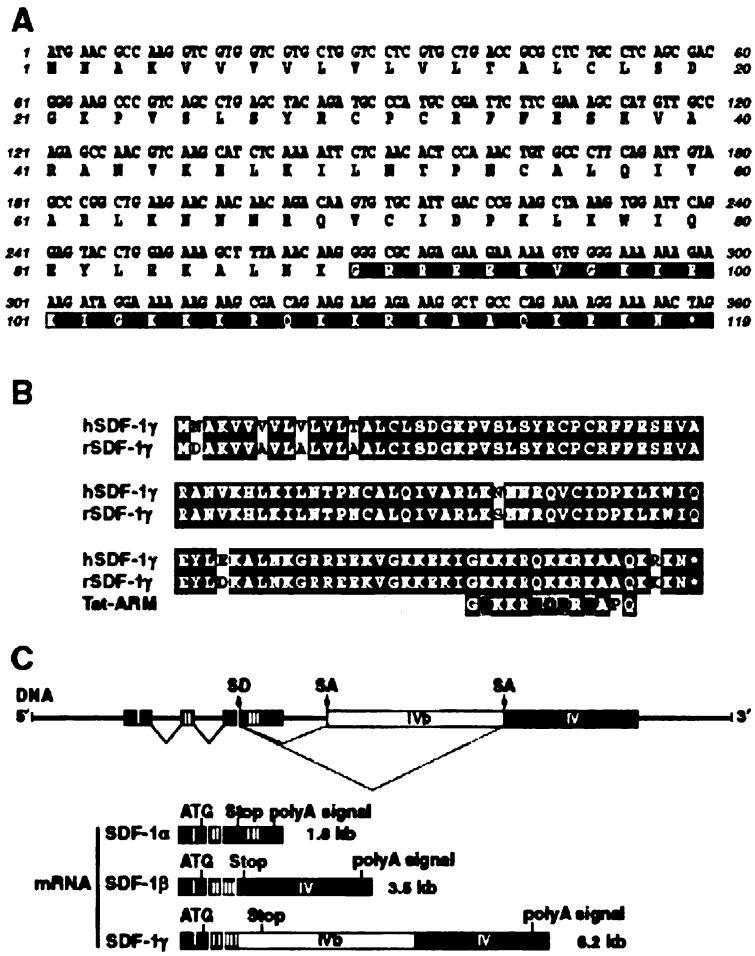


Figure 2.11 – Tissue-specific expression and function of SDF-1gamma.

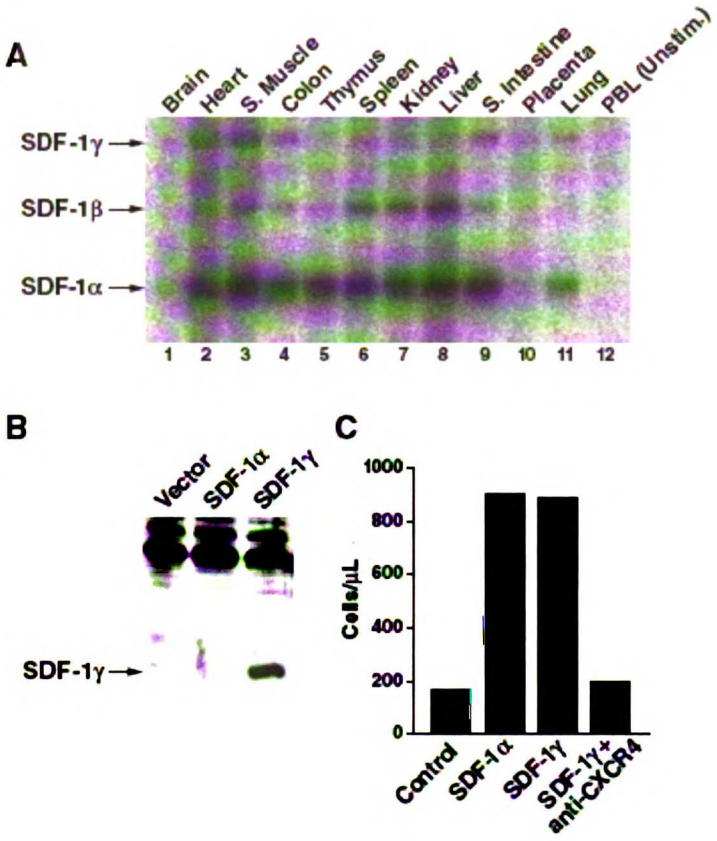


Figure 2.12 – SDF-1gamma is predominantly a cytoplasmic protein with a discrete nucleolar localization.

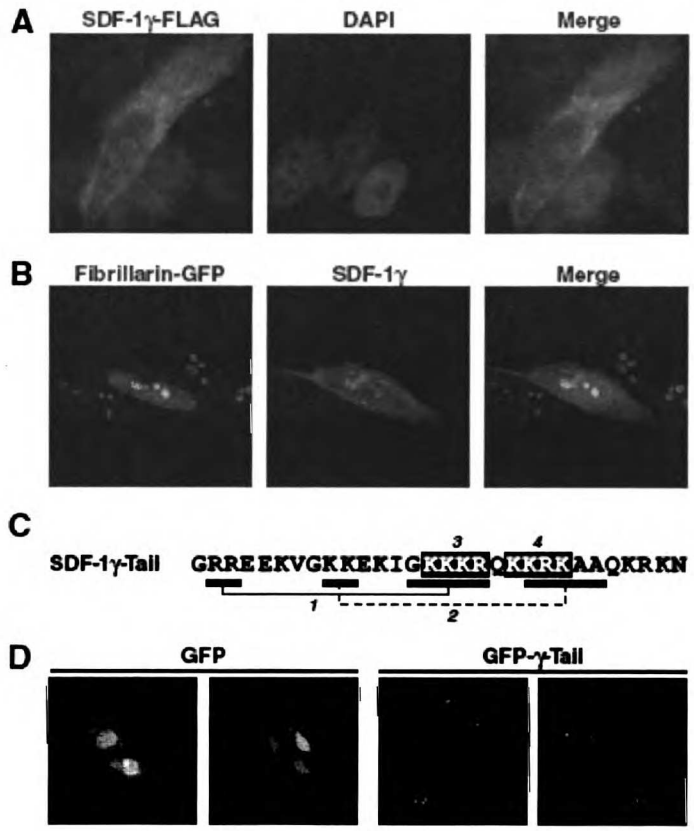


Figure 2.13 – SDF-1gamma binds specifically to TAR RNA in vitro.

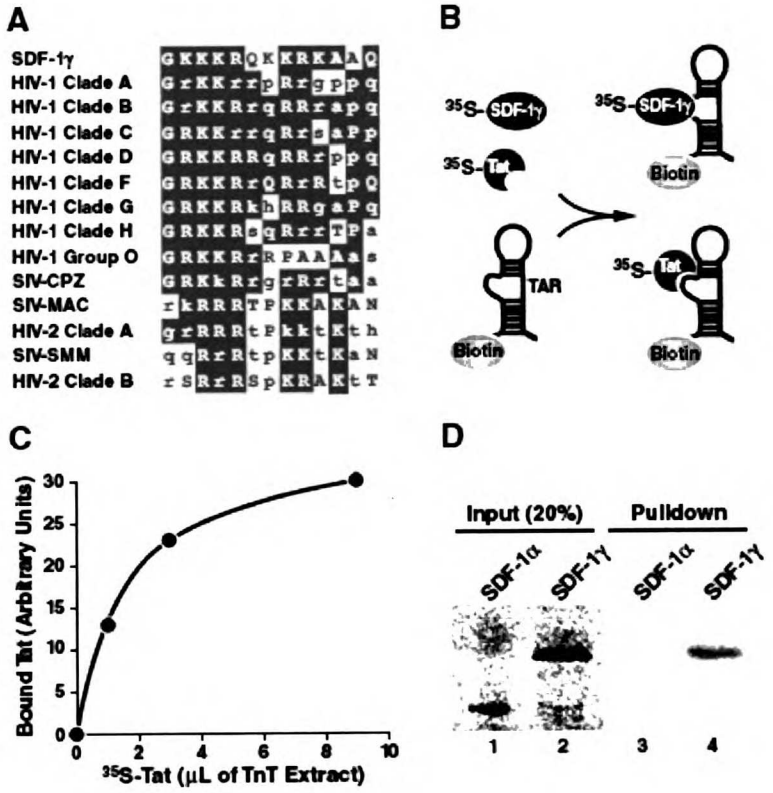


Figure 2.14 – SDF-1gamma blocks Tat-dependent LTR transactivation.

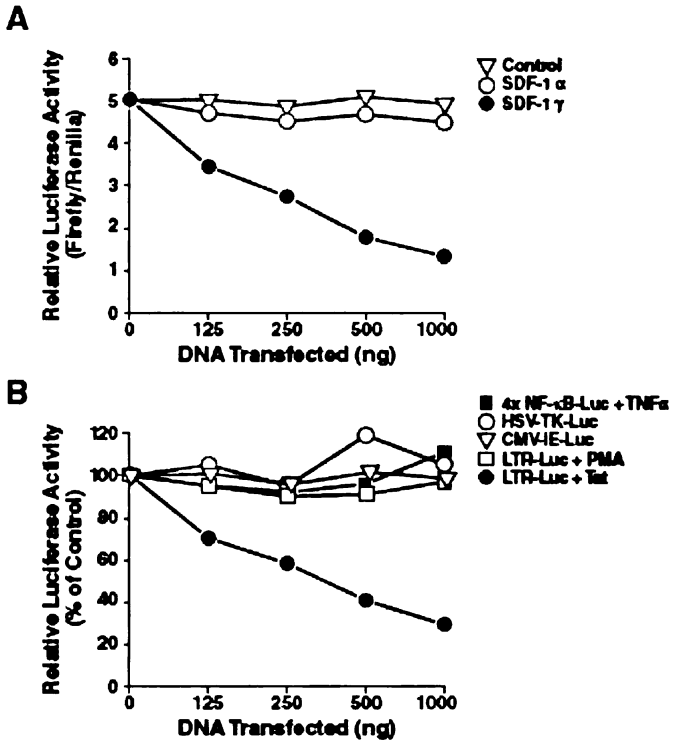


Figure 2.15 – SDF-1gamma blocks both X4 and R5 virus infection in GHOST cells.

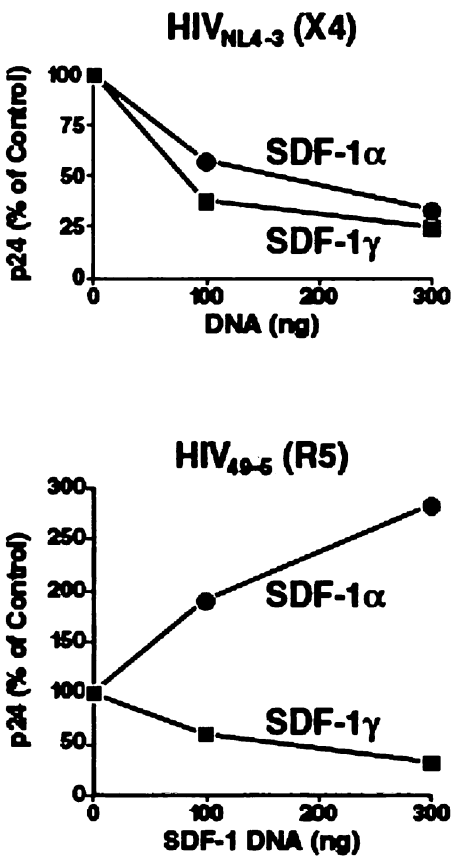


Figure 2.16 - Tat and nicotinamide cause T-cell hyperactivation

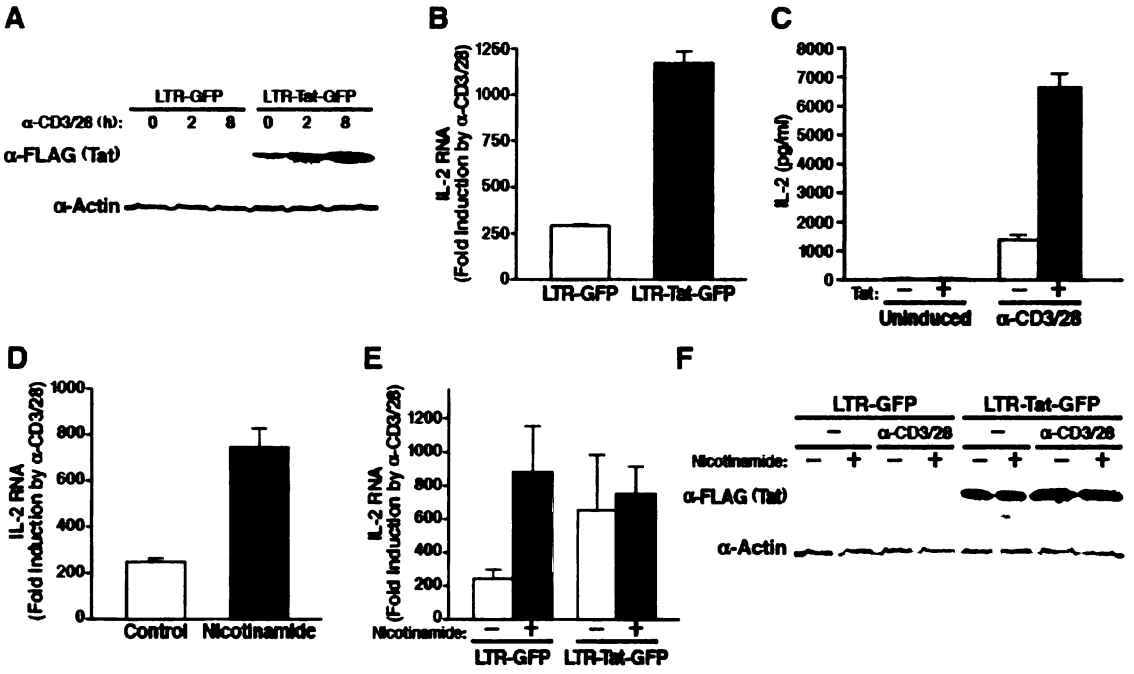


Figure 2.17 - Tat-mediated superinduction of NFκB response genes requires SIRT1

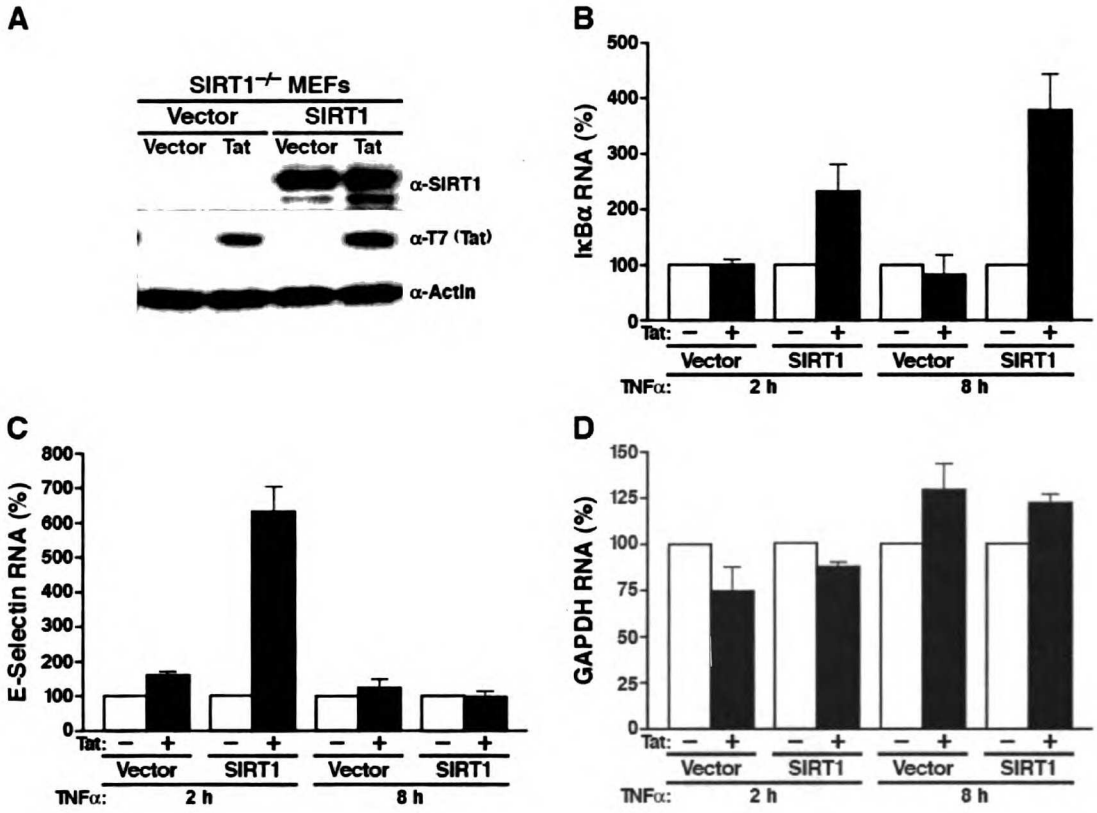


Figure 2.18 - Tat neutralizes SIRT1 inhibition on NFκB promoter activity

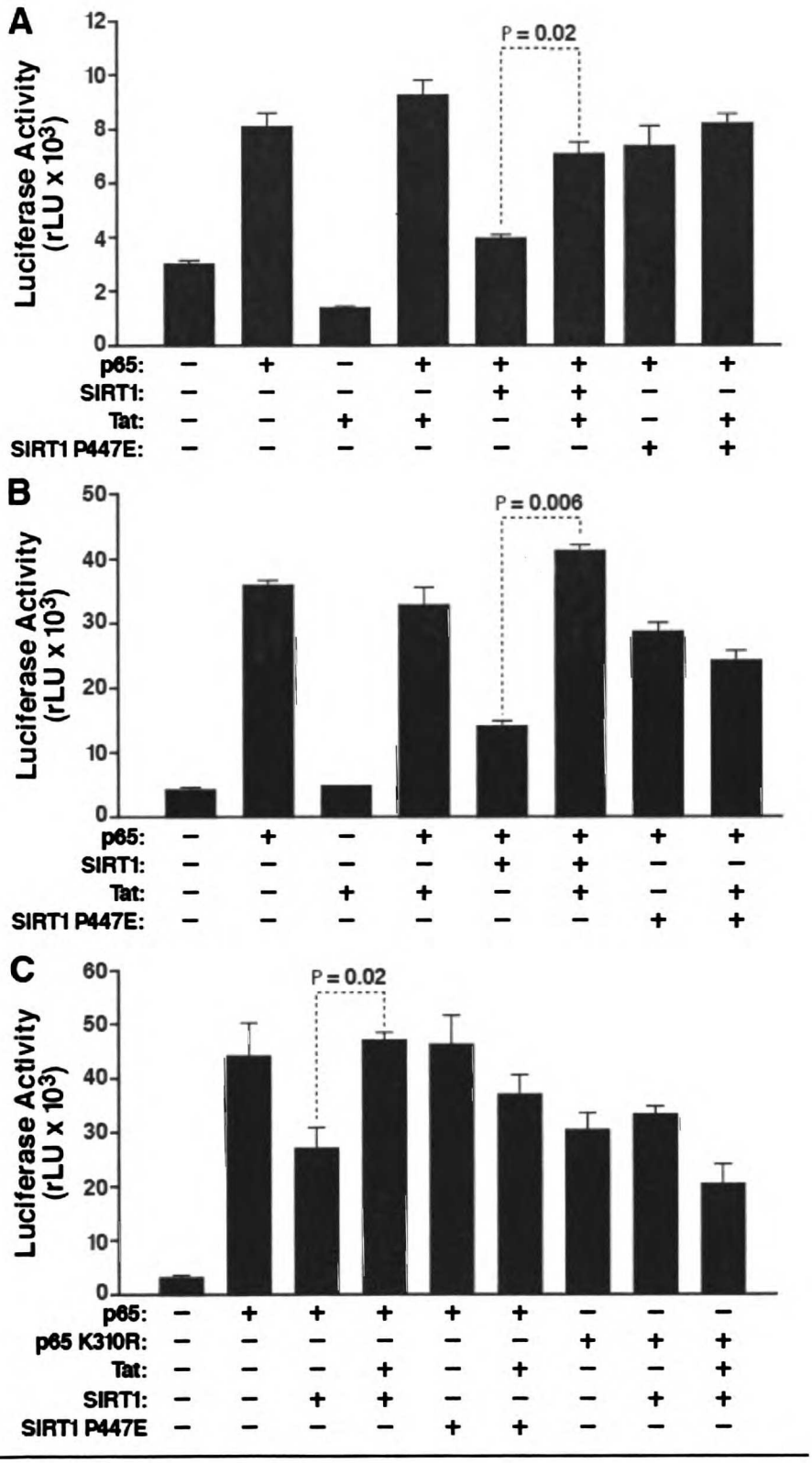


Figure 2.19 - Tat binds to the acetyl lysine-binding site in SIRT1

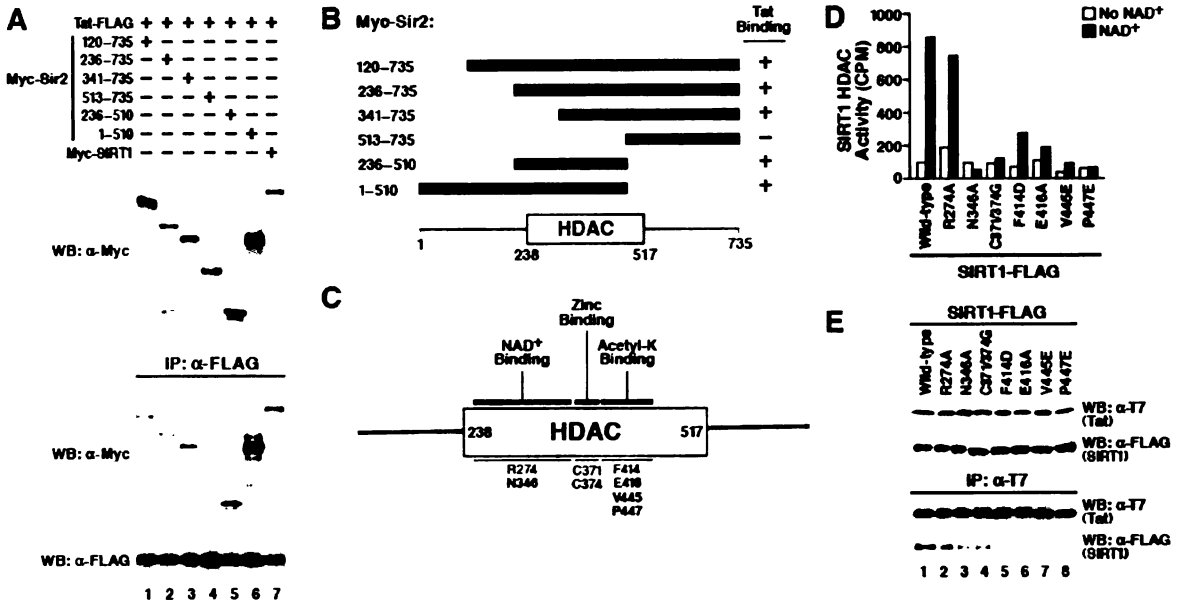


Figure 2.20 - Tat inhibits the SIRT1 deacetylase activity

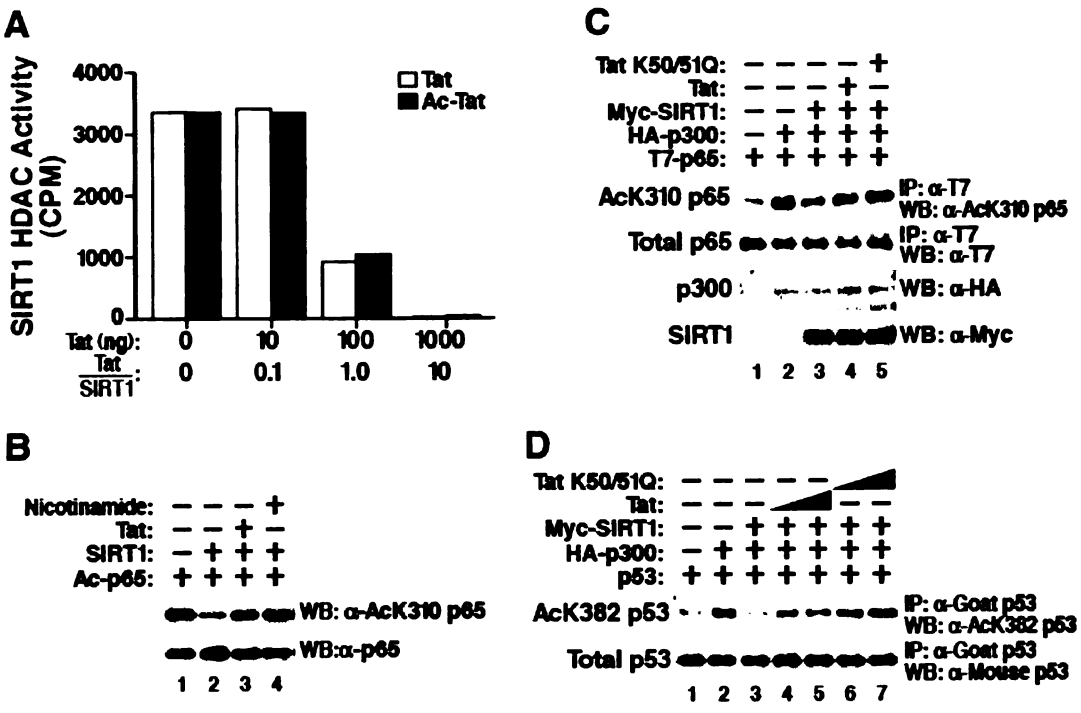


Figure 2.21 - Tat blocks the SIRT1 deacetylase activity and superinduces T-cell activation and HIV transcription via NFκB.

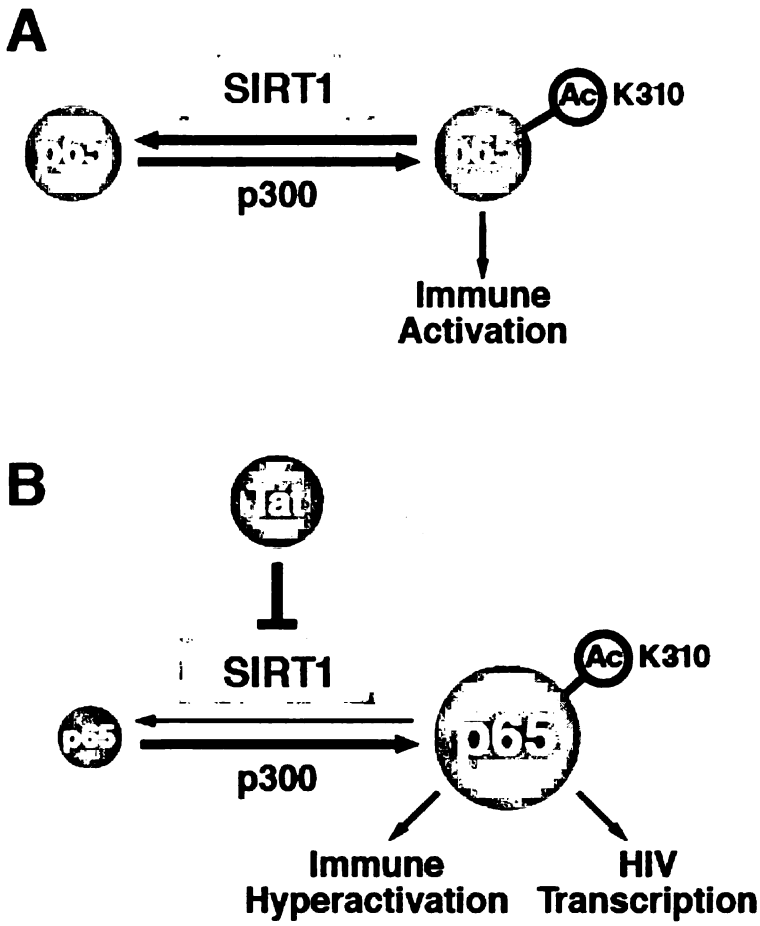


Figure 3.1 - Primary X4 viruses UG021 and J130 do not use CCR5 as a coreceptor and require CXCR4 for infection.

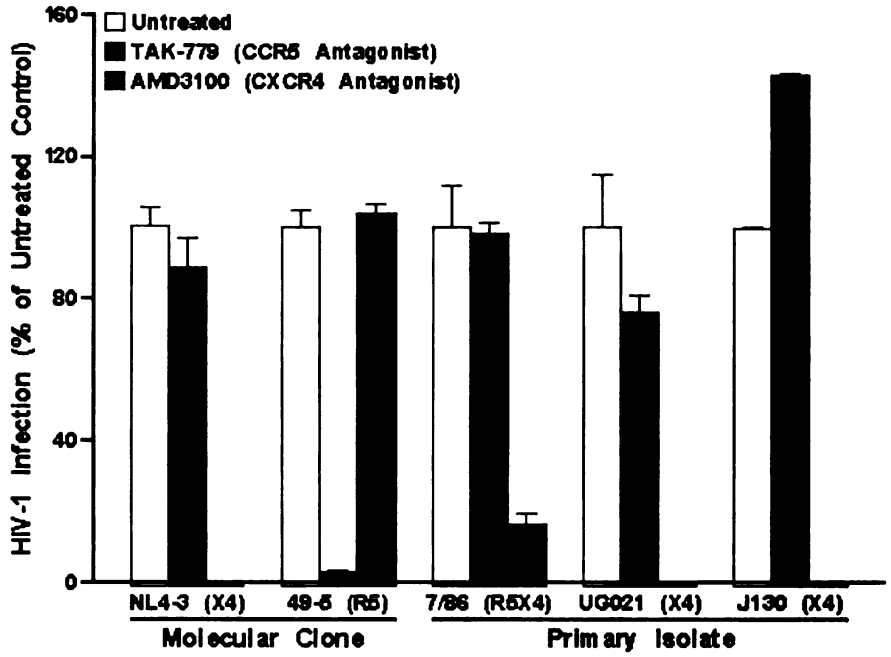


Figure 3.2 - Primary X4 viruses replicate in *ex vivo* human tonsil histocultures.

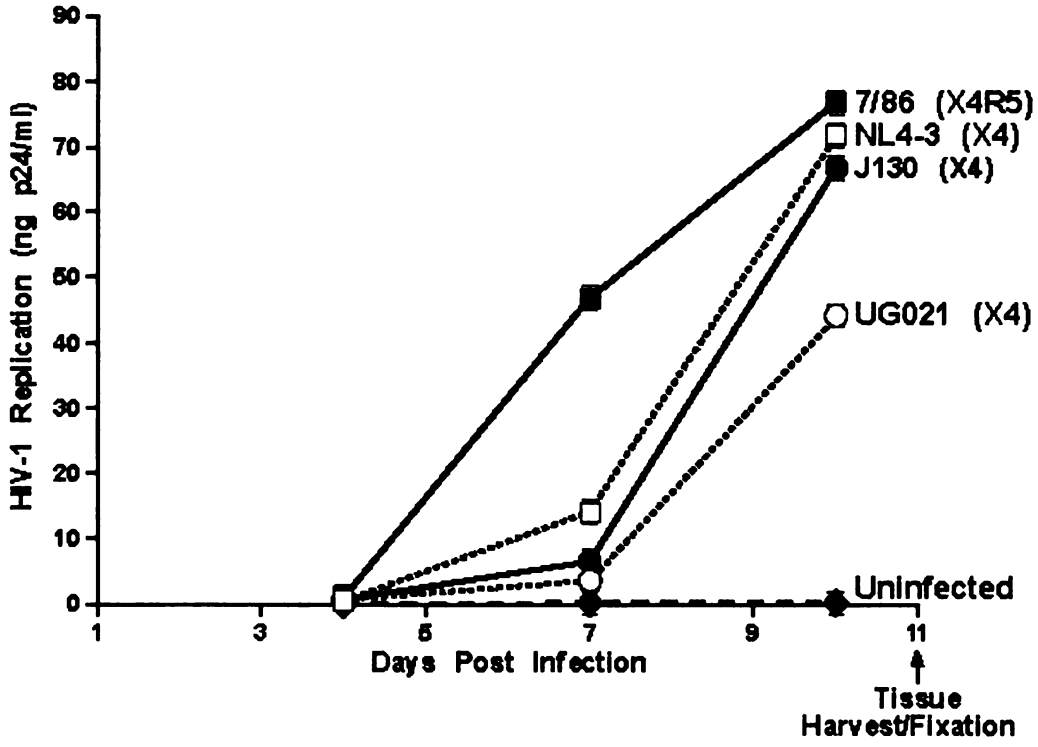


Figure 3.3 - Primary X4 isolates UG021 and J130, but not the TCLA-X4 strain NL4-3, productively infect tissue macrophages in human tonsil histocultures.

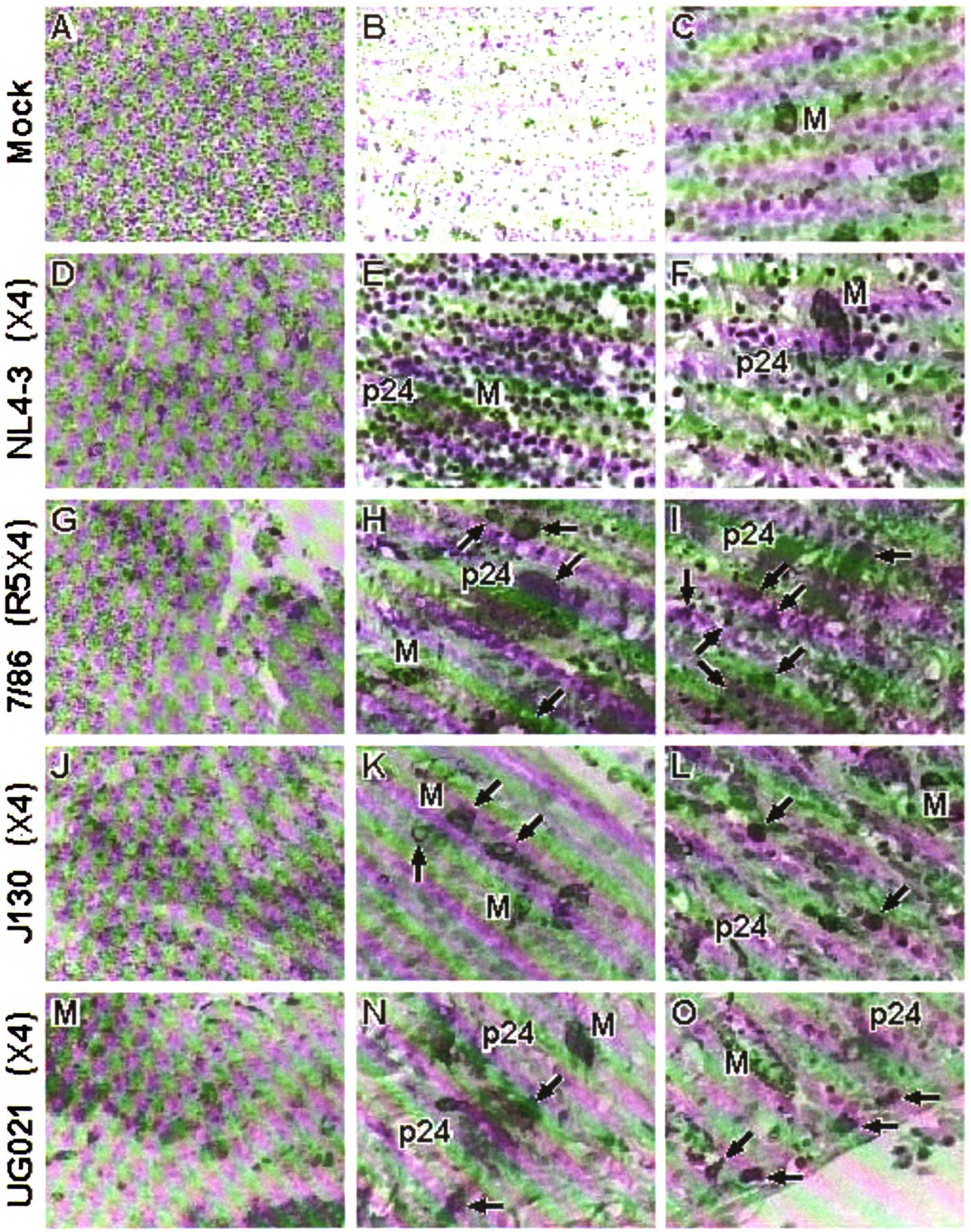


Figure 3.4 - Tissue-resident macrophages in tonsils histocultures infected with primary X4 isolates are HIV-1 RNA positive.

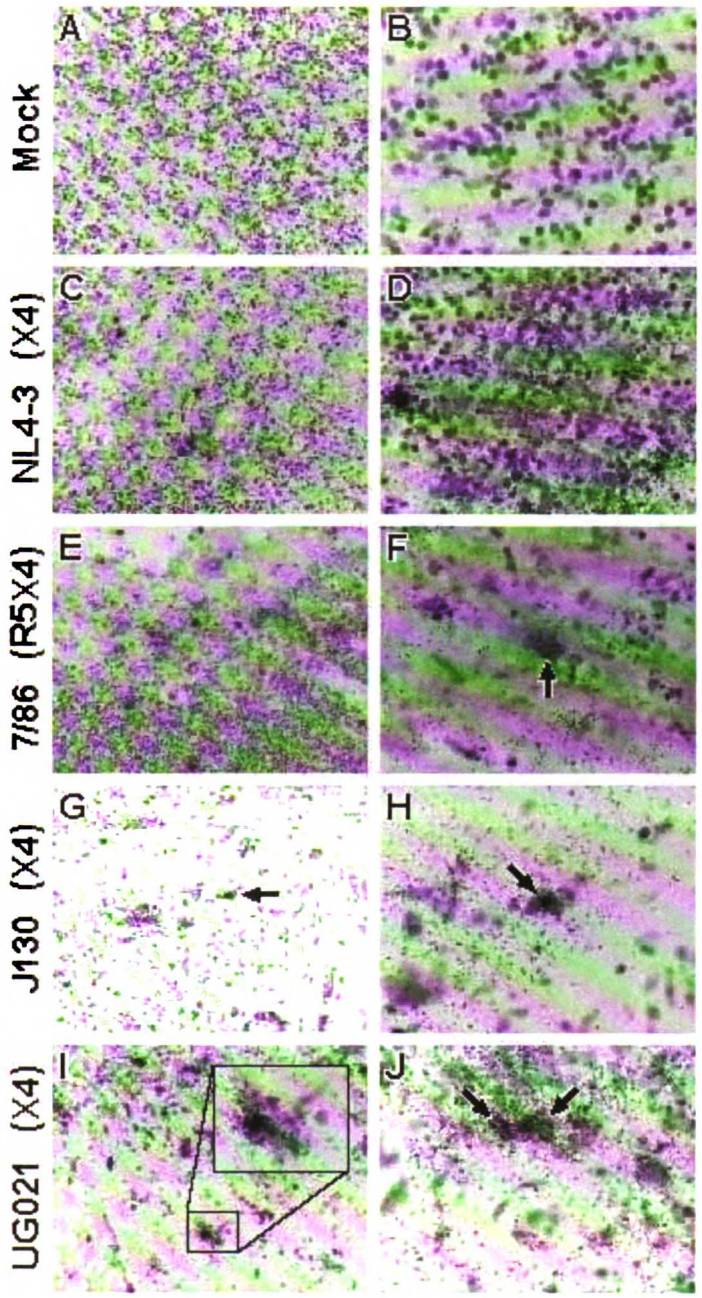


Figure 3.5 - Confocal double-immunofluorescence microscopy allows determination of frequency of infected tissue macrophages.

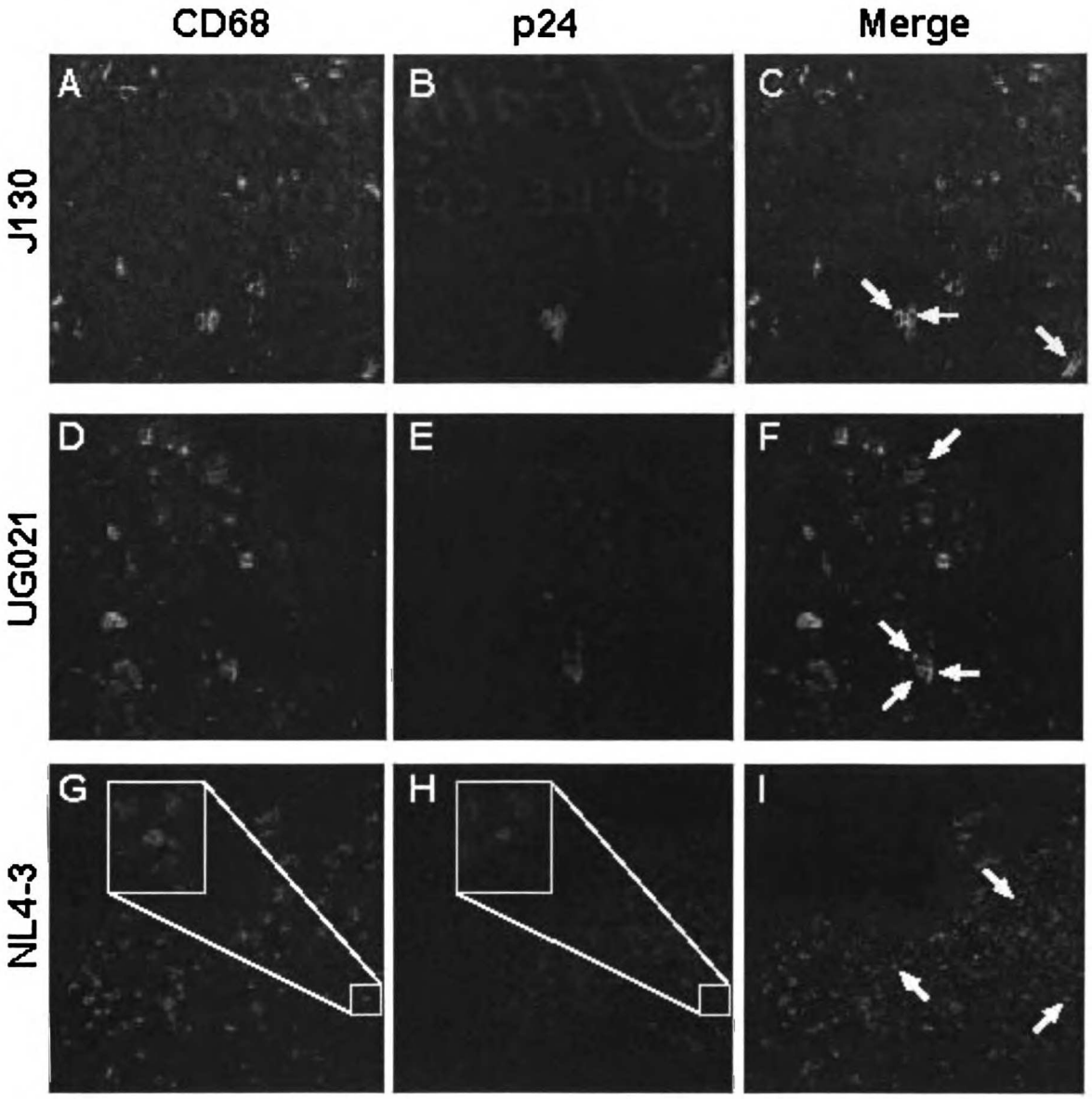


Figure 3.6 - Markers for T cells and macrophages do not colocalize in infected tonsil histocultures.

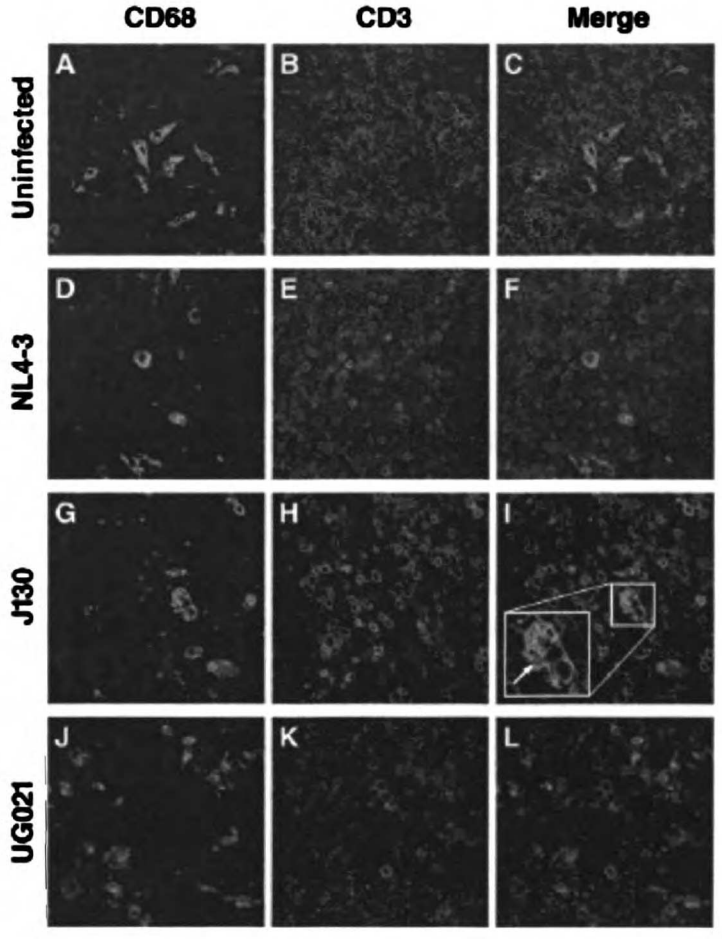
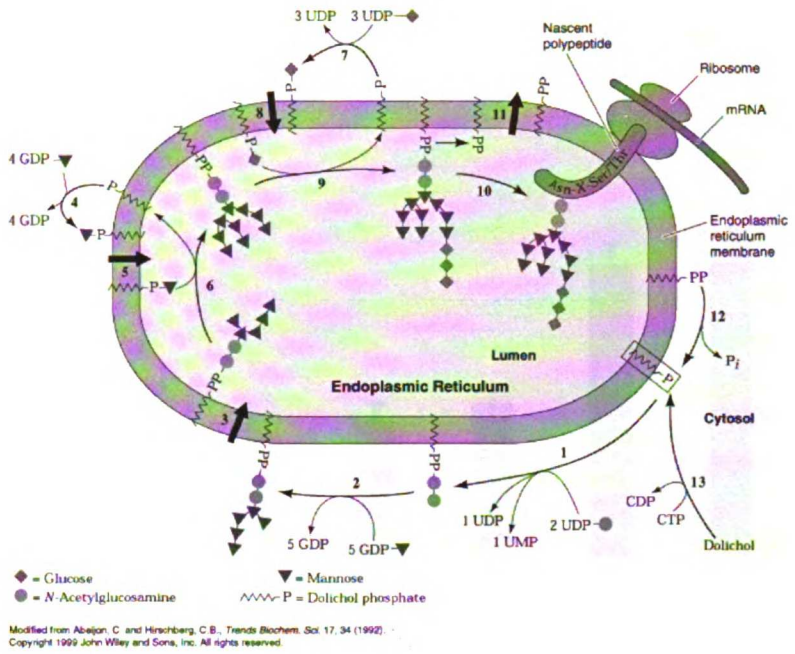


Figure 3.7 – N-glycosylation pathway



From http://www.mun.ca/biochem/courses/3107/images/VVP/Ch15/15-34.jpg

Figure 3.8 – X4 dualtropic viruses NL4-3 and glycosylation mutants infect HeLa CD4-CCR5 cells.

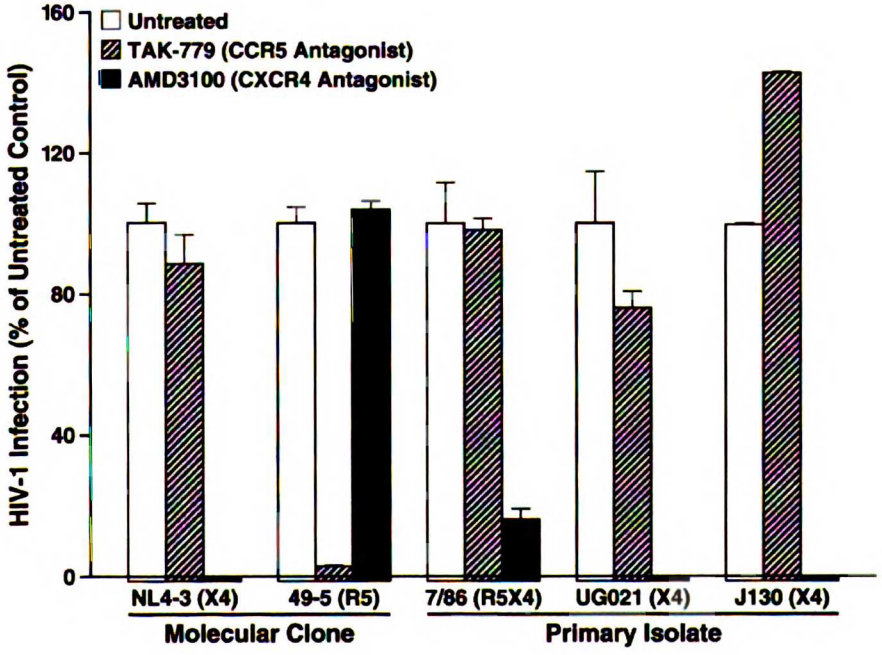


Figure 3.9 – X4 dualtropic viruses and glycosylation mutants infect GHOST-CD4 cells.

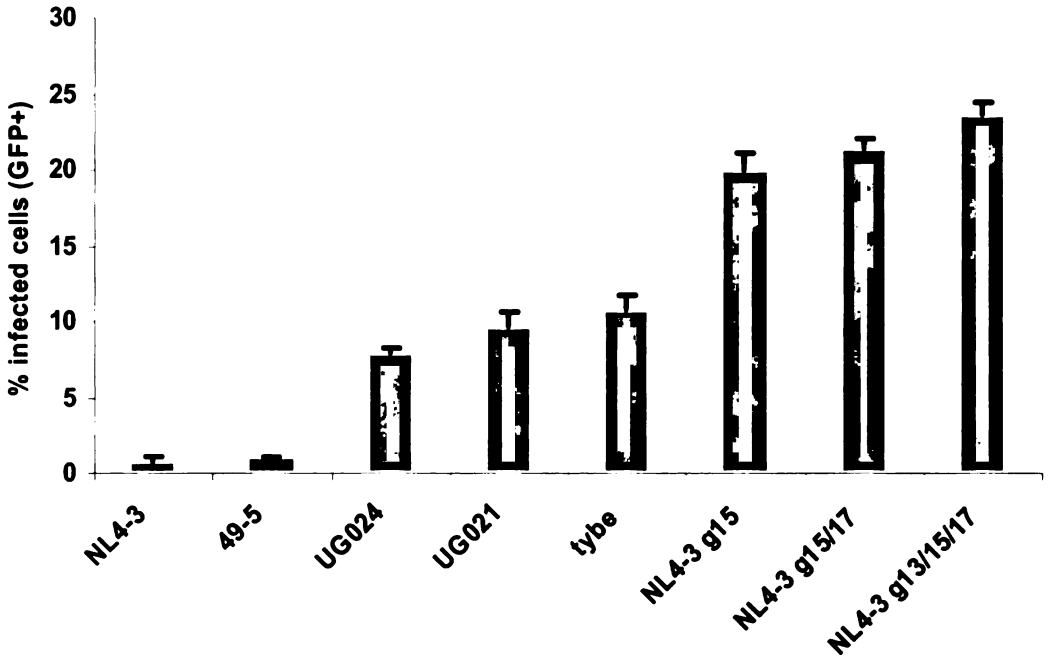


Figure 3.10 – Glycosylation mutants fail to infect monocyte-derived macrophages.

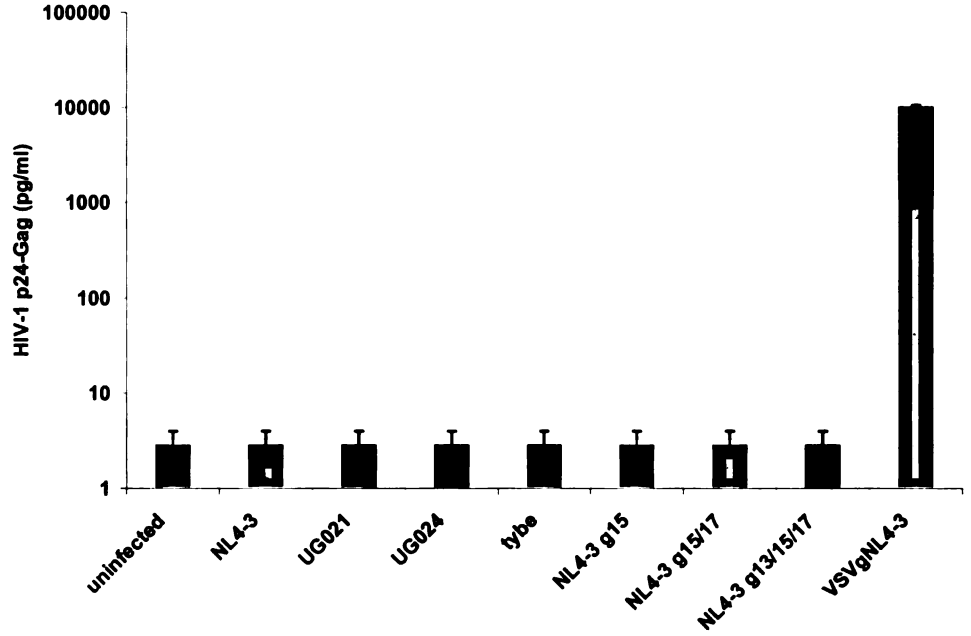


Figure 4.1 – Schematic of virus preparation and infection.

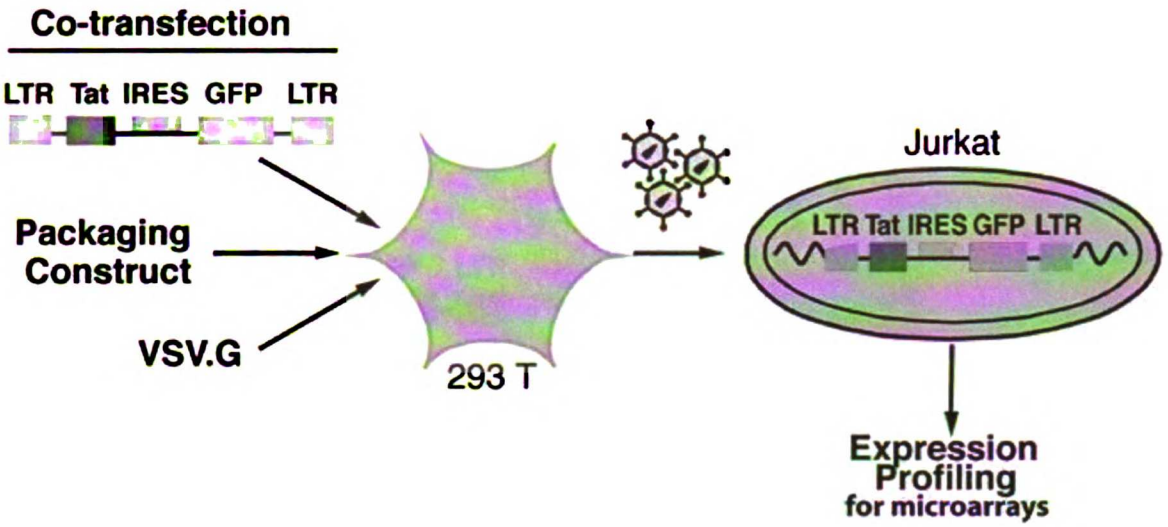


Figure 4.2 – Tat expression in infected cells and GFP mean fluorescence intensity in Tat-infected cells

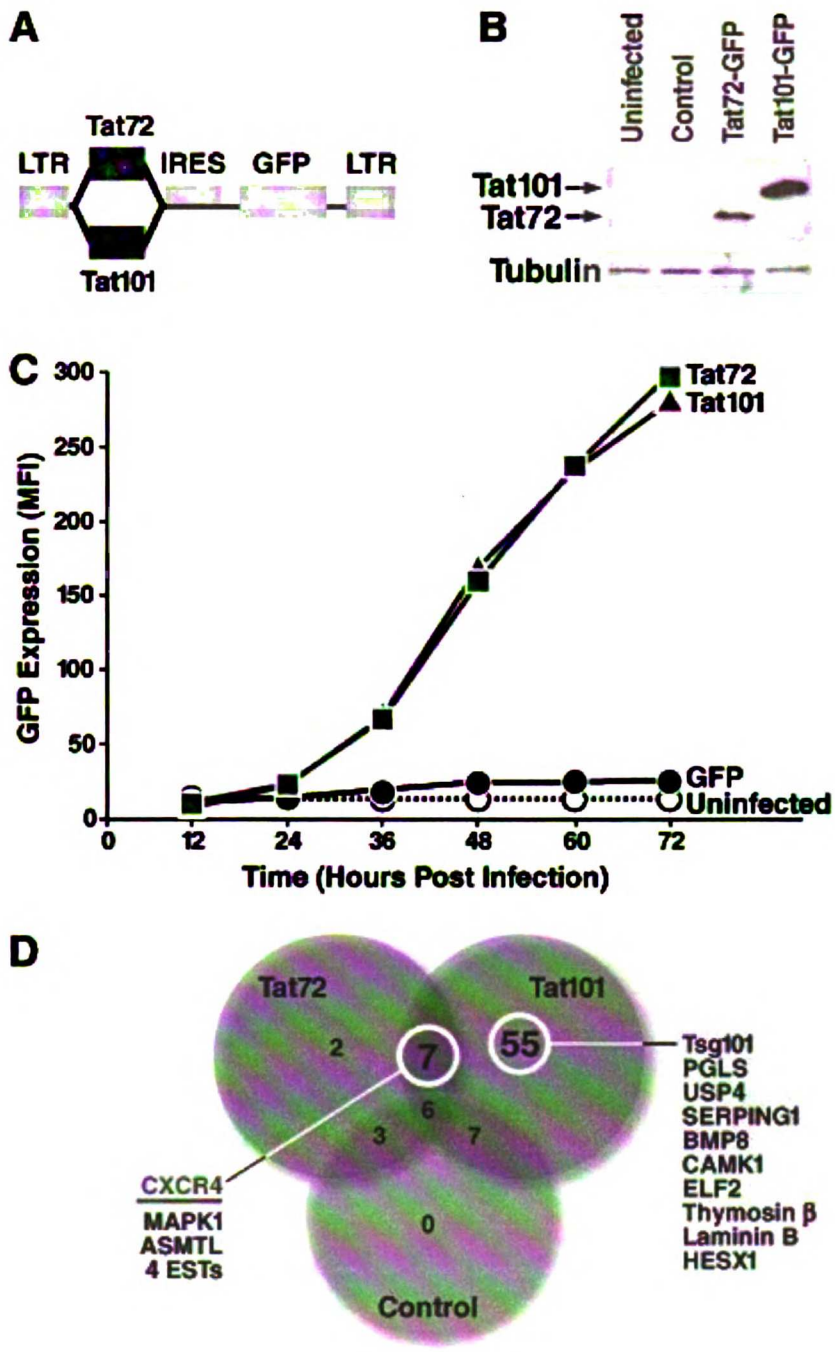


Figure 4.3 – Activation of GFP expression by extracellular Tat.

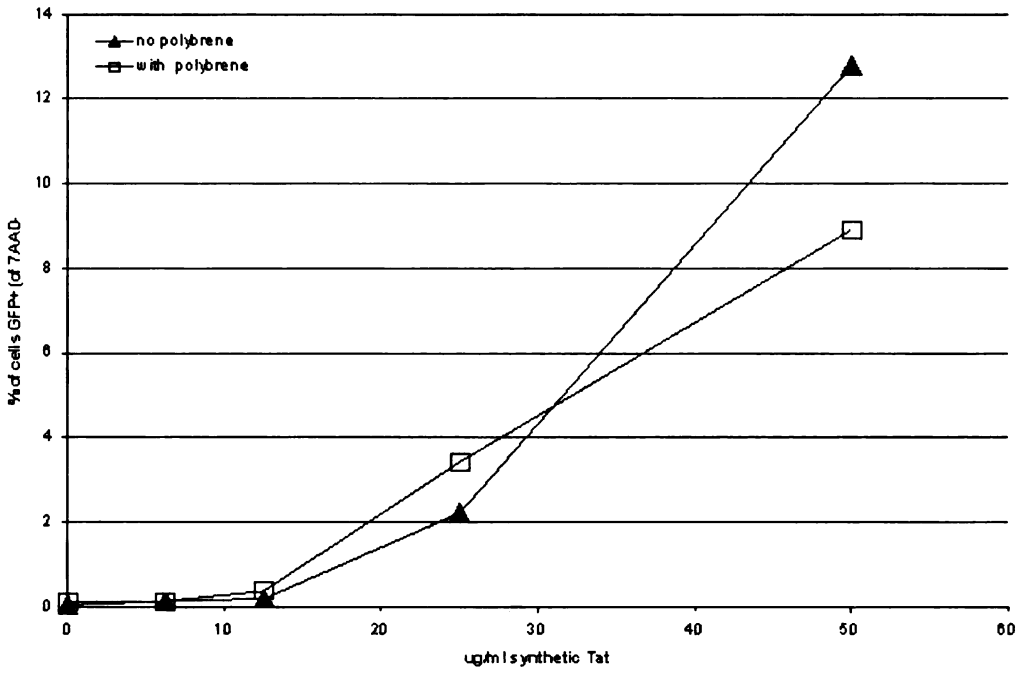


Figure 4.4 – Microarray comparisons

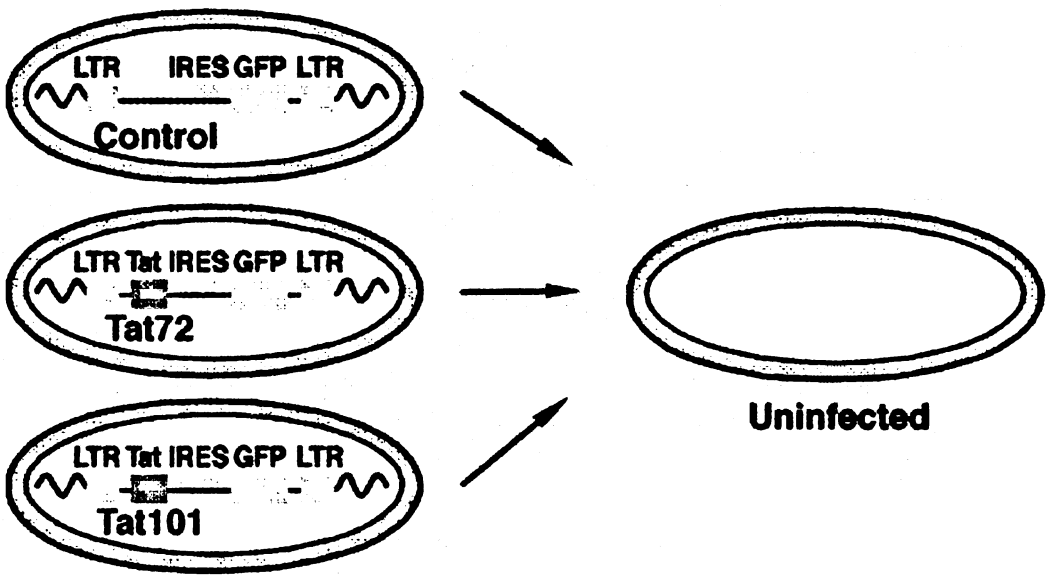


Figure 4.5 – Microarray preparation

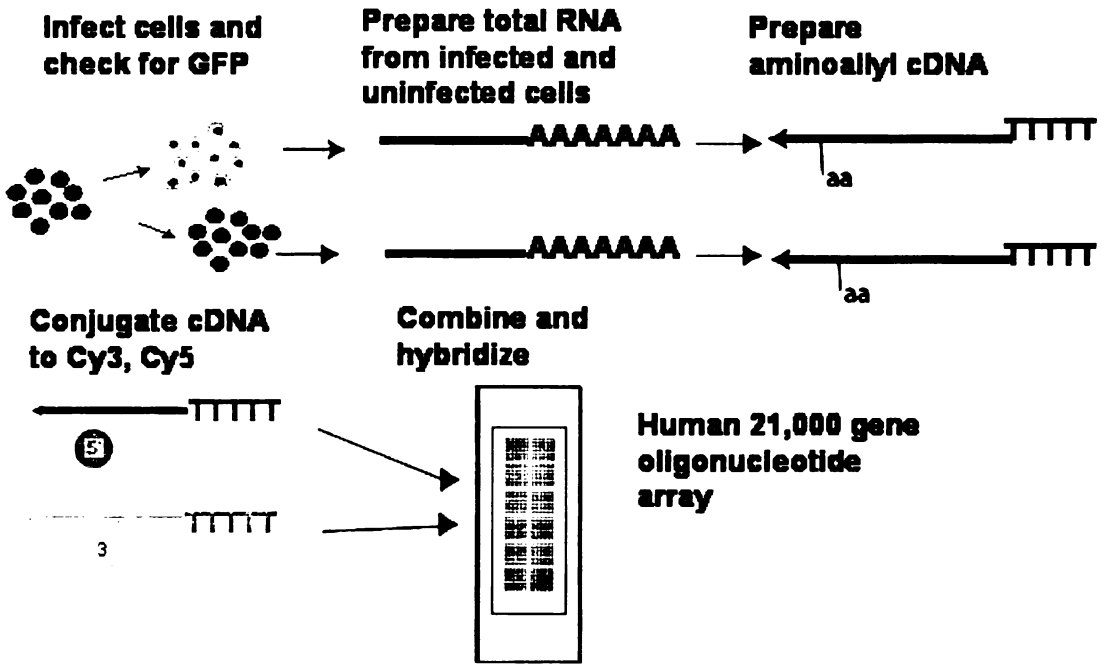


Figure 4.6 – CXCR4 RNA and protein are increased by Tat72 and Tat101

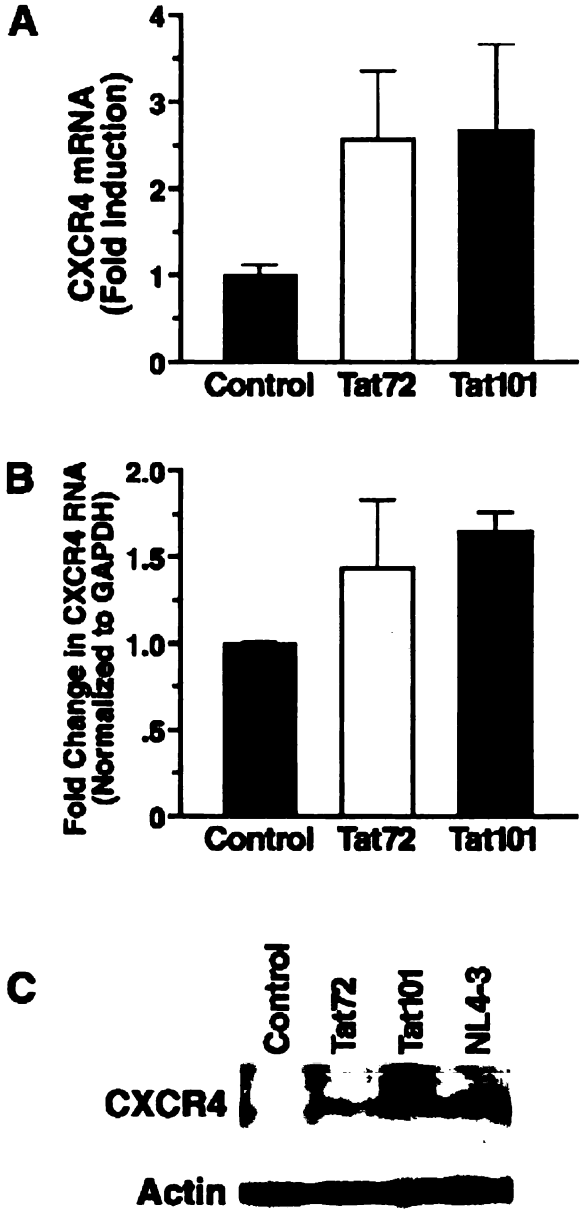


Figure 4.7 – Tat increases CXCR4 cell surface expression in infected cells

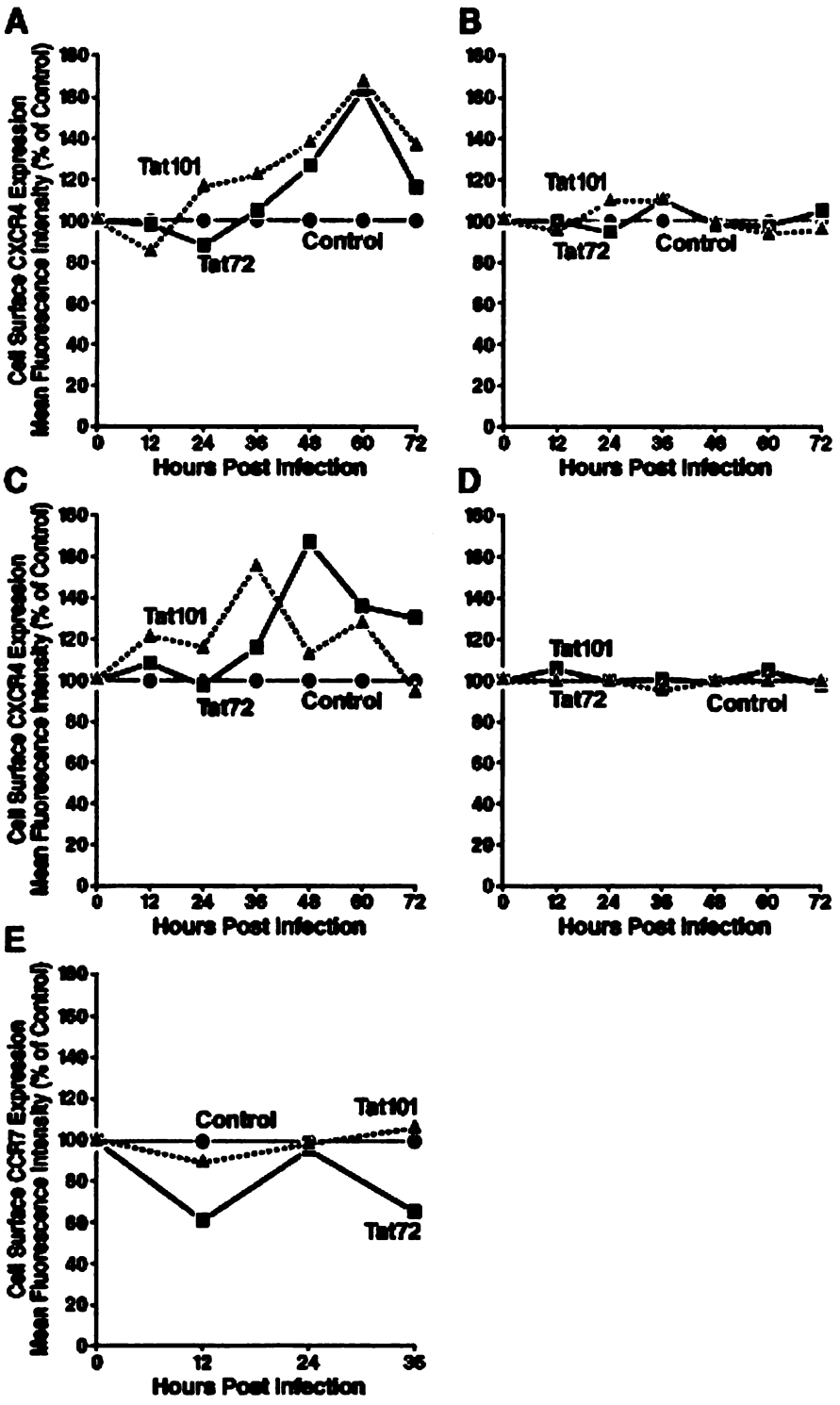


Figure 4.8 – Tat improves migration of T cells to SDF1alpha.

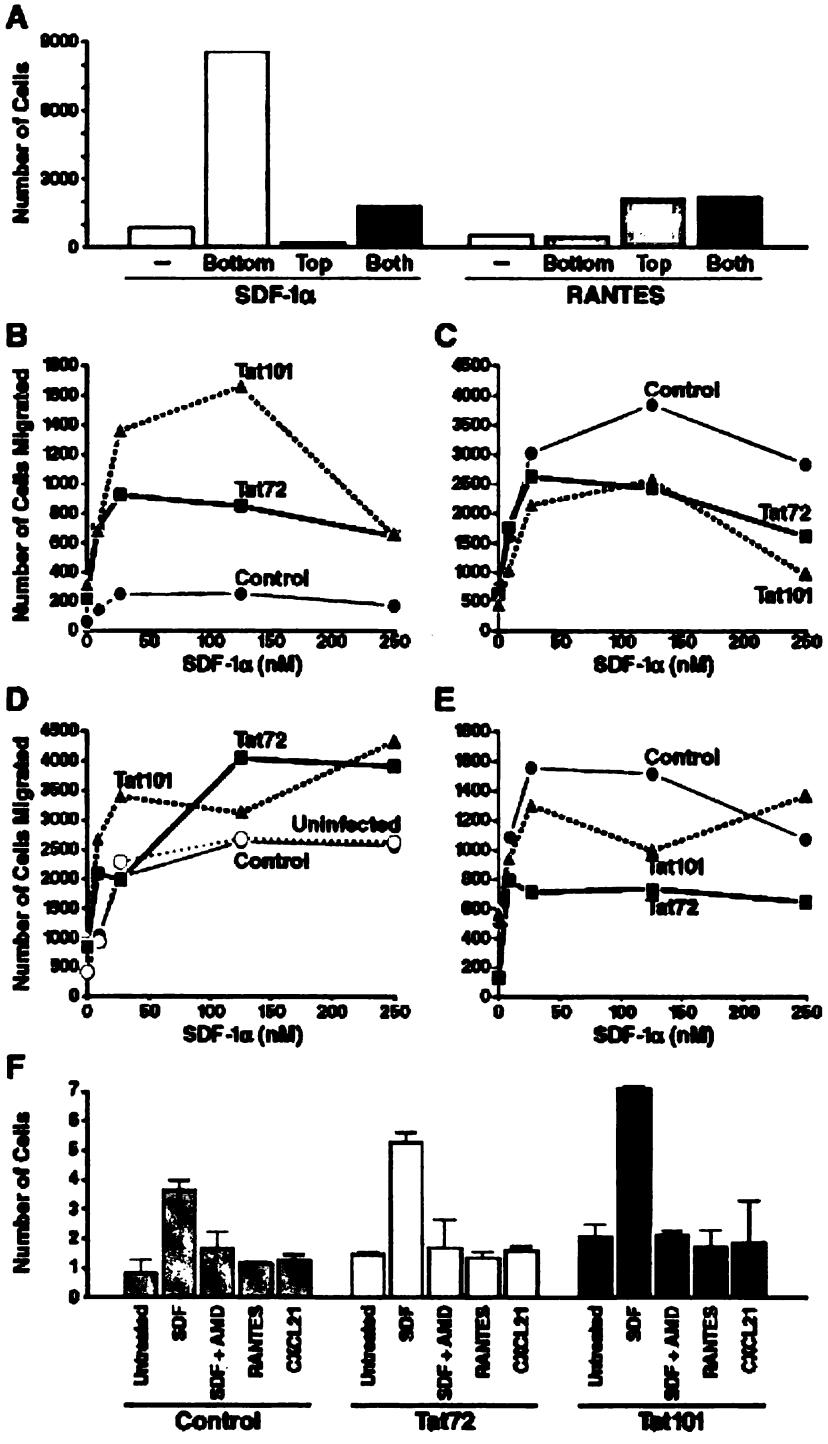


Figure 4.9 – Tat activates the CXCR4 promoter.

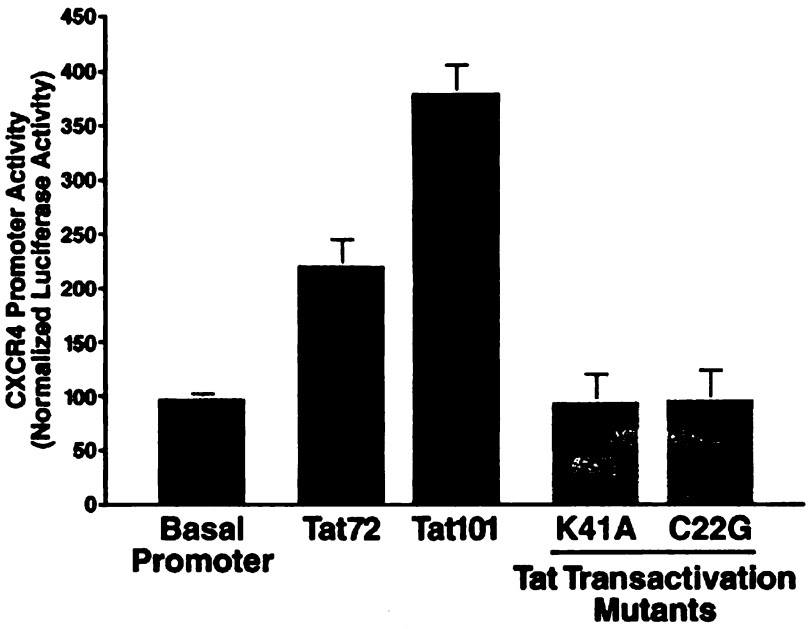


Figure 4.10 – The Tat-responsive element in CXCR4 is located between –600 and –357 upstream of the start site.

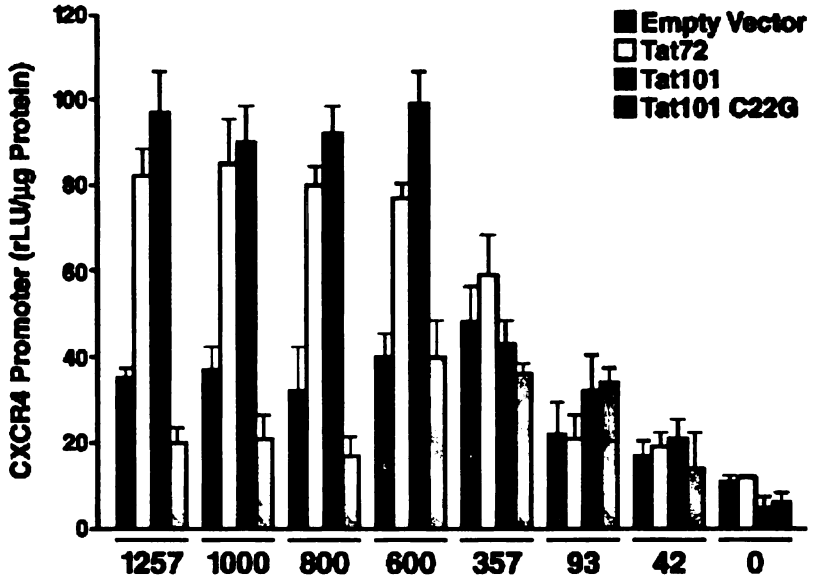


Figure 4.11 – A conserved region in the Tat-responsive element of CXCR4

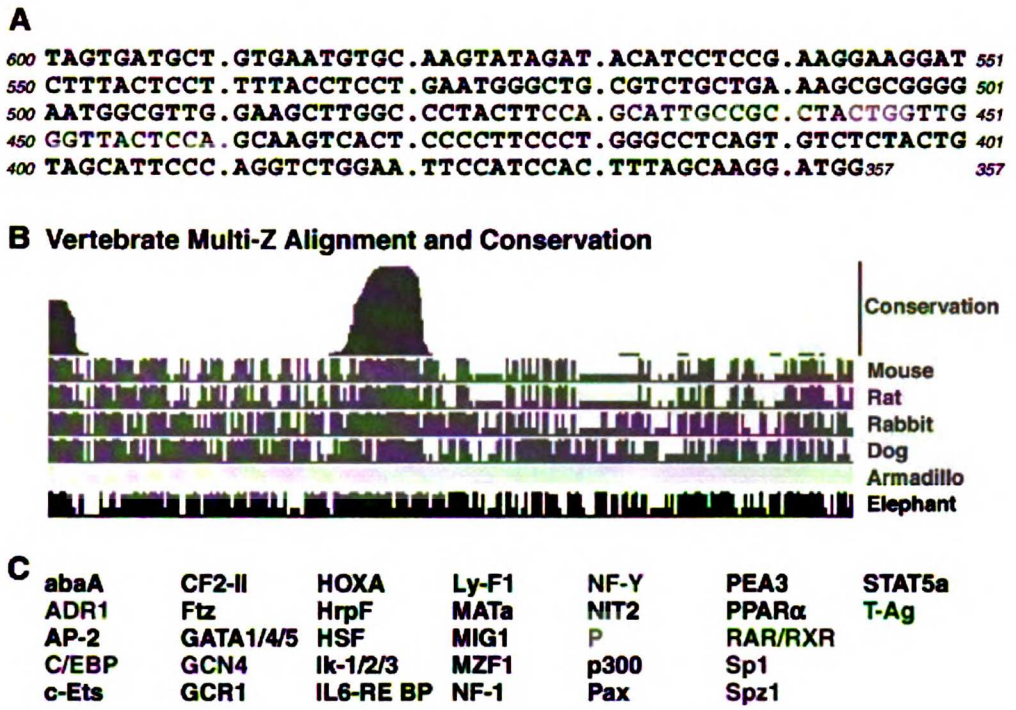


Figure 4.12 – Model of in vivo CXCR4 upregulation.

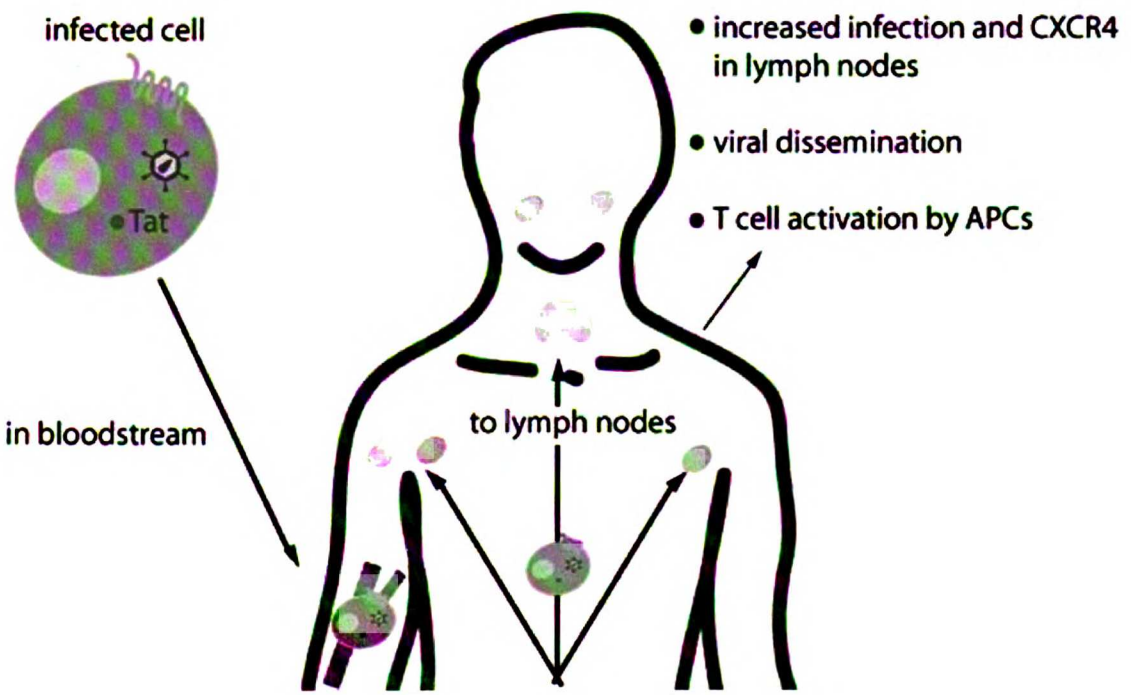


Figure 5.1 – ESCRT complexes

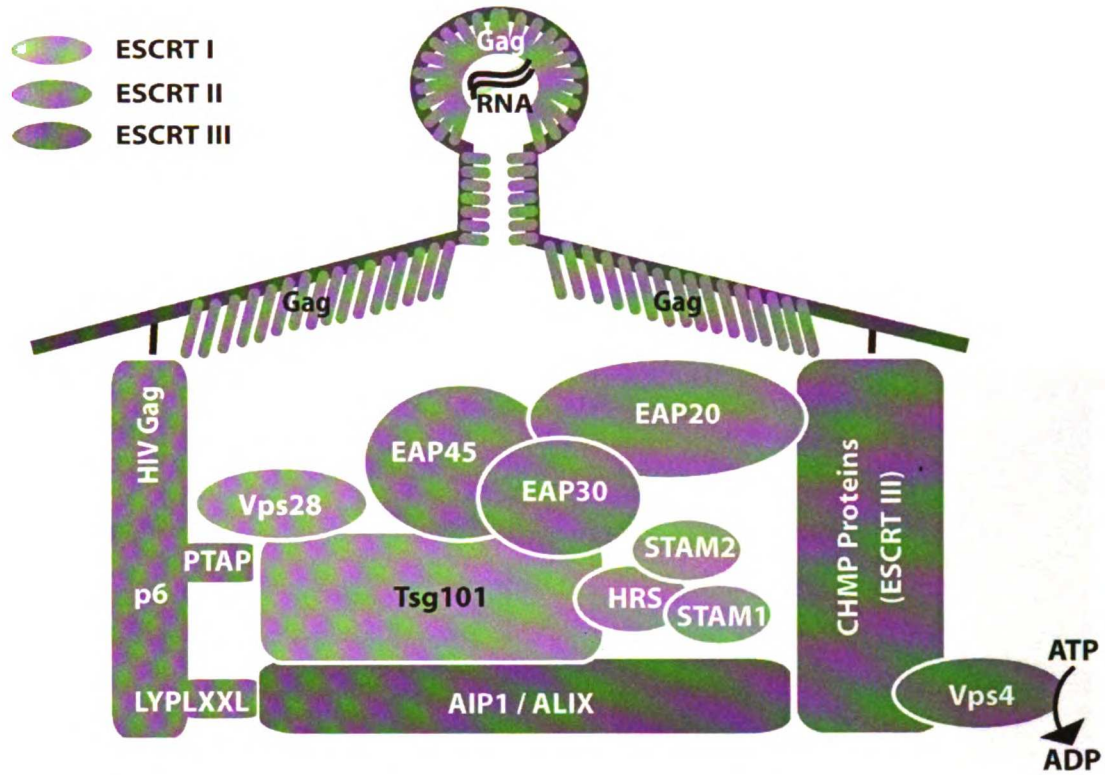


Figure 5.2 – Tat upregulates Tsg101 RNA levels

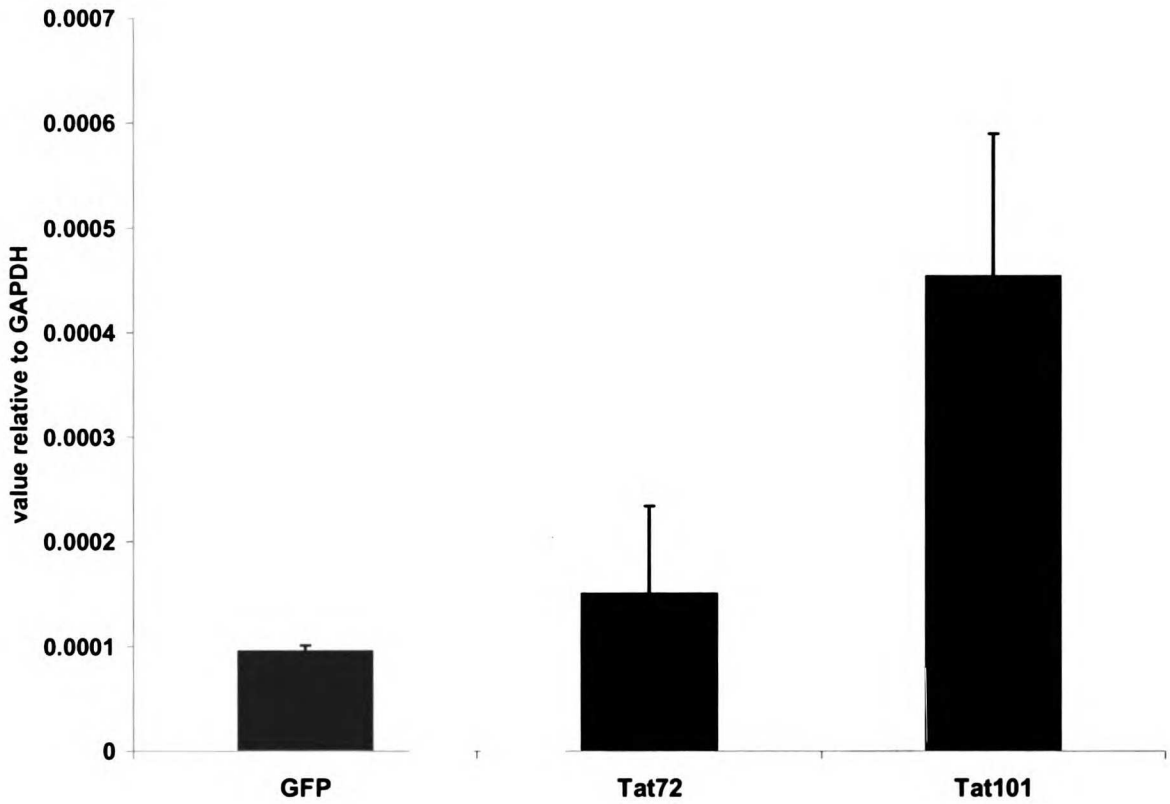


Figure 5.3 – Tat101 slightly increases Tsg101 protein levels.

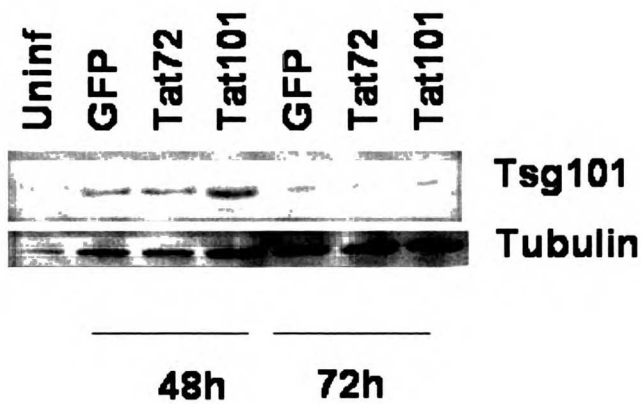


Figure 5.4 – Tat101 has no effect on Tsg101 levels in exosomes.

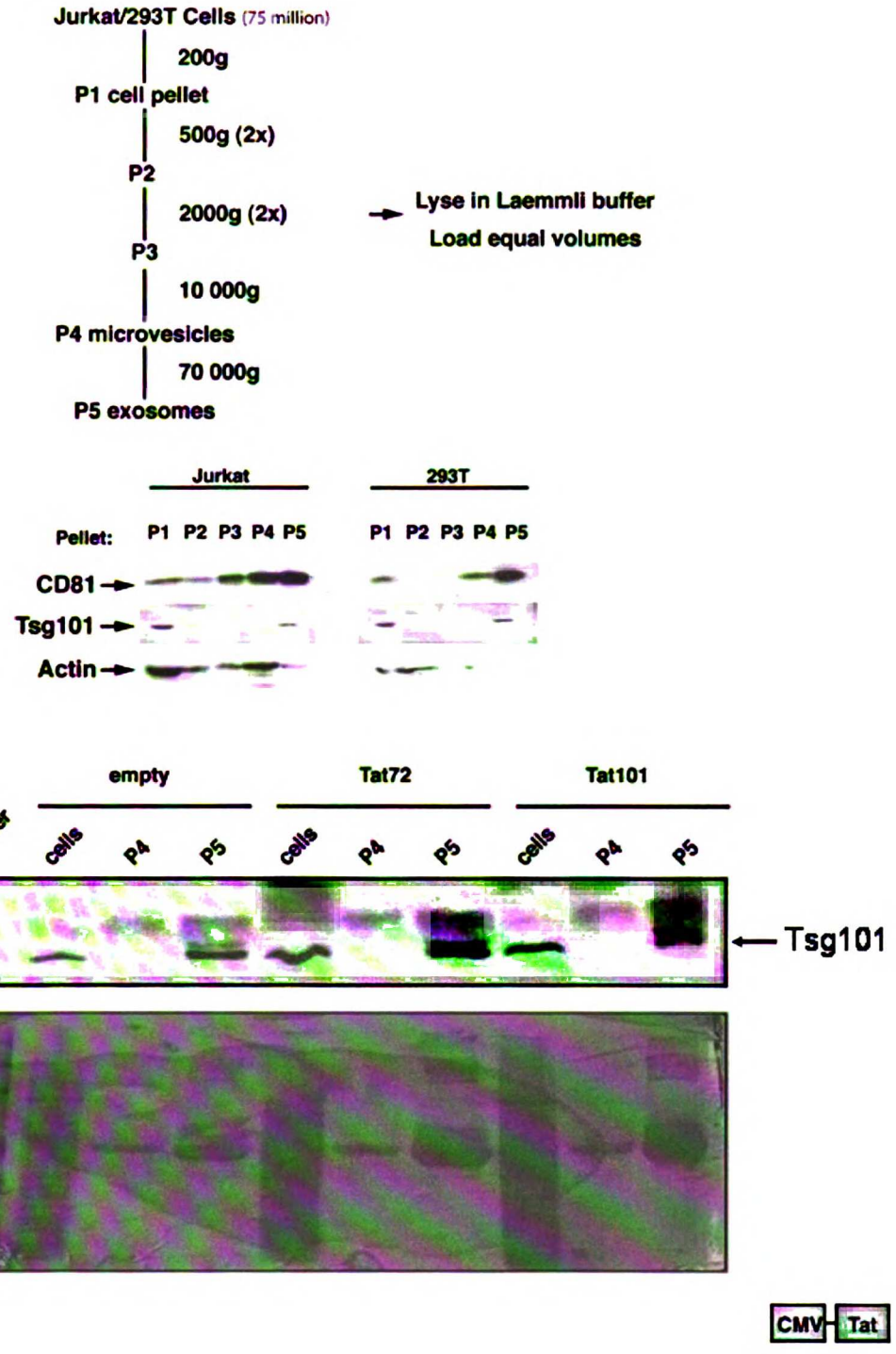


Figure 5.5 – Tat101 improves HIV budding from 293T cells.

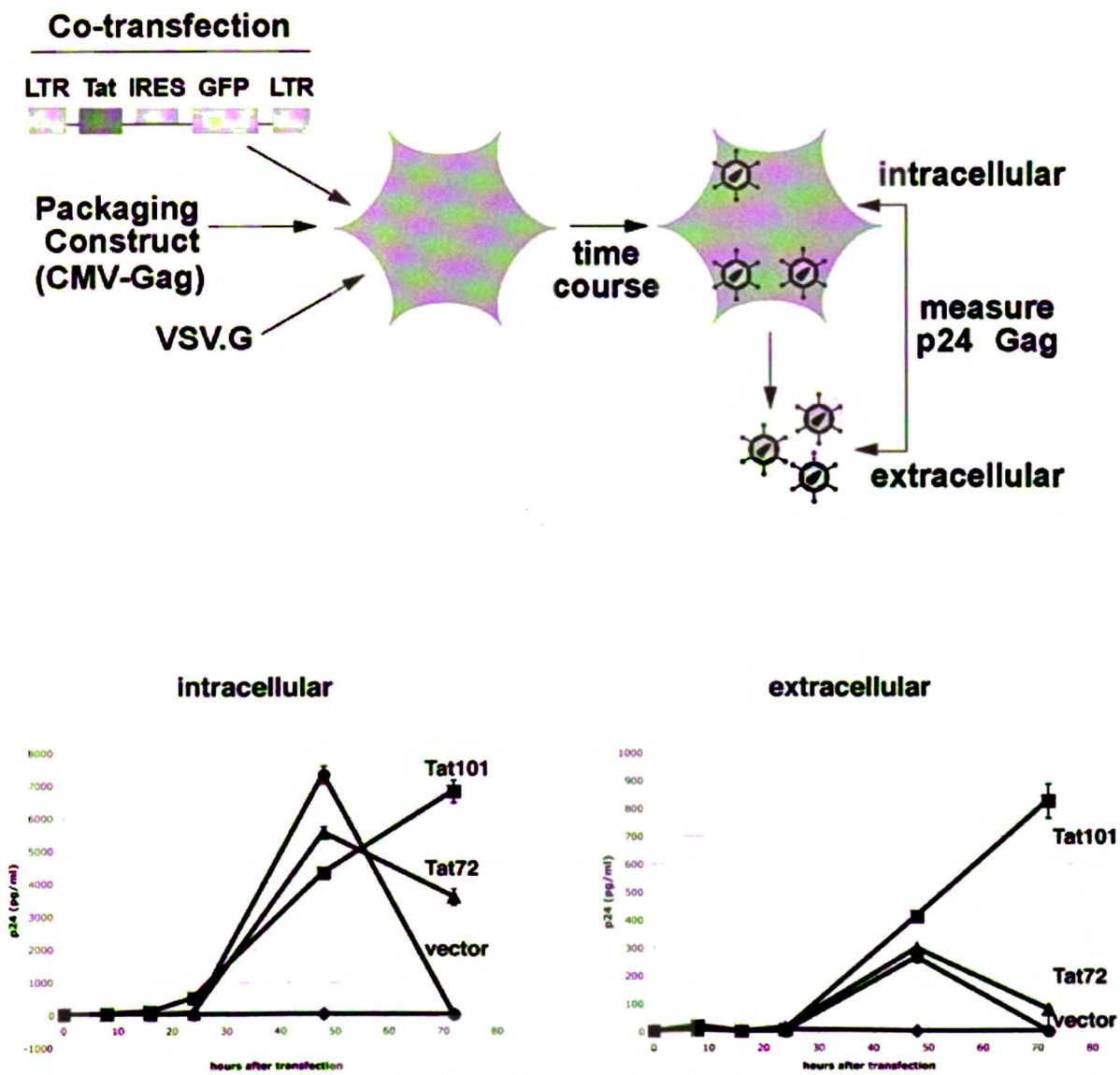
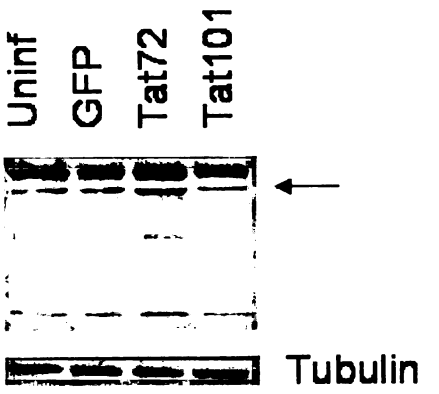
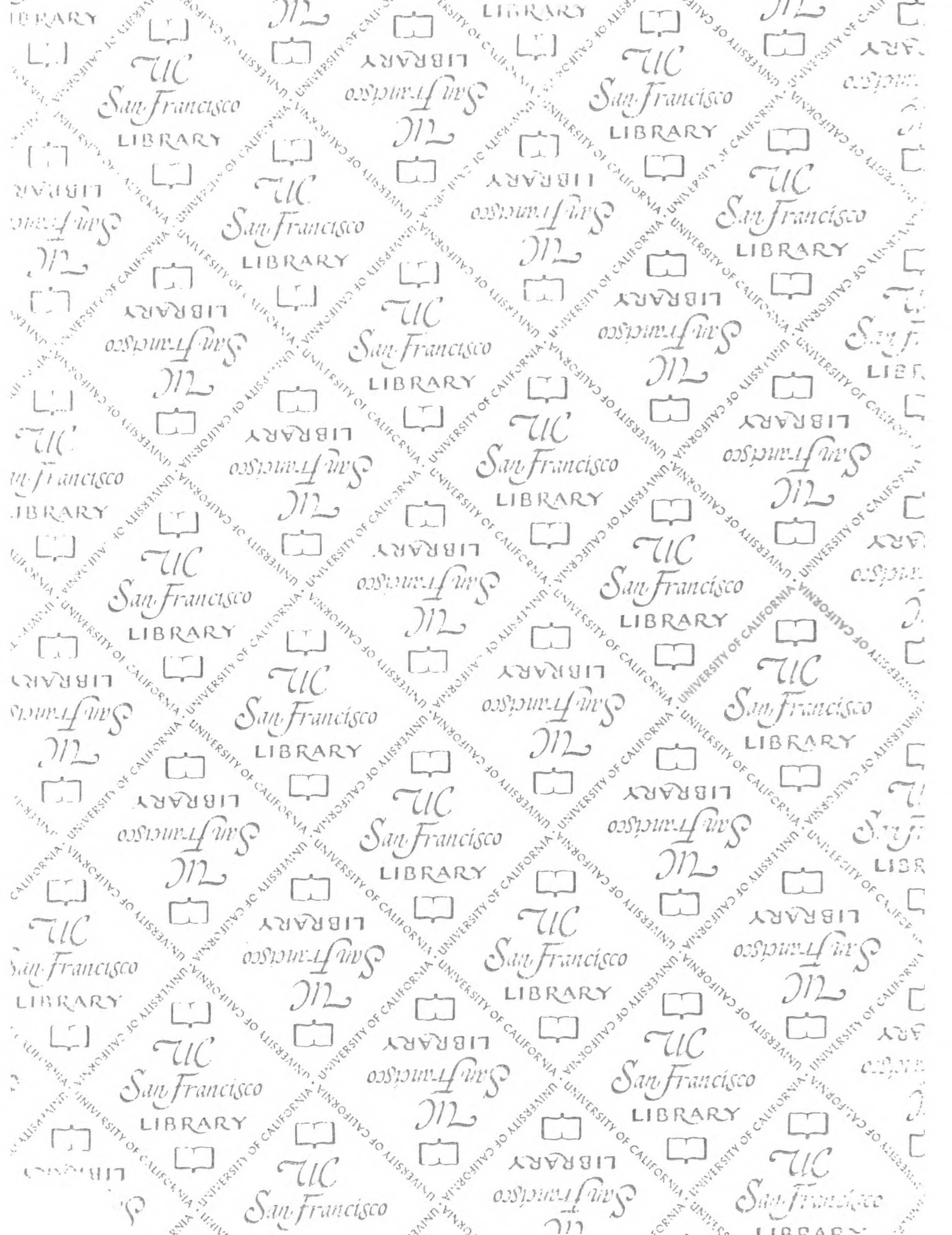


Figure 5.6 – AIP1 protein levels in response to Tat.

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