HIV ASSOCIATED NEUROCOGNITIVE DISORDERS AND LENTIVIRAL VECTOR-MEDIATED STABLE EXPRESSION OF ANTI-HIV-1 TAT INTRABODIES IN HUMAN MACROPHAGE, NEURONAL, AND PRIMARY PERIPHERAL BLOOD MONONUCLEAR CELLS AS A POTENTIAL THERAPY FOR NEUROAIDS

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SUMMARY

HIV-1 Tat is required for HIV replication and is also a known potent neurotoxin causing HIV-Associated Dementia. To test whether stable production of secreted Tat antibody in the brain could be an effective approach to inactivate Tat and thus provide protection from neuroAIDS, our research integrates HIV-1 Tat single chain variable fragment (scFv) intrabodies with a novel gene therapy approach utilizing monocytes, which naturally cross the blood-brain barrier, for gene delivery.

HIV-based defective lentiviral vectors were constructed to express one of two different HIV-Tat scFv antibodies or control scFvs with a CMV promoter and Fc-fusion protein and GFP as indicator genes. High titer vectors ($2x10^7$) were generated through calcium phosphate precipitation mediated transfection of human embryonic kidney 293T packaging cells, and tested for transduction of established human neuroblastoma (HTB-11) and microglial (CHME-5) cells, as well as primary peripheral blood mononuclear cells (PBMC). Expression of anti-HIV-Tat scFv in transduced cell lines was detected using optimized ELISA, Western Blot, and Immunofluorescent staining. Immunoblot and Neuroprotection assays were performed to assess anti-HIV-1 Tat scFv function and multiplex genetic expression analysis of 24 common reference genes was utilized to determine any cellular gene expression changes.

Efficient transduction ranging from 80% to 100% in HTB-11 and CHME-5 cell lines, determined by GFP quantification, was achieved at a multiplicity of

infection of 10 and confirmed by PCR. Long-term observation of transduced cells revealed no apparent change as compared to normal cells in terms of cell growth and morphology. Multiplex genetic expression analysis revealed similar gene expression levels in non-transduced and transduced cells. The expression of transgenes (GFP and anti-HIV-1 Tat scFv) in transduced cells was stable long term (>20 cell passages) and intracellular production of these genes was confirmed through Immunofluorescent staining. Western Blot assays confirmed anti-HIV-1 Tat scFv expression and ELISA quantitatively assessed secreted anti-HIV-1 Tat scFv concentrations to range from 350ng/mL in transduced CHME-5 to 700ng/mL in transduced HTB-11 cell lines. In addition, Immunoblot assays demonstrated the accurate biological function of secreted anti-HIV-1 Tat scFv by its specific binding to HIV-1 Tat protein in vitro and Neuroprotection assays against HIV-1 Tat and gp-120 demonstrated that anti-HIV-1 Tat scFv, both in transduced cells and conditioned media provided significant protection from both neurotoxins ($p<0.01$).

Primary PBMCs were isolated and transduced at a multiplicity of infection of 10, achieving 10% transduction efficiency as determined by GFP quantification and confirmed by PCR. ELISA detected secreted anti-HIV-1 Tat scFv, which provided significant protection from HIV-1 Tat and Gp120 neurotoxins ($p<0.01$). Multiplex genetic expression analysis revealed similar gene expression levels in both transduced and non-transduced PBMC cultures.

Findings from this study support the in-depth study of anti-HIV-1 Tat scFv, which will facilitate the development and potential use of the constructed lentiviral

vectors to deliver anti-HIV-1 Tat scFv into the brain for neuroprotective intervention using genetically modified macrophage cells as a vehicle.

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%	percentage
°C	Degree centigrade
AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
AIDS-KS	acquired immunodeficiency syndrome related Kaposi's sarcoma
AMP	ampicillin
ANI	asymptomatic neurocognitive impairment
APOE ε4	apolipoprotein E epsilon4 isoform
ART	antiretroviral treatment
ARV	antiretroviral
ATCC	America Tissue Cell Culture
BBB	blood brain barrier
BMDM	bone marrow monocyte derived macrophages
bp	base pair(s)
BSA	bovine serum albumin
C31S	Tat mutation of cysteine 31 to serine
CA3	hippocampal cornu ammonis field 3
Ca	calcium
$CaCl_2$	calcium chloride
CART	combined antiretroviral therapy
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CDC	Center for Disease Control and Prevention
CDK9	cyclin dependent kinase 9
cDNA	complementary DNA
CHARTER	CNS HIV Anti-Retroviral Therapy Effects Research

CHME-5	human microglial immortalized cell line
cm	centimeter
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CPE	CNS penetration effectiveness
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
CVD	cardiovascular disease
CXCR4	C-X-C chemokine receptor type 4
DAB	3,3-diaminobenzidine tetrahydrochloride
ddH_2O	double distilled water
DEPC	Diethylpyrocarbonate
DHHS	Department of Health and Human Services
DLV	defective lentiviral vector
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DPBS Ca-Mg	Dulbecco's phosphate buffered saline with calcium and magnesium
DTI	diffusion-tensor imaging
e. coli	*Escherichia coli*
eGFP	enhanced green fluorescent protein
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
Env	HIV-1 envelope protein
EtBr	ethidium bromide
FBS	fetal bovine serum
FD	fold difference
FDA	Food and Drug Administration
FDC	follicular dendritic cell

g	gravity
Gag	HIV-1 gag protein
GeXP	genetic expression
GFP	green fluorescent protein
gp120	HIV-1 glycoprotein 120
h	hour(s)
HAART	highly active antiretroviral therapy
HAD	HIV associated dementia
HAND	HIV associated neurocognitive disorders
HBS	Hepes Buffered Saline
HBV	hepatitis B virus
HCV	hepatitis C virus
HDS	HIV dementia scale
HEK 293T	human embryonic kidney 293 SV40 large T-antigen
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type I
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HTB-11	human neuroblastoma immortalized cell line
HTLV-III	Human T-cell Leukemia Virus III
IACUC	The Institutional Animal Care & Use Committee
ICTV	International Committee on Taxonomy of Viruses
IDU	injecting drug user
IgG	immunoglobulin G
IRES	internal ribosome entry site
IU	infectious units
Kan^r	kanamycin RNA
kb	kilobase
kDa	kilodalton
L	liter
LB	Luria-Berntani
LTR	long terminal repeat

M	molar
mA	milliamp
mAb	monoclonal antibody
MBL-2	mannose binding lectin 2
MCP-1	monocyte chemoattractant protein 1
MDM	monocyte derive macrophages
MEM	Eagle's minimal essential medium
MEM-10	Eagle's minimal essential medium with 10% FBS
meth	methamphetamine
µg	microgram
mg	milligram
min	minute(s)
µL	microliter
mL	milliliter
mm	millimeter
mM	millimolar
MND	mild neurocognitive disorder
moi	multiplicity of infection
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRS	magnetic resonance spectroscopy
MSK	Memorial Sloan-Kettering dementia severity scale
MSM	men who have sex with men
MTCT	mother-to-child transmission
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
$NaCl_2$	sodium chloride
$NaHCO_3$	sodium bicarbonate
Nef	HIV-1 negative regulatory factor
NeuroIRIS	neurologic immune reconstitution inflammatory syndrome
ng	nanogram
NIMH	National Institute of Mental Health
nm	nanometer

NMDA	N-methyl-D-aspartate
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with 5% Tween-20
PCR	polymerase chain reaction
PE	phycoerythrin
PEP	post-exposure prophylaxis
PEPFAR	President's Emergency Plan for AIDS Relief
PMTCT	prevention of mother-to-child transmission
Pol	HIV-1 pol protein
PPV	positive predictive value
PrEP	pre-exposure prophylaxis
RANTES	regulated upon activation, normal T cell expressed, secreted
Rev	HIV-1 regulator of virion expression
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI-1640	Roswell Park Memorial Institute medium
RRE	HIV-1 Rev response element
RT	reverse transcription
s	second(s)
scFv	single chain variable fragment
SDF-1	stromal derived factor-1
SDS	sodium dodecyl sulfate
TAR	trans-activation response
Tat	trans-activator of transcription
TB	tuberculosis
TBS	tris buffered saline
TBS-T	tris buffered saline with 5% Tween-20

TC	tissue culture
TE	Tris EDTA
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor alpha
U	Units
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
V	Volts
v/v	volume to volume
Vif	HIV-1 viral infectivity factor
Vpr	HIV-1 viral protein R
Vpu	HIV-1 viral protein U
VSV-G	vesicular stomatitis virus glycoprotein
w/v	weight to volume
WHO	World Health Organization
Zn	Zinc

CHAPTER 1

INTRODUCTION

1. Human Immunodeficiency Virus

1.1. Epidemiology of HIV

The first clinical cases of Human Immunodeficiency Virus (HIV), the virus that is now known to cause acquired immunodeficiency syndrome (AIDS), were reported in the United States in 1981 (CDC, 1981; Friedman-Kien *et al.*, 1981; Gottlieb *et al.*, 1981; Hymes *et al.*, 1981; Masur *et al.*, 1981). In the years following the initial reported cases, HIV was quickly recognized as a global epidemic. The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that 34 million people were living with HIV in 2010, an increase from the estimated 28.6 million in 2001 attributed in part to new infections, extended life expectancy of HIV infected individuals, and general population growth (UNAIDS, 2011c). HIV infection incidence declined by 15% from 2001 and 20% from 1997 to 2010, with only 2.7 million new infections in 2010, the equivalent of 7,000 new infections each day (UNAIDS, 2011b). Despite continuing new infections, the global prevalence among those aged 15 to 49 years has remained 0.8% since 2001 (UNAIDS, 2011a). Globally, nearly 30 million individuals died of AIDS-related causes since 1981. Of these, an estimated 1.8 million died in 2010, representing a 21% decrease from 2005 (UNAIDS, 2011c) mostly due to increased access to antiretroviral treatment (ART). However, HIV remains the leading cause of death in Africa (WHO, 2006).

While HIV infection leaves individuals vulnerable to many different opportunistic infections, the epidemic has particularly led to a resurgence of tuberculosis (TB), which is currently the prominent cause of death among HIV infected individuals worldwide and 13% of new TB cases occur in HIV infected individuals (UNAIDS, 2011c; WHO 2011).

The face of the HIV epidemic differs across varying regions. Almost all HIV infected individuals (97%) reside in middle- and low-income countries and 42% of new infections occur in young adults, ages 15-24 (UNAIDS, 2011b). In the United States and Europe, an estimated 2.2 million individuals are infected with HIV with the epidemic predominantly affecting injecting drug users (IDU) and men who have sex with men (MSM) (UNAIDS, 2011b). However, in sub-Saharan Africa, which is home to 67% of the world's HIV infected population and almost 15 million AIDS orphans, heterosexual contact and vertical mother-to-child transmission (MTCT) account for most new infections (UNAIDS, 2011c). Globally, an estimated 3.4 million children are infected with HIV, 91% residing in sub-Saharan Africa, with 390,000 new infections and 250,000 AIDS related deaths in 2010 (UNAIDS, 2010; UNAIDS, 2011c). Globally, women represent almost half of all HIV infections and 59% of infections in sub-Saharan Africa as a result of gender inequalities, sexual violence, limited access to healthcare, and increased biological susceptibility (UNAIDS 2011a). Many risk factors have been identified for HIV infection as a result of cohort studies of individuals seeking treatment as well as discordant couples. Some risk factors found to increase the risk of acquiring HIV infection include abuse of alcohol and IDU (de Azevedo *et al.*, 2007; Ruzagira *et al.*, 2011),

concurrent infection with a sexually transmitted disease (Ruzagira *et al.*, 2011; Steen *et al.*, 2004), engaging in unprotected intercourse, especially anal intercourse (Boily *et al.*, 2009; Weir *et al.*, 1999), increasing number of sexual partners (Baggaley *et al.*, 2010), being born to an HIV infected mother (Whitmore *et al.*, 2012), and lack of circumcision among males (Bailey *et al.*, 2007; Gray *et al.*, 2007).

1.2. Biology of HIV infection

HIV was first isolated in 1983 and named Human T-cell Leukemia Virus III (HTLV-III) by Robert Gallo of the University of Maryland in Balitmore, Maryland (Gallo *et al.*, 1983), and also separately yet simultaneously isolated by Luc Montagnier and Francoise Barre-Sinoussi of the Pasteur Institute in Paris, France (Barre-Sinoussi *et al.*, 1983) from a patient considered at risk for AIDS. As a compromise to the co-discoverers, the isolated virus was renamed Human Immunodeficiency Virus. HIV, classified as a lentivirus in the retroviridae family (ICTV, 2009), is composed of an RNA genome containing three sets of viral proteins including structural proteins (Gag, Pol, Env), regulatory proteins (Tat, Rev, Nef), and maturation proteins (Vif, Vpu, Vpr) (Bunnell *et al.*, 1998). Cellular CD4 receptor is necessary for HIV infection in conjunction with at least one chemokine co-receptor including CXCR4, the receptor for stromal derived factor-1 (SDF-1) and CCR5, the receptor for RANTES (He *et al.*, 1997; Premack *et al.*, 1996). Early after infection, widespread dissemination of HIV occurs in association with large number of virions seeded in lymphoid organs and trapped on follicular dendritic cells (FDCs) located in the lymph nodes (Steinman, 2000). FDCs maintain HIV infection and actively convert neutralized HIV into infectious virus (Burton *et al.*, 2002). This

is associated with a rapid increase in plasma viremia (Embretson *et al.*, 1993; Pantaleo *et al.* 1993), in excess of 1 million RNA molecules per milliliter (Rosenberg *et al.*, 1997), followed by a reduction in viral replication to a steady-state viral set-point (Daar *et al.*, 1991; Piatak *et al.*, 1993) as a result of broad HIV-1-specific cytotoxic T lymphocyte (CTL) response (Borrow *et al.*, 1997; Musey *et al.*, 1997). Individuals with high viral set-points have more rapid rates of disease progression (Mellors *et al.*, 1996) while individuals with a more successful CTL response and subsequently lower viral set-point have an associated slower progression to AIDS and death (Pantaleo *et al.*, 1997). HIV infection depletes gut-associated memory T cells, leading to massive CD4 depletion and exhaustion of homoeostatic T-cell responses following chronic immune activation (McArthur *et al.*, 2010). Opportunistic infections and cancers develop as a result of induced severe cellular immunodeficiency.

A reverse transcriptase enzyme reverse transcribes the HIV RNA genome into proviral DNA which is integrated into the host cell genome where it can remain latent without affecting cellular function for many years. Subsequent cellular activation triggers provirus production of retrovirus mRNA and viral replication is active in most infected individuals even during clinical latency following initial infection (Michael *et al.*, 1992; Ho *et al.*, 1995). Some HIV infected individuals, termed elite controllers, are able to control HIV replication without treatment (Pereyra *et al.*, 2009). Figure 1 compares disease progression of normal rapid progressors and elite controllers. Epidemiological and molecular studies of elite

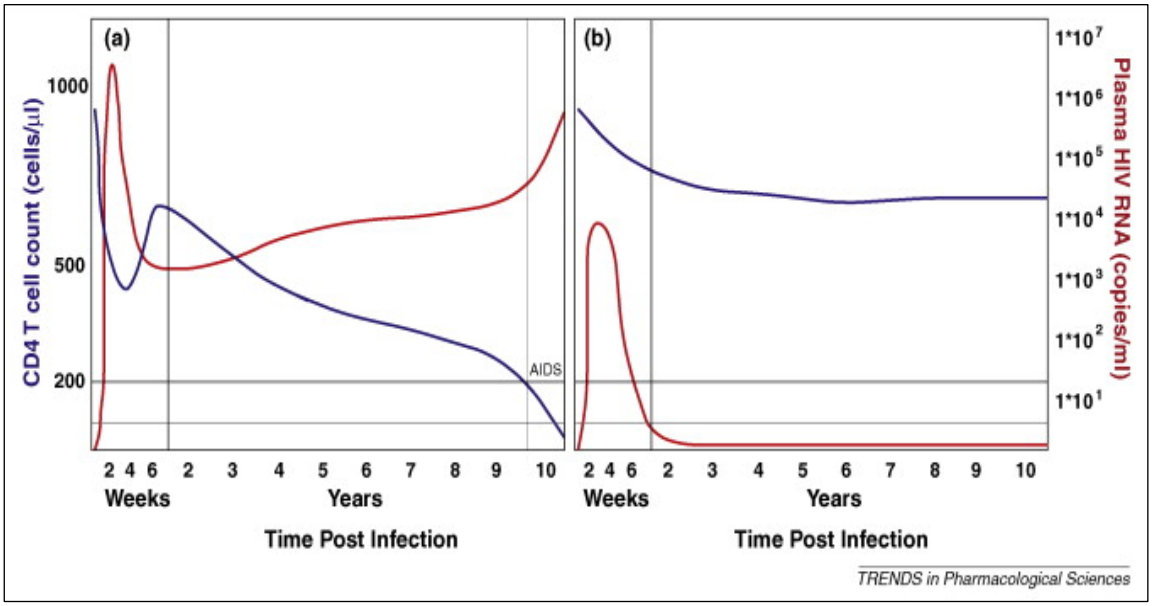

Figure 1. Plasma Viral Load in Normal Progressors and Elite Controllers.

Following HIV infection in a normal progressor (a) plasma viral load (red) sharply spikes and then slowly increases over time and CD4 T cell count (blue) varies inversely while in an Elite Controller (b) plasma viral load is quickly suppressed and never exceeds CD4 T cell count, which is consistently maintained over many years (O'Connell *et al.*, 2009).

controllers, less than 1% of the HIV infected population (Okulicz *et al.*, 2009, Okulicz *et al.*, 2011), have revealed important immune differences associated with prolonged periods, at least 10 years, of spontaneously controlled viremia. Genetically, studies have overall found an overrepresentation of protective HLA alleles (Han *et al.*, 2008; Migueles *et al.*, 2010; The International HIV Controllers Study, 2010) as well as a reduced capacity for viral reservoirs, demonstrated by much lower quantities of HIV DNA isolated from peripheral blood mononuclear cells (PBMC) (Lambotte *et al.*, 2005; Sajadi *et al.*, 2007). Also of interest is the adaptive immune response of HIV-1 specific CD8 T cells in elite controllers. This response is predominantly against the HIV-1 Gag protein (Emu *et al.*, 2005; Saez-Cirion *et al.*, 2009), the CD8 T cells are polyfunctional (Betts *et al.*, 2006; Lopez *et al.*, 2011), and produce perforine to suppress viral replication (Hersperger *et al.*, 2010; Migueles *et al.*, 2002). Additionally, elite controllers have been found to have higher plasma levels of antibodies to HIV-1 Tat (Re *et al.*, 2001a; Van Baalen *et al.*, 1997; Zagury *et al.,* 1998). These findings provide important implications for the development of vaccines and treatments for HIV infected individuals.

1.3. Prevention and treatment

Antiretroviral (ARV) agents have been developed to inhibit HIV. However, less than half of HIV infected individuals have access to this expensive therapy, especially in resource-limited countries, which bear the majority of the burden of the HIV epidemic (UNAIDS, 2011a). Therefore, behavioral risk reduction interventions play an important role in reducing HIV incidence. Recently, several studies in sub-Saharan Africa have found that male circumcision significantly

reduces the risk of HIV infection among men (Bailey *et al.*, 2007; Gray *et al.*, 2007; Williams *et al.*, 2006). Other interventions include male and female condom use (Wariki *et al.*, 2012), needle exchange programs (Uuskula *et al.*, 2011), pre-exposure prophylaxis such as vaginal microbicides and topical antiretrovirals (Agashe *et al.*, 2012; Kiser *et al.*, 2012), and exclusive breastfeeding for children born to infected mothers (Read, 2012). Discordant couple studies have revealed that a lower viral load in the infected partner is associated with lower risk of infection for the sero-negative partner (Cohen, *et al.*, 2010). Coupled with the finding that treatment of infected mothers has resulted in significantly increased prevention of MTCT (PMTCT) (Coovadia, 2004), there is currently an emphasis on promotion of treatment as prevention.

Theoretically, the HIV replication cycle can be interrupted by blocking or inhibiting cellular entry, the function of one or more of the key viral proteins, or viral packaging and release (Bunnell *et al.*, 1998). Currently, there are seven classes of ARV agents, five of which are approved by the US Food and Drug Administration (FDA) including chemokine receptor antagonists, nucleoside (NRTI) and non-nucleoside (NNRTI) reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors, and maturation inhibitors (Adamson *et al.*, 2008). Figure 2 outlines these classes and the target mechanism of each. The first ARV, zidovudine, was produced in 1987. Subsequently, the FDA has approved more than 30 ARV agents. In 2005, the US Department of Health and Human Services (DHHS) released federal guidelines for ARV drug usage in HIV infected adults and adolescents, recommending that Highly Active Antiretroviral Therapy (HAART), typically a

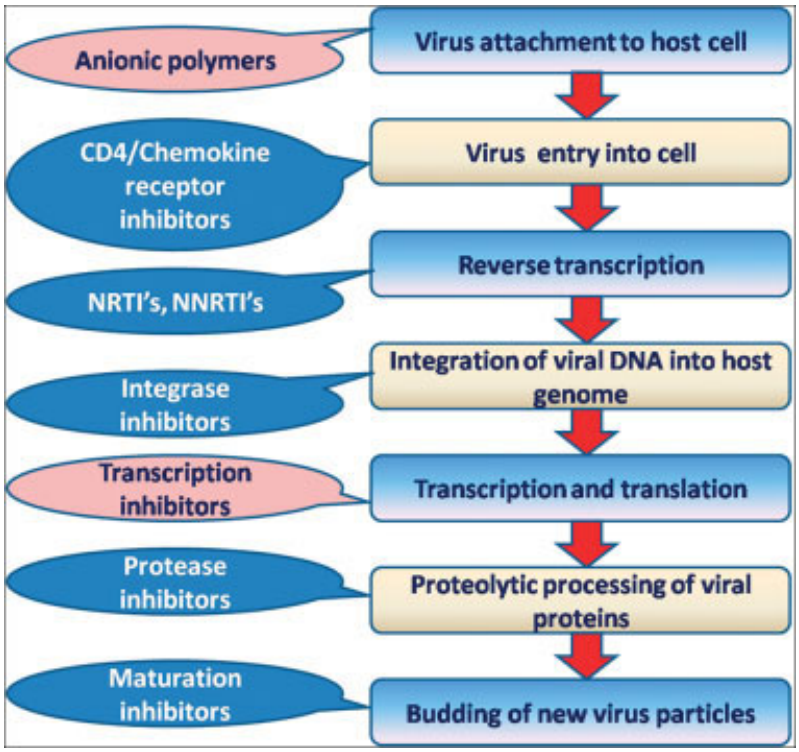

Figure 2. ARV target mechanisms of the HIV-1 life cycle.
There are seven potential classes of antiretroviral agents. Five are currently available and approved by the FDA (blue) and two are theoretical and currently undergoing research (pink) (McArthur *et al.*, 2010).

combination of 3 or 4 ARV agents, is initiated when a patient's CD4 count drops below 350/mm^3 or plasma HIV RNA exceeds 100,000 copies per milliliter (DHHS, 2005). However, numerous studies have demonstrated the benefits of earlier initiation of HAART (Zolopa *et al.*, 2009).

In 2009, the World Health Organization (WHO) recognized for the first time that incidence rates for HIV/AIDS were beginning to decline. This is mostly attributed to reduced transmission as a result of HAART as well as increased awareness of HIV and successful preventative interventions (McArthur *et al.*, 2010). Access to HAART has dramatically increased from less than half a million individuals in 2001 to 6.6 million in 2010 (UNAIDS, 2011c). One major success in the HAART era has been the dramatic decrease in the rates of HIV/AIDS in children acquired through MTCT as a result of the administration of HAART or even single dose ARV to pregnant women, decreasing MTCT rates by 25% (Anoje *et al.*, 2012). The proportion of eligible pregnant women receiving HAART for prevention of MTCT has increased from only 15% in 2005 to 48% in 2010 (UNAIDS, 2011c). A second success has been the global distribution of HAART, particularly in sub-Saharan Africa, made possible through the President's Emergency Plan for AIDS Relief (PEPFAR). PEPFAR was initiated in 2002 and is responsible for supplying ARV agents to millions of eligible individuals at a projected cost of $7.2 billion in 2012. In sub-Saharan Africa, the number of individuals receiving HAART increased 20% from 2009 to 2010 (UNAIDS, 2011c).

Despite the successes of HAART, it is not a cure for HIV and only 47% eligible individuals are currently receiving treatment (Cobos-Jimenez *et al.*, 2011;

UNAIDS, 2011c). Additionally, as a result of the millions of replication cycles occurring daily and the high error rate in HIV RNA transcription, viral mutants readily develop. While most have reduced replication fitness, mutants do arise that have a high level of fitness and are resistant to multiple antiretrovirals, mostly as a result of inconsistent ARV regime adherence (McArthur *et al.*, 2010). Additionally, antiretrovirals have a limited ability to cross the blood brain barrier (BBB), leaving the central nervous system (CNS) a reservoir for HIV and a site for unchecked HIV replication (Pardridge, 2002). HAART is not an effective therapy for suppressing HIV in the CNS. Therefore, new treatments that will be unimpeded by the BBB are vital for combating the HIV epidemic and the consequences thereof.

2. HIV Associated Neurocognitive Disorders

2.1. Etiology

HIV enters the CNS within the first two weeks of primary HIV infection (Davis *et al.*, 1992) predominantly through infected macrophage cells that migrate across the BBB, known as the "Trojan horse" hypothesis (Peluso *et al.*, 1985), and to a much smaller extent as cell free virus by infecting the endothelial cells of the BBB and diffusing into the CNS (Argyris *et al.*, 2003). HIV-1 replication in hematopoietic progenitor cells induces activation of circulating monocytes and monocytes in the bone marrow whose subsequent circulation in the blood appears to be the most critical step for brain entry (Alexaki *et al.*, 2008). Additionally, monocyte ingress is facilitated by the expression of an amyloid precursor protein within the brain and on circulating monocytes (Vehmas *et al.*, 2004). HIV infected monocytes that cross

the BBB differentiate into macrophages and subsequently infect other CNS cells such as microglia, astrocytes and perivascular macrophages through direct contact (Brack-Werner, 1999; Clay *et al.*, 2007). Microglia and macrophages, which sustain a productive infection without cellular activation (Schnell *et al.*, 2011), become cellular centers for further viral replication within the CNS (Gonzalez-Scarano *et al.*, 2005) and macrophages have been observed to function as long-lived reservoirs during latent infection (Masliah *et al.*, 1992a). Studies have shown that in later stages of infection, HIV isolated from the CNS is often phylogenentically distinct from virus in the plasma, most likely due to the fact that microglia almost exclusively express the HIV co-receptor CCR5, whereas outside of the CNS HIV utilizes co-receptors CCR5 or CXCR4 for cell infection (Schnell *et al.*, 2010).

Neurons are not infected by HIV, but are severely impacted by the direct and indirect effects of CNS HIV infection (Rackstraw, 2011). Cognitive impairment underlying HIV associated neurocognitive disorders (HANDs) is a result of neuronal damage from either direct and/or indirect neurotoxic effects of HIV infection, classified as primary HAND, or opportunistic infections and treatment related effects, classified as secondary HAND (Kaul *et al.*, 2001). The main source of direct neuronal damage is neurotoxic viral proteins HIV-1 Tat (Rappaport *et al.*, 1999) and gp120 (Giulian *et al.*, 1993), which are secreted by HIV infected cells within the CNS and have the capability of being transported along axonal pathways and causing damage remotely (Bruce-Keller *et al.*, 2003). Brief exposure to these viral proteins is sufficient to cause neuronal damage and induce cellular activation through positive feedback loops which leads to a self sustaining and long lasting

cascade of events, termed the "hit and run phenomenon" (Chauhan *et al.*, 2003; Williams *et al.*, 2002; Nath *et al.*, 1999). Neuronal apoptosis, programmed cell death, and necrosis contribute to reduced neural cell viability and survival (Jones *et al.*, 2006). Indirect damage is attributed to a chronic inflammatory process induced by the presence of HIV and HIV infected cells in the CNS, leading to dysregulation and eventual death of neurons and astrocytes (Gonzalez-Scarano *et al.*, 2005; Gorry *et al.*, 2003). Chronic immune activation and depletion of regulatory T cells associated with HIV disease progression leads to overproduction of various proinflammatory cytokines and chemokines within the CNS resulting in the dysregulation of macrophages (Genis *et al.*, 1992). A recent study found a correlation between immunoproteasome induction during HIV infection and neurological deficits, possibly as a result of perpetuation of inflammatory responses (Nguyen *et al.*, 2010). Figure 3 illustrates HIV neuroinvasion and the subsequent neuronal damage.

2.2. Clinical presentations and pathology

The clinical presentation, diagnosis, and treatment of HANDs have evolved since HIV was first recognized, with the most distinct change occurring after the introduction of HAART. In the pre-ART era, a variety of opportunistic infections and tumors of the CNS with a high mortality rate were recognized as AIDS defining illnesses (Snider *et al.*, 1983). A large number of newly diagnosed HIV infected patients presented with diseases that affected the CNS and although the neurocognitive state was affected, patients rarely lived for long periods of time with

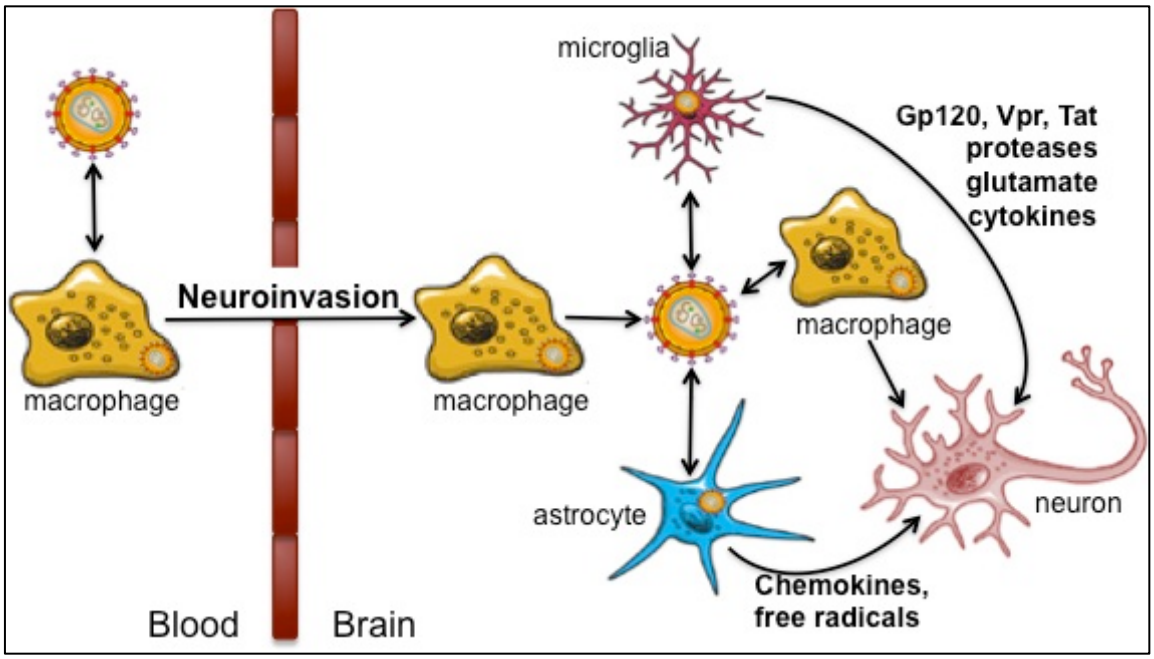

Figure 3. Mechanism and Consequence of HIV Neuroinvasion.

HIV-1 crosses the blood-brain barrier via infected macrophages. Infected macrophages, astrocytes, and microglia in the brain release HIV-1 proteins, such as Gp120, Tat, and Vpr, and host chemokines and cytokines, which act as neurotoxins, leading to inflammation and neuronal apoptosis, the underlying cause of HAND (adapted from Power *et al*., 2009b).

cognitive impairments. As it became evident that opportunistic infections were not the sole source of CNS damage, but that HIV was able to enter the CNS (Ho *et al.*, 1985), causing disabling cognitive and motor dysfunction that progressively worsened and eventually led to death (Navia *et al.*, 1986), a syndrome named the AIDS dementia complex or HIV dementia, the Centers for Disease Control (CDC) revised its surveillance case definition for AIDS to include this syndrome as an AIDS-defining illness (CDC, 1987). AIDS dementia complex presented as a subcortical dementia and was described as similar to the dementia observed in Parkinson's disease rather than cortical dementias such as Alzheimer's disease (Navia *et al.*, 1986). Morphometric studies found neuronal density decrease of 40% in the frontotemporal areas (Everall *et al.*, 1991; Ketzler *et al.*, 1990; Masliah *et al.*, 1992a) and 50% to 90% in the hippocampus (Masliah *et al.*, 1992b). A system for grading the severity of AIDS dementia complex was proposed in 1988, based on presenting clinical features, ranging from 0-normal to 4-end-stage (Price *et al.*, 1988) and the suggested method for distinguishing AIDS dementia complex from other opportunistic infections was autopsy (CDC, 1987). Table 2 lists common nervous system syndromes and opportunistic infections associated with HIV infection.

Following the introduction of HAART, the clinical characteristics of AIDS dementia complex began to change as it was observed to occur at much higher CD4 counts, was milder but still prevalent, and was suggested to likely be the first indication of AIDS-defining illness (Dore *et al.*, 1999; McArthur, 2004; Sacktor *et al.*, 2002). In response to the AIDS dementia complex evolution, the National Institutes

Table 1. Selected primary and secondary nervous system syndromes associated with HIV-1 infection.

There are many recognized central and peripheral nervous system syndromes that result from opportunistic infections, antiretroviral toxicities, and direct HIV-related damage (Power *et al.*, 2009).

Primary HAND:	
Central Nervous System:	Aseptic Meningitis Asymptomatic neurocognitive impairment Mild neurocognitive disorder HIV-associated dementia Primary HIV-Induced Headache
Neuromuscular disorders:	Mononeuritis Multiplex Diffuse Infiltrative Lymphocytosis Syndrome Guillian-Barré Syndrome Motor Neuron Disease Syndrome Entrapment/Mono-neuropathies Neuropathies Autonomic neuropathy Myopathies
Secondary HAND:	
Opportunistic Infections:	Toxoplasmic encephalitis Cryptococcal Meningitis Progressive Multifocal Leuko-encephalopathy Primary CNS lymphoma CNS tuberculosis Cytomegalovirus encephalitis and radiculitis Multidermatomal herpes zoster
Antiretroviral Toxicities:	Exacerbated distal sensory polyneuropathy Myopathies

of Health and Mental Health (NIMH) in the United States redefined the case definitions in 2005 for HIV-associated neurocognitive disorders (HAND) (Antinori *et al.*, 2007) to encompass a hierarchy of progressively more severe disease, ranging from asymptomatic neurocognitive impairment (ANI) and minor neurocognitive disorder (MND), to the most severe HIV-associated dementia (HAD). HANDs must be caused entirely by HIV, with no pre-existing conditions, and in the absence of delirium. Neurocognitive domains that are known to be affected by HIV infection include simple motor skills or sensory perception abilities, complex perceptual motor skills, language, abstraction executive skills, information processing, memory including learning and recall, and attention. ANI is defined as acquired impairment in cognitive functioning in two or more of the above mentioned domains at least one standard deviation below the mean for age and education adjusted population norms that does not interfere with everyday functioning. MND is similarly defined, however the cognitive impairment produces at least mild interference in daily function and the neuropsychological assessment typically corresponds to a Memorial Sloan-Kettering (MSK) scale stage of 0.5 – 1.0. HAD is defined as acquired impairment in cognitive functioning in at least two of the above mentioned domains, each two standard deviations or greater from age and education adjusted population norms, typically corresponding to an MSK scale stage of 2.0 or greater, with marked interference in daily functioning (Antinori *et al.*, 2007).

HAND progresses slowly, with the exception of neurological immune reconstitution inflammatory syndrome (NeuroIRIS), which is characterized by

inflammation in the CNS as a result of infiltration by CD8 T lymphocytes in response to opportunistic infections, and occasionally HIV, as the immune system reconstitutes (Langford *et al.*, 2002; Langford *et al.*, 2006; Venkataramana *et al.*, 2006). Clinical presentation in the early stages of HAND includes apathy, mental slowing, reading and comprehension difficulties, and short-term memory loss. Later stage clinical presentations include gait disturbance with stumbling and tripping, tremor and impairment of fine manual dexterity, impaired rapid eye movements, hyper-reflexia, and release signs (McArthur *et al.*, 2010). Since the introduction of HAART, HANDs have evolved from a predominately subcortical disorder to a mixed pattern of both subcortical and cortical (Dore *et al.*, 2003; Masliah *et al.*, 2000). Most productive HIV infection occurs within the basal ganglia, brainstem, and deep white matter (Brew *et al.*, 1995; Kure *et al.*, 1991), with neuropathological changes most prominent in the basal ganglia (Everall *et al.*, 1995). Neuronal loss and synaptodendritic simplification are common pathological features (Thompson *et al.*, 2001). Pathogenic events in HIV infected children differ slightly from adults and include high HIV production, prominent basal ganglia calcification, and a more florid infection of subcortical astrocytes (da Cunha *et al.*, 1997). Clinical features of HANDs in HIV infected children include delayed or regressing developmental milestones, microcephaly, and spastic paraparesis (Brew, 2009). Prior to the introduction of HAART, survival was limited to 6 to 24 months (Epstein *et al.*, 1988).

2.3. Epidemiological trends

Globally, HIV associated neurocognitive disorders are the most common form of young-age dementia (Wright *et al.*, 2008). In the pre-ART era, AIDS dementia complex, a common presentation of end-stage disease, was estimated to affect up to 40% of patients dying of AIDS (Rackstraw, 2011) and 30% of congenitally infected children (Mintz *et al.*, 1989) although autopsy studies suggest more than of 90% of HIV/AIDS patients exhibited neuropathological changes (Johnson, 1998). Introduction of the first licensed anti-retroviral, zidovudine, in 1987 led to a reduction in the incidence of AIDS dementia complex and increased the length of survival following diagnosis (Portegies *et al.*, 1989; Portegies *et al.*, 1993). An even more dramatic decrease, approximately 75%, in the incidence of AIDS dementia complex as well as opportunistic infections and AIDS-defining cancers of the CNS, was observed after the introduction of HAART (Sacktor *et al.*, 2001b). HAART also reduced the severity of dementia from 7% in 1989 to 1% in 2000 (McArthur *et al*, 2004). Despite the decrease in incidence, the overall prevalence of HANDs increased due to the increasing incidence of HIV infection as well as the drastically improved survival benefit conferred by HAART which lead to the development of HANDs in individuals with less advanced immunosuppression (Dore *et al.*, 2003; Maschke *et al.*, 2000; McArthur *et al.*, 2003).

A recent study, the CNS HIV Anti-Retroviral Therapy Effects Research project (CHARTER), followed a cohort of 1,500 HIV infected patients from across the United States from 2003-2007, and found that while 53% of patients were diagnosed with HANDs, only 2% met the case definition criteria for HAD (Heaton *et*

al., 2010). Figure 4 illustrates the findings of the CHARTER study in comparison to pre-HAART estimates of HAND prevalence. HIV infected individuals suffering from ANI, approximately 30%, are more likely to develop a more severe form of HANDs (Ellis *et al.*, 2007). MND, identified in 20% to 30% of HIV infected individuals, is associated with shortened survival, reduced adherence to anti-retrovirals, and is predictive of HIV encephalitis (Cherner *et al.*, 2002; Hinkin *et al.*, 2002; Janssen *et al.*, 1989). HAD has been identified in 2% to 8% of individuals receiving HAART, and has been found to shorten survival to one third that estimated for individuals without HANDs (Power, 2009; Sacktor *et al.*, 1996). Globally, cohorts from countries with high HAART accessibility have demonstrated similar findings, with HAND diagnosis ranging from 20-85% (Bonnet *et al.*, 2009; Dulioust *et al.*, 2009; Vassallo *et al.*, 2009; Ciccarelli *et al.*, 2010; Simioni *et al.*, 2010). Although knowledge of the epidemiology of HANDs in resource-limited settings, such as Africa and the Asia-Pacific region, is limited, available data from recent cohorts suggest that HANDs occur as frequently as in developed countries (Nakasujja *et al.*, 2005; Wong *et al.*, 2007; Wright *et al.*, 2008a).

Despite ongoing research, several important questions pertaining to the recent shift in HANDs incidence and prevalence trends remain controversial. It has been suggested, but not thoroughly explored, that the reduced incidence of HANDs, which has largely been attributed to the effects of HAART, may naturally occur through innate control mechanisms, as seen in elite controllers (Pereyra *et al.*, 2009). Finally, and perhaps most critical, is the question of whether all individuals with well-controlled HIV infection will inevitably develop HANDs, given adequate

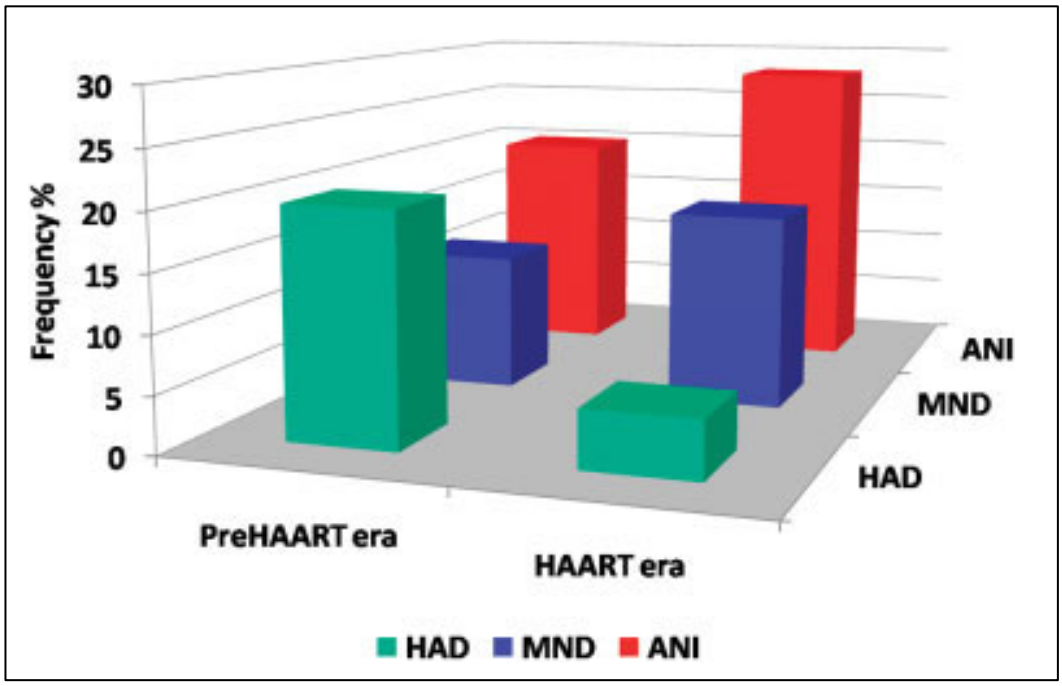

Figure 4. HAND prevalence in HAART era remains high despite a decrease in severity as compared to the pre-HAART era.
(Heaton *et al.* 2010)

time (McArthur *et al.*, 2010). For example, the CHARTER study demonstrated that the rate of HANDs in those with no detectable plasma viral load and nadir CD4≥200 was almost half that in patients with less successful virological control, suggesting that cognitive function may be preserved in those with well-controlled HIV viremia (Cole *et al.*, 2007). However, other studies have identified HANDs in up to 34% of aviremic patients with nadir CD4<200, suggesting that there may be a nonreversible component of neurological injury (Cysique *et al.*, 2006; Robertson *et al.*, 2007; Sevigny *et al.*, 2007).

2.4. Risk factors

HIV neuroinvasion occurs in all infected individuals. However, clinically evident neurological disease, or neurovirulence, only affects a subset of patients, most likely due to specific susceptibility variables (Power *et al.*, 2009b). Prior to the introduction of HAART, low CD4 count and high levels of plasma viremia were the two most prominent predictors of HANDs (Childs *et al.*, 1999). In the HAART era, it has become evident that nadir CD4 count, which may represent the point at which immune dysregulation within the CNS is established, is also important (Munoz-Moreno *et al.*, 2008). Cerebrospinal fluid (CSF) HIV DNA levels greater than that in the plasma are correlated to the development of neurocognitive impairment (Ragin *et al.*, 2010; Sevigny *et al.*, 2004). Additional risk factors include co-morbidities, injection drug use, age, low body mass index, systemic symptoms, female sex, and several genetic factors (Chiesi *et al.*, 1996; Childs *et al.*, 1999; McArthur *et al.*, 2004). Figure 5 explores the relationship between HANDs and co-morbidities, nadir CD4 count, and plasma viral load.

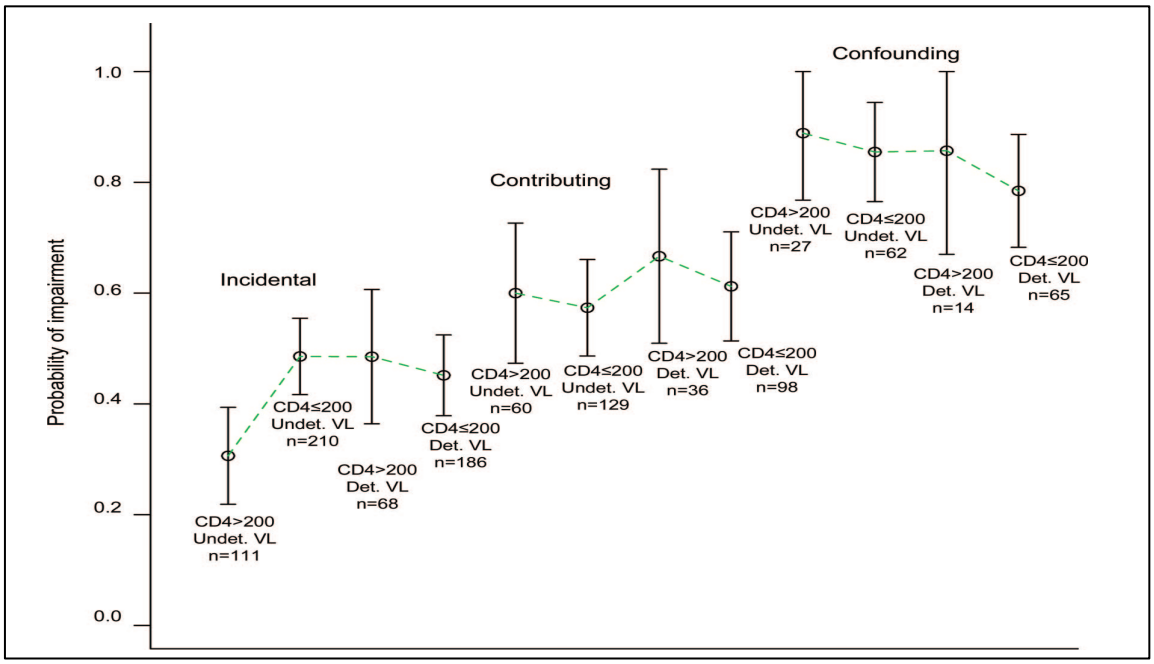

Figure 5. HAND risk factors.

The CHARTER Project explored the probability of neurocognitive impairment in HIV-1 infected individuals receiving HAART stratified by comorbidity group, plasma HIV-1 viral load (UD = undetectable, Det = detectable), and nadir CD4 count (Heaton *et al.*, 2010).

Higher rates of HAND have been found in HIV infected individuals with comorbid illnesses including hepatitis B virus (HBV), hepatitis C virus (HCV), diabetes, and cardiovascular disease (CVD) (Heaton et al, 1995). HIV infected individuals have a higher risk of infection with HBV and HCV, as all three viruses have similar modes of transmission. HCV is independently associated with neurocognitive impairment, which affects one third of those infected with HCV (Forton *et al.*, 2002; Hilsabeck *et al.*, 2002). Individuals co-infected with HIV and HCV are almost twice as likely to develop neurocognitive impairment compared to individuals infected with HIV alone, suggesting an additive effect of co-infection (Letendre *et al.*, 2005; Lu *et al.*, 2009; Perry *et al.*, 2005; Ryan *et al.*, 2004).

Intravenous drug use (IDU) also increases the risk of developing HANDs in HIV infected individuals as well as increasing the risk of contracting HCV. Immune activation is one proposed mechanism for the independent association between IDU and HANDs in the HIV infected (Ancuta *et al*., 2008) and particularly injecting stimulants, such as methamphetamine (meth), has been found to increase this risk (Rippeth *et al.*, 2004). Chronic meth use is known to induce structural changes in the hippocampus, correlating to memory loss (Sharma *et al.*, 2009). A study based in the United States examined the rates of neurocognitive impairment between HIV-negative non-meth users, HIV-negative meth users, HIV-positive non-meth users, and HIV-positive meth users, revealing that only HIV-negative non-meth users exhibited no increase. Similar levels of cognitive impairment were found in HIV-positive non-meth users and HIV-negative meth users, but even higher rates

were detected in HIV-positive meth users suggesting that meth use increases the risk of cognitive impairment in HIV infected individuals (Rippeth *et al.*, 2004).

HAART currently extends the life span of HIV infected individuals by 10.6 years for those with AIDS and 21.5 years for those without AIDS (Fang *et al.*, 2007). The aging HIV-infected population is affected by many of the same comorbidities as the aging non-infected population (Alisky, 2007) and recent studies have concluded that aging increases the risk of development of HANDs in HIV infected adults (Valcour *et al.*, 2004b; Valcour *et al.*, 2004c). High rates of HANDs are similarly observed in HIV-infected adolescents who acquired HIV infection from maternal transmission, possibly reflecting long term neurological damage caused by HIV and its interaction with the developing brain (Wood, *et al.*, 2009; Paramesparan *et al.*, 2010). Low blood CD8+ T-lymphocytes and high circulating monocytes are also identified risk factors for HANDs in HIV infected children (Sanchez-Ramon *et al.*, 2003). Other co-morbidities such as diabetes and traditional cardiovascular risk factors have been found to be associated with increased rates of HANDs (Valcour *et al.*, 2005; Wright *et al.*, 2010).

Neurosusceptibility is also impacted by host genetic factors. Severity of dementia is associated with the epsilon4 (E4) isoform for apolipoprotein E (APOE) among older HIV infected individuals, possibly through increasing neuronal vulnerability to oxidative stress (Corder *et al.*, 1998; Valcour *et al.*, 2004a). A recent study found that HANDs were more prevalent in those with the APOE E4 and MBL-2 O/O haplotypes in a Chinese cohort who contracted HIV infection from infected blood products (Spector *et al.*, 2010). Additionally, high concentrations of CSF CCL2

(monocyte chemoattractant protein 1) and plasma tumor necrosis factor α (TNF-α) are predictors of HAND development (Eugenin *et al.*, 2006). Specifically, polymorphisms in the TNF-α promoter and CCL2 (MCP-1) or its receptor CCR2 (64-I allele) are associated with HAD, possibly by affecting neuronal vulnerability of TNF-α toxicity and monocyte infiltration into the CNS, respectively (Gonzalez *et al.*, 2002; Singh *et al.*, 2004; Quasney *et al.*, 2001).

2.5. Diagnosis and Treatment

Diagnosing HANDs can be challenging, and ANI and MND especially are commonly overlooked unless specifically screened for. Delayed diagnosis inevitably occurs in patients presenting with symptoms of neurocognitive impairment who have not previously been diagnosed with HIV infections, particularly those not believed to be at high risk for HIV infection (Navia *et al.*, 1987). Even among individuals with known HIV infection, lack of symptom disclosure and the non-specific nature of mild HAND symptoms can also lead to delayed diagnosis (Rackstraw, 2011). Erratic clinic attendance and poor ART adherence are often indications for HANDs screening (Andrade *et al.*, 2005; Zogg *et al.*, 2010). While adherence levels to medication regimes are affected by many factors, an unimpaired, functioning prospective memory is required for good adherence. Other functional tasks affected by HANDs include driving, meal preparation, and managing household finances (Benedict *et al.*, 2000; Marcotte *et al.*, 2004). Poor job performance and unemployment are also associated with HANDs (Heaton *et al.*, 1994), illustrating the importance of psychosocial history documentation.

Expanding knowledge of the functional consequences of HANDs, most notably poor ARV adherence, which leads to increased morbidity and mortality (Paterson *et al.*, 2000), has increased awareness of the benefits of screening for HANDs. However, the number of HIV infected individuals receiving treatment continues to increase and providing a thorough screening for each patient is not economically feasible. In response, tools and guidelines are being developed and validated for efficient screening. For example, the European AIDS Clinical Society has established guidelines suggesting that screening be provided only for patients who have detectable plasma HIV RNA, CD4 count nadir of less than 200, ongoing depression, or are taking anti-retroviral regimes with limited CNS penetration (European AIDS Clinical Society, 2009). One screening tool, validated in English and Spanish, the HIV dementia scale (HDS), consists of four short tests of memory, attention, psychomotor speed, and construction. Scores of 10 or less out of 16 are indicative of HAD with a sensitivity of 80%, specificity of 91%, and positive predictive value (PPV) of 78% (Power *et al.*, 1995; Wojna *et al.,* 2007). Increasing the score for indication of a positive screening result from 10 to 14 identifies both HAD and MND with a sensitivity of 83%, specificity of 63%, and PPV of 92% and ANI with a sensitivity of 88%, specificity of 67%, and PPV of 82% (Simioni *et al.*, 2010). A variation of the HDS, the International HIV dementia scale has also been cross-culturally validated in Uganda but tests only memory, motor speed, and psychomotor speed with a sensitivity of 80% and specificity of 57% (Sacktor *et al.*, 2005b). Studies are currently underway for developing validated computerized screening tests for HAD as well as MND and ANI (Cysique *et al.*, 2006).

Currently there are no clinically available biomarker based tests for definitive HAND diagnosis and one major factor in HAND diagnosis is exclusion of opportunistic infections. For example, CSF analysis is used to exclude cryptococcal tuberculous meningitis in febrile or encephalopathis patients. Several CSF markers based on inflammatory byproducts associated with HAND severity (Price *et al.*, 2007) currently being evaluated as diagnostic biomarkers including neopterin (Yilmaz *et al.*, 2008), soluble Fas (Towfighi *et al.*, 2004), protein carbonyls (Turchan *et al.*, 2003), sphingolipid products (Haughey *et al.*, 2004), and nitrosulated proteins (Li *et al.*, 2008).

Brain imaging is often utilized to exclude CNS opportunistic infections, but is also used to confirm and track the progression of CNS damage in HANDs. Magnetic resonance imaging (MRI) scans demonstrate characteristic subcortical and cortical atrophy as well as confluent signal abnormalities within the deep white matter which represent increased water content and are reversible with HAART (McArthur *et al.*, 2010). Research MRI scan sequences, such as volumetric analysis, diffusion-tensor imaging (DTI) and magnetic resonance spectroscopy (MRS), have been evaluated, but not fully validated. Longitudinal volumetric analysis has found an accumulation of white matter abnormalities over time, despite status of virological control (Taylor *et al.*, 2009) as well as a correlation between nadir CD4 and duration of HIV infection with cerebral atrophy (Cohen *et al.*, 2010) and continued white matter volume loss in patients on HAART suggesting continued cerebral injury despite systemic virological control (Cardenas *et al.*, 2009). Abnormalities in white matter, which correspond with elevation of chemokines

such as MCP-1 and neurocognitive impairment, can also be demonstrated using DTI (Ragin, 2009). MRS evaluation of myoinositol, choline, and N-acetyl aspartate in HAART-naïve HIV-infected patients has demonstrated deviations from normal metabolite levels, reflecting astrocytosis and neuronal injury (Brew *et al.*, 1989), and correlating strongly with HAND severity, measured cognitive function, CD4 count, and plasma and CSF viral load (Chang, 1999; Paul *et al.*, 2008). Follow-up MRS after 9 months of HAART reveal normalized metabolite levels, however these changes lag behind improvements in CD4 count and CSF HIV RNA concentrations and do not always correlate with neurocognitive improvement, confirming recent clinical suspicions that systemic virological control does not necessarily equate to HAND reversal (Chang *et al.*, 2001).

Several methods for treating HANDs have been suggested and are currently being explored including HAART, treatment of co-morbidities, and adjunctive treatments. Soon after the introduction of HAART, studies reported that initiating therapy in patients with HANDs led to sustained neurocognitive improvement in all domains correlated with increases in CD4 count and decrease of plasma viral load (Tozzi *et al.*, 1999). However, larger extended studies observed sustained neurocognitive improvement in less than 50% of patients with HANDs (Tozzi *et al.*, 2007). Detailed recommendations have been developed for preferred initial, second, and salvage HAART regimens. However, no official guidelines for HAART specifically address HAND prevention or reduction. As researchers attempt to understand the interaction between HAART and HANDs, several important discoveries have been made. Neurocognitive improvements have been

demonstrated to correlate with reductions in CSF viral load (Marra *et al.*, 2003). Macrophages, the principle target of HIV within the CNS, require much higher ARV concentrations to inhibit replication than T-lymphocytes (Aquaro *et al.*, 2002). Additionally, ARV concentrations differ in the CNS and plasma due to the limited ability of anti-retrovirals to cross the blood-brain barrier and maintain adequate levels as a result of efflux pumps, such as P-glycoprotein, actively removing ARV agents from the brain (Kim *et al.*, 1998; Thomas, 2004). In response, a CNS penetration effectiveness (CPE) scoring system for antiretrovirals was developed through analysis of ARV chemical characteristics (likelihood of entering CNS), pharmacokinetic properties (achievement of concentrations above IC50 in CSF), and pharmacodynamics data (clinical ability to decrease CSF viral load) (Letendre *et al.*, 2008). This validated scoring system classifies ARV agents as non-penetrators (score of 1), poor penetrators (score of 2), good penetrators (score of 3) or super penetrators (score of 4) with the total regime score calculated as the sum of the score of the individual ARV agents (Letendre *et al.*, 2010). Table 2 outlines the classifications of common current antiretrovirals.

Certain combinations of ARV agents have been confirmed to have better CNS penetration and suppression of CSF HIV RNA (Cysique *et al.*, 2004; Marra *et al.*, 2009; Sacktor *et al.*, 2001). However, conflicting results have been observed in the correlation of CPE score and neurocognitive improvement in patients with HANDs. For example, a study of 37 patients with HANDS found a correlation between high HAART regime CPE score and neurocognitive improvement (Cysique *et al.*, 2009). Another study of a mixed population of cognitively impaired and unimpaired HIV-

Table 2. CPE scoring classifications for ARVs currently available.
(Adapted from McArthur *et al.*, 2010).

	Very Good	**Good**	**Fair**	**Poor**
NRTIs	Zidovudine	Abacavir Emtricitabine	Lamivudine Stavudine	Didanosine Tenofovir Zalcitabine
NNTRIs	Nevirapine	Delavirdine Efavirenz	Etravirine	
Protease Inhibitors	Indinavir-r	Darunavir-r Fosamprenavir-r Indinavir Lopinavir-r	Atazanavir Atazanavir-r Fosamprenavir	Nelfinavir Ritonavir Saquinavir Saquinavir-r Tirpranavir-r
Entry Inhibitors	Vicriviroc	Maraviroc		
Integrase Inhibitors		Raltegravir		

r = ritonavir boost

infected patients showed a decrease in neurocognitive abilities in HANDs patients receiving a HAART regime with a high CPE score (Marra *et al.*, 2009). This reversal of correlation may be explained by recent in vitro data that demonstrated an increased level of toxicity in certain ARV agents with high CPE scores, suggesting further research is needed to determine a therapeutic window in which toxicity does not overcome good CNS penetration (Liner *et al.*, 2010). This suggestion is also supported by data from neurological sub-studies of recent ARV trials (Winston *et al.,* 2010).

While opportunistic infections of the central and peripheral nervous systems, which arise as a consequence of HIV-induced immunosuppression, are rarely observed in patients receiving HAART (Power *et al.*, 2009b), antiretroviral drug toxicity frequently exacerbates primary HAND (Power *et al.*, 2002) both in developed and resource-limited countries (Ferrando *et al.*, 1998; Sacktor *et al.*, 2000; Sacktor *et al.*, 2005). The most common HAART regime in resource-limited countries is a generic combination of stavudine (a potentially neurotoxic reverse transcriptase inhibitor) with nevirapine and lamivudine. In Uganda, for example, this combination was found to have a beneficial effect on cognition, but also frequently increased peripheral neuropathy, raising the concern of enhanced neurotoxicity in stavudine (Sacktor *et al.*, 2009a). In addition to antiretroviral drug toxicity, a subset of HIV-infected individuals experience NeuroIRIS which is the onset or worsening of neurological impairment following initiation of HAART that usually occurs in patients with extremely low CD4 counts and concurrent CNS opportunistic infection and presents as ataxia, hemiparesis and confusion, CSF

pleocytosis and abnormalities in white matter and cortex (McCombe *et al.*, 2009; Roberson *et al.*, 2011; Veld *et al.*, 2011).

Another method of intervention is treating co-morbidities that increase the risk of developing HANDs. For examples, HIV-infected individuals with cardiovascular risk factors or hepatitis C have an increased risk of developing HANDs. Improvement in neurocognitive function has been observed in patients with sustained hepatitis C virological response to interferon and ribavirin treatment (Thein *et al.*, 2007). Similarly, neurocognitive function improved in patients who received treatment of previously untreated risk factors for cerebrovascular disease (Foley *et al.*, 2010).

Lastly, many adjunctive treatments are being explored with varying levels of success. These adjunctive treatments target, through many different mechanisms, interference with the dysregulation of cytokines and brain metabolism that normally results in the neuronal dysfunction causing HANDs (Rumbaugh *et al.*, 2008). Thus far, only small studies with short follow-up periods have been conducted for drugs which are already approved for use in other applications such as sodium valproate, lithium, memantine and selegiline (Schifitto *et al.*, 2006; Schifitto *et al.*, 2007; Schifitto *et al.*, 2009; Zhao *et al.*, 2010; Zink *et al.*, 2005). These treatments are generally well tolerated and show slight trends towards neurocognitive improvement, but further research is necessary. Other important potentially neuroprotective adjunctive treatments are those targeting Tat and gp120, the known neurotoxic proteins of HIV. However, in any form of treatment, the difficulty of crossing the blood-brain barrier remains (Pardridge, 2002)

The introduction of HAART has transformed HAD, and more generally HANDs, from an invariably fatal disorder to a manageable yet still degenerative disease (Vivithanaporn *et al.*, 2011; Wright, 2011). HAND caused directly by HIV infection is now among the most common CNS disorders in industrialized countries, but is frequently under-recognized due to subtle symptoms, concurrent illness, and drug-related effects. Despite treatment with HAART, HIV-infected individuals can still exhibit sustained inflammation and subsequent neuronal damage in the brain (Anthony *et al.*, 2008). HANDs are often complicated by mental health issues and result in worsened employability, survival, and overall quality of life representing substantial personal, economic, and societal burdens (Pandya *et al.*, 2005). Development of novel and efficient treatments for HANDs are vital to improving the lives of millions of individuals currently living with HIV and HANDs.

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CHAPTER 2

STUDY DESIGN

1. Gene Therapy

Gene therapy is the introduction of specific genetic material into the cells of an individual with the intention of producing a therapeutic benefit and is generally used as an alternative treatment for diseases not amenable to conventional therapies (Anderson, 1992; Baltimore 1988; Bunnell *et al.*, 1998). Gene therapy is currently being investigated for treatment of a wide variety of infectious diseases including HBV (Kumar *et al.*, 2011), HCV (Zi *et al.*, 2012), Epstein-Barr virus (Hong *et al.*, 2011), HIV, (Di Nunzio *et al.*, 2012) TB (Woong *et al.*, 2011), and West Nile virus (Chang *et al.*, 2008), cancers (Marukawa *et al.*, 2012), CVD (Katz *et al.*, 2012), and CNS diseases including Alzheimer's and Parkinson's (Nobre *et al.*, 2011). Gene therapy targeting infectious diseases utilizes genes designed to block or inhibit the expression or function of gene products vital for replication or infectivity by the infectious agent on both intra- and extra-cellular levels. There are numerous approaches to gene therapy for infectious disease which fall into one of three broad categories: immunotherapy involving pathogen-specific lymphocytes or genetic vaccines, nucleic acid moieties including RNA decoys, antisense DNA and RNA, and ribozymes, and protein therapies such as single-chain antibodies (Bunnell *et al.*, 1998). Successful gene therapy is contingent upon selection of an appropriate target, the gene delivery system employed, stability and appropriate expression and regulation of gene therapy product, and the efficiency of the gene therapy

product to inhibit the selected target or infectious agent replication. The current study utilizes single-chain variable fragment (scFv) antibodies produced by defective lentiviral vector transduced human cells to target HIV-1 Tat, which is vital for HIV-1 replication and is a potent neurotoxin within the CNS. Expression levels and stability of the gene therapy product will be monitored along with inhibition of HIV-1 Tat-mediated neurotoxicity. Overall, the anti-HIV-1 Tat scFv gene will be transferred to primary monocytes which will deliver antibodies to the CNS via their natural ability to cross the BBB.

1.1. HIV-1 *trans*-activator of transcription

HIV-1 Tat is a 101 residue 11 kDa regulatory protein that is essential for HIV-1 replication, specifically transcription from the long terminal repeat (LTR) promoter (Fujisawa *et al.*, 1985; Jeang *et al.*, 1999). HIV-1 transcription occurs at a rate several hundred-fold higher in the presence of Tat (Berkhout *et al.*, 1992). Tat binds to the trans-activation response (TAR) region, a short nascent stem-bulge loop leader RNA, via its basic domain and recruits the complex cyclin T1 and cyclin-dependent kinase 9 (CDK9) to form the positive transcription elongation factor B complex (Ammosova *et al.*, 2006; Bres *et al.*, 2002; Cullen, 1990). Tat is encoded by two exons and is divided into six functional regions (Kuppuswamy *et al.*, 1989). Tat retains functionality with up to 40% sequence variation (Cambpell *et al.*, 2007; Opi *et al.*, 2002). However, the basic domain is well conserved across isolates from different HIV-1 strains (Goldstein, 1996; Gregoire *et al.*, 1996).

HIV-1 Tat is actively secreted from HIV-1-infected cells (Gatignol *et al.*, 2000) and has been detected in *ex vivo* culture supernatants as well as serum of

HIV-1-infected individuals at concentrations as high as 40ng/mL (Ensoli *et al.*, 1990; Noonan *et al.*, 2000; Xiao *et al.*, 2000). Extracellular Tat binds to a variety of cellular receptors and therefore performs a wide variety of functions, illustrated in Figure 7, including entering and activating the transcription of HIV-1 in latently infected cells as well as promoting HIV-1 coreceptor expression, mainly CXCR4 in the CNS, thereby inducing a self-perpetuating permissiveness to HIV-1 infection (Secchiero *et al.*, 1999). Tat contributes to immune suppression by acting on cells of the immune system (Caputo *et al.*, 1999; Cohen *et al.*, 1999) and has been implicated in development of Kaposi's Sarcoma in AIDS patients (AIDS-KS) through its role in inducing neovascularization and its activity as a growth factor (Barillari *et al.,* 1999a; Barillari *et al.*, 1999b; Ensoli *et al.*, 1993; Trinh *et al.*, 1999). Extracellular Tat also has the ability to transactivate the viral genomes of human herpes virus 8 (Huang *et al.*, 2001), HCV (Ferbeyre *et al.*, 1997) and human cytomegalovirus (Toth *et al.*, 1995), thereby promoting secondary infections in HIV-1-infected individuals.

Perhaps most importantly, extracellular Tat is a neurotoxin implicated in the pathogenesis of HANDs (Agrawal *et al.*, 2011; Dewhurst *et al.*, 1996). Tat is secreted by infected cells within the CNS and also easily enters the CNS by crossing the BBB (Banks *et al.*, 2005) where it performs functions consistent with an extracellular chemokine (Albini *et al.*, 1998) as well as upregulating a number of

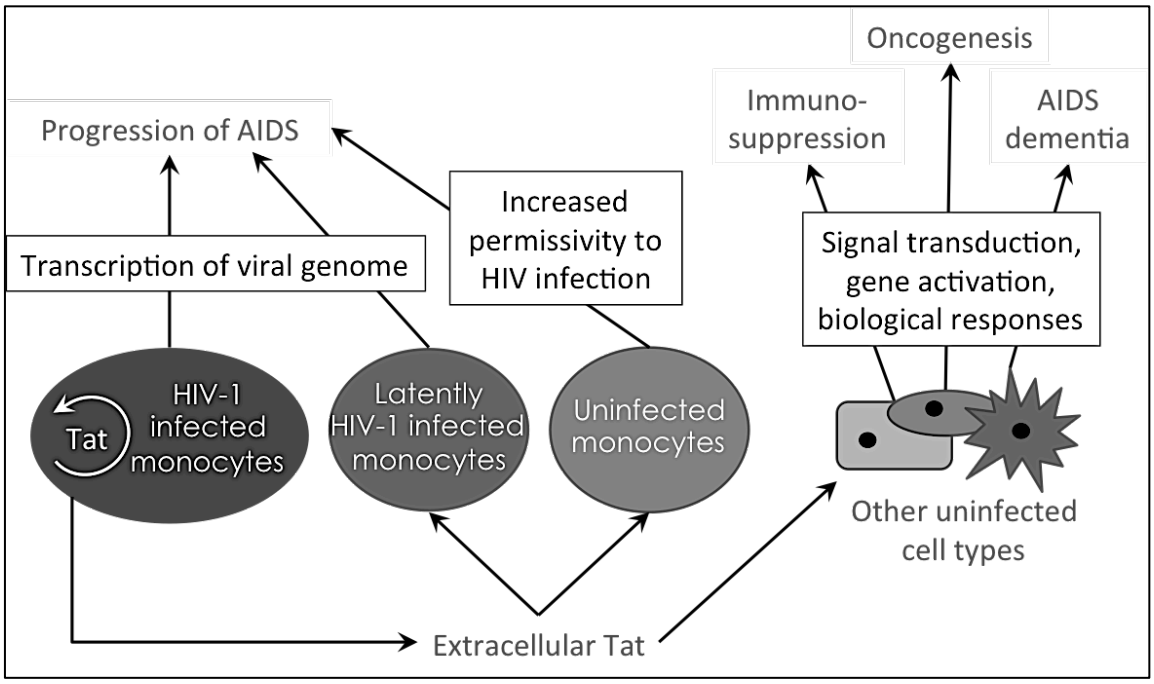

Figure 6. HIV-1 Tat has many intracellular and extracellular functions.

HIV-1 Tat promotes HIV-1 replication in infected and latently infected monocytes and increases the permissivity of uninfected monocytes to HIV-1 infection. Extracellular Tat affects other cell types causing AIDS dementia, onocogenesis as seen in AIDS-KS, and increased immunosuppression (adapted from Rusnati *et al.*, 2002).

cytokines, chemokines, growth factors and receptors (Xiao *et al.*, 1998). Extracellular Tat increases the expression of nitric oxide synthase and release of nitric oxide in microglia, astrocytes, and brain endothelial cells (Kim *et al.*, 2003) and increases BBB permeability through disruption of tight junction distribution (Weiss *et al.*, 1999). Tat increases the release of tumor necrosis factor (TNF) from monocytes and microglial cells in the CNS (Campbell *et al.*, 2004), corresponding to studies in which individuals with HANDs have been found to have an increased expression of TNF and TNF receptors on activated macrophages and monocytes in brain white matter and sera (Brabers *et al.*, 2006). Authophagy, apoptosis, and activation-induced cell death (AICD) are hallmarks of HIV and HAND progression (Ameisen *et al.*, 1991; Espert *et al.*, 2006; Noraz *et al.*, 1997). Tat, along with other mechanisms, is responsible both directly and indirectly for many of these cellular-death activities through numerous identified pathways (Chen *et al.*, 2002; Gulow *et al.*, 2005; Li *et al.*, 1995; Patik *et al.*, 1996). For example, in hippocampal neurons, Tat acts as a neurotoxin through potentiation of N-methyl-D-aspartate (NMDA) – mediated death by disinhibiting Ca^{2+}-permeable NMDA receptors from Zn^{2+}-mediated antagonism (Chandra *et al.*, 2005) in the CA3 region and the dentate gyrus (Maragos *et al.*, 2003). Within the CNS, extracellular Tat can also travel along axonal pathways causing injury at distant sites. For example, Tat produced in the striatum has been identified in the substantia nigra (Bruce-Keller *et al.*, 2003), where it can lead to loss of synapses and glial cell activation with or without causing cell death (Kim *et al.*, 2008). Tat-treated animals have demonstrated long-lasting mitochondrial morphologic abnormalities as well as pathological dilation of

the endoplasmic reticulum similar to findings in AIDS brain tissue (Norman *et al.*, 2008).

HIV-1 Tat is an ideal target for gene therapy for several reasons. First, Tat is the most conserved HIV-1 protein (Korber *et al.*, 1995). However, a reduction in neurovirulence has been consistently observed in HIV-1 Clade C (Sacktor *et al.*, 2007), prevalent in India where HANDs are rare (Sacktor *et al.*, 2009b; Siddappa *et al.*, 2006). The proposed mechanism behind this reduction is a mutation of cysteine 31 to serine, C31S in Region II of the Tat protein, which prevents Tat from binding to CCR2 and inducing a transient flux in cytosol Ca^{2+}, thereby abrogating Tat's ability to act as a chemoattractant for macrophages and monocytes (Campbell *et al.*, 2007b; Mishra *et al.*, 2008), whose CNS infiltration is an important component in the etiology of HANDs (Albini *et al.*, 1998). Second, high levels of immune response against Tat have been found to correlate with better prognosis in HIV-1-infected individuals (Addo *et al.*, 2001; Re *et al.*, 2001b; Van Baalen *et al.*, 1997). For example, elite controllers have been observed to have higher levels of circulating Tat antibodies than rapid progressors (Re *et al.*, 2001a; Van Baalen *et al.*, 1997; Zagury *et al.,* 1998). Also, the basic region of Tat is often not recognized by antibodies to Tat produced by HIV-1-infected individuals (Campbell *et al.*, 2007a), likely due to its sequence similarity to human protamine, which is only expressed with sexual maturation. It is suggested that for this reason, two-thirds of children born to HIV-1-infected mothers are able to sero-revert (Singh *et al.*, 2009), because they are not subject to the immune system repression of the ability to recognize Tat that exists in adults (Campbell *et al.*, 2009). Third, HIV-1 Tat has been implicated in

the development of AIDS-KS and altering cellular and viral gene expression to favor secondary infections. Lastly, Tat is responsible, both directly and indirectly, for a large portion of the neurological damage resulting in HANDs (Gallo, 1999; Nath *et al.*, 1996). Additionally, it increases permissiveness for HIV infection in non-infected cells and dramatically enhances HIV transcription in infected cells (Li *et al.*, 1997).

Previous attempts using intracellular immunization to inhibit Tat, including the overexpression of TAR-containing sequences as TAR decoys in the form of a TAR transcription unit (Sullenger *et al.*, 1990) or a tandem repeat (Graham *et al.*, 1990), Tat-antisense RNA (Chang *et al.*, 1994), anti-Tat ribozyme RNA (Lo *et al.*, 1992), and trans-dominant mutants of Tat (Modesti *et al.*, 1991) met with no major success. Subsequently, the development of intrabodies, intracellular antibodies, in the early 1990's allowed for the exploration of gene therapy based HIV-1 Tat antibodies.

1.2. Single chain variable fragment intrabodies

Single chain variable fragment intrabodies, generated by cloning the genes encoding the heavy and light chains of an antibody to a specific protein, are the smallest structural domain which retain complete binding-site capabilities and antigen specificity of the parental antibody (Chen *et al.*, 2009). A flexible polypeptide linker connects the re-arranged heavy and light chain variable regions (Weisser *et al.*, 2009). The small size of scFv antibodies, approximately one sixth that of a monoclonal antibody (mAb), allows escape from certain defense mechanisms, such as the steric occlusion to which mAbs to HIV-1 proteins have

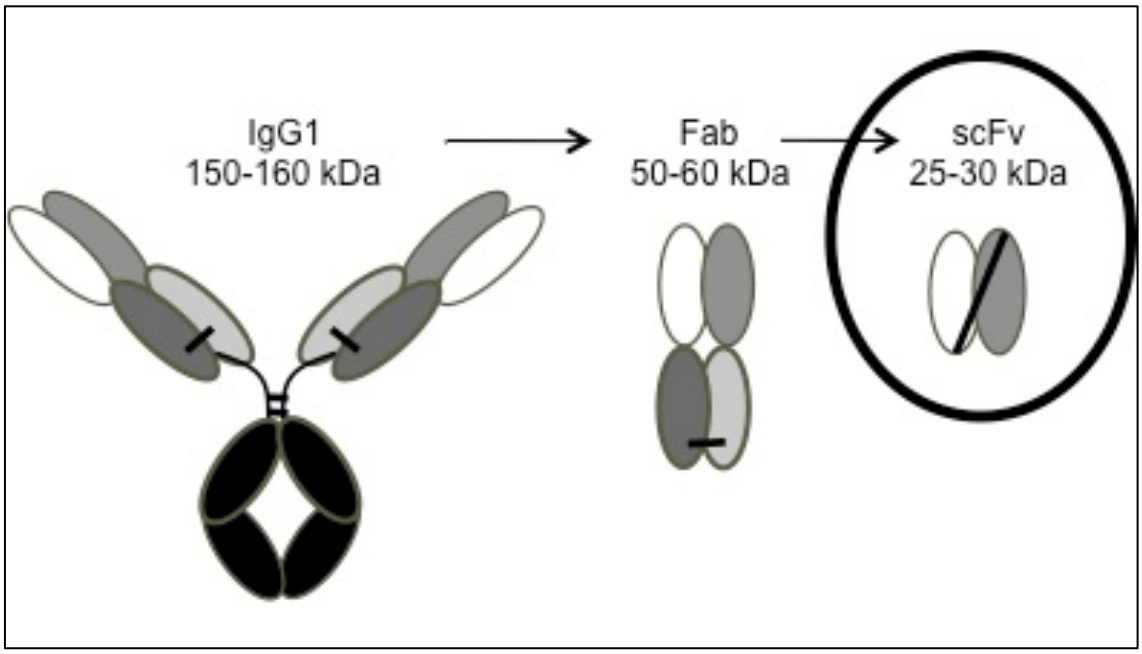

Figure 7. Single chain variable fragment antibodies.

The smallest structural domain which retains complete antigen specificity and binding site capabilities of the parental antibody, scFv are roughly one-sixth the size of IgG. This small size provides many advantages, such as the escape from steric occlusion often presented by HIV-1 (adapted from Chen *et al.*, 2009).

typically been observed to encounter (Wei *et al.*, 2003), resulting in more efficient neutralization by scFv (Choudhry *et al.*, 2006; Labrijn *et al.*, 2003). Figure 8 illustrates the relationship of scFv and parental mAb. ScFv antibodies are more stable than RNA therapies such as antisense RNA or ribozymes and require much lower concentrations than other protein therapies, such as dominant negative inhibitors, which require excessive concentrations to effectively compete with native species. Additionally, scFv antibodies can be designed to react with a wide variety of targets including nucleic acids, proteins, carbohydrates, lipids, and other cellular components (Dana Jones *et al.*, 1998). Provided that the intended target of the scFv antibody is not essential for cell survival, both cultured and primary cells have demonstrated tolerance of high scFv expression levels without exhibiting morphological or growth kinetics changes (Richardson *et al.*, 1995). In 1995, Marasco *et al.* engineered and optimized anti-Tat scFvs that were highly expressed in mammalian SupT cells and successfully bound to HIV-1 Tat, inhibiting Tat-mediated HIV-1 LTR transactivation, with the most successful, completely humanized intrabody termed sFvHutat2 (Marasco *et al.*, 1999; Mhashilkar *et al.* 1995; Mhashilkar *et al.*, 1999). Single chain variable fragment antibodies have many advantages over naturally produced antibodies as well as the other approaches available for gene therapy and therefore a modified version of Marasco et al.'s characterized sFvHutat2 intrabody is used as the initial scFv sequence in this study.

1.3. Defective lentiviral vectors

HIV-1-based defective lentiviral vectors (DLV) have been identified as an ideal gene transfer method for developing anti-HIV gene therapy for several reasons. Foremost, lentiviral vectors have the unique ability to transduce a wide variety of cells regardless of cellular division status (Bukrinsky *et al.*, 1997; Daly *et al.*, 2000; Klimatcheva *et al.*, 1999) including monocytes and monocyte derived macrophages (MDM) (Lee *et al.*, 2003; Lu *et al.*, 2003) which are important targets for gene therapy (Mordelet *et al.*, 2002; Nabel, 2004). As with other retroviral vectors, lentiviral vectors also have the ability to stably integrate proviral DNA into host cell genome, thus establishing long-term transgene expression (Kay *et al.*, 2001; Zhu *et al.*, 2001). Several studies assessing the efficacy of DLV-mediated gene transfer have interestingly found that these HIV-1 derived vectors, even in the absence of anti-HIV-1 genes, inhibit HIV-1 replication (Bukovsky *et al.*, 1999; Klimatcheva *et al.*, 2001; Zeng *et al.*, 2006a) possibly through interference with reverse transcription and packaging or a TAR and RRE decoy effect (An *et al.*, 1999; Corbeau *et al.*, 1998).

Typically, DLV are produced by transient cotransfection of human embryonic kidney (HEK) 293T cells with transfer, envelope, and packaging plasmids (Lemiale *et al.*, 2009; Tiscornia *et al.*, 2006; Zufferey *et al.*, 1998). To allow expression of more than one open reading frame (ORF), an internal ribosome entry site (IRES) from the encephalomyocarditis virus was included as a translational *cis*-acting element (Rees *et al.*, 1996; Stripecke *et al.*, 2000), an internal cytomegalovirus (CMV) immediate-early promoter was included to drive

transcription of bicistronic mRNA (Reiser *et al.*, 2000), and an Fc-fusion protein and enhanced green fluorescent protein (eGFP) (Persons *et al.*, 1997) were included as reporter genes in the transfer plasmid construct utilized in this study. The packaging and envelope plasmids used in this study to produce DLV, pCMVΔR8.2Δvpr and pCMV-VSV-G respectively, have previously demonstrated high titer vector production following cotransfection with transfer plasmids (Akkina *et al.*, 1996; An *et al.*, 1999; Mautino, 2002; Zeng *et al.*, 2006a).

1.4. Macrophages targeting the central nervous system

Monocytes, which are produced by hematopoietic stem cells in the bone marrow, circulate in the bloodstream for one to three days and then migrate into various tissues where they differentiate into resident macrophages or dendritic cells. Monocyte derived macrophages (MDM) play an important role in both innate and adaptive immunity and also stimulate lymphocytes and other immune cells. Monocytes naturally cross the BBB and enter the CNS, a phenomenon enhanced in response to inflammation (Lui *et al.*, 2008) or following transient chemical disruption of the BBB (Borlongan *et al.*, 2003; Rapoport, 2000). In the central nervous system, macrophages serve as an important reservoir (Brown *et al.*, 2006) and are a major source of HIV recombination and diversity (Lamers *et al.*, 2009). HIV-1 infection of the CNS often results in the development of HANDs, despite treatment with HAART. Therefore, due to the role of MDM in HIV infection of the CNS, the ability of MDM to transverse the BBB and mature into long-lived resident macrophages of the CNS (Wu *et al.*, 2006), and the capacity of MDM for efficient DLV transduction (Zeng *et al.*, 2006b), monocytes are potentially an important gene

delivery vehicle for anti-HIV or neuroprotective gene therapy targeting the CNS (Burke *et al.*, 2002). The overarching goal of this study is to assess the potential therapeutic benefit of utilizing DLV transduced monocytes as a novel gene delivery method to combat HANDs in the CNS.

1.5. Functional evaluation

The current study focuses on the construction, transduction, and expression assessment of anti-HIV-1 Tat scFv in immortalized human cell lines as well primary human peripheral blood mononuclear cells (PBMC), the first step in achieving the overarching study goal. Western Blot, Immunofluorescence staining, and ELISA monitored appropriate scFv regulation and expression in transduced cells long-term. Immunoblot assays confirmed anti-HIV-1 Tat scFv biological specificity to recombinant HIV-1 Tat and neuroprotection assays evaluated the ability of anti-HIV-1 Tat scFv to inhibit HIV-1 Tat and gp120-mediated neurotoxicity. Figure 8 illustrates the theoretical mechanism through which the designed anti-HIV-1 Tat scFv bind to and effectively inhibit HIV-1 Tat. Lastly, the regulation of twenty-four reference genes were monitored through genetic expression analysis of control and transduced cells.

2. Significance

Human immunodeficiency virus (HIV) was first identified in 1983 and in 2010 the World Health Organization estimated that 33.3 million people were living with HIV (UNAIDS, 2010). HIV-associated neurocognitive disorders (HANDs) occur in more than 50% of infected individuals and are the most common disorders

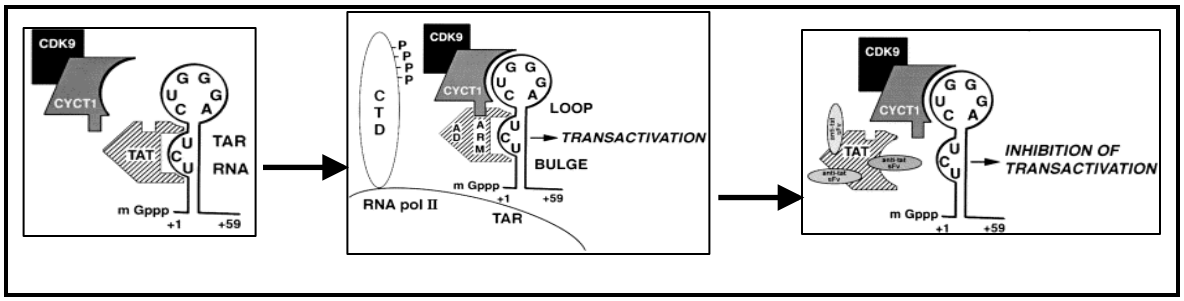

Figure 8. Schematic inhibition of HIV-1 replication by anti-HIV-1 Tat scFv. (Marasco *et al.*, 1999)

among people infected with HIV in spite of antiretroviral therapy (McArthur *et al.*, 2005; McArthur *et al.*, 2010). Autopsy studies have found that as many as 90% of HIV positive patients exhibit signs of neurological changes (Johnson, 1998). HANDs vary among infected individuals and can develop at all stages of infection. There are three main categories of HANDs: opportunistic infections, primary neurologic disorders, and undesirable treatment related effects (Power *et al.*, 2009). The most common primary neurologic disorders, which are the result of direct damage by HIV and its neurotoxic products to neuronal cells affecting the brain, spinal cord, and peripheral nerves, include asymptomatic neurocognitive impairment, minor neurocognitive disoorder, and HIV-associated dementia (AIDS Dementia Complex, HIV encephalopathy) (Johnson, 1998). Highly active actiretroviral therapy has increased the life expectancy of HIV positive individuals with acquired immunodeficiency syndrome by 10.6 years and for those without AIDS by 21.5 years (Fang *et al.*, 2007). The extended life provided by HAART has led to an increased overall prevelance of HANDs which results in worsened quality of life and substantial social and economic burdens (Pandya *et al.*, 2005). Many potential neuroprotective agents against HIV neurotoxicity are being developed and tested, but results from clinical trials have shown no significant protection to date (Rumbaugh *et al.*, 2008; Theisen *et al.*, 2006).

In infected individuals, the brain acts as a reservoir for HIV due to the limited ability of antiretrovirals to cross the blood-brain barrier. Therefore, despite the potency of HAART, HIV continues to replicate freely in the brain (Brack-Werner, 1999). Additionally, HIV infected macrophages, which naturally migrate across the

BBB, have been implicated in the development of HANDs. Infected macrophages in the brain release chemokines, inflammatory proteins, and apoptosis factors that cause damage to neighboring neuronal cells, which cannot be infected by HIV (Weiss *et al.*, 1999). Macrophage proteins up-regulated by HIV disturb the BBB, leading to an influx of macrophages. This unnatural influx of macrophages, along with increased inflammatory proteins, causes inflammation in the central nervous system which is responsible for neuronal damage (Yang *et al.*, 2010). Apoptosis factors secreted from infected macrophages also cause damage and death of nearby neuronal cells (Weiss *et al.*, 1999).

There are many identified neurotoxins secreted by infected macrophages in the brain, one of which is HIV-1 *trans*-activator of transcription (Tat), which is essential for HIV-1 replication (Jeang *et al.*, 1999). HIV-1 Tat is present both intra- and extracellularly in HIV-1 infected individuals (Rusnati *et al.*, 2002). Extracellular Tat induces neuronal apoptosis and is responsible for disturbing the BBB (Banks *et al.*, 2005; Kim *et al.*, 2003). Intracellular Tat has been identified in both infected and uninfected cells, suggesting that it plays an important role in initial cellular infection (Albini *et al.*, 1998). Tat is a 101 residue protein which can mutate up to 40% without a change in function and is highly conserved among all of the HIV-1 subtypes (Butto *et al.*, 2003; Campbell *et al.*, 2009; Jeang *et al.*, 1999). These characteristics, along with the observation that HIV infected individuals who are classified as elite controllers have high levels of serum HIV-1 Tat antibodies compared to HIV infected individuals classified as rapid progressors, make HIV-1

Tat an ideal target for treatment to eliminate HIV replication and HANDs (Campbell *et al.*, 2009; Re *et al.*, 2001b).

Currently, gene therapy is being explored for treatment of many infectious diseases. Gene therapy is used to introduce new genes which are designed to inhibit or limit replication of the target infectious agent. The newly introduced genes block gene expression or the function of gene products of the infectious agent (Bunnell *et al.*, 1998). One of the most successful methods for the desired inhibition has been through the introduction of single-chain variable fragment intrabodies (Weisser *et al.*, 2009). Single-chain variable fragment intrabodies (scFv) are the smallest structural domain of an antibody retaining complete antigen specificity and binding site capabilities of the parental antibody (Bunnell *et al.*, 1998). ScFv intrabodies are more versatile and stable than RNA based therapies and are more efficient than whole antibodies due to their smaller size (Bunnell *et al.*, 1998; Chen *et al.*, 2009). ScFv intrabodies also require lower concentrations than other protein-based therapies and have been used in many applications such as inhibiting oncogene function and autoimmunity during transplantation (Dana Jones *et al.*, 1998).

Gene therapy efficacy is determined by four strategy components. These include the selection of an appropriate target; efficiency of the gene delivery system; appropriate expression, regulation, and stability; and efficiency of inhibition of replication (Bunnell *et al.*, 1998). The target selected for this study is HIV-1 Tat due to its role in HIV-1 replication and HANDs (Marasco *et al.*, 1999; Mhashilkar *et al.*, 1995; Mhashilkar *et al.*, 1997; Mhashilkar *et al.*, 1999). The

efficiency of the gene delivery system, monocyte-derived macrophages (MDM) transduced by an HIV-based defective lentiviral vector system, has previously been assessed (Zeng *et al.*, 2006a; Zeng *et al.*, 2006b). Macrophages were selected due to their natural ability to cross the BBB and their role in HANDs and as HIV reservoirs in the CNS (Wu *et al.*, 2006). The specific aim of this study is to analyze the appropriate expression, regulation, and stability of anti-HIV-1 Tat scFv in transduced cell lines using Western Blot, ELISA, Immunofluorescence staining, and Immunoblot assays both short and long term (20 passages). Regulation of the anti-HIV-1 Tat scFv gene as well as 24 other genes will be monitored through gene expression analysis of control and transduced cells. Functionality assays to determine the efficiency of inhibition of HIV-1 Tat- and gp120-mediated neurotoxicity will be conducted using Immunoblot and Neurotoxicity Assays.

Despite the widespread treatment of HIV with HAART, development of HANDs remains likely for many HIV infected individuals. Anti-HIV-1 Tat scFv gene therapy can potentially prevent the HIV-induced neuronal damage that causes HANDs and eliminate the use of the CNS by HIV as a reservoir from treatment. The success of this therapeutic approach would preserve the quality of life for millions of HIV infected individuals and compliment HAART by attacking HIV in an area where current antiretrovirals have very limited access.

3. Specific Aims

The brain is a key component in HIV pathogenesis due to its role as a reservoir for HIV as a result of the limited ability of antiretroviral treatments to cross the

blood-brain barrier as well as its role in facilitating an influx of monocytes across the blood-brain barrier which both directly and indirectly leads to HIV-associated neurocognitive disorders in more than 50% of HIV infected individuals. To combat this issue, the proposed research integrates previously developed anti-HIV-1 Tat single-chain variable fragment antibodies with a novel gene therapy method utilizing monocytes for gene delivery. There are five specific aims for this research:

1. Construction of vectors designed to express one of two different anti-HIV-1 Tat scFv antibodies or control scFvs and assessment of the transduction efficiency and stability of these vectors.

2. Assessment of expression levels and biological specificity of target genes in transduced cell lines.

3. Assessment of the ability of anti-HIV-1 Tat scFv antibodies from transduced cells to protect neurons from HIV-1 Tat- and gp120-mediated neurotoxicity.

4. Identification of any gene expression variations in transduced cell lines that could possibly result from the lentiviral vector transduction method employed.

5. Isolation and efficient transduction of primary peripheral blood mononuclear cells followed by assessment of anti-HIV-1 Tat scFv expression levels, biological specificity, neuroprotection capabilities, and housekeeping gene expression analysis.

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CHAPTER 3

MATERIALS AND METHODS

1. Transduction of Human Neuroblastoma and Microglial Cells

Successful gene therapy must employ an efficient gene delivery method. For this study, a defective lentiviral vector system was used to integrate the gene of interest into the DNA of the target cell lines. Defective lentiviral vectors were chosen as the method of gene delivery due to the fact that lentiviral vectors are able to efficiently transduce cells regardless of dividing status (Zang *et al.*, 2006a). Lentiviral vectors have also displayed great stability and have thus far not demonstrated any adverse effects on the expression of housekeeping or oncogenes (Lu *et al.*, 2003). The development of this lentiviral vector system included constructing a transfer plasmid using molecular cloning methods, co-transfection of HEK 293T cells for vector production, vector collection and concentration, and transduction of target cell lines. The anti-HIV-1 Tat scFv constructs used in the construction of plasmids were designed to target HIV-1 Tat Domain 1 due to the fact that Domain 1 is highly conserved among HIV-1 subtypes and contains most of the immunogenic epitopes (Kuppuswamy *et al.*, 1989). High transduction efficiency is vital for the success of this gene therapy strategy and is dependent upon the production of high titer vectors which can only be achieved through high transfection efficiency of HEK 293T packaging cells.

1.1. Plasmids

1.1.1. Molecular cloning

Four transfer plasmids were constructed to contain two different anti-HIV-1 Tat scFv (Hutat2, E46) and two controls (A3H5, Fc). Constructs include a CMV promoter, green fluorescent protein (GFP) as an indicator gene, and an Fc fusion protein for identification in protein assays. TOPO TA cloning (Invitrogen) was performed following manufacturer's instructions. First, the construct of interest was amplified by polymerase chain reaction (PCR) using primers that included restriction digestion sites for BamH1 and Xho1 (Table 3). The PCR product was then used to transform competent cells, which were plated onto LB Agar plates with 10 mg/mL ampicillin (Amp) and cultured at 37 degrees centigrade (°C) for 12 hours (h). X-gal was added and colonies with positive transformation were selected and inoculated into 3 mL LB media with AMP. After 12 h culture at 37°C with shaking at 30 rpm, cultures were subjected to restriction enzyme digestion to confirm integration of the gene of interest into the E. coli plasmid. Additionally, transfer plasmid backbones were digested with restriction enzymes. Restriction enzyme digestion was performed by gently mixing restriction enzymes Xho1 and BamH1 with 10ug plasmid, 100x buffer NEB4, and BSA and incubating at 37°C for 2 h.

The digested plasmids were then separated on 1% agarose gel for 45 minutes (min) at 130 volts (V) and gel extraction was performed to recover the purified transfer plasmid backbone and gene of interest using QIAquick Gel Extraction Kit (QIAGEN, CA, catalogue #28706). Briefly, the plasmid backbone DNA

and the amplified genes of interest were excised from the agarose gel with a scalpel and placed in a clear centrifuge tube. Buffer QG was added and incubated at 50°C for 10 minutes to dissolve the agarose gel. Isopropanol was added and the DNA was transferred and bound to a QIAquick column. The column was subsequently washed with Buffer PE and the DNA was eluted with water. The purified DNA was then ligated by combining plasmid backbone and gene of interest with ligation buffer and T4 DNA ligase and incubating at 16°C overnight. The resulting plasmids were separated with 1% agarose gel electrophoresis for 45 mins at 130V to confirm successful ligation. Results were visualized with the Molecular Imager Gel Doc XR+ System (BioRad Laboratories, CA). E. coli competent cells were transformed with the constructed plasmid by incubating 10 µL ligated plasmids and 100 µL competent cells on ice for 60 min followed by heat shock at 42°C for 90 s and a subsequent incubation on ice for 10 min. Competent cells were centrifuged for 1 min at 5000 rpm, resuspended in SOC medium, and incubated at 37°C for 60 min. Transformed cells were then streaked onto an LB Agar plate with Amp and incubated at 37°C overnight for colony formation. Several medium-sized colonies were selected for inoculation in 3 mL LB media with AMP at 37°C and shaking at 30 RPM. Plasmids were extracted from each culture after 12 h and restriction enzyme digestion with Xho1 and BamH1 was performed as previously described to confirm successful transformation.

1.1.2. Production

Production of the DLV used to transduce target cell lines required large quantities of each transfer, envelope, and packaging plasmid. Therefore, one 3 mL

transformed competent cell culture positive for each plasmid, as determined by restriction enzyme digestion, was selected for a maxi-preparation. 500 µL of each selected 3 mL culture was separately inoculated into 250 mL LB medium with Amp and cultured for 12-16 h at 37°C with shaking at 30 rpm. Plasmids were then extracted using QIAfilter Plasmid Maxi Kit (QIAGEN, CA, catalogue #12263). Cells were pelleted by centrifugation at 10,000 rpm and resuspended in buffer P1 and then lysed with buffer P2. After inversion to ensure adequate lysis, the solution was neutralized with buffer P3. Cell debris was removed using a Maxi separation column and plasmid DNA was subsequently captured in a Maxi spin column. The column was washed twice and then buffer TE was used to elute the plasmid DNA. Packing and envelope plasmids, VSV-G and ΔR-8.2 respectively, were also amplified using the maxi-preparation and extraction protocol. All plasmids were quantified using a DU800 UV/Vis spectrophotometer (Beckman Coulter, CA), evaluated for quality through agarose gel separation, and stored at 4°C until used for vector production.

1.2. Defective lentiviral vectors

1.2.1. Calcium-phosphate precipitation transfection of HEK293T cells

Human embryonic kidney 293T cells were transiently co-transfected with transfer, envelope, and packaging plasmids using an optimized calcium-phosphate precipitation protocol for defective lentiviral vector production. HEK 293T cells were maintained at 37°C with 5% CO_2 with Delbuccio's minimum essential medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 1.0 g/L glucose, 2mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma

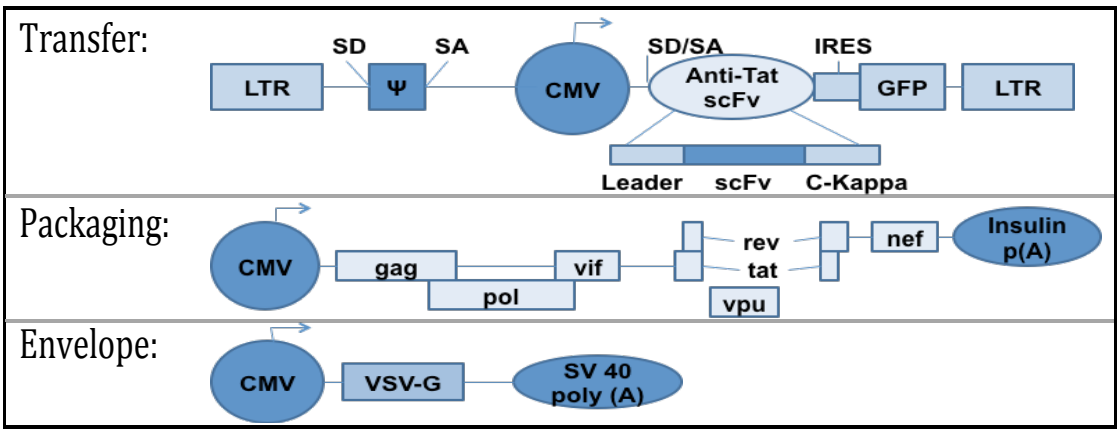

Figure 9. Transfer, packaging, and envelope plasmid constructs.

Plasmids were constructed and amplified for use in HEK 293T transfection for defective lentiviral vector production. LTR: long terminal repeat; SD: splice donor; Ψ: packaging signal; SA: splice acceptor; CMV: cytomegalovirus promoter; IRES: internal ribosome entry site; GFP: green fluorescent protein

Aldrich), and 10% fetal bovine serum (FBS) (HyClone, Logan, UT). HEK 293T cells were passaged to a density of 70% using ethylenediaminetetraacetic acid (EDTA) with 10% trypsin. Media was replaced after 36 h and cells were subsequently passaged 24 h later.

HEK 293T cells were transfected at a density of 80% within 24 h of passaging. Media was replaced 2-4 h prior to transfection. Packaging, envelope, and transfer plasmids were combined at a ratio for 3 μg, 13.5 μg, and 13.5 μg respectively, for each T75 flask transfected. Molecular grade water was added to bring the total plasmid volume to 163 μL. 69 μL 2.0 M $CaCl_2$ was added and the solution was mixed thoroughly. Polybrene was added to a final volume of 8 mg/mL and incubated on ice for 5 min. 558 μL 2x HBS was added drop-by-drop with vigorous agitation and then incubated on ice for 20 min. The DNA solution was slowly mixed with the HEK 293T culture media and the cells were incubated at 37°C with 5% CO_2 for 14-16 h. The culture media was then discarded and fresh media was added. Vector was collected along with the culture media every 24 h and fresh media was added for eight consecutive days. Culture supernatant was centrifuged at 4,000 RPM for 30 min to remove any cell debris and cytotoxins and then stored at -20°C until ultracentrifugation.

1.2.2. Vector concentration by ultracentrifugation

Recent studies have demonstrated that higher moi, achievable through vector concentration, result in increased transduction efficiency (Zeng *et al.*, 2006b). Therefore, defective lentiviral vectors produced by transfected HEK 293T cells were ultraconcentrated to increase transduction efficiency and limit the

concentration of cytotoxins present in the DLV suspension to which target cell lines would be exposed, thereby sustaining the health of the transduced cells. DLV were collected from transfected HEK 293T cell culture media of every 24 h from day 2 - 8 post-transfection, filtered using a 0.2 mm membrane and concentrated using an ultracentrifuge (Beckman Coulter) at 25,000 rpm for 3 h with a sucrose cushion. Pelleted DLV was resuspended in 100 μL aliquots of serum free DMEM (Sigma-Aldrich, St. Louis, MO) with 1 mg/mL glucose, 2mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma Aldrich) and stored at -80°C until use.

Concentrated DLV were titered on HEK 293T cells in 96 well tissue culture (TC) plates. Vector was serial diluted and added to each well in triplicate with polybrene at a final concentration of 8 mg/mL. Cells were incubated at 37°C with 5% CO_2 for 72 h. Vector titer was calculated by averaging the number of GFP positive cells in each well from the dilution that resulted in transduction of 20-100 cells and dividing by the corresponding dilution factor. Titration was performed for all vector constructs and both concentrated and unconcentrated vectors.

1.3. Cell culture

1.3.1. Cell growth and passage

Human neuroblastoma (HTB-11) and human microglial (CHME-5) cells were transduced with each of the four constructed vectors. HTB-11 cells were maintained at 37°C, 5% CO_2 with Minimum Essential Media (MEM) (Sigma-Aldrich, St. Louis, MO) containing 1.0 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma Aldrich), and 10% FBS

(HyClone, Logan, UT) (MEM-10). Culture media was replaced every 2-3 days and cells were passaged when they reached a monolayer with EDTA containing 10% trypsin and seeded at a density of 70%, according to America Tissue Cell Culture (ATCC) guidelines. CHME-5 cells were maintained at 37°C, 5% CO_2 with DMEM (Sigma-Aldrich, St. Louis, MO) containing 4.5 g/L glucose, 2mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma Aldrich), and 10% FBS (HyClone, Logan, UT). Cells were passaged every 4 days with EDTA containing 10% trypsin and seeded at a density of 70%.

1.3.2. Transduction

HTB-11 and CHME-5 cells were transduced with concentrated DLV at a multiplicity of infection (moi) of 10 in the presence of polybrene. Cell cultures at a density of 80% were trypsanized during exponential growth phase, pelleted, and then $1x10^5$ cells were incubated in 0.1 mL DLV (1×10^7 IU/mL) and polybrene at a final concentration of 8 mg/mL in a 1.5 mL tube for 3 h at 37°C, 5% CO_2 with shaking every 15 min. Transduced cell cultures were seeded into a 25 cm^2 TC flask. Medium was replaced 24 h post infection and cultures were maintained under normal conditions. After 72 h, transduction efficiency was evaluated using GFP quantification. For transduction efficiencies below 90%, a second transduction was performed following an identical protocol.

1.3.3. Green fluorescent protein quantification

After 72 h post-transduction, GFP positive cells were quantified as an indicator of transduction efficiency. Briefly, five fields of the transduced cell culture containing at least 100 cells each were randomly selected and visualized under a

fluorescence microscope (Nikon Eclipse TE2000-U). The number of GFP positive cells visible at 495 nm was summed and then divided by the total number of cells in the field visible under normal light. This percentage was averaged over the five fields. Transduction efficiency was monitored through GFP quantification for 25 passages to confirm long-term stability.

1.3.4. Polymerase chain reaction confirmation

To confirm successful transfer of the gene of interest from DLV to the target cell genome, DNA was extracted from transduced and non-transduced cells and subjected to PCR utilizing specifically designed primers to detect each gene of interest as well as the GFP indicator gene. Cellular DNA of transduced and non-transduced HTB-11 and CHME-5 cells was extracted using Qiagen FlexiGene DNA kit (cat#51204). Cell cultures were harvested by trypsinization and centrifuged for 5 min at 300 x g. Supernatant was removed and the cell pellet was resuspended in lysis buffer. Protease was added and mixed by gentle inversion. The cells were then incubated for 10 min at 65°C in a heating block. DNA was precipitated with the addition of 100% isopropanol followed by thorough inversion and centrifugation for 3 min at 10,000 x g. The supernatant was discarded and 70% ethanol was added to the pellet and vortexed for 5 s and centrifuged for 3 min at 10,000 x g. The supernatant was discarded and the DNA pellet was allowed to air-dry for at least 5 min. The DNA pellet was resuspended in provided buffer by vortexing for 5 s then incubating for 30 min at 65°C in a heating block.

Extracted DNA concentrations were determined using a DU800 UV/Vis spectrophotometer (Beckman Coulter, CA). PCR was performed on each DNA

Table 3. Oligonucleotide primers utilized in construction of transfer plasmids (Fc), detection of gene of interest (Hutat2, E46, A3H5) and GFP as well as screening extracted RNA for the presence of DNA (β-actin).

Primer	Sequence (5' ➔3')	Amplicon
F-Fc	CCGCTCGAGCGGGCCGGCCATGGCCCAGGTGCA	~1.5 kb
R-Fc	CGCGGATCCGCGTTAAATCATTTACCCGGAGACAGG	
F-Hutat2	ACATCTGTGGTTCTTCCTTCTCCT	213 bp
R-Hutat2	TCACTCCATATCACTCCCAGCCACTC	
F-E46	CTGGGGCTGAGGTGAAGAGG	316 bp
R-E46	TTGCCCCAGACGTCCATGTAGTAGTA	
F-A3H5	TATTAGTAGTGATGGGGGTAGCACAT	189 bp
R-A3H5	TAGTCAAAGAAGTGCCGGTAATAACCACTAC	
F-GFP	GGTGAGCAAGGGCGAGGAG	155 bp
R-GFP	GCCGGTGGTGCAGATGAACT	
F-β-actin	GGCCACGGCTGCTTC	207 bp
R-β-actin	GTTGGCGTACAGGTCTTTGC	

sample to detect the presence of the inserted scFv and GFP genes using designed primers (Table 3). DNA extracted from non-transduced HTB-11 and CHME-5 cells served as the no template control. Briefly, each 1.0 µL DNA template was combined with 24 µL mixture containing 1X Standard Taq Reaction Buffer (Mg^{2+} free) (New England Biolabs, MA), 1.5 mM $MgCl_2$ (New England Biolabs, MA), 200 µM of each dNTA (Sigma Aldrich, MO), 0.2 µM forward and reverse primers (IDT, IA), and 2 units of *Taq* polymerase (provided by Dr. Tung T. Huang, University of Hawaii at Manoa). PCR amplification was performed with a MasterCycler Gradient (Eppendorf North America) starting with an initial denaturation at 94°C for 5 min, followed by cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 90 s. After a final extension at 72°C for 7 min, PCR amplicons were separated using 2% agarose gel electrophoresis stained with ethidium bromide (EtBr) alongside a 100 bp DNA marker (New England Biolabs, MA). Results were visualized using a Molecular Imager Gel Doc XR+ System (BioRad Laboratories, CA).

2. Intrabody Expression

2.1. Immunofluorescence assay

Immunofluoresence staining was optimized and employed to visualize the intracellular presence of scFv produced by transduced cells. Transduced and non-transduced HTB-11 and CHME-5 cells were seeded in 24 well TC plates for 24 h at 37°C, 5% CO_2 and then fixed with 4% paraformaldahyde at room temperature. Cells were washed with phosphate buffered saline (PBS) and incubated in Triton X

100, to puncture the cell membranes allowing antibodies to enter, followed by blocking solution (0.2% gelatin in PBS), each for 10 min. Cells were then incubated with primary antibody, rabbit anti-human (H+L) Fc (Rockland, PA) for 2 h, washed, and then incubated with secondary antibody, goat anti-rabbit IgG Fc-Rhodamine (Rockland, PA) for 40 min. BisBenzimide was added to stain the cell nuclei as a positive control for 5 min, preceded and succeeded by washing. Results were visualized at 450 nm under a fluorescent microscope (Nikon Eclipse TE2000-U).

2.2. Western Blot

Western Blot was optimized and utilized to confirm the presence of each scFv in transduced cell culture media. Cell culture media from transduced and non-transduced HTB-11 and CHME-5 cells was collected after 48 h incubation in serum free media at 37°C, 5% CO_2. Samples were diluted 1:2 in non-reducing Laemmli sample buffer (Biorad, CA) and 15 μL of diluted sample was loaded per well in a 4% stacking / 8% separating SDS-polyacrylamide gel. Samples were separated at 120 V, 200 mA for 1 h. Proteins were then transferred to a nitrocellulose membrane at 100 V, 200 mA for 1.5 h. The nitrocellulose membrane was blocked for 1 h at room temperature in 0.5% blotto in TBS-T containing 10 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 0.05% Tween 20. Incubations with primary antibody, rabbit anti-human IgG (H+L) (Rockland, PA), followed by secondary antibody, goat anti-rabbit IgG Fc-HRP (Rockland, PA), were 1.5 h each at room temperature on an orbital shaker. Washing with TBS-T was repeated three times between each step. Finally, metal enhanced 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (Pierce) was used to visualize protein bands.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Moderate to high long-term scFv expression is vital for successful neutralization of Tat. ELISA was optimized and used to quantify scFv expression levels in cell culture media every 5 passages to monitor long-term expression stability. Capture antibody, goat anti-human IgG Fc (KPL), in a 0.5 M Carbonate-Bicarbonate, pH 9.6, coating buffer was incubated in a 96 well plate for 2 h at 4°C and then blocked with TBS blocking buffer containing 1% BSA for 1 h at room temperature on an orbital shaker. Conditioned culture media from transduced and non-transduced HTB-11 and CHME-5 cells was collected after 48 h incubation at 37°C, 5% CO_2. Samples were diluted 1:50 with TBS dilution buffer containing 0.05% Tween-20 and 1% BSA and then loaded and incubated for 1.5 h at room temperature with shaking. Detection antibody, goat anti-human IgG Fc-biotin (Rockland, PA), followed by streptovidin-HRP (Rockland,PA), were incubated at room temperature for 1.5 h with shaking. Washing with TBS-T was repeated 5 times between each step. Finally, one-Step Ultra TMB (tetramethylbenzidine) substrate (Pierce) was incubated with protection from light for 5-10 min and stopped with 2 M Sulfuric Acid. Results were read at 450 nm using an ELISA reader (Beckman Coulter AD340). Human IgG Fc standard (Bethyl, TX) was used to develop a standard curve for every assay for the calculation of scFv concentration.

3. Intrabody Function

HIV-1 Tat is vital for HIV replication and is a known neurotoxin. The anti-HIV-1 Tat scFv in this study was designed to address both of these important

functions of HIV-1 Tat. The anti-HIV-1 Tat scFv should not only bind specifically to HIV-1 Tat, but also significantly reduce HIV-1 Tat-mediated neurotoxicity in neuronal cells, which are especially sensitive to the toxic effects of Tat (Bohan *et al.*, 1992).

3.1. Immunoblot

Immunoblot was employed to assess the ability of anti-HIV-1 Tat scFv to bind specifically to HIV-1 Tat. Serial dilutions of recombinant HIV-1 Tat protein (NIH AIDS Reagents) were blotted and dried onto a 45 nm nitrocellulose membrane and then blocked with 5% blotto in TBS-T for 30 min at room temperature on an orbital shaker. Buffer utilized for the reconstitution of the HIV-1 Tat protein was blotted as a negative control. The nitrocellulose membrane was exposed to 4 mL HTB-11 or CHME-5 transduced cell culture media containing approximately 2.5 mg scFv as determined by ELISA for 2 h at room temperature with shaking. The membrane was then incubated with detection antibody, mouse anti-Human IgG Fc-HRP (SouthernBiotech, AL), and bands were visualized using metal enhanced DAB substrate (Pierce). Washing with TBS-T was repeated three times between each step.

3.2. Neuroprotection assay

3.2.1. HIV-1 Tat-mediated neurotoxicity

Neuroprotection assay was utilized to assess the degree to which anti-HIV-1 Tat scFv inhibited the neurotoxic properties of HIV-1 Tat. HTB-11 cells were exposed 500 ng/mL recombinant HIV-1 Tat protein (NIH AIDS Reagents #2222), and conditioned culture media diluted 1:10 in MEM-10 from either transduced or

non-transduced cells. Additionally, transduced HTB-11 cells were exposed to 500 ng/mL recombinant HIV-1 Tat protein under the same conditions. Controls included cells receiving an HIV-1 Tat concentration of 0 ng/mL, cells receiving antiserum to recombinant HIV-1 Tat protein (NIH AIDS Reagents #4357), and cells receiving conditioned media from non-transduced cell cultures. After 48 h, MTT assay was employed to determine cell viability. Cells were washed thoroughly with PBS and then incubated with 5 mg/mL MTT at 37°C for 2 h. After adequate crystal formation, DMSO was added with shaking for 15 min to dissolve precipitate and remove bubbles. Results were read at 570 nm using a microplate reader (Beckman Coulter AD340). T-tests were used to assess any significant differences in cell viability between transduced and non-transduced cells or cell receiving conditioned media from transduced or non-transduced cells.

3.2.2. HIV-1 gp120-mediated neurotoxicity

Antibodies to HIV-1 Tat and DLV have previously been demonstrated to provide protection, either directly or indirectly, from HIV-1 gp120-mediated neurotoxicity. Therefore, neuroprotection assay was also utilized to assess to degree to which anti-HIV-1 Tat scFv inhibited the neurotoxic properties of HIV-1 gp120. HTB-11 cells were exposed 250 ng/mL recombinant HIV-1 gp120 (NIH AIDS Reagents #2968), and conditioned culture media diluted 1:10 in MEM-10 from either transduced or non-transduced cells. Additionally, transduced HTB-11 cells were exposed to 250 ng/mL recombinant HIV-1 gp120 under the same conditions. Controls included cells receiving an HIV-1 gp120 concentration of 0 ng/mL, cells receiving antiserum to recombinant HIV-1 Tat protein (NIH AIDS

Reagents #4357), and cells receiving conditioned media from non-transduced cell cultures. After 48 h, MTT assay was employed to determine cell viability. Cells were washed thoroughly with PBS and then incubated with 5 mg/mL MTT at 37°C for 2 h. After adequate crystal formation, DMSO was added with shaking for 15 min to dissolve precipitate and remove bubbles. Results were read at 570 nm using a microplate reader (Beckman Coulter AD340). T-tests were used to assess any significant differences in cell viability between transduced and non-transduced cells or cell receiving conditioned media from transduced or non-transduced cells.

4. Genetic Alteration

4.1. Growth kinetics and morphology

It is vital for future clinical applications that our lentiviral vector system does not alter the expression of any genes in transduced cells, including but not limited to housekeeping genes, oncogenes, and other stress-related genes. This assessment was conducted in two ways. First, the growth kinetics of transduced cells and non-transduced cells were monitored and compared to visualize any changes in morphology or growth rate. Transduced and non-transduced HTB-11 and CHME-5 cultures were monitored for any change in morphology or growth kinetics over a period of twenty passages (60 to 80 days, respectively).

4.2. Genetic expression analysis

The second method for monitoring any potential adverse genetic expression effects of the DLV system employed utilized an XP-PCR multiplex to comparatively assess the expression levels of twenty housekeeping genes in transduced and non-

transduced cells. RNA was extracted from HTB-11 and CHME-5 transduced and non-transduced cells using Qiagen RNeasy Kit (cat# 74104). First, cells were tryspanized and pelleted through centrifugation at 4,000 RPM for 5 min. The supernatant was discarded and the cell pellet was resuspended in resuspension buffer. The cells were lysed with lysis buffer and then neutralized with neutralization buffer. Cell debris was removed and RNA was captured using an RNeasy spin column, washed twice with wash buffer, and then eluted with RNase free molecular grade water. RNA concentration was determined via spectrophotometer at 450 nm.

PCR was performed using primers specific to β-actin (Table 3) on the extracted RNA to confirm the absence of DNA contamination. Briefly, each 1.0 µL RNA template was combined with 24 µL mixture containing 1X Standard Taq Reaction Buffer (Mg^{2+} free) (New England Biolabs, MA), 1.5 mM $MgCl_2$ (New England Biolabs, MA), 200 µM of each dNTA (Sigma Aldrich, MO), 0.2 µM forward and reverse primers (IDT, IA), and 2 units of *Taq* polymerase (provided by Dr. Tung T. Huang, University of Hawaii at Manoa). PCR amplification was performed with a MasterCycler Gradient (Eppendorf North America) starting with an initial denaturation at 94°C for 5 min, followed by cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 90 s. After a final extension at 72°C for 7 min, PCR amplicons were separated using 2% agarose gel electrophoresis stained with EtBr alongside a 100 bp DNA marker (New England Biolabs, MA). Results were visualized using a Molecular Imager Gel Doc XR+ System (BioRad Laboratories, CA). If DNA contamination was present, the RNA was

subjected to DNase treatment (Invitrogen). Briefly, 1 μg RNA was combined with 1 μL 10X DNAse reaction buffer, 1 μL DNAse, and DEPC-treated water to 10 μL and incubated for 30 min at 37°C. The DNAse was inactivated by addition of 1 μL 25 mM EDTA followed by incubation for 10 min at 65°C. The RNA was aliquoted and stored at -80°C until use.

Human Reference RNA (Beckman Coulter, A54267) was used to develop a standard curve for a human reference multiplex (Tables 4-5). XP-PCR was performed on sample RNA with reverse transcriptase minus and no-template reactions as negative controls and Reference RNA as a positive control. Kan^r RNA (Beckman Coulter, #A85017) was added to each sample as an internal control and three genes were assigned as reference genes. XP-PCR was conducted in two stages. The first was the RT reaction in which sample RNA was incubated with DNase/RNase free water, 5X RT buffer, Reverse Transcriptase, and the reverse primer plex for 1 min at 48°C, 60 min at 42°C, and 5 min at 95°C. Next, the PCR reaction was performed in which cDNA from the RT reaction was incubated with 5X PCR buffer, 25 mM $MgCl_2$, DNA polymerase, and the forward primer plex for 10 min at 95°C and 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 70°C. Finally, amplicons were combined with DNA Size Standard-400 and Sample Loading Solution (Beckman Coulter, #608098 and #608082) and subjected to Capillary Electrophoresis. Results, analyzed by eXpress Profiler and Quant Tool (Beckman Coulter), were normalized to the internal control Kan^r to eliminate any inter-capillary differences and then normalized a second time to the pre-selected reference genes. Lastly, results were compared to the standard curve created for the specific multiplex and

Table 4. GeXP Reference Multiplex genes, accession numbers, and functions (NCBI gene bank).

Gene	ID	Function
EZR	X51521	Plays key role in cell surface structure adhesion, migration, and organization; implicated in various human cancers
QARS	X76013	Catalyzes aminoacylation of tRNA
HDAC1	U50079	Plays key role in regulation of eukaryotic gene expression
TRFC	BC001188	
ILF2	U10323	Transcription factor required for expression of interleukin 2 gene
CASC3	X80199	Functions in nonsense-mediated mRNA decay
GK	NM_203391	Regulation of glycerol uptake and metabolism
PSMB6	D29012	Cleaves peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway
RPL37A	L06499	Ribosomal protein; catalyzes protein synthesis
18s-rRNA	M10098	
UBE2D2	U39317	Ubiquitination of tumor-suppressor protein p53
EEF1A1	NM_001402	Enzymatic delivery of aminoacyl tRNAs to the ribosome
PPIA (cyclophilin A)	BC000689	Cyclosporin binding-protein; role in cyclosporine A-mediated immunosuppression
HYAL2	AJ000099	GPI-anchored cell surface protein
TAF7	X97999	TATA box binding protein; required for transcription
ACTB (beta-actin)	NM_001101	Involved in cell mobility, structure, and integrity
GAPDH	NM_002046	Catalyzes an important energy-yielding step in carbohydrate metabolism
ATP50	X83218	ATP synthase involved in transmission of conformational changes
SRP14	NM_003134	Signal recognition particle; RNA binding protein
HPRT1	M31642.1	Plays central role in generation of purine nucleotides through purine salvage pathway
B2M	NM_004048	Serum protein found on the surface of nearly all nucleated cells
CAPN2	M23254	Large subunit of the ubiquitous enzyme, calpain 2
RPLP0	NM_001002	Acidic ribosomal protein
GUSB	NM_000181	Degrades glycosaminoglycans

Table 5. GeXP Reference Multiplex gene abbreviations and expected amplicon sizes.

Gene	Abbreviation	Expected Amplicon Size (w/ universal sequence)
Ezrin	EZR	150
QRSHs glutaminyl-tRNA synthetase	QARS	160
Histone deacetylase 1	HDAC1	165
Transferrin Receptor	TRFC	172
Nuclear factor NF45	ILF2	186
Cancer susceptibility candidate 3	CASC3	197
Glycerol kinase	GK	201
Proteasome subunit Y	PSMB6	211
Ribosomal protein L37a	RPL37A	214
18s-rRNA	18s-rRNA	220
E2 Ubiquitin conjugating enzyme UbcH5B	UBE2D2	225
Elongation factor EF-1-alpha	EEF1A1	233
cyclophilin A	PPIA	237
Lysosomal hyaluronidase	HYAL2	253
Transcription Factor IID	TAF7	258
beta-actin	ACTB	267
GAPDH	GAPDH	277
ATP synthase	ATP50	281
18kDa Alu RNA binding protein	SRP14	291
Hypoxanthaine ribosyl transferase	HPRT1	305
Beta 2 microglobulin	B2M	314
Ca2-activated neutral protease large subunit	CAPN2	317
Acidic Ribosomal Protein	RPLP0	330
Beta-glucuronidase	GUSB	338
Kanamycin resistance	KAN^r	325

fold changes and significance differences between samples were calculated using t-tests.

5. Primary Human Peripheral Blood Mononuclear Cells

The overall objective of this study is to utilize DLV transduced MDM as a novel gene delivery method for anti-HIV-1 Tat scFv in the CNS. Established immortalized cell lines are ideal for in vitro research. However, these cell lines lose many characteristics of the primary cells from which they are derived. Therefore, to assess the efficiency and stability of DLV-mediated transduction and scFv expression in MDM, primary human PBMCs were isolated, transduced and subjected to ELISA, Neurotoxicity assays and GeXP analysis.

5.1. Isolation and Cell Culture

Primary human PBMCs were isolated from 50 mL whole blood collected from a healthy donor by intravenous puncture in BD Vacutainers ACD (Beckman Dickson). Aliquots were pooled and diluted with DPBS to a ratio of 1:2. Slowly, 25 mL diluted blood was layered over 20 mL Ficoll-Paque Plus in a 50 mL polypropylene centrifuge tube. The blood cells were centrifuged for 30 min at 1,000 x g, with the break off. The upper layer was then removed by aspiration and the white layer at the interface containing PBMCs was collected and pooled into a fresh 50 mL centrifuge tube. The pooled PBMCs were diluted 1:2 with DPBS and centrifuged for 15 min at 460 x g. The supernatant was removed and the cell pellet was resuspended in 20 mL DPBS and subsequently centrifuged for 8 min at 220 x g. The PBMCs were seeded at a density of 1.5×10^7 cells/12.5 cm^2 primary TC flask in RPMI-1640 growth medium supplemented with 20% defined FBS, 10% human

serum, 2mM L-glutamine, 100 IU/mL penicillin (Sigma-Aldrich), and 0.1 mg/mL streptomycin (Sigma Aldrich), and maintained at 37°C with 5% CO_2. Prior to seeding, $7x10^6$ cells were labeled with CD14 MACS microbeads or CD11b MACS microbeads and seeded in a 12 well plate to determine if additional cell sorting would benefit PBMC culture purity and growth. After 48 hrs, non-adherent cells were removed, attached MDM were washed three times with DPBS, and fresh RPMI-1640 growth medium was added. This was repeated every 2 days for the duration of the culture. PBMC culture purity was verified by staining the cells for 1 h with anti-human CD14 monoclonal antibodies conjugated with R-phycoerytherin diluted 1:100 in DPBS and examining the results under an inverted fluorescent microscope (Nikon Eclipse TE2000-U).

5.2. Transduction

On day 7 post isolation, MDM were washed three times with DPBS and then incubated at 37°C with 5% CO_2 with 0.4 mL vector stock ($1x10^7$IU/mL) in the presence of 8 μg/mL polybrene for 2 h with gentle rocking every 15 min. Transduced cells were washed twice with DPBS and given fresh RPMI-1640 growth medium. Transduction efficiency was determined by GFP quantification 5 days post infection. Cells were harvested by scraping and DNA was extracted and used in PCR confirmation as previously described.

5.3. ScFv Expression and Function

Expression of scFv from transduced MDM was quantified relative to non-transduced cells by ELISA. Recombinant HIV-1 Tat (NIH AIDS Reagents #2222), in a 0.5 M Carbonate-Bicarbonate, pH 9.6, coating buffer was incubated in a 96 well

plate for 2 h at 4°C and then blocked with TBS blocking buffer containing 1% BSA for 1.5 h at room temperature on an orbital shaker. Cell culture media from transduced and non-transduced primary human MDM was collected after 48 h incubation at 37°C, 5% CO_2. Samples were diluted 1:20 with TBS dilution buffer containing 0.05% Tween-20 and 1% BSA and then loaded and incubated for 1 h at room temperature with shaking. Direct and indirect labeling was utilized with detection antibodies, mouse anti-human IgG-HRP (SouthernBiotech, AL) or goat anti-human IgG Fc-biotin (Rockland, PA), followed by streptovidin-HRP (Rockland, PA), were incubated at room temperature for 1 h with shaking. Washing with TBS-T was repeated 5 times between each step. Finally, one-Step Ultra TMB (tetramethylbenzidine) substrate (Pierce) was incubated with protection from light for 5-10 min and stopped with 2 M Sulfuric Acid. Results were read at 450 nm using an ELISA reader (Beckman Coulter AD340) and the OD of transduced MDM was compared to that of non-transduced MDM.

Neuroprotection from HIV-1 Tat- and gp120-mediated neurotoxicity conferred by the scFv in conditioned MDM culture media was also assessed. HTB-11 cells were exposed to 500 ng/mL recombinant HIV-1 Tat protein (NIH AIDS Reagents #2222) or 250 ng/mL recombinant HIV-1 gp120 (NIH AIDS Reagents #2968), and conditioned culture media diluted 1:10 in MEM-10 from either transduced or non-transduced MDM. Controls included cells receiving an HIV-1 neurotoxin concentration of 0 ng/mL and cells receiving antiserum to recombinant HIV-1 Tat protein (NIH AIDS Reagents #4357). After 48 h, MTT assay was employed to determine cell viability. Cells were washed thoroughly with PBS and

then incubated with 5 mg/mL MTT at 37°C for 2 h. After adequate crystal formation, DMSO was added with shaking for 15 min to dissolve precipitate and remove bubbles. Results were read at 570 nm using a microplate reader (Beckman Coulter AD340). T-tests were used to assess any significant differences in cell viability between cells receiving conditioned media from transduced or non-transduced MDM.

5.4. Genetic Expression Analysis

RNA was extracted from transduced and non-transduced MDM and subjected to DNAse treatment as previously described. XP-PCR and capillary electrophoresis were performed using the Human Reference Multiplex as previously described. Results were analyzed using eXpress Profiler and Quant Tool (Beckman Coulter) and gene expression fold changes in transduced MDM were calculated relative to non-transduced MDM.

6. References

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The reagent HIV-1 Tat protein (catalogue#2222) was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Tat protein from Dr. John Brady and DAIDS, NIAID.

The reagent HIV-1 Tat antiserum (catalogue#4357) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: EIA V Tat Antiserum from Dr. Wendy Maury.

The reagent HIV-1 gp120 recombinant protein was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 gp120 CM envelope protein (Cat #2968).

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CHAPTER 4

RESULTS AND DISCUSSION

1. Transduction of Human Neuroblastoma and Microglial Cells

1.1. Plasmid preparation and verification

Four transfer plasmids were constructed and amplified to produce the DLV necessary for gene transfer to target cell lines. Two anti-HIV-1-Tat scFv and two control sequences were amplified with PCR and subsequently ligated into a plasmid backbone containing as a CMV promoter and GFP and Fc fusion protein as indicator genes. Plasmids were subjected to restriction enzyme digestion using BamH1 and Xho1 and separated using 1% agarose gel electrophoresis to identify the inserted genes of interest, approximately 1.5 kb (Figure 8). Successful plasmid construction and transformation of e. coli competent cells was confirmed and transformed cultures were grown in several 250 mL cultures for maxi preparation and extraction of plasmids. Maxi preparation and extraction was also performed for packaging (pCMVΔR8.2Δvpr) and envelope (pCMV-VSV-G) plasmids. All plasmids were aliquoted and stored at 4°C for use in transfection of HEK 293T cells.

1.2. High titer vector stock

Defective lentiviral vectors were produced by transient co-transfection of HEK 293T cells with transfer, packaging, and envelope plasmids using a calcium-phosphate precipitation protocol optimized in our laboratory. Transfection efficiency was assessed by GFP quantification 12 h post transfection to be 100% for all four transfer plasmids (Figure 9). Media from transfected HEK293T cells

containing DLV was collected every 24 h for 8 days post transfection. Titration of un-concentrated vector on HEK 293T cells revealed a moderately high vector titer of 1–2x10^7 IU/mL (Table 6). Collected vector was filtered and concentrated using an ultra-centrifuge. Titration of concentrated vector on HEK 293T cells revealed a titer of 1-2x10^7 (Table 6). Concentrated vector was stored in aliquots at -80°C for use in transduction of HTB-11, CHME-5, and primary PBMC cell cultures.

1.3. Transduction efficiency

Defective lentiviral vectors were used to transduce 1x10^5 HTB-11 or CHME-5 cells at a moi of 10. Transduction efficiency was determined by GFP quantification three days post infection to be 100% in HTB-11 cells for all four DLV types (Table 7). Initial transduction of CHME-5 cells resulted in transduction efficiencies less than 100% for all four DLV types. Therefore, a second transduction was performed for all CHME-5 cells resulting in 90-98% transduction efficiency as determined by GFP quantification (Table 8). This is consistent with previously published data using similar DLV to transduce HTB-11 and CHME-5 cell lines (Cao *et al.*, 2011). Transduced cell lines were subsequently renamed based on the parental cell line and DLV construct, i.e., HTB-11-Hutat2, CHME-5-E46. DNA was extracted from each transduced cell line and subjected to PCR using primers specific to each DLV construct as well as the GFP indicator gene. DNA was also extracted from non-transduced HTB-11 and CHME-5 cells to represent a no-template control. No amplicons were visualized for any gene of interest or GFP in non-transduced cells (Figure 10). Strong bands were visible for each transduced cell line using gene-specific primers as well as GFP primers (Figures 11-12).

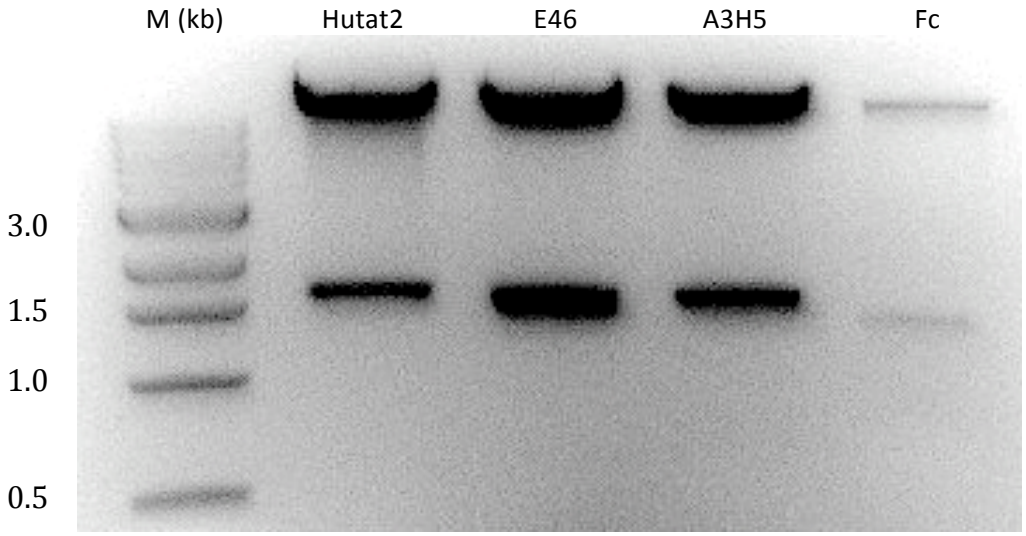


Figure 10. Restriction enzyme digestion of constructed plasmids.
Hutat2, E46, A3H5, and Fc plasmids were digested with Xho1 and BamH1 restriction enzymes and separated on a 1% agarose gel confirming successful ligation and transformation of e. coli competent cells.

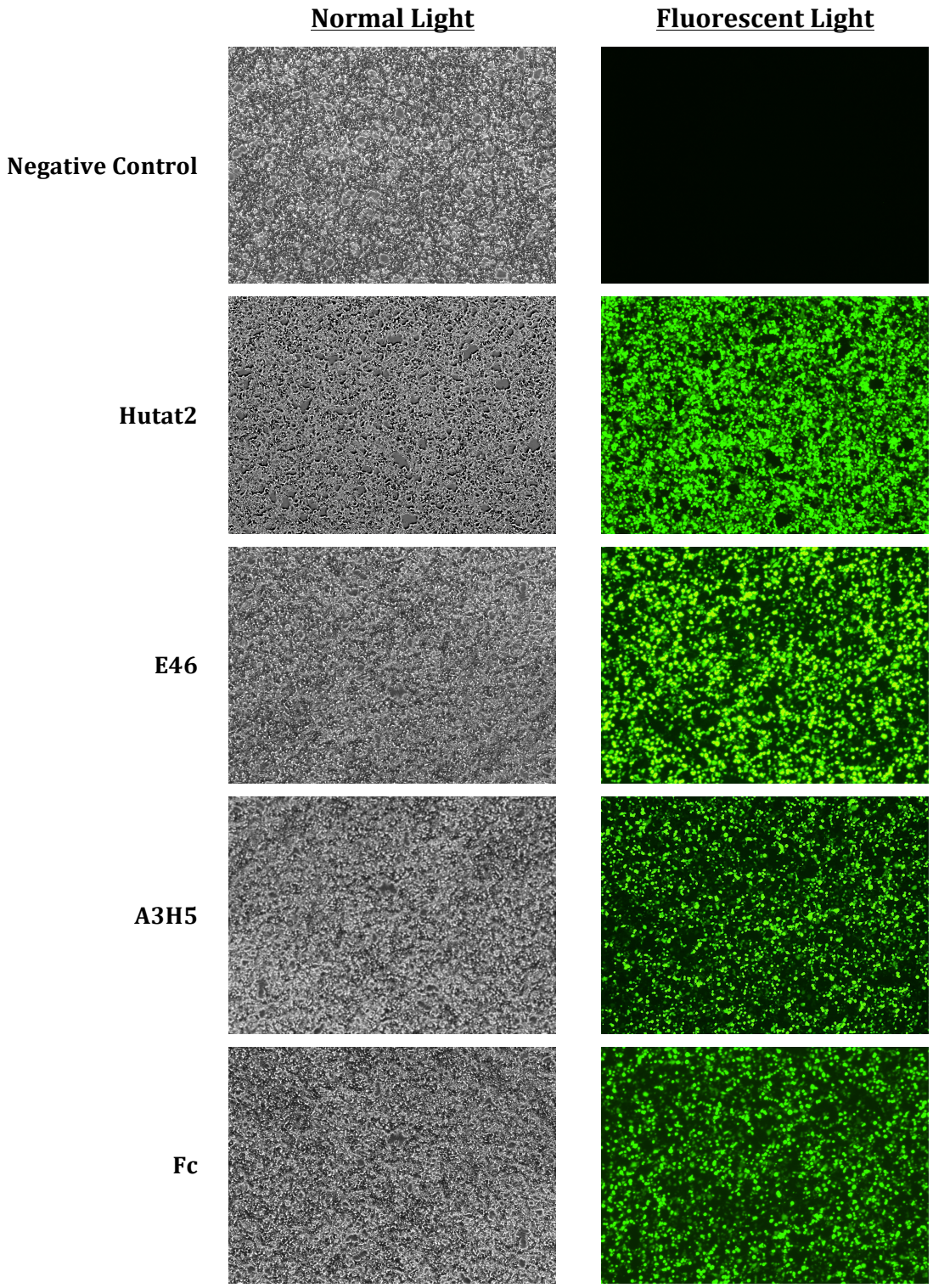


Figure 11. Transient co-transfection of HEK 293T cells for DLV production.
HEK 293T packaging cells were co-transfected with packaging, envelope, and transfer plasmids using an optimized calcium-phosphate precipitation transfection protocol. Transfection efficiency was calculated to be 100% by GFP quantification.

Table 6. Defective Lentiviral Vector Titers on HEK 293T Cells.

DLV titers were determined to range from $1x10^6$ IU/mL pre-concentration to $2x10^7$ IU/mL post-concentration by serial titration on HEK 293T cells. Vector produced by the transfected HEK 293T cells was collected every 24 hours on days 1 through 8 post-transfection and then concentrated using ultracentrifugation.

Vector	Un-concentrated (IU/mL)	Concentrated (IU/mL)
Hutat2	$1x10^6$	$1x10^7$
E46	$2x10^6$	$2x10^7$
A3H5	$1x10^6$	$1x10^7$
Fc	$1x10^6$	$1x10^7$

Table 7. Transduction Efficiency in HTB-11 Cell Cultures.

Human neuroblastoma (HTB-11) cells were transduced with 1 mL un-concentrated vector in the presence of polybrene for 3 hours with gentle mixing every 15 minutes. Transduction efficiency was determined by GFP quantification.

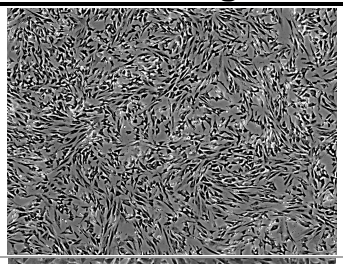
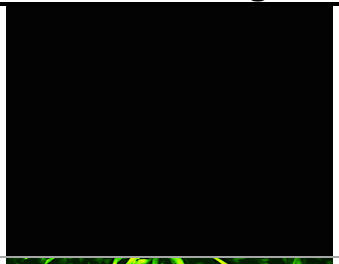
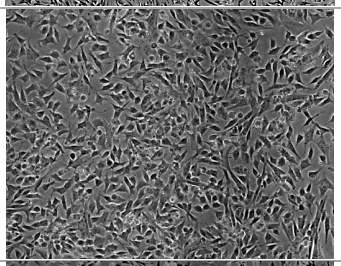
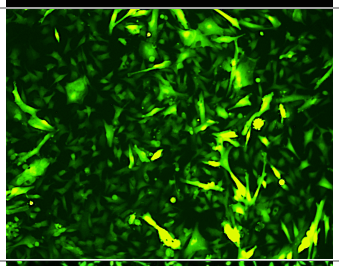
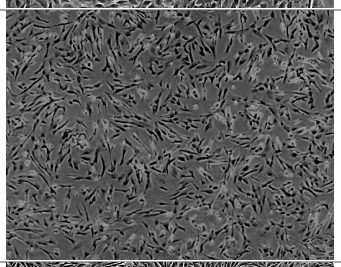
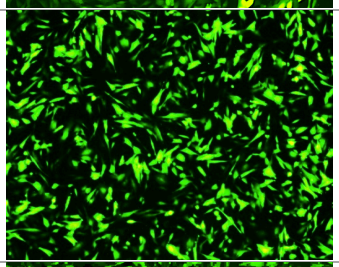
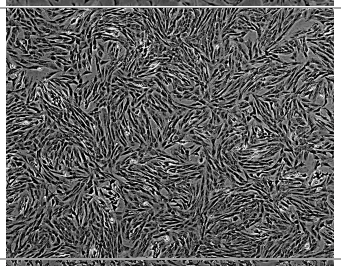
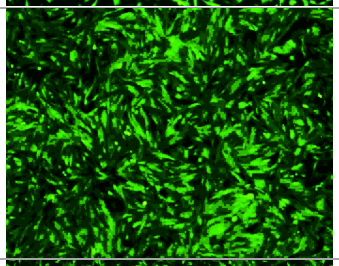
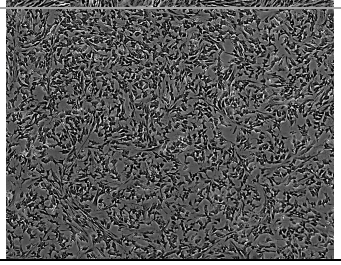
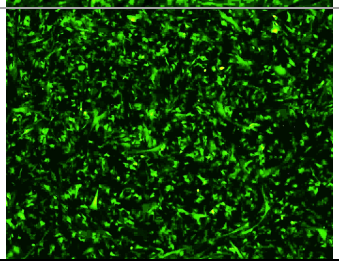
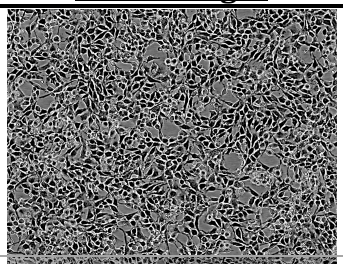
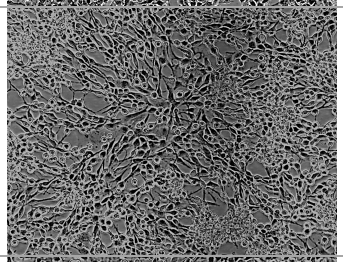
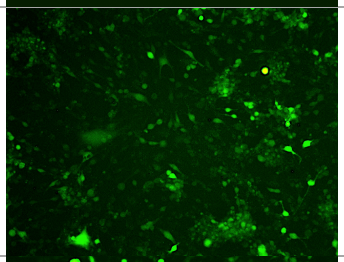
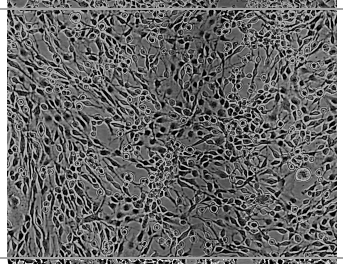
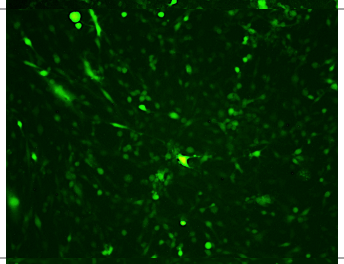
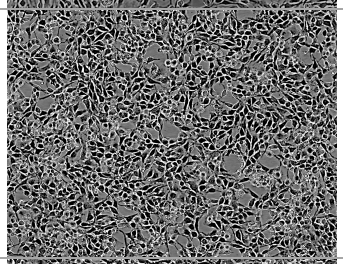
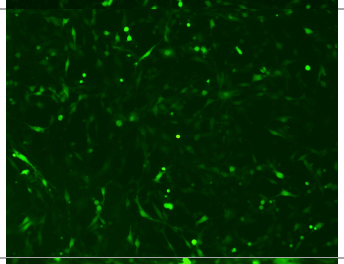
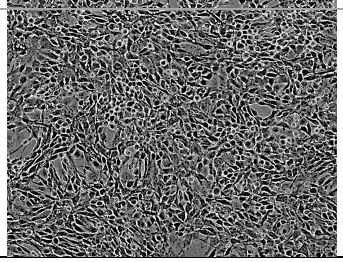
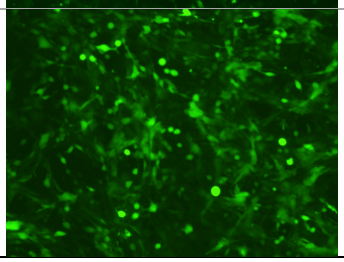
Construct	Normal Light	Fluorescent Light	GFP%
Non Transduced			N/A
Hutat2			100%
E46			100%
A3H5			100%
Fc			100%

Table 8. Transduction Efficiency in CHME-5 Cell Cultures.

Human microglial cells (CHME-5) were transduced with 0.1 mL concentrated vector in the presence of polybrene for 3 hours with gentle mixing every 15 minutes. Transduction efficiency was determined by GFP quantification.

Construct	Normal Light	Fluorescent Light	GFP%
Non Transduced			N/A
Hutat2			90%
E46			98%
A3H5			90%
Fc			95%

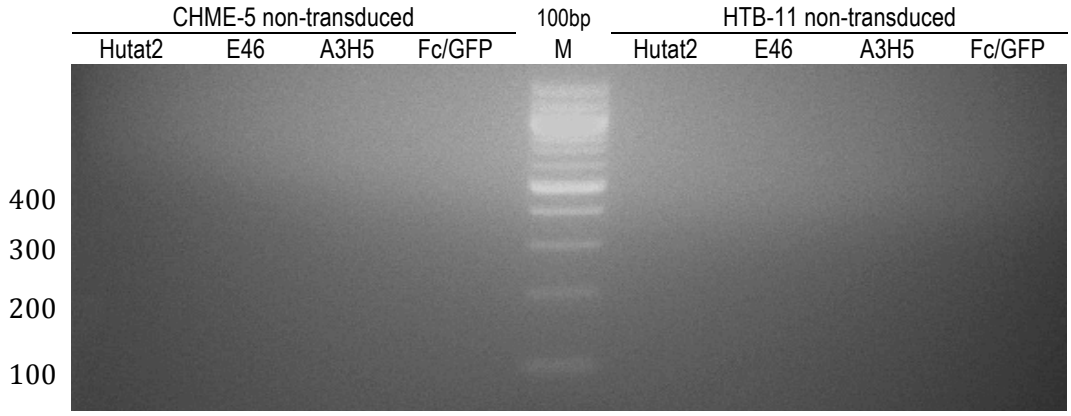


Figure 12. PCR demonstrated no amplification of gene of interest or eGFP in non-transduced CHME-5 and HTB-11 cells.

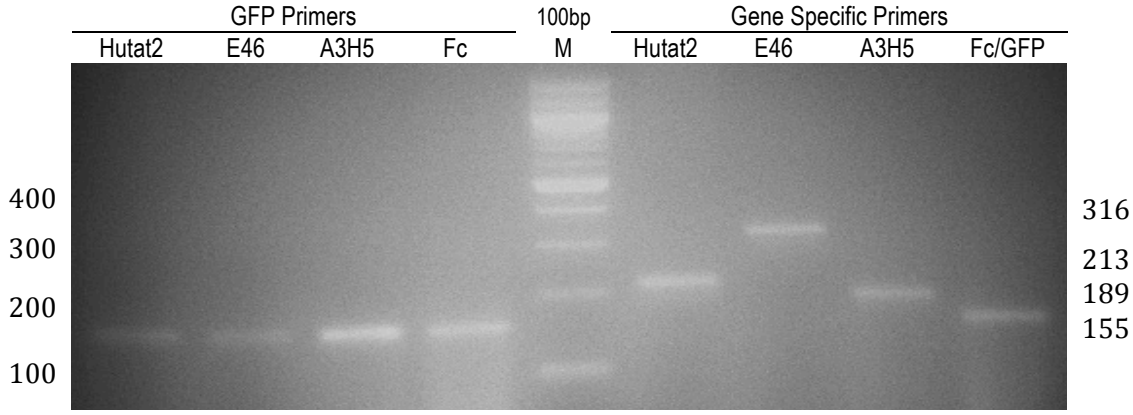


Figure 13. PCR confirmation of eGFP and gene of interest in transduced HTB-11 cells.

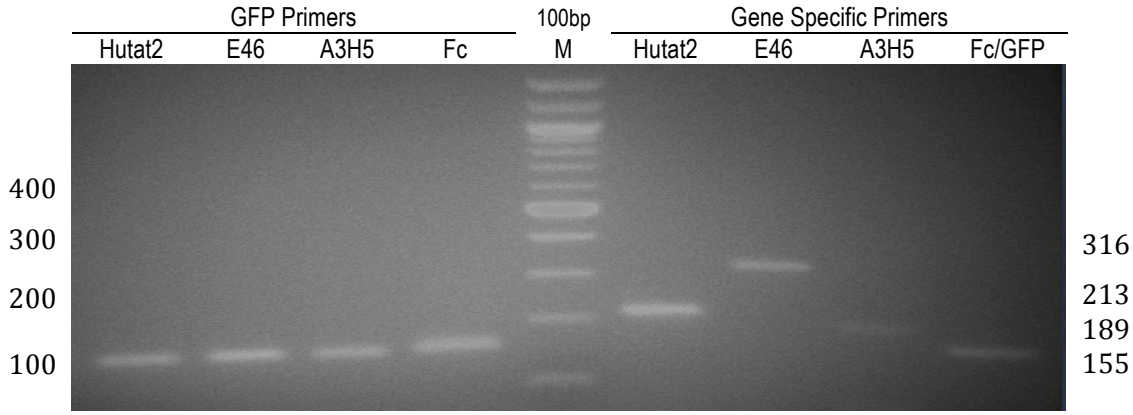


Figure 14. PCR confirmation of eGFP and gene of interest in transduced CHME-5 cells.

2. Intrabody Expression

2.1. Intracellular scFv detection

HIV-1 Tat is present both intracellularly and extracellularly in the CNS. Therefore, the anti-HIV-1 Tat scFv assessed in this study was designed to remain in the cell as well as to be secreted. The intracellular presence of expressed scFv in transduced HTB-11 and CHME-5 cells was observed through immunofluorescence staining. An optimized protocol utilizing antibodies targeting the Fc-fusion protein of the scFvs clearly identifies GFP positive cells as also positive for the Fc-fusion protein in all transduced cell lines, with no GFP or Fc-fusion protein detected in the non-transduced HTB-11 or CHME-5 cells (Figures 12-13) as expected.

2.2. Extracellular scFv detection

Extracellular scFv was detected using an optimized Western Blot protocol utilizing antibodies targeting the Fc-fusion protein of the scFvs secreted in conditioned cell culture media. Due to high background caused by cross-reaction between the primary antibody (rabbit anti-Human IgG (H+L)) and IgG present in the FBS, cells were incubated in serum free media for 48 h prior to collection of the media for processing in the Western Blot. Due to this 48 h period of essentially starving the cells, the protein bands blotted onto the nitrocellulose membrane are not as strong as they may be when the cells are growing under optimal conditions. The expected size of the scFvs ranges from 55-65 kDa. However, under the non-reducing conditions utilized in this study, it has previously been found (data unpublished) that the scFv form dimers and trimers, thereby separating as 110 to 130 or 165 to 195 respectively as is demonstrated in Figures 14-15.

2.3. Quantification of secreted scFv

ELISA was employed to quantify scFv expression levels in transduced cells and to monitor stability of expression long-term (20 passages). Expression of scFv was found to be stable long term in both HTB-11 and CHME-5 cell lines. Moderate to high levels of scFv expression were observed in transduced HTB-11 cells ranging from 808–941 ng/mL in HTB-11-Hutat2 cells, 112–129 ng/mL in HTB-A3H5 cells, 86–124 ng/mL in HTB-11-E46 cells, and 77–85 ng/mL in HTB-Fc cells (Figure19). Moderate levels of scFv expression were observed in transduced CHME-5 cell ranging from ng/mL in CHME-5-Hutat2 cells, ng/mL in CHME-5-A3H5 cells, ng/mL in CHME-5-E46 cells, and ng/mL in CHME-5-Fc cells (Figure 20). These differences can possibly be attributed to the integration of more than one set of scFv genes into the genome of HTB-Hutat2 cells, thereby markedly increasing detectable levels of scFv in conditioned media. Overall, these findings demonstrate that DLV-mediated transduction of HTB-11 and CHME-5 cell lines results in stable long-term moderate to high levels of scFv expression.

3. Intrabody Function

3.1. Specific binding to HIV-1 Tat

After confirming stable moderate to high scFv expression levels, Immunoblot assay was employed to assess the ability of secreted anti-HIV-1 Tat scFv to bind specifically to HIV-1 Tat. Recombinant HIV-1 Tat, supplied by the NIH AIDS Research & Reference Reagents Program, was serial diluted and blotted onto a nitrocellulose membrane using the dilution buffer as a negative control.

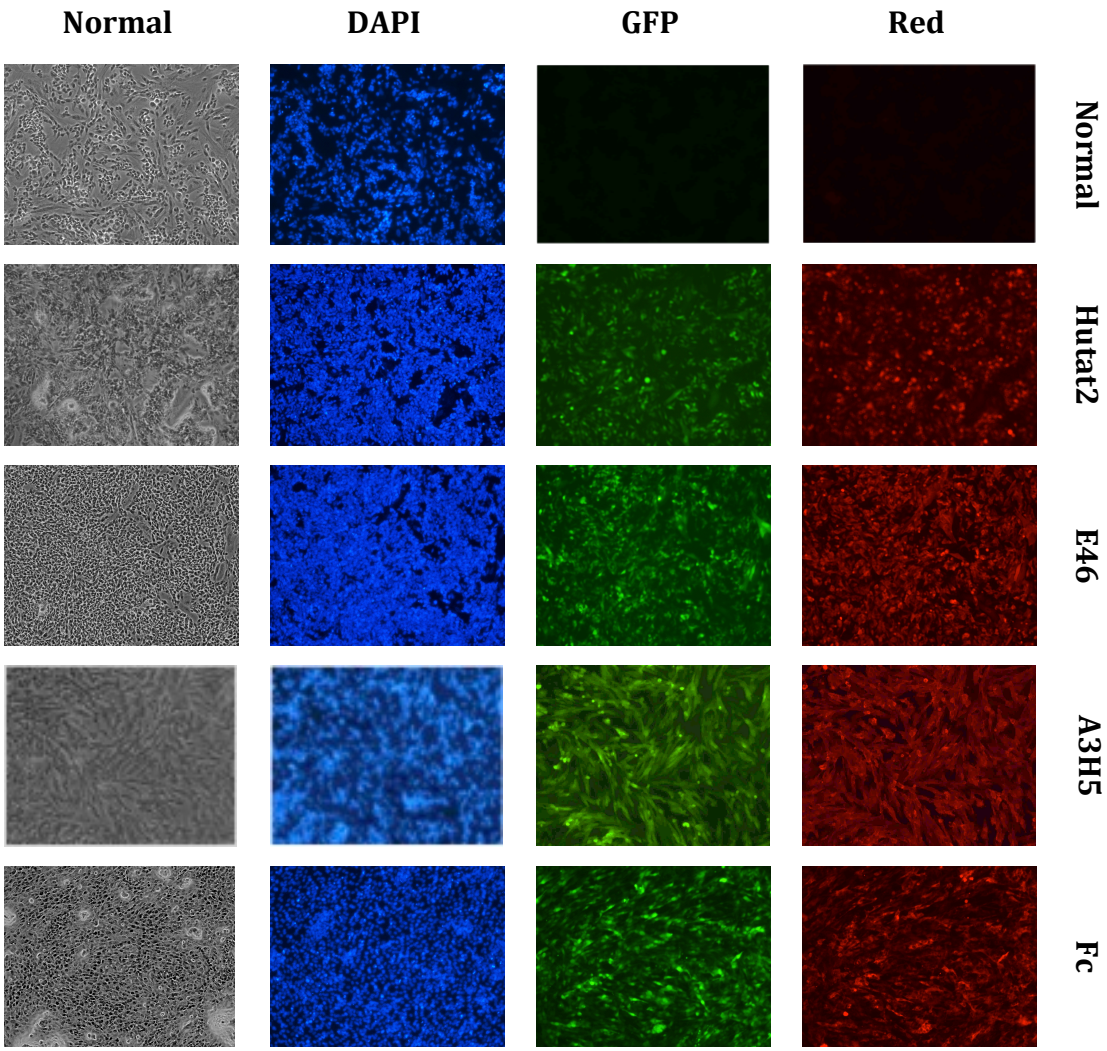


Figure 15. Immunofluorescent staining of scFv in HTB-11 cells.

Immunofluoresence staining visualized the intracellular presence of scFv in transduced HTB-11 cells. Cells were seeded in 24 well TC plates for 1 day and then fixed with 4% paraformaldahyde at room temperature. Cells were washed with PBS and incubated in Triton X 100 followed by blocking solution. Primary and secondary antibodies were rabbit anti-human IgG (H+L) and goat anti-rabbit IgG-R, respectively. BisBenzimide was added and results were visualized under a fluorescent microscope using a wavelength corresponding to the conjugation of the secondary antibody.

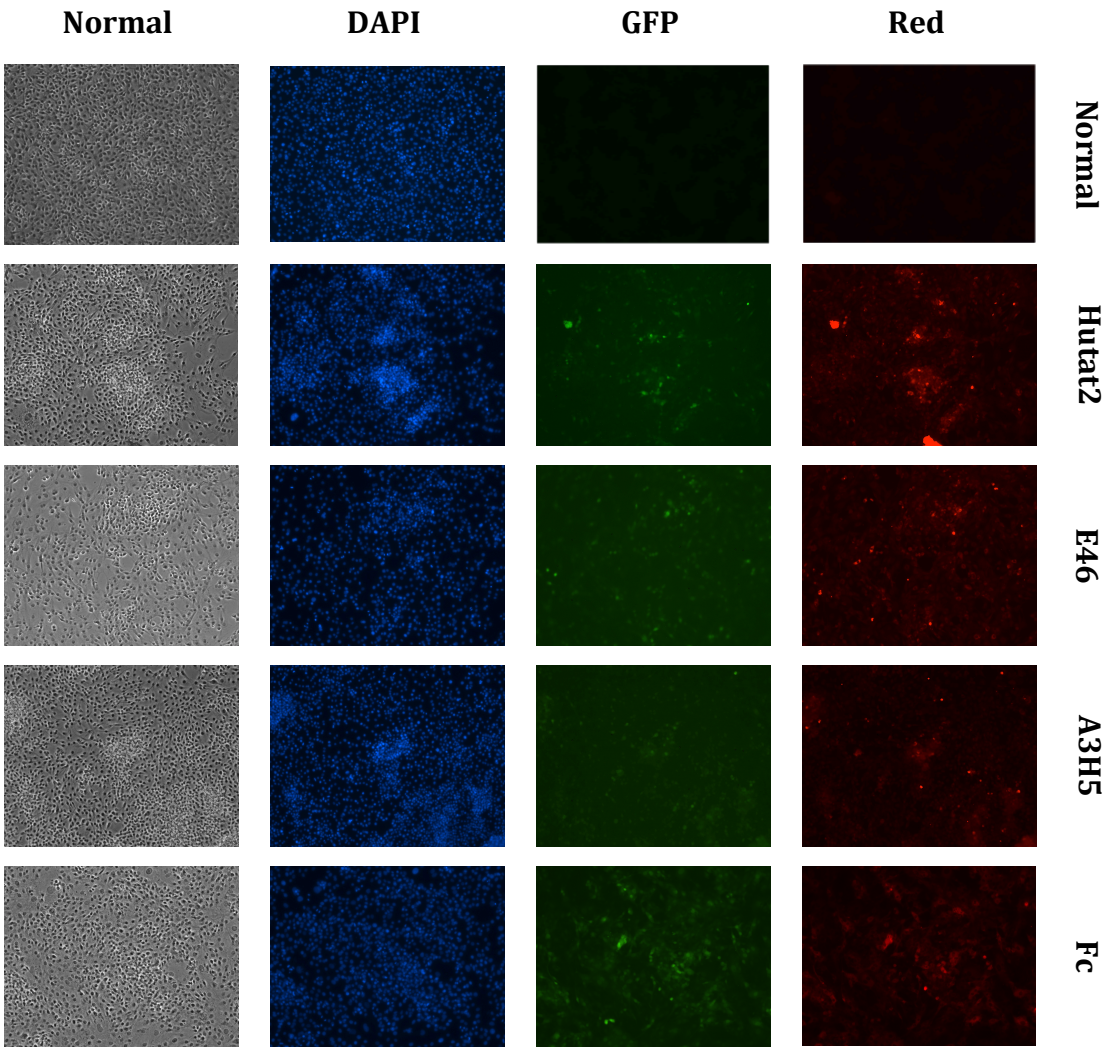


Figure 16. Immunofluorescent staining of scFv in CHME-5 cells.

Immunofluoresence staining visualized the intracellular presence of scFv in transduced CHME-5 cells. Cells were seeded in 24 well TC plates for 1 day and then fixed with 4% paraformaldahyde at room temperature. Cells were washed with PBS and incubated in Triton X 100 followed by blocking solution. Primary and secondary antibodies were rabbit anti-human IgG (H+L) and goat anti-rabbit IgG-R, respectively. BisBenzimide was added and results were visualized under a fluorescent microscope using a wavelength corresponding to the conjugation of the secondary antibody.

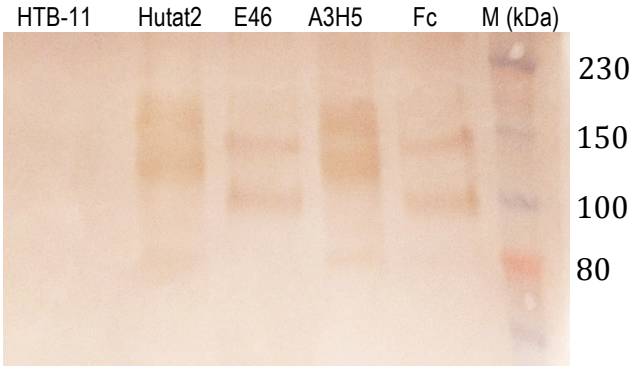


Figure 17. Western Blot detection of secreted scFv in HTB-11 culture media.
Secreted scFv were detected in the culture media of transduced HTB-11 cells at approximately 120-140 kDa by Western Blot under non-reducing conditions. Primary and secondary antibodies were rabbit anti-human IgG (H+L) and goat anti-rabbit IgG-Fc-HRP, respectively.

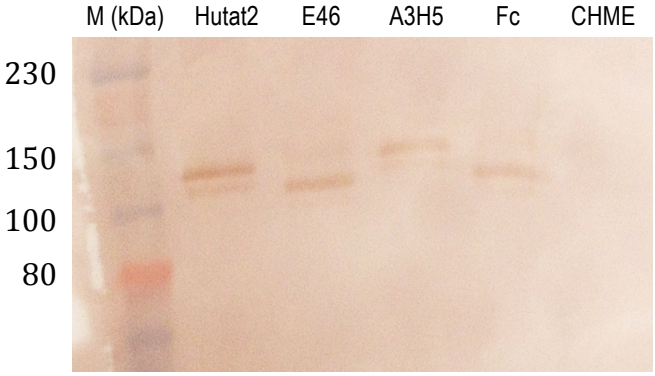


Figure 18. Western Blot detection of secreted scFv in CHME-5 culture media.
Secreted scFv were detected in the culture media of transduced CHME-5 cells at approximately 120 kDa by Western Blot under non-reducing conditions. Primary and secondary antibodies were rabbit anti-human IgG (H+L) and goat anti-rabbit IgG-Fc-HRP, respectively.

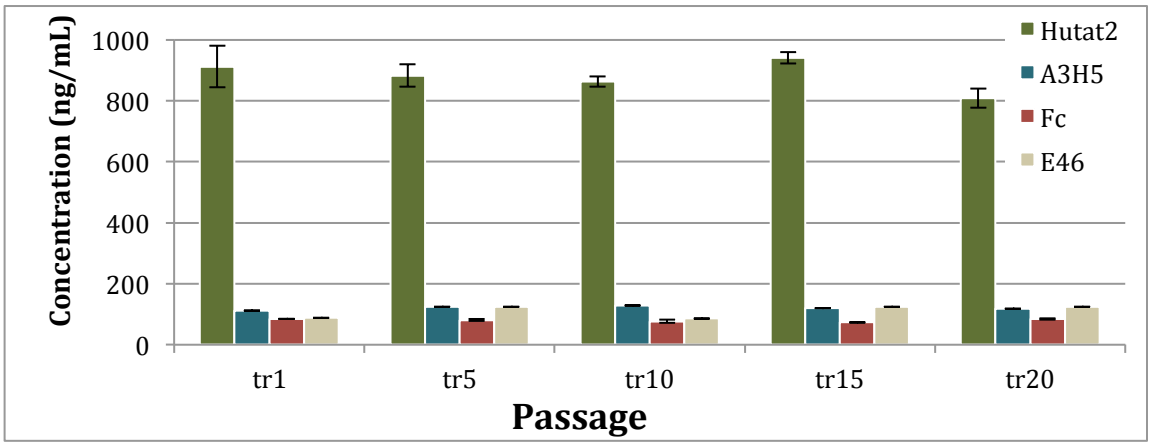


Figure 19. Moderate to high levels of secreted scFv in transduced HTB-11 conditioned media quantified by Enzyme Linked Immunosorbant Assay.

Moderate to high levels of expression are detected in transduced HTB-11 cells (Hutat2, A3H5, E46, Fc). Capture and detection antibodies were goat anti-human IgG Fc, goat anti-human IgG Fc-Biotin, and streptovidin-HRP, respectively. ELISA was performed every 5 passages for 20 passages to assess scFv gene expression stability.

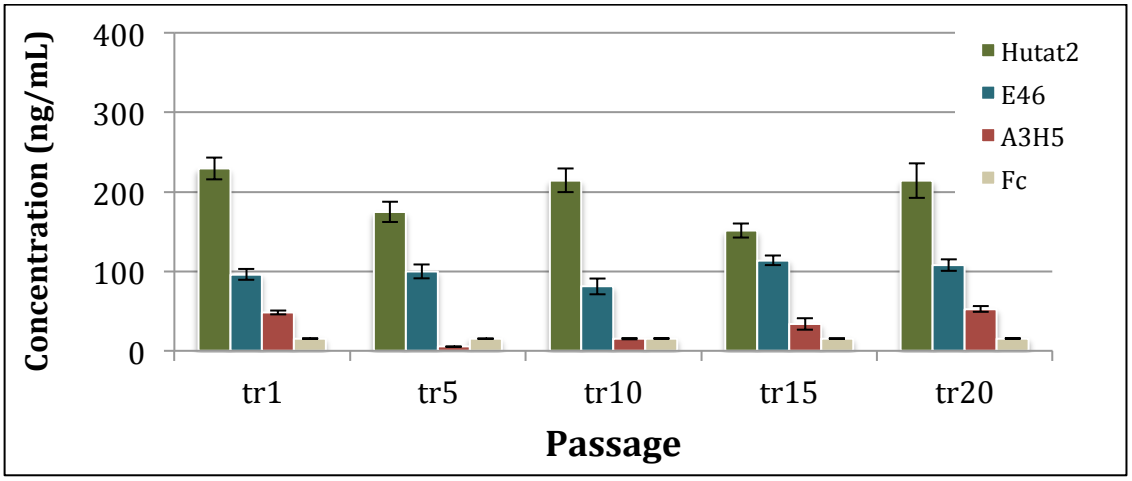


Figure 20. Moderate to high levels of secreted scFv in transduced CHME-5 conditioned media quantified by Enzyme Linked Immunosorbant Assay.

Moderate to high levels of expression are detected in transduced CHME-5 cells (Hutat2, A3H5, E46, Fc). Capture and detection antibodies were goat anti-human IgG Fc, goat anti-human IgG Fc-Biotin, and streptovidin-HRP, respectively. ELISA was performed every 5 passages for 20 passages to assess scFv gene expression stability.

Conditioned media from HTB-11 and CHME-5 cell lines transduced with anti-HIV-1 Tat scFv (Hutat2 and E46) bound specifically to HIV-1 Tat with a noticeable dose-response gradient with no binding to the negative control. However, secreted control scFv (A3H5 and Fc) did not bind to HIV-1 Tat nor to the negative control (Figures 21-22). This demonstrates that the secreted levels of scFv are sufficient to bind to Tat and that the anti-HIV-1 Tat scFv bind specifically, as designed.

3.2. Protection from HIV-1 Tat-mediated neurotoxicity

The next important step was to determine whether the binding of anti-HIV-1 Tat scFv to HIV-1 Tat successfully inhibits the neurotoxic properties of HIV-1 Tat that lead to neuronal apoptosis and the development of HANDs. Transduced and non-transduced HTB-11 cells were exposed to 500 ng/mL HIV-1 Tat in conjunction with conditioned media from transduced and non-transduced HTB-11 and CHME-5 cells or anti-serum to HIV-1 Tat, supplied by the NIH AIDS Research & Reference Reagents Program. Cell viability as assessed by MTT assay revealed that anti-HIV-1 scFv Hutat2 and E46 provide significant protection in transduced cells ($p<0.01$ and $p<0.05$, respectively) to neurons from HIV-1 Tat-mediated neurotoxicity (Figure 23). Also, anti-HIV-1 Tat scFv Hutat2 and E46 present in the conditioned media from transduced HTB-11 and CHME-5 cells provided significant protection ($p<0.01$) to neurons from HIV-1 Tat-mediated neurotoxicity (Figure 24). Control scFv A3H5 and Fc from conditioned media in HTB-11 and especially CHME-5 cells also provided protection, though not significant, from HIV-1 Tat-mediated neurotoxicity as demonstrated in higher cell viability than the neuronal cells receiving HIV-1 Tat treatment alone. Most likely this is a result of the use of DLV in

cell transduction. Several studies have observed that HIV-1 based DLV, despite the absence of any anti-HIV gene inserts, inhibits HIV-1 replication and associated neurotoxicities (Zeng *et al.*, 2006a).

3.3. Protection from HIV-1 gp120-mediated neurotoxicity

Previous studies have observed that therapies which inhibit HIV-1 Tat-mediated neurotoxicity also have the ability to inhibit HIV-1 gp120-mediated neurotoxicity, although the mechanism is unknown (Cao *et al.*, 2011). To assess the ability of anti-HIV-1 Tat scFv Hutat2 and E46 to inhibit the neurotoxic properties of HIV-1 gp120, transduced and non-transduced HTB-11 cells were exposed to 250 ng/mL HIV-1 gp120, supplied by the NIH AIDS Research & Reference Reagents Program, in conjunction with conditioned media from transduced and non-transduced HTB-11 and CHME-5 cells or anti-serum to HIV-1 Tat, supplied by the NIH AIDS Research & Reference Reagents Program. Cell viability as assessed by MTT assay revealed that anti-HIV-1 scFv Hutat2 and E46 provide significant protection in transduced cells ($p<0.01$) to neurons from HIV-1 gp120-mediated neurotoxicity (Figure 25). Also, anti-HIV-1 Tat scFv Hutat2 and E46 present in the conditioned media from transduced HTB-11 and CHME-5 cells provided significant protection ($p<0.01$) to neurons from HIV-1 gp120-mediated neurotoxicity (Figure 26). Control scFv A3H5 and Fc from conditioned media in HTB-11 and especially CHME-5 cells also provided protection, from HIV-1 gp120-mediated neurotoxicity as demonstrated in higher cell viability than the neuronal cells receiving HIV-1 gp120 treatment alone. Protection conferred by the scFv is due in part to the use of DLV in cell transduction, as previously discussed (Lu *et al.*, 2003). However,

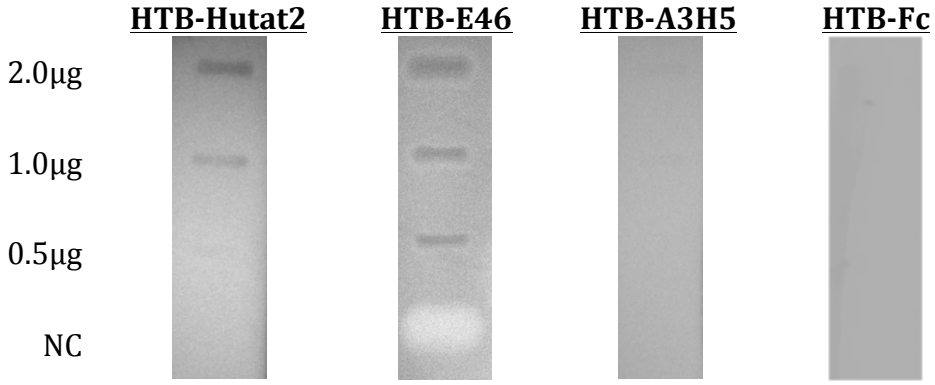


Figure 21. HIV-1 Tat immunoblot of transduced HTB-11 cell culture media.
Anti-HIV-1 Tat scFvs Hutat2 and E46 secreted into HTB-11 culture media bind specifically to serial dilutions of HIV-1 Tat blotted on a nitrocellulose membrane while control scFvs A3H5 and Fc do not. This demonstrates the biological specificity of the anti-HIV-1 Tat scFvs.

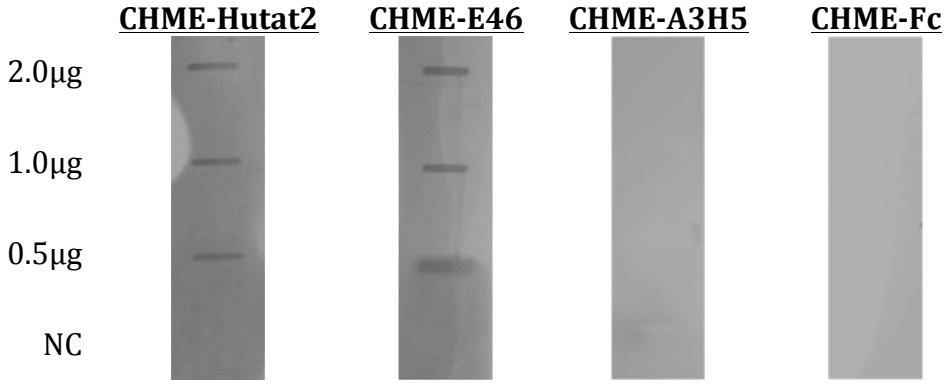


Figure 22. HIV-1 Tat immunoblot of transduced CHME-5 cell culture media.
Anti-HIV-1 Tat scFvs Hutat2 and E46 secreted into CHME-5 culture media bind specifically to serial dilutions of HIV-1 Tat blotted on a nitrocellulose membrane while control scFvs A3H5 and Fc do not. This demonstrates the biological specificity of the anti-HIV-1 Tat scFvs.

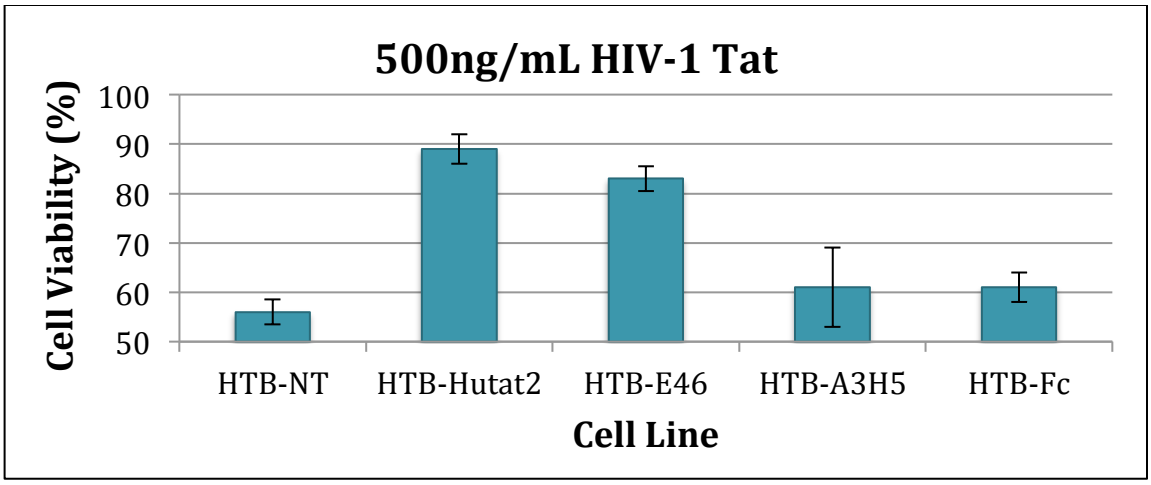


Figure 23. HIV-1 Tat-mediated neurotoxicity in transduced neuronal cells.
Following exposure to 500ng/mL HIV-1 Tat, a known neurotoxin, cell viability was significantly higher among HTB-11 cells transduced with anti-HIV-1 Tat scFv constructs (HTB-Hutat2, HTB-E46) than those transduced with control scFv constructs (HTB-A3H5, HTB-Fc) or non-transduced (HTB-NT) cells.

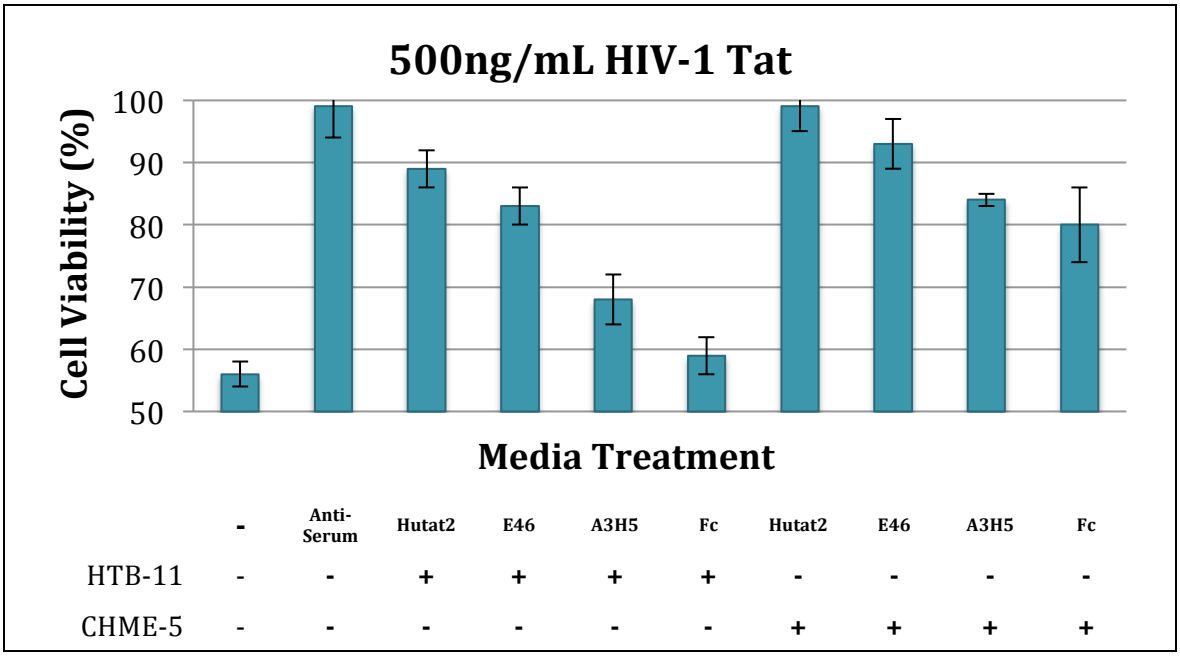


Figure 24. HIV-1 Tat-mediated neurotoxicity in non-transduced neuronal cells.
Cell viability was significantly higher among HTB-11 cells exposed to 500ng/mL HIV-1 Tat in combination with conditioned culture media containing anti-HIV-1 Tat scFvs (Hutat2, E46) than conditioned culture media containing control scFvs (A3H5, Fc) or from non-transduced cells from both CHME-5 and HTB-11 cell lines.

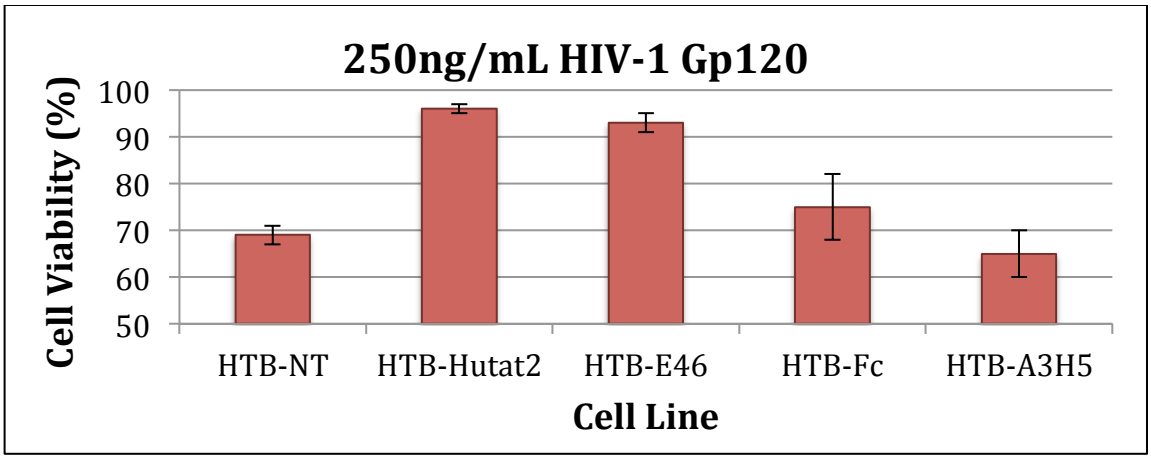


Figure 25. HIV-1 gp120-mediated neurotoxicity in transduced neuronal cells. Following exposure to 250ng/mL HIV-1 gp120, a known neurotoxin, cell viability was significantly higher among HTB-11 cells transduced with anti-HIV-1 Tat scFv constructs ($p<0.01$) than non-transduced (HTB-NT) cells while cell viabilities for control scFv constructs (HTB-A3H5, HTB-Fc) were not significantly higher than HTB-NT.

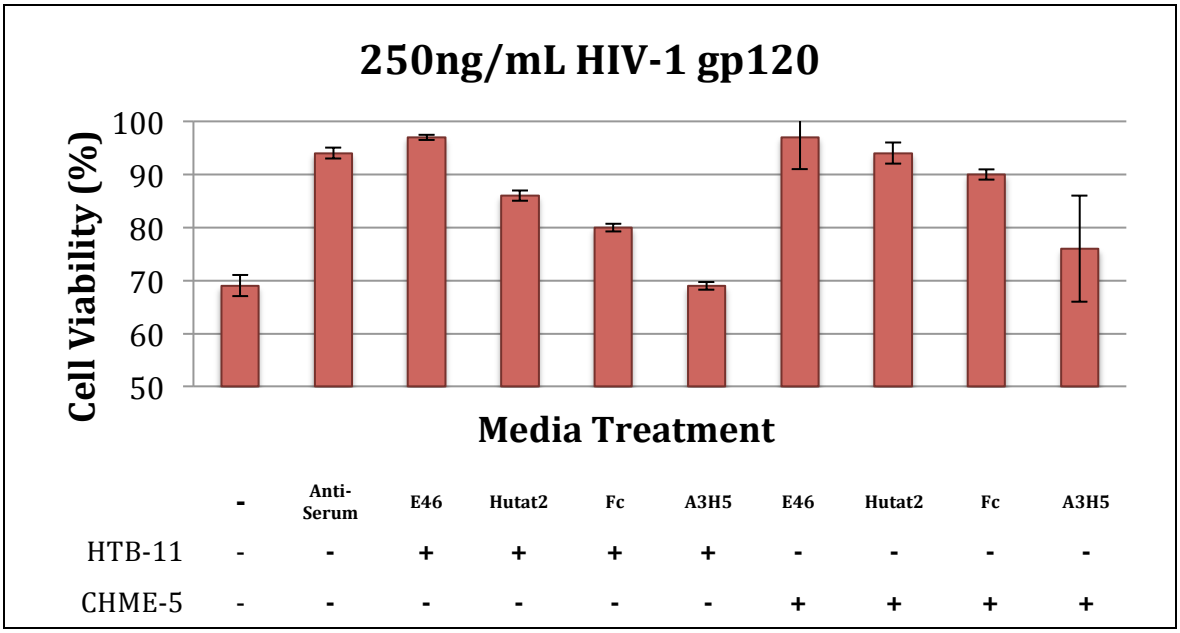


Figure 26. HIV-1 gp120-mediated neurotoxicity in non-transduced neuronal cells. Cell viability was significantly higher among HTB-11 cells exposed to 250ng/mL HIV-1 gp120 in combination with conditioned culture media containing anti-HIV-1 Tat scFvs (Hutat2, E46) than conditioned culture media containing control scFvs (A3H5, Fc) or from non-transduced cells from both CHME-5 and HTB-11 cell lines.

neuroprotection is higher among anti-HIV-1 Tat scFv, suggesting there may be another mechanism involved which warrants further study.

4. Genetic Alteration

A vital component of gene therapy is ensuring that neither the method of gene delivery nor the subsequent gene expression has any adverse effects on the target cell line or tissue. Transduced cell lines were monitored for 20 passages during which time no changes in growth kinetics or morphology were observed. In addition to visual observation, multiplex analysis of expression of 24 reference genes was performed on transduced and non-transduced HTB-11 and CHME-5 cell lines (Figures 27-31). Kan^R spike was used in all samples for intercapillary normalization.

All transduced and non-transduced HTB-11 cell lines were normalized with SRP14, 18s-rRNA, and HDAC1 and all 100% of the Human Reference Multiplex genes were detected. Overall, very little expression fold difference (FD) was observed between transduced and non-transduced cells. HTB-Hutat2 cells displayed significantly higher RPL37A expression (FD 0.612 ± 0.032; $p<0.05$) and significantly lower CAPN2 expression (FD -0.565 ± 0.045; $p<0.05$). HTB-E46 cells displayed significantly lower CASC3 expression (FD -0.531 ± 0.033; $p<0.05$) and RPLP0 expression (FD -1.44 ± 0.319; $p<0.05$). RPLP0 expression was also significantly lower in HTB-A3H5 cells (FD -8.657 ± 1.493; $p<0.01$). HTB-Fc cells displayed significantly lower CASC3, UBE2D2, and HYAL2 expression (FD -0.483 ± 0.038; -0.542 ± 0.034; -0.281 ± 0.005; $p<0.05$) and significantly higher B2M

expression (FD 5.490 ± 0.411; $p<0.01$). With the exception of RPLP0 in HTB-Hutat2 and –A3H5 and B2M in HTB-Fc, all significant EFDs were less than 1 fold, representing minor changes that may result in negligible differences in cell function and viability. However, any change should be monitored throughout the study to ensure safety of the gene therapy method employed.

All transduced and non-transduced CHME-5 cell lines were normalized with UBE2D2 and PPIA. Only 50% of the Human Reference Multiplex genes were detectable in the CHME-5 cell lines. This could be attributed to the difference in general expression profiles between neuronal and microglial cells. Additionally, immortalization of the CHME-5 cell line may have altered baseline expression. Overall, CHME-5 gene expression was consistent among transduced and non-transduced cells with the exception of 18s-rRNA (FD 0.556 ± 0.031; $p<0.01$), RPL37A (FD 0.542 ± 0.034; $p<0.01$), and PSMB6 (FD 0.226 ± 0.010; $p<0.05$) which were significantly higher in CHME-Fc cells. Although statistically significant, these differences in expression between transduced and non-transduced CHME-5 cells were less than 1 fold for every gene detected, suggesting that the detected differences may not significantly alter cell function or viability. An increase in expression of RPL37A, which is responsible for catalyzing ribosomal protein synthesis, was detected in both HTB-11 and CHME-5 transduced cells. Expression of CASC3, which functions in nonsense-mediated mRNA decay, and RPLP0, which is an acidic ribosomal protein, was significantly decreased in more than one HTB-11

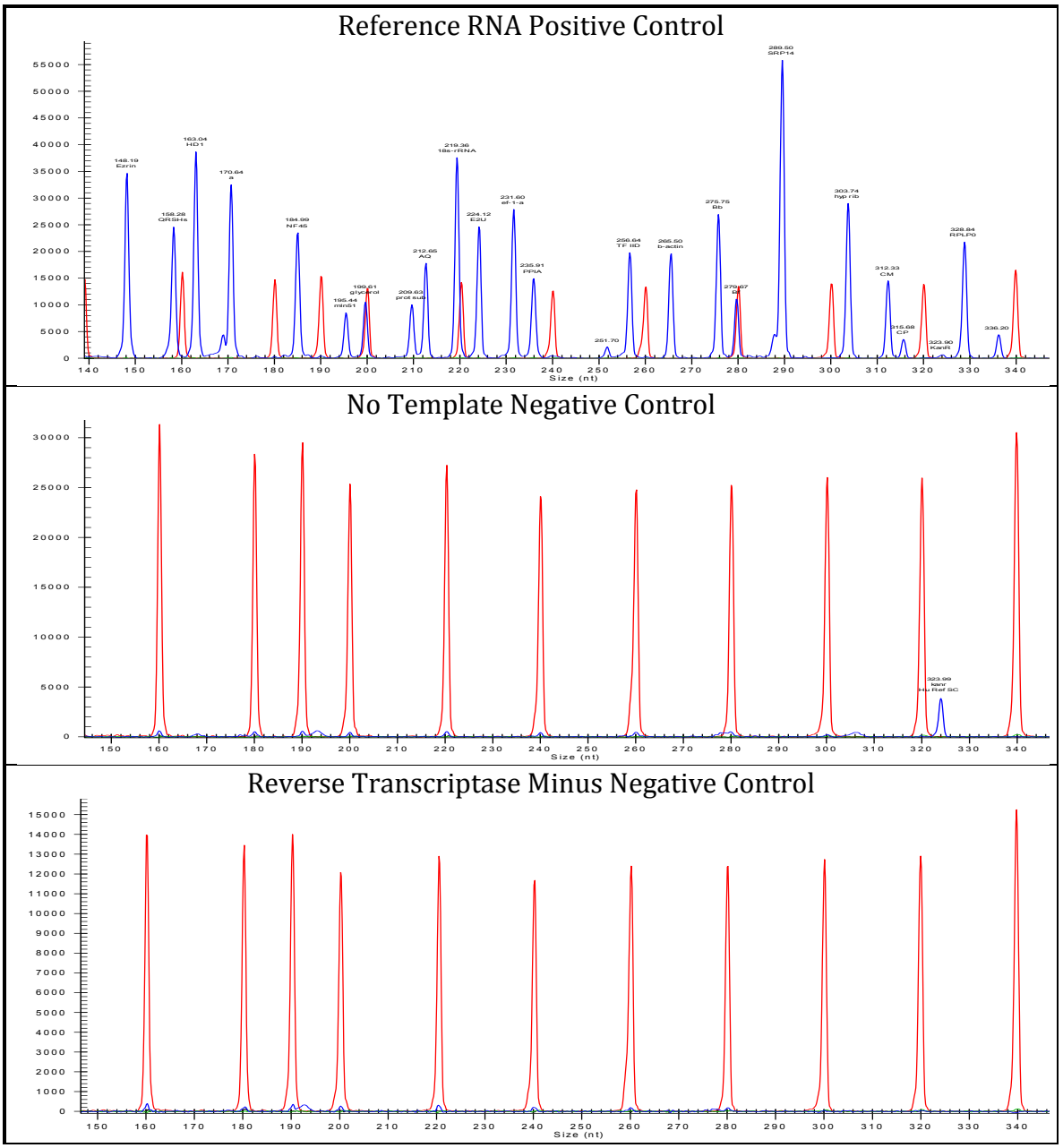


Figure 27. Genome Lab analysis of Human Reference Multiplex positive and negative controls.

Genome lab analysis of expression levels of 24 common reference genes using Human Derived Reference RNA (supplied by Beckman Coulter) as a positive control, no template with the addition of Kan^R RNA (internal control) and reverse transcriptase minus as negative controls. XP-PCR was performed and amplicons were separated by capillary electrophoresis.

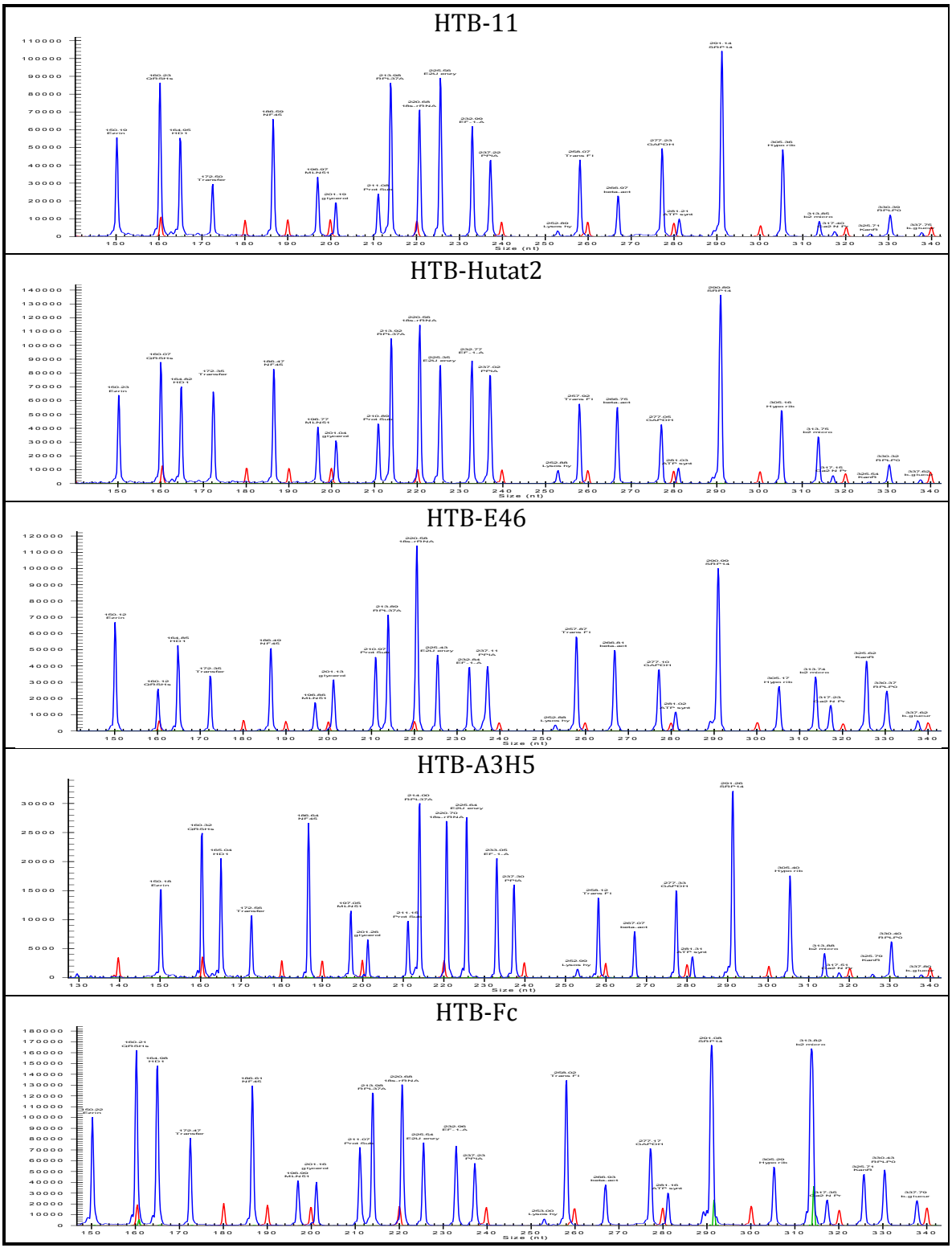


Figure 28. Genome Lab analysis of Human Reference Multiplex in transduced and non-transduced HTB-11 cells.
XP-PCR was performed and amplicons were separated by capillary electrophoresis.

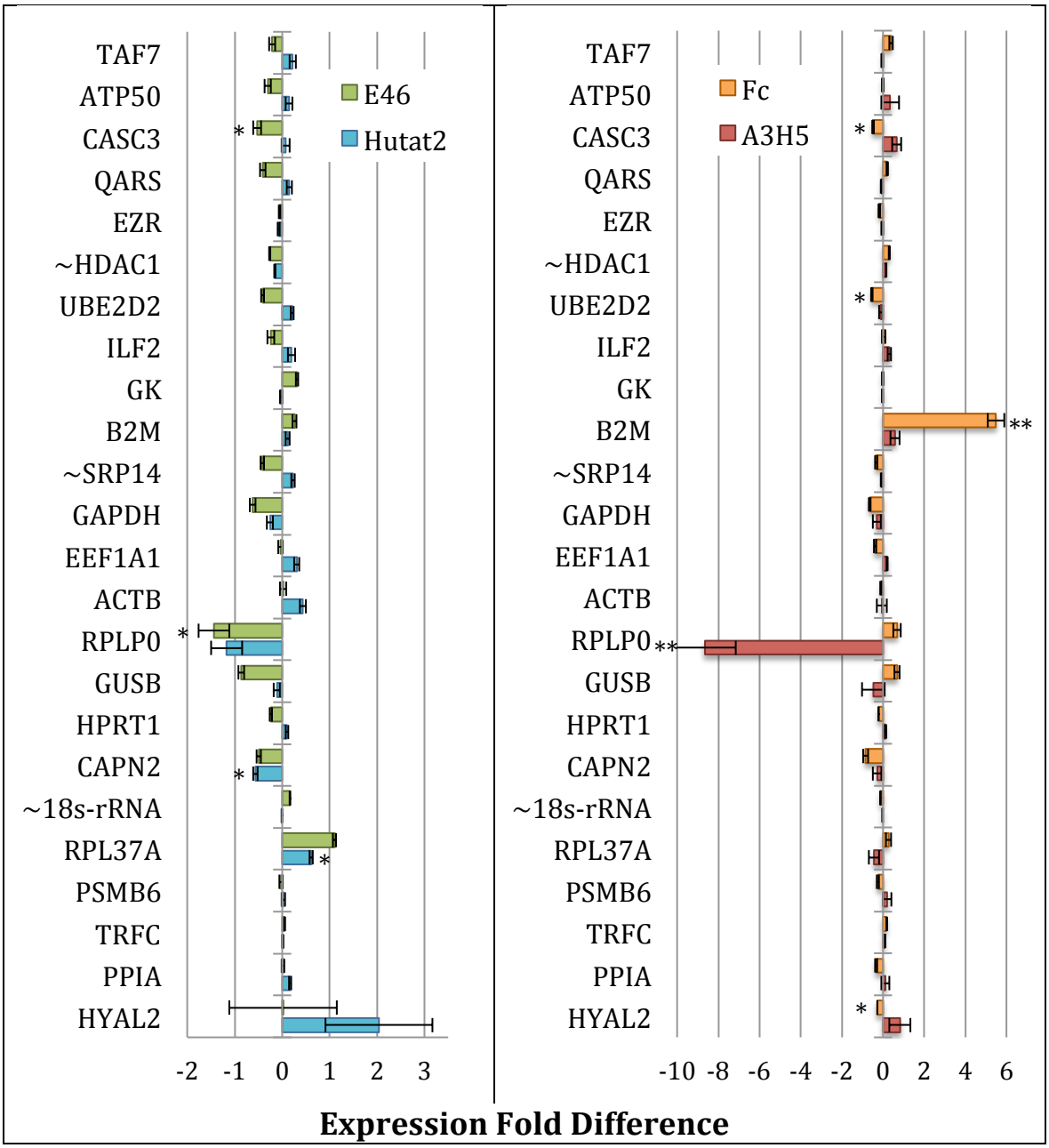


Figure 29. Express Profiler analysis of Human Reference Multiplex in transduced and non-transduced HTB-11 cells.

Kan^R spike was used for normalization followed by normalization with srp14, 18s-rRNA, and HDAC1 (~). Overall, gene expression was consistent among transduced and non-transduced cells with the exception of RPLP0 which was significantly higher ($p<0.05$) and CAPN2 which was significantly lower ($p<0.05$) in HTB-Hutat2, CASC3 which was significantly lower ($p<0.05$) in HTB-E46, RPLP0 which was significantly lower in HTB-E46 ($p<0.05$) and HTB-A3H5 ($p<0.01$), and CASC3, UBE2D2, and HYAL2 which were significantly lower ($p<0.05$) and B2M which was significantly higher ($p<0.01$) in HTB-Fc cells.

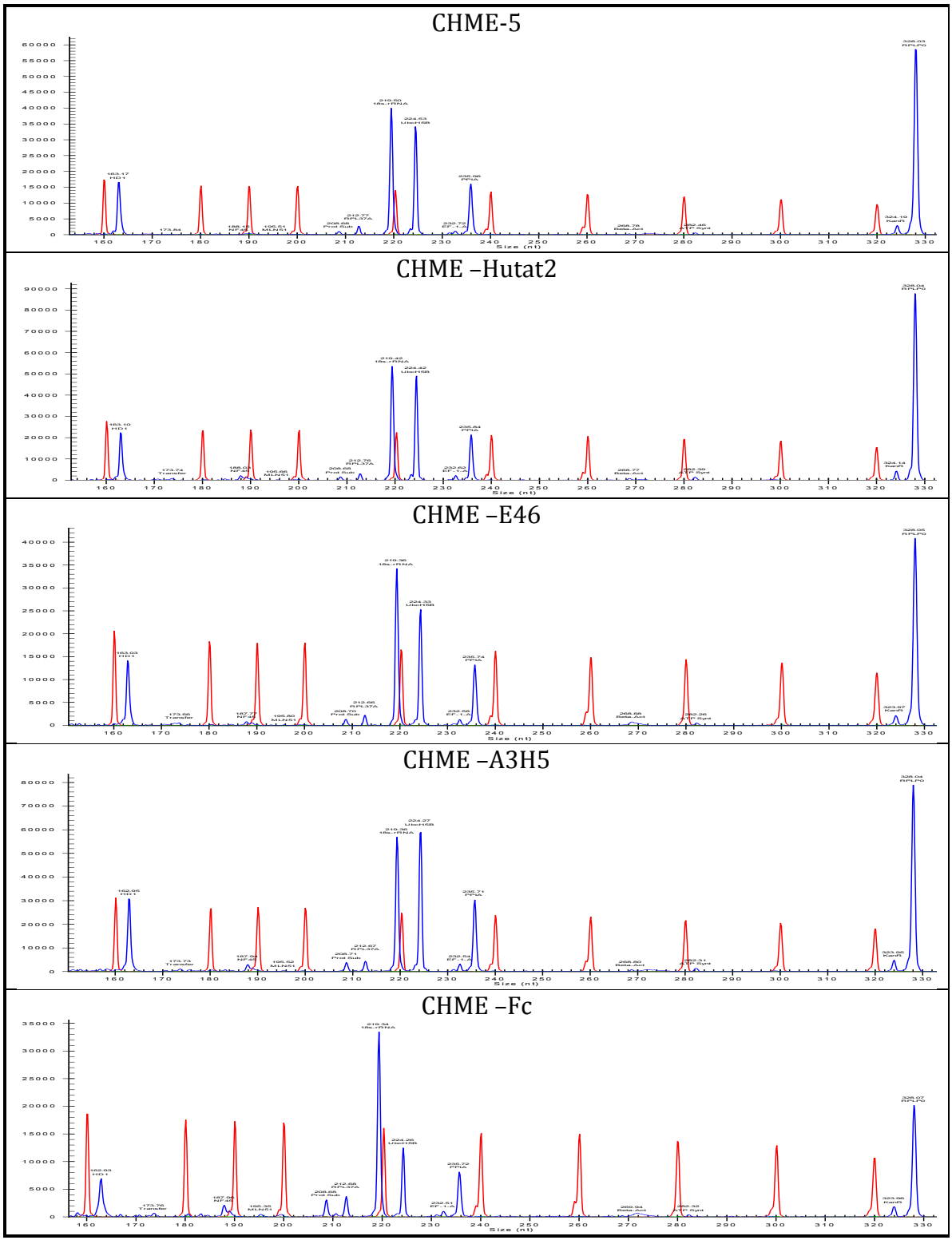


Figure 30. Genome Lab analysis of Human Reference Multiplex in transduced and non-transduced CHME-5 cells.
XP-PCR was performed and amplicons were separated by capillary electrophoresis.

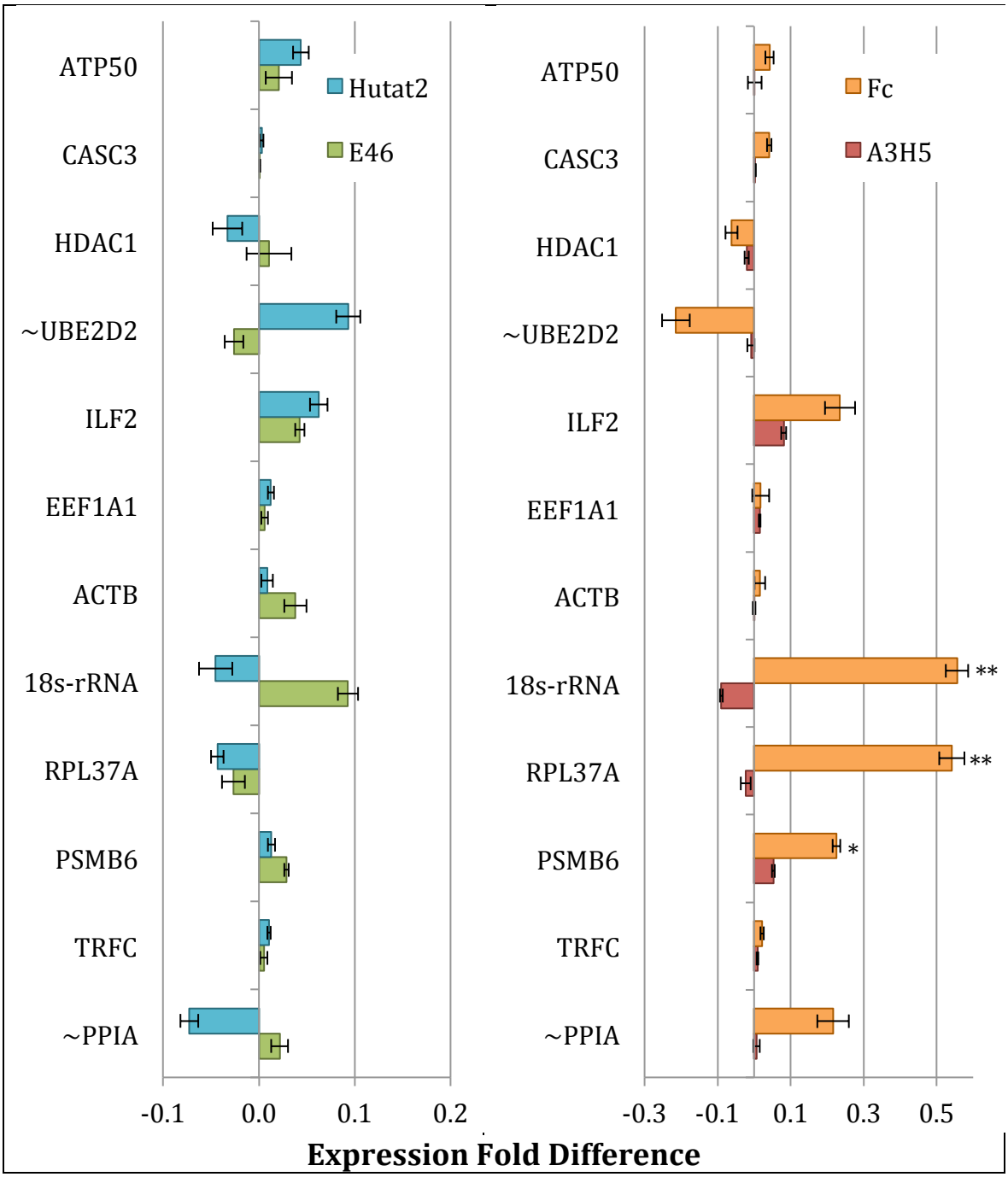


Figure 31. Express Profiler analysis of Human Reference Multiplex in transduced and non-transduced CHME-5 cells.

Kan^R spike was used for normalization followed by normalization with UBE2s2, and PPIA (~). Overall, gene expression was consistent among transduced and non-transduced cells with the exception of 18s-rRNA ($p<0.01$), RPL37A ($p<0.01$), and PSMB6 ($p<0.05$), which were significantly higher in CHME-Fc cells.

transduced cell line, suggesting that these three genes should be explored more in-depth and monitored throughout the study.

5. Primary Human Peripheral Blood Mononuclear Cells

5.1. Isolation and Transduction

Primary human PBMC were isolated from healthy donor blood and seeded at a density of 1.5 x 10^7 cells/12.5 cm^2 primary TC flask (Figure 32). A small portion of the isolated PBMC were labeled with CD14 MACS microbeads or CD11b MACS microbeads and seeded in a 12 well plate to determine if additional cell sorting would benefit PBMC culture purity and growth. Initially, the ratio of attached to non-attached cells in the cultures separated with either microbead type was much higher than the cells seeded without separation. However, after the first wash there appeared to be no difference in MDM growth or purity (Figure 33). MDM were cultured without growth factor for four weeks in good condition. On day 7 post isolation, MDM were transduced with 0.4 mL vector stock (1x10^7 IU/mL). GFP quantification determined transduction efficiency to be 12% ± 2% on day 5 post infection (Figure 34). Possibly, a second transduction performed on day two post infection could significantly increase the transduction efficiency, as suggested by Mhashilkar et al. (Mhashilkar *et al.*, 1999), and should be explored further. DNA was extracted from transduced and non-transduced MDM and subjected to PCR, confirming successful integration of the Hutat2 gene into the MDM genome (Figure 35).

5.2. ScFv Expression and Function

Hutat2 expression in transduced MDM was quantified by ELISA. Transduced and non-transduced MDM were cultured in RPMI-1640 media supplemented with 10% human serum, resulting in very high background when subjected to the ELISA protocol optimized for established cell lines HTB-11 and CHME-5 which utilizes anti-human IgG Fc antibodies to detect the Fc fusion protein on the scFv. Currently, there are no commercial antibodies available to detect the unique Hutat2 scFv intrabody. Therefore, to prevent the necessity of culturing transduced and non-transduced MDM in serum free media, which would be very detrimental to the sensitive MDM, recombinant HIV-1 Tat was employed as the capture antibody for the new ELISA protocol, greatly reducing the background IgG detected from the human serum incorporated in the RPMI-1640 growth medium. However, this unique ELISA design also resulted in the lack of availability of a standard curve. Therefore, OD was calculated relative to non-transduced MDM, a method also utilized in a previous publication characterizing Hutat2 in transduced SupT1 cells (Mhashilkar *et al.*, 1999). Both direct and indirect labeling methods were utilized (Figure 36). As expected, in-direct labeling amplified Hutat2 detection.

5.3. Neuroprotection from HIV-1 Tat and gp120

The ability of anti-HIV-1 Tat scFv Hutat2 produced by transduced MDM to inhibit the neurotoxic properties of HIV-1 Tat and gp120, was assessed through the exposure of human neuronal cells to 500 ng/mL HIV-1 Tat or 250 ng/mL HIV-1 gp120, both supplied by the NIH AIDS Research & Reference Reagents Program, in

conjunction with conditioned media from transduced or non-transduced MDM or anti-serum to HIV-1 Tat, supplied by the NIH AIDS Research & Reference Reagents Program. Cell viability as assessed by MTT assay revealed that anti-HIV-1 scFv Hutat2 provides significant protection to neurons from HIV-1 Tat-mediated neurotoxicity ($p<0.01$) and HIV-1 gp120-mediated neurotoxicity ($p<0.01$) compared to neuronal cells receiving conditioned media from non-transduced MDM (Figure 37).

5.4. Genetic Expression Analysis

Kan^R spike was used for intercapillary normalization followed by normalization with ATP50, and HPRT1 (~). Sixty-three percent of the 24 Human Reference Multiplex genes were detected in MDM, most likely due to extremely lower levels of expression of some genes rendering them undetectable. Overall, gene expression was consistent among transduced and non-transduced cells with the exception of UBE2D2 and RPLP0, which were significantly lower and higher, respectively, in PBMC-Hutat2 cells ($p<0.01$) (Figures 38-39). UBE2D2 expression, which is responsible for ubiquitination of tumor-suppressor protein p53, was significantly lower in transduced HTB-11 and MDM cultures. RPLP0 expression, which is an acidic ribosomal protein, was also altered in HTB-11 GeXP analysis, although in the opposite direction as MDM, suggesting that RPLP0 may easily vary due to internal cellular conditions (Gresner *et al.*, 2011). RPLP0 and UBE2D2 should be monitored throughout the study and explored more in-depth to ensure gene transfer safety.

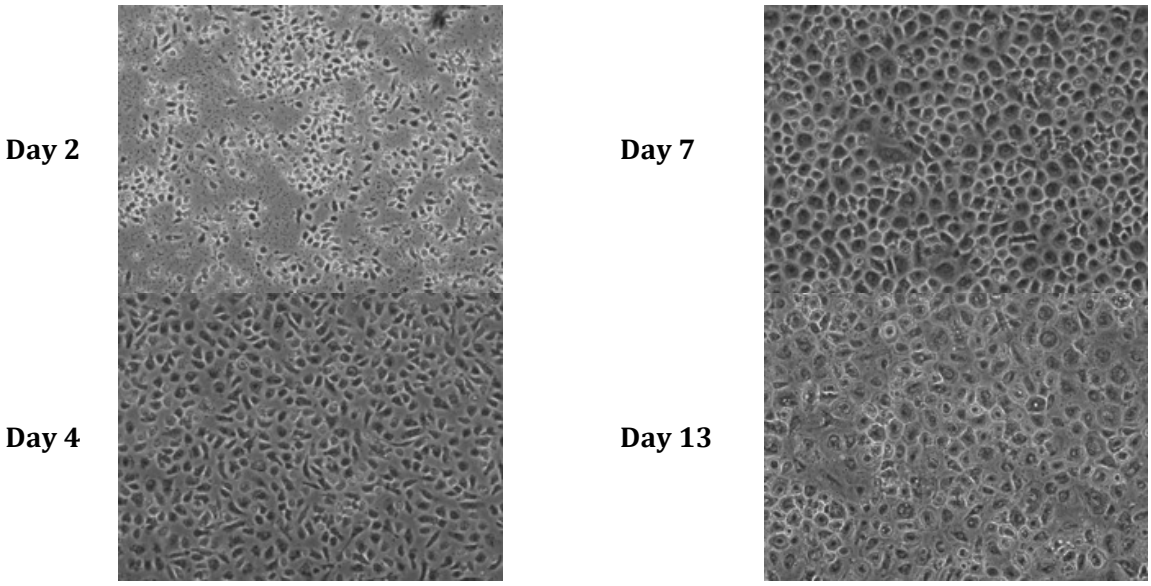


Figure 32. Peripheral Blood Mononuclear Cell culture.
PBMC cultures were monitored for two weeks for optimal growth and morphology. Under optimal conditions, MDM were cultured for four weeks in good condition.

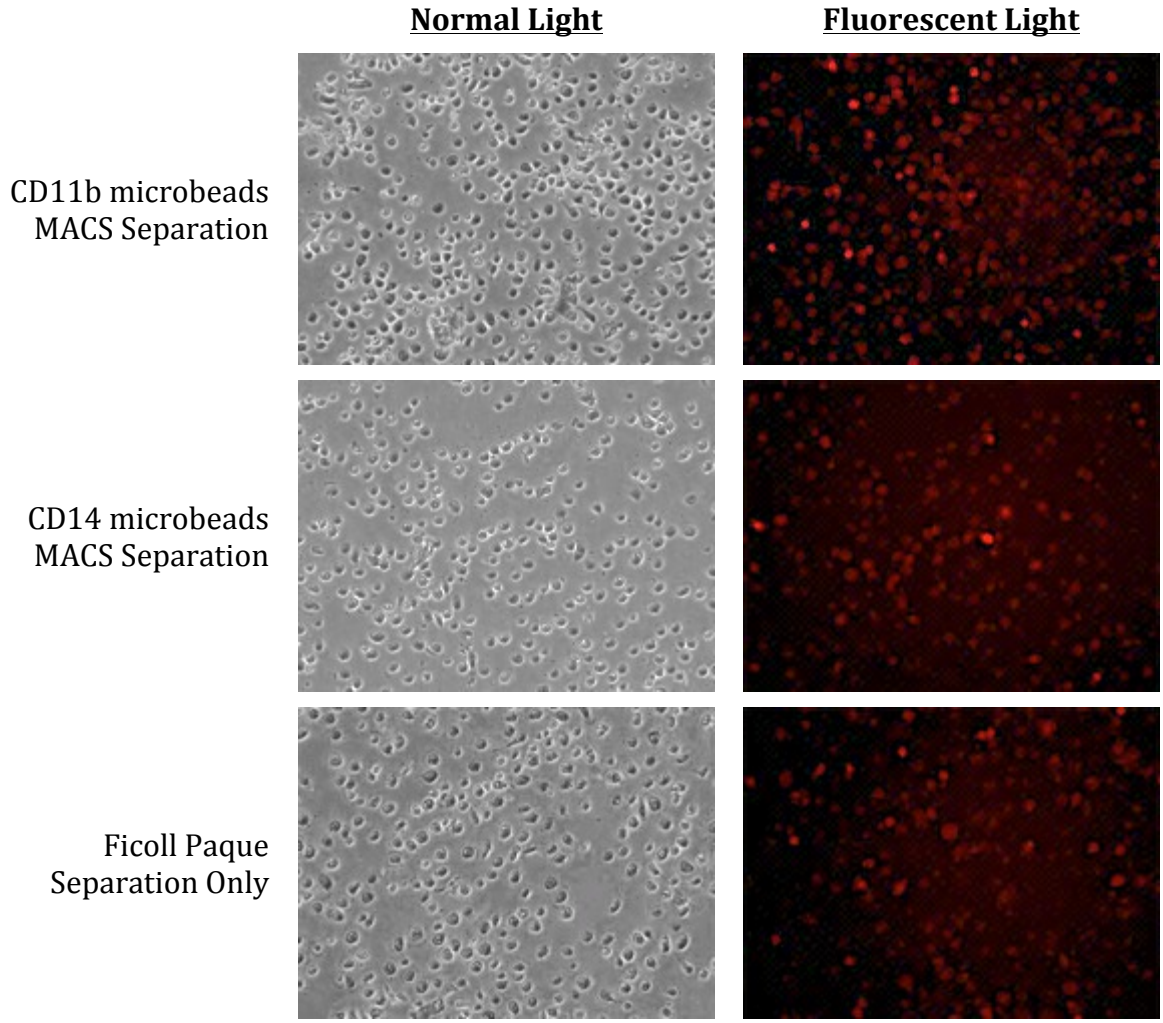


Figure 33. PBMC purity verification.

Immunofluorescent staining with goat anti-human CD-14 Rhodamine labeled antibodies confirmed the purity of PBMC culture, which was similar among PBMC isolated with ficoll paque only and PBMC subjected to additional separation with microbeads. Original magnification: 100X.

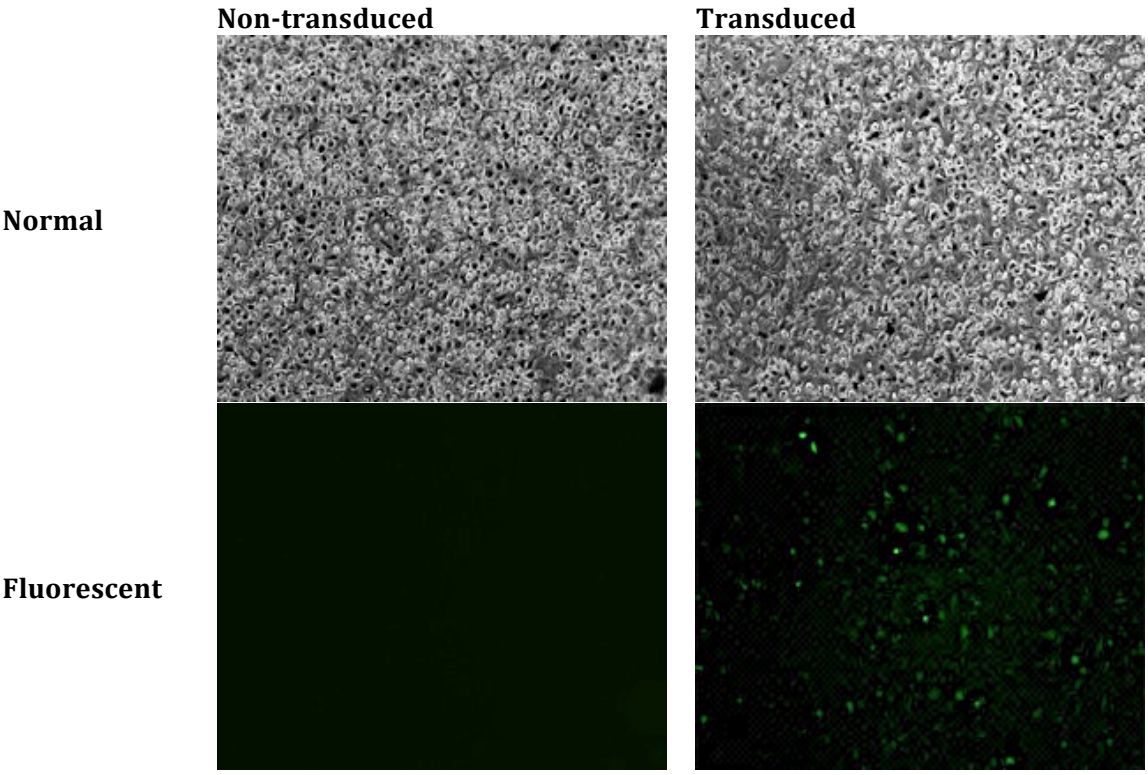
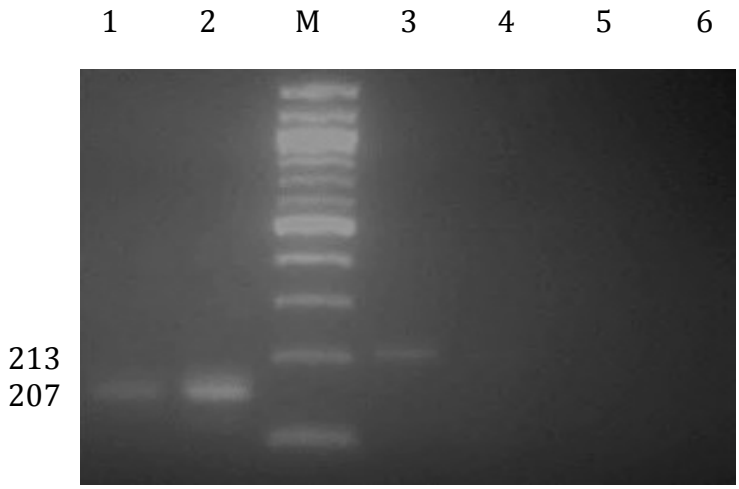


Figure 34. Hutat2 transduction of PBMC.

PBMCs were transduced with Hutat2 defective lentiviral vector. Transduction efficiency was determined by GFP quantification to be 12% ± 2%. Original magnification: 40X.



1 – B-actin primers: PBMC-Hutat2
2 – B-actin primers: PBMC
M – 100 bp DNA ladder
3 – Hutat2 primers: PBMC-Hutat2
4 – Hutat2 primers: PBMC
5 – Hutat2 primers: No Template
6 – B-actin primers: No Template

Figure 35. PCR confirmation of PBMC transduction.

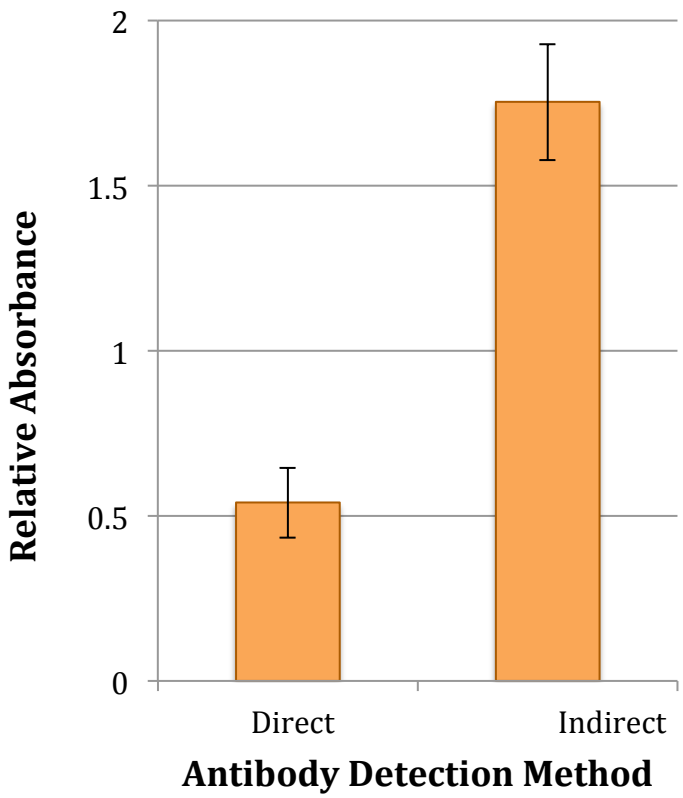


Figure 36. ELISA detection of secreted anti-HIV-1 Tat scFv Hutat2 in transduced primary human MDM culture.
Recombinant HIV-1 Tat served as the capture antibody and Hutat2 scFv was detected using direct or indirect labeling.

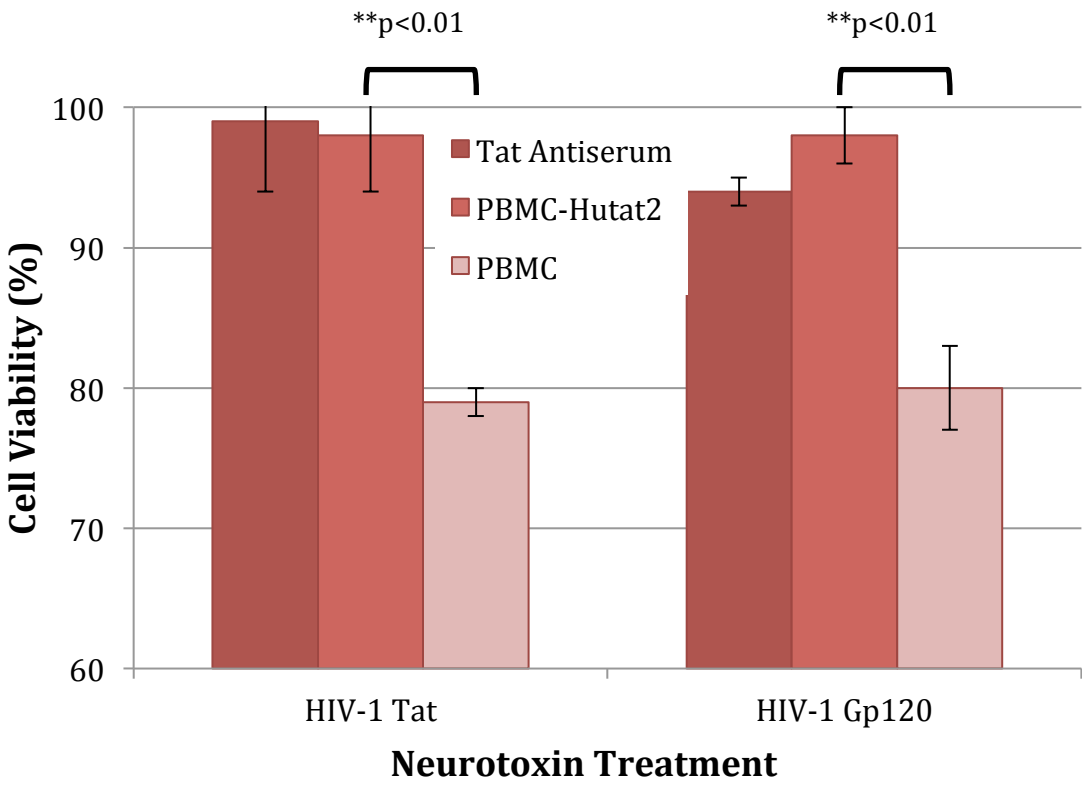


Figure 37. Conditioned media from anti-HIV-1 Tat scFv Hutat2 transduced MDM conferred significant protection to human neuronal cells exposed to HIV-1 neurotoxins Tat and gp120.

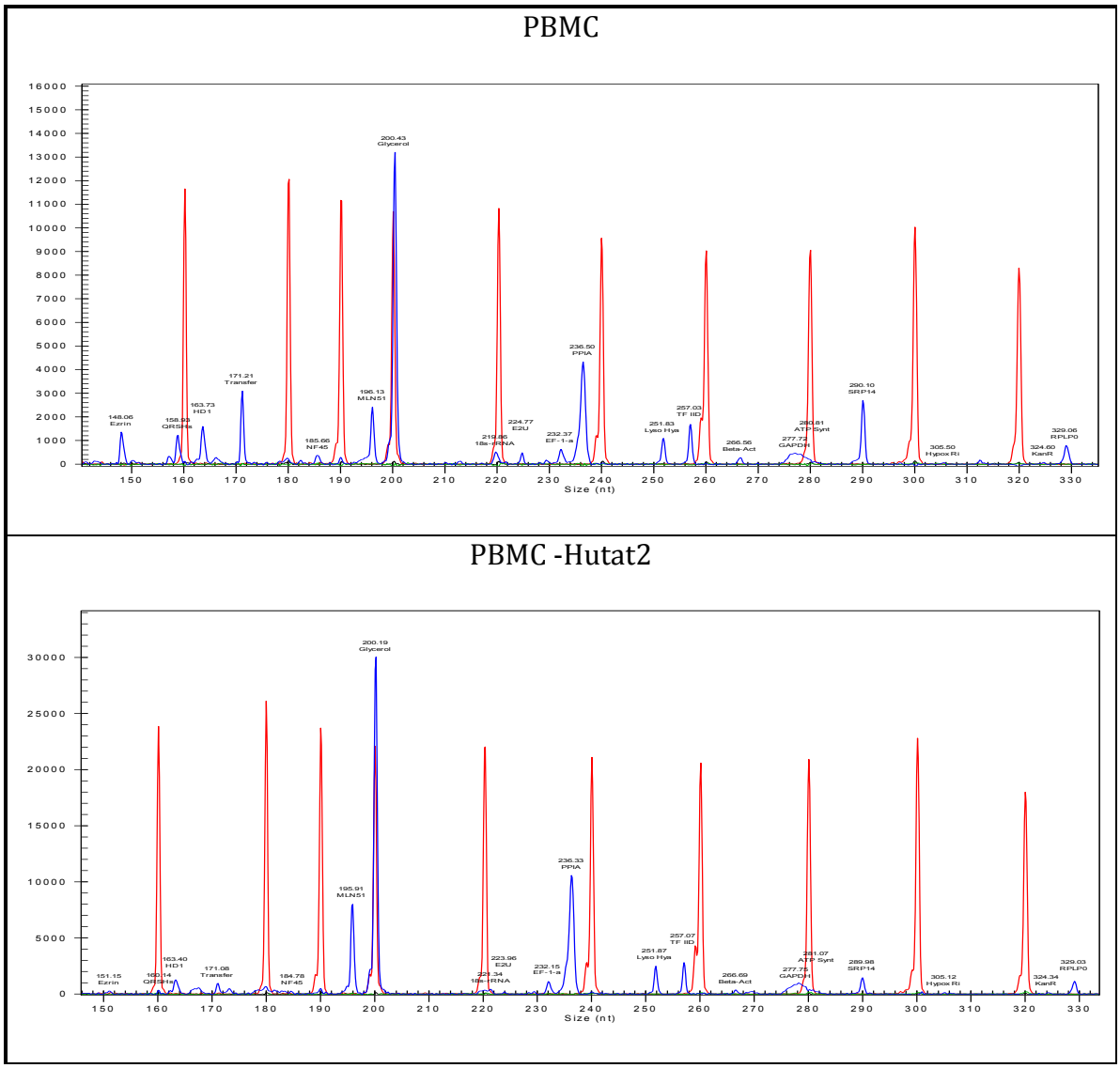


Figure 38. Genome Lab analysis of Human Reference Multiplex in transduced and non-transduced MDM cells.
XP-PCR was performed and amplicons were separated by capillary electrophoresis.

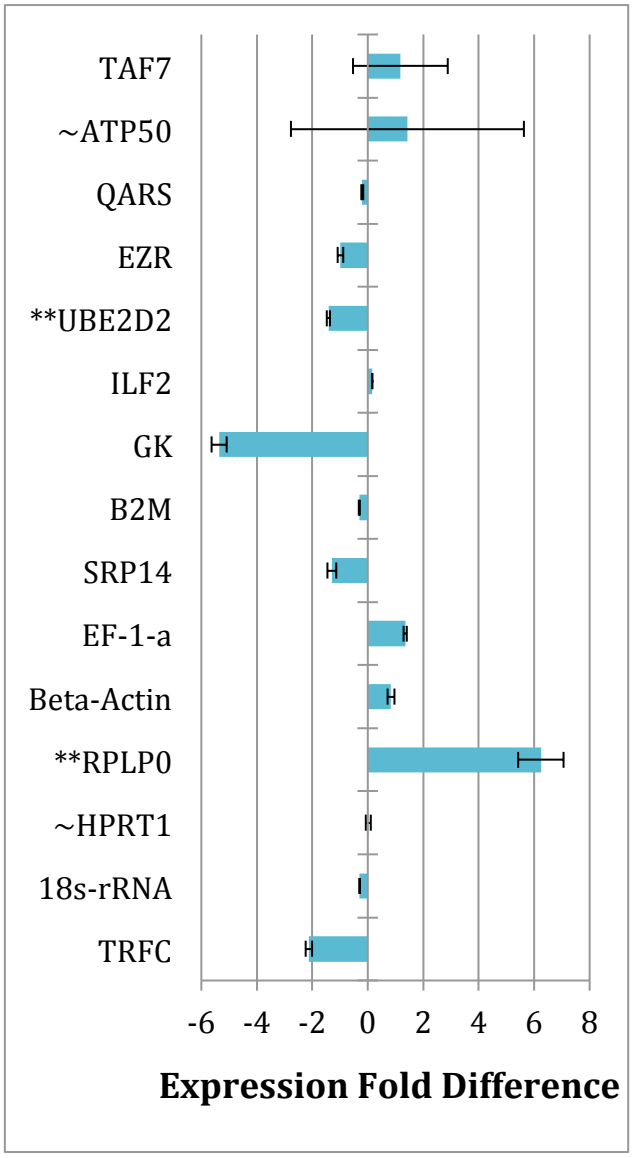


Figure 39. Express Profiler analysis of transduced and non-transduced PBMC cultures.

Kan^R spike was used for normalization followed by normalization with ATP50, and HPRT1 (~). Overall, gene expression was consistent among transduced and non-transduced cells with the exception of UBE2D2 and RPLP0, which were significantly lower and higher, respectively, in PBMC-Hutat2 cells ($p<0.01$).

6. Conclusion

Overall, these results from the transduction and characterization of anti-HIV-1 Tat scFv expression and function in established human neuroblastoma and microglial cell lines as well as primary human MDM suggest that moderate to high levels of transduction efficiency and scFv expression can be obtained in both immortalized and primary human cells utilizing HIV-1 based DLV. Furthermore, the anti-HIV-1 Tat scFv produced by transduced HTB-11, CHME-5, and MDM provides significant protection from HIV-1 Tat- and gp120-mediated neurotoxicity without altering the growth kinetics, morphology, or expression of the majority of human reference genes assessed. These are all vital aspects to successful gene therapy and lend anti-HIV-1 Tat Hutat2 scFv and DLV-mediated gene transfer as ideal candidates for further study in developing anti-HIV gene therapy targeting HANDs in the CNS utilizing transduced MDM as a novel delivery method across the BBB. The ability to effectively combat HIV and related neuronal damage in the CNS would enormously improve the lives of millions of HIV infected individuals worldwide and the targeted approach assessed this study contributes to the progress of this vital treatment.

7. References

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