HIV-1 TRANSACTIVATOR OF TRANSCRIPTION (TAT) PROTEIN CAUSES NEUROTOXICITY VIA ASTROCYTE ACTIVATION

By

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Under the supervision of

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PREFACE AND DECLARATION

The experimental studies carried out in this thesis were conducted in the Neuroscience Lab, Department of Human Physiology at the University of Kwa-Zulu Natal (Westville Campus) and Kwa-Zulu Natal Research Institute for TB and HIV (K-RITH), Leslie lab, under the supervision of Prof W.M.U. Daniels and co-supervision of Dr D. Ramsuran.

This study represents original work by the author and has not otherwise been submitted to UKZN or any other tertiary institution for purposes of obtaining a degree or any other academic qualification.

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Prof W.M.U Daniels

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DEDICATION

To

My Late Dad

(Mr R. Ganga)

PLAGARISM DECLARATION

I, Yashica Ganga declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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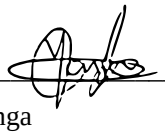
Signed:  Date: 30-03-16

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Prof W.M.U Daniels

ABSTRACT

HIV is most well-known for its negative effects on the immune system and the resulting development of AIDS, however it also has severe damaging effects on the central nervous system. Many infected individuals exhibit neuropsychological and behavioral dysfunctions which are collectively referred to as HIV-associated dementia (HAD). One of the worrying aspects of HAD is the fact that current anti-retroviral therapy, while being effective in managing the onslaughts of HIV on the immune system, is less efficient in addressing the impact of HIV on the CNS. The HIV-1 regulatory protein, transactivator of transcription (Tat), is responsible for the transactivation of viral transcription, and has been identified as a possible etiological factor of HAD. Neurotoxicity caused by HIV-1 is an indirect effect since the virus is unable to infect neurons directly. We subsequently hypothesized that HIV-1 infects non-neuronal cells in the CNS which leads to their activation, resulting in the release of cytokines that are detrimental to neurons. The aims of this study was therefore to (i) determine whether Tat activates astrocytes, (ii) establish whether astrocytes exposed to Tat result in the release of IL-6 and TNF-α, and to (iii) assess whether these cytokines can induce apoptosis of neuronal cells. Our study has shown that Tat does activate astrocytes and that activated astrocytes do indeed release cytokines IL-6 and TNF-α into their growth medium. Tat treated cells release more than double the amount of IL-6 than the control group of untreated astrocytes. We also observed that exogenous administration of these cytokines (individually or collectively) to neurons has the ability to cause neuronal apoptosis. Interestingly in combination, these cytokines show no cooperative effect. Our data also showed that neurons, when exposed to the culture medium of astrocytes that were subjected to Tat, exhibit hallmarks of apoptosis similar to that induced by IL-6 and TNF-α. Our findings led us to conclude that in individuals with HIV-infection, the virus activates astrocytes possibly via the production and release of Tat. This causes the astrocytes to secrete pro-inflammatory cytokines (e.g. TNF-α and IL-6) that may induce apoptotic cell death of neurons. This mechanism may explain the development of HAD.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Acquired immune deficiency syndrome (AIDS) was first observed in 1981 amongst the young homosexual men of New York and California (Fauci, 2003). This disease was characterized by a progressive decline in cell mediated immunity, mainly the $CD4^+$ T-cells (Lane, 2010). However, soon diverse groups of individuals were infected, indicating that the disease was caused by an infectious microorganism (Fauci, 2003). By 1983, both epidemiological and virological evidence was established showing that human immunodeficiency virus (HIV), a member of the retrovirus family, was the causative agent of AIDS (Fauci, 2003).

The 2013 fact sheet of the Joint United Nations Program on HIV/AIDS (UNAIDS) 2013, reported that in 2012 there were 35.3 million people living with HIV, 2.3 million new infections globally, 1.6 million deaths from AIDS- related causes and 9.7 million people accessing treatment (Table 1). The UNAIDS global and regional statistics show that in 2012, Sub-Saharan Africa had the largest population of people living with HIV (25 million), new HIV infections (1.6 million) and AIDS-related deaths (1.2 million).

HIV-1 can be divided into 3 different and highly divergent groups : major, outlier and non-major/outlier (Bagashev and Sawaya, 2013). Within the major group, there are 9 subtypes/clades, with subtype C predominating worldwide (Bagashev and Sawaya, 2013). Globally, clade C HIV-1 accounts for 56% of HIV infections as compared to other clades A,B and D-J (Bagashev and Sawaya, 2013; Ranga *et al.*, 2004; Siddappa *et al.*, 2006). Clade C has been successful in establishing rapidly growing epidemics in most populations of Sub-Saharan Africa, India, China and Brazil (Siddappa *et al.*, 2006). Even though clade C causes the most infections compared to the other subtypes, little is understood about their molecular and pathogenic properties (Siddappa *et al.*, 2006). Currently the knowledge of HIV-1 pathogenesis is mainly obtained from clade B strains, prevalent in the US and Europe (Alaeus, 2000; Siddappa *et al.*, 2006) and the relevance of this knowledge to Sub-Saharan African where clade C is most prevalent, has been questioned.

The human immunodeficiency virus is spherically shaped with an approximate diameter of 120nm (Freed, 1998). Its diploid genome is encased by a protective protein coat called the capsid. Between the genome lies the essential proteins required for the completion of the viral life cycle. These include reverse transcriptase (RT), protease, polymerase and integrase (Figure1) (Freed, 1998; Pomerantz and Horn, 2003).

Table 1. Global HIV/AIDS statistics as reported by UNAIDS

	2001	2005	2008	2009	2010	2011	2012
People living with HIV in millions	30	32.5	33.5	34	34.4	34.9	35.3
New HIV infections in millions	34	2.9	2.6	2.6	2.5	2.5	2.3
AIDS-related deaths in millions	1.9	2.3	2.1	2.0	1.9	1.8	1.6
People accessing treatment in millions		1.3	4.1	5.3	6.6	8.1	9.7

The viral envelope (env) protein, encodes for the surface glycoprotein 120 (gp120) as well as the transmembrane glycoprotein 41 (gp41) (Figure 1) (Freed, 1998; Tiwari *et al.*, 2012). These proteins function mainly in host cell attachment. Group specific antigen (Gag) proteins make up the capsid, matrix and nucleocapsid proteins (Freed, 1998). Polymerase (pol) encodes for RT, proteases and integrase, all of which have functions in the life cycle (Freed, 1998). The virus also has regulatory proteins, Tat and Rev, as well as accessory proteins Nef, Vpu and Vpr (Pomerantz and Horn, 2003; Sierra *et al.*, 2005; Tiwari *et al.*, 2012).

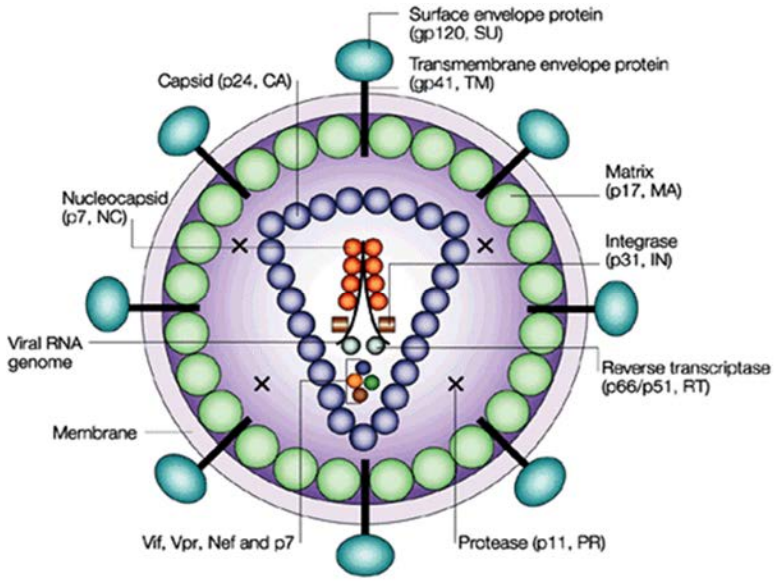


Figure 1. A schematic illustration of the HIV virion (Robinson, 2002).

1.2 HIV-1 Life Cycle, Pathogenesis and Therapeutic Approaches

HIV is an obligate parasite relying solely on host cell machinery for its replication and survival (Collins and Collins, 2014). It's simple structure does not contain any energy producing or biosynthetic machinery (Collins and Collins, 2014). HIV initially targets the immune cells and a drop in these cells' numbers renders an HIV-infected patient more susceptible to several other infections compared to an uninfected individual (Lane, 2010; Sierra *et al.*, 2005). As the virus persists, many infections manifest due to the weakening of the immune system (Lane, 2010). The body is able to control the virus for a period of time until the circulating $CD4^+$ count drops below 200 cell/mm^3. This markedly reduced amount of immune cells results in a poor host immune response towards the virus, allowing ongoing viral replication (Lane, 2010) and viral persistence (Ross, 2001).

1) Viral attachment and fusion

Once the virus enters the body it binds to $CD4^+$ T-cell receptors and co-receptors, CCR5 or CXCR4 on the host target cells (these cells include $CD4^+$ T-cells, macrophages or dendritic cells) (Frankel and Young, 1998). At this stage receptor blockers can be used to inhibit the binding process, however, these drugs are not widely available. The virus then fuses to the host cell and penetrates through the host cell membrane entering the host cytoplasm (Frankel and Young, 1998). Fusion and entry can be hindered using fusion and entry inhibitors. Uncoating allows the virus to expose its genome and virally encoded proteins (Frankel and Young, 1998).

2) Reverse transcription

The enzyme reverse transcriptase, reverse transcribes the diploid viral RNA into double stranded DNA by using one strand of RNA as a template (Sierra *et al.*, 2005). This process can be blocked by reverse transcriptase (RT) inhibitors that prevent the lifecycle of the virus from proceeding. A RT inhibitor is commonly one of the components of antiretroviral therapy (ART) (Frankel and Young, 1998).

3) Integration

Newly transcribed double stranded DNA enters into the host nucleus where integrase nicks the host genome for the integration of the newly transcribed DNA (Sierra *et al.*, 2005). The virus integrated into the host is called the provirus, which replicates each time the host cell divides. Integration can be restrained by the use of integrase inhibitors, which are at this stage not widely available (Pomerantz and Horn, 2003).

4, 5) Transcription and Translation

The pro-viral genome is transcribed into messenger RNA which is translated into viral proteins gag, pol and env (Freed, 1998). The immature virus is at this point called a virion. Maturation of the virion occurs once essential viral proteins are cleaved by proteases (Frankel and Young, 1998).

6) Assembly, Budding and Maturation

Subsequent to the viral proteins being cleaved by the protease enzyme into smaller, active, structural polypeptides, the mature virus buds away from the host cell and is free to infect other cells (Freed, 1998; Stevenson, 2003). The maturation and assembly of HIV can be prevented by the use of protease inhibitors, which together with RT inhibitors, are the backbone of ART (Pomerantz and Horn, 2003).

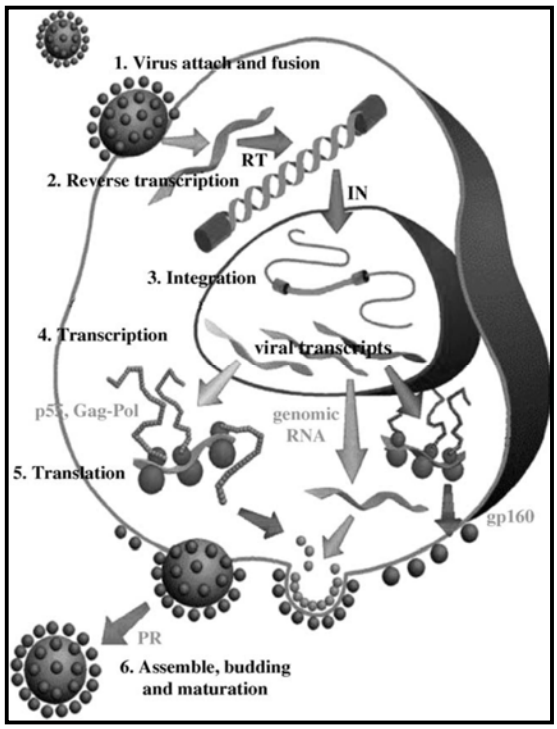


Figure 2. A schematic representation of the HIV-1 life cycle (Sierra et al., 2005).

The pathogenesis of the virus depends on the viral replication, the reservoirs that support it and the host response to the virus (Stevenson, 2003). All retroviruses require a $CD4^+$ receptor for attachment, however the co-receptor could either be CCR5 (if virus is M tropic) or CXCR4 (if virus is T tropic) found on macrophages and primary T-cell lines respectively (Stevenson, 2003). The virus is unable to infect cells that do not have a $CD4^+$ receptor (Stevenson, 2003). Cells possessing $CD4^+$ receptors include macrophages, T lymphocytes and glial cells (Pomerantz and Horn, 2003). Once the virus has infected the surveillance cells, the infection migrates to the lymph nodes where there is a burst of viral replication causing extreme

viremia (Stevenson, 2003). At this stage of the infection the $CD4^+$ and $CD8^+$ T lymphocytes are activated keeping the viral load low (Stevenson, 2003). However, as the $CD4^+$ cell count continues to drop the body's immune response becomes too weak to control the virus (Stevenson, 2003).

Clinically an HIV-infected person displays flu-like symptoms (Sierra *et al.*, 2005; Stevenson, 2003). During this initial stage the body's HIV-1 specific immune response and the synthesis of HIV-1 specific antibodies lead to a decline in the plasma viral load and a stage of clinical latency may be reached (Sierra *et al.*, 2005). This stage may last 8-10 years and the individual may appear normal (Stevenson, 2003). However once the $CD4^+$ T-cell count drops below 200cells/mm^3 a patient experiences overt AIDS (Stevenson, 2003). During this final stage the virus completely takes over the immune system, the patient is faced with life-threatening AIDS-defining diseases which eventually leads to death (Pomerantz and Horn, 2003; Sierra *et al.*, 2005).

1.3 HIV-1 Tat Protein

Essential, regulatory as well as accessory viral proteins play an integral role in the pathogenesis of HIV-1 infection (Nath *et al.*, 1998). The transactivator of transcription (Tat) protein is a key viral transactivator regulating gene expression from the viral promoter (Nath *et al.*, 1998; Wallace, 2006). Tat is expressed early in the viral life cycle from multiple spliced viral transcripts (Schwartz *et al.*, 1990). It is one of the first proteins to be expressed after infection and unlike typical DNA binding transcription factors, Tat is a RNA binding protein (Bagashev and Sawaya, 2013). Tat was first characterized in 1985 and was shown to consist of 86 or 101 amino acid and a weight of 14-16 kDa (Pugliese *et al.*, 2005). HIV-1 Tat has the ability to translocate to the nucleus of the infected cells where it plays a pivotal role in enhancing viral transcription (Tiwari *et al.*, 2012). Tat can also act as an exogenous factor, having the ability to elicit a range of biochemical effects in various cell types (Brigati *et al.*, 2003; Pugliese *et al.*, 2005). The pathogenicity of the virus is aggravated by the ability of Tat to effortlessly enter target cells (Herce and Garcia, 2007).

1.3.1 Structure of Tat protein

Tat consists of two exons divided by an intron (Tiwari *et al.*, 2012). The first encodes for 1-72 amino acids constituting five domains; an acidic domain with amphipathic characteristics, a cysteine-rich domain which is a metal chelating dimerization regulatory domain, a core domain which is involved in tubulin binding and apoptosis, the basic domain consisting of TAR-RNA binding properties and finally the glutamine-rich domain involved in Tat mediated apoptosis (Jeang, 1996; Tiwari *et al.*, 2012). The second exon encodes for amino acids 73-101 and plays a vital role in cell adhesion and viral transcription (Jeang, 1996; Tiwari *et al.*, 2012). According to Boykins *et al.* (1999), the 20 residue core domain sequence of Tat is sufficient to transactivate and induce HIV replication.

1.3.2 Production of Tat protein

Tat is essential for HIV-1 gene expression, replication and infectivity (Gavioli *et al.*, 2002). The transcription process of HIV-1 is controlled primarily by Tat (Li *et al.*, 2009; Rosen *et al.*, 1988). Intracellularly, Tat augments viral transcription as its main function and exogenous Tat plays a role in altering gene expression and activating viral transcription in uninfected bystander cells (Tiwari *et al.*, 2012). Once the virus is integrated in the host genome, transcription of the HIV-1 pro-virus is characterized by an early and a late, Tat-independent phase of transcription (Li *et al.*, 2009). Tat protein is produced during this Tat-independent phase (Li *et al.*, 2009). In the absence of Tat, a series of short transcripts are produced as a result of inefficient RNA polymerase II elongation capability (Li *et al.*, 2009). This results in the synthesis of a small amount of full-length viral transcripts, responsible for the production of Tat protein (Li *et al.*, 2009; Yedavalli *et al.*, 2003).

The Tat-dependent phase involves the augmentation of transcription, where Tat binds to the bulge of TAR (Transactivation response element) which is the major gene expression control centre (Figure 3) (Tiwari *et al.*, 2012). TAR is found on the hairpin-loop of the 5' terminus of the viral transcripts (Tiwari *et al.*, 2012). This binding results in the formation of a stable RNA stem loop at the 5' end of the viral transcript (Siddappa *et al.*, 2006). Following this is the recruitment of the positive elongation complex (p-TEF) consisting of cyclin-dependent kinase 9 (CDK9) and cyclin T1 which hyperphosphorylates the carboxyl-terminal domain of RNA polymerase II, an enzyme that enhances elongation of transcription from the viral promoter (Siddappa *et al.*, 2006; Tiwari *et al.*, 2012). This series of events lead to the production of more Tat forming a positive regulatory loop (Tiwari *et al.*, 2012). Binding of Tat to TAR, cyclin T1 or CDK9 occurs within the nanomolar range (Debaisieux *et al.*, 2012). In the presence of Tat, gene expression from the viral promoter is upregulated several hundred fold (Siddappa *et al.*, 2006).

1.3.3 Functions of Tat protein

Tat has effects inside the cell, where it is produced, as well as other cells that are invaded by secreted Tat. Inside the producing cell, Tat is able to stimulate efficient reverse transcription (Harrich *et al.*, 1997). Enhanced initiation of reverse transcription occurs when Tat binds to TAR RNA, altering the structure of TAR (Li *et al.*, 2009). Studies have shown that mutations on the Tat gene decreases the initiation of reverse transcription of HIV-1 replication by several thousand fold (Li *et al.*, 2009). Furthermore, viruses lacking Tat are defective in endogenous assays of reverse transcription (Li *et al.*, 2009). Together these results show that Tat is not only fundamental for regulating gene expression, but also for efficient HIV-1 reverse transcription (Li *et al.*, 2009). Tat enhances the processing ability of RNA polymerase II complexes that would otherwise produce short transcripts, and is therefore required to stimulate proficient elongation of viral transcripts (Bagashev and Sawaya, 2013; Ott *et al.*, 2004).

Tat, by virtue of its structure, can be released from infected cells and enter uninfected bystander cells. In the central nervous system these cells include astrocytes, glial cells and macrophages, where it elicits multiple biochemical effects, thereby exacerbating immune compromise (Pugliese *et al.*, 2005). HIV-1 Tat leaves infected cells via a leaderless but energy dependent pathway and extracellular Tat re-enters cells by binding to heparin sulfate proteoglycans (HSP) found on almost all cell surfaces (Chang *et al.*, 1997). Thereafter transmembrane endocytosis occurs via the caveolae pathway (Fittipaldi *et al.*, 2003; Vendeville *et al.*, 2004). Once extracellular Tat is taken up by cells, it reaches the nucleus of the cell and modulates the expression of diverse cellular genes including cytokines, chemokine co-receptors, major histocompatibility complex (MHC) and many others, hence contributing to the overall pathogenicity of the virus (Siddappa *et al.*, 2006). Tat therefore plays a vital role in HIV-1 pathogenesis, due to firstly its role in transactivation of viral replication and secondly, due to its action as an extracellular toxin (Campbell and Loret 2009).

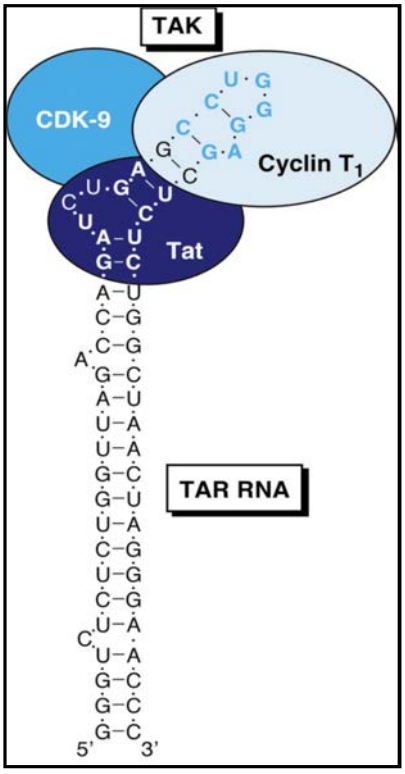


Figure 3. Schematic representation of Tat-TAR binding (Karn, 1999).

1.3.4 Neurotoxic effects of Tat

In the central nervous system (CNS) infection with HIV-1 is often associated with the development of dementia, a neurological disorder that is a critical cause of morbidity in a growing number of infected patients (Bradford *et al.*, 1986; Price *et al.*, 1988). HIV-1 Tat protein has been identified as a potential cause of this cognitive abnormality (Price *et al.*, 1988). However, the exact mechanism by which Tat causes the dementia is unknown (Brabers and Nottet, 2006). Initially, studies on HIV-associated dementia (HAD) were directed towards an underlying opportunistic infection, however, it was later discovered that HIV itself infects the brain and directly causes damage to the brain (Nielsen *et al.*, 1984; Price *et al.*, 1988). The virus enters the brain during the early stages of infection, causing neuropathological changes including monocyte infiltration, glial cell activation and loss of neurons (Gendelman *et al.*, 1994; Ghafouri *et al.*, 2006; Nath *et al.*, 1998; Price *et al.*, 1988).

The concern around HAD stems from its debilitating effect on the quality of life of HIV infected persons. It disrupts normal function at work, daily life and even self-care (Price *et al.*, 1988). This is due to the dampening and loss of motor and cognitive function which includes exaggerated tremor, unsteadiness, impaired fine and rapid motor control, slowed and inaccurate mental performance and other behavioral changes (Price *et al.*, 1988).

The pathophysiological features associated with HAD include increased microglia, elevated TNF-α messenger RNA in astrocytes, presence of excitotoxins, decreased synaptic and dendritic density, and neuronal loss possibly via apoptosis (Kaul *et al.*, 2001). The brain structures affected include the cerebrum, basal ganglia, thalamus, brain stem, the spinal cord and hippocampus, which explains the motor and cognitive dysfunction related to HAD (Price *et al.*, 1988). Interestingly, neurons themselves are rarely infected directly by HIV, while astrocytes and macrophages are the most commonly infected cells in the brain (Nath, 2002; Pocernicha *et al.*, 2005). Furthermore, the relation between HIV disease severity and viral load is not credible, while neuronal apoptosis has been found to correlate well with microglial activation (Adle-Biassette *et al.*, 1999; Petito *et al.*, 1994; Saha and Pahan, 2003). Taken together these observations suggest that the abnormal functioning of neurons in HAD very likely stem from indirect toxic mechanisms rather than HIV itself. A similar suggestion has been proposed by others (Mattson *et al.*, 2005; Saha and Pahan, 2003), however the exact mechanisms has not been elucidated.

Tat protein is released from infected cells to initiate a surge of positive feedback loops by activating uninfected astrocytes and microglia, causing the release of a variety of toxic substances postulated to induce neuronal dysfunction and cell loss (Li *et al.*, 2009; Nath, 2002). Previous studies have suggested that soluble products released from infected cells, be it virotoxins or cytokines, may be toxic to neurons (Nath, 2002). Evidence for Tat protein involvement comes from studies that show Tat transcripts to be elevated in HIV-1 demented brains, and both full length and first exon derived Tat are produced by HIV-1 infected cells of the central nervous system (Nath *et al.*, 1998; Wesselingh *et al.*, 1993a). The neurotoxic effects of Tat have now been shown to include neuronal activation, impaired synaptic function, increased production/release of pro-inflammatory cytokines, direct depolarization of neurons, loss of neurites and eventual neuronal cell death (Li *et al.*, 2009; Nath *et al.*, 1998). Interestingly, for these cytotoxic effects to occur, Tat need not be present continually (Nath *et al.*, 1998). Instead, it seems as if the effects of Tat protein can be induced rapidly. *In vitro* studies have shown that Tat protein causes prolonged depolarizations of neuronal cells within a second after exposure and the secretion of cytokines from glial cells and macrophages can occur within minutes of exposure (Nath *et al.*, 1998). Furthermore, Tat protein is capable of inducing its neurotoxic effects at picomolar levels (Kim *et al.*, 2003). These studies show that transient exposure of very low levels of Tat is enough to cause immense neurotoxicity.

1.4 Astrocytes

Astrocytes are specialized type of glial cells which span the entire CNS (Sofroniew and Vinters, 2010). Astrocytes forms a homogenous cell population having a stellate morphology with extended end-feet processes that surround neighboring neurons and blood vessels (Sofroniew and Vinters, 2010). It also has intermediate filaments called glial fibrils (Kimelberg and Nedergaard, 2010; Sofroniew and Vinters, 2010). There are two main types of astrocytes, fibrous, which populate the white matter and protoplasmic, the gray matter (Molofsky *et al.*, 2012). Fibrous astrocytes have consistent contours, cylindrical processes and dense glial filaments, whilst protoplasmic astrocytes have more irregular processes, few glial filaments, and they sheathe synapses (Molofsky *et al.*, 2012; Sofroniew and Vinters, 2010).

Astrocytes play a vital role in various essential CNS functions, including a supportive role to electrically active neurons (Kimelberg and Nedergaard, 2010; Sofroniew and Vinters, 2010). They integrate neuronal inputs and modulate calcium excitability, thereby influencing the activity of neighboring neurons (Araque *et al.*, 2001). Being the most numerous cell type in the CNS, astrocytes have the potential to impact a wide range of physiological and pathological functions (Claycomb *et al.*, 2013).

Astrocytes lack axons and the capability to form action potentials and were thought to merely function as "brain glue" supporting neuronal activity (Wang and Bordey, 2008). However, astrocytes are now recognized to have various critical functions that contribute significantly to the form and function of the CNS (Wang and Bordey, 2008). Below are brief descriptions of a few well-established astrocytic functions.

1.4.1 Astrocytes and ion homeostasis

Astrocytes have the ability to regulate the ionic content of the extracellular space (ECS) (Wang and Bordey, 2008). If this function is not controlled during normal neuronal activity, as a result of neurotransmission, the buildup of potassium ions (K^+) in the ECS will cause neuronal depolarization, hyperexcitability and seizures (Wang and Bordey, 2008). Astrocytes take up the excess K^+ ions, distributing them through gap junction-coupled syncytia, and extrude ions at low extracellular K^+ sites, such as directly into the blood stream via end-feet connections into capillaries (Figure 4) (Magistretti and Ransom, 2002; Wang and Bordey, 2008). There are two proposed mechanisms by which this is done, namely by transport mechanisms/Donnan forces and by K^+ redistribution, known as spatial buffering (Magistretti and Ransom, 2002).

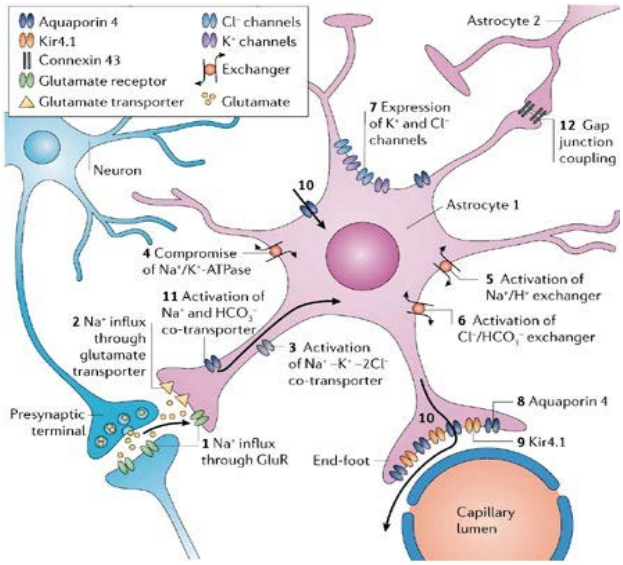


Figure 4. Diagram illustrating astrocyte morphology and function (Seifert *et al*., 2006).

1.4.2 Astrocytes and neurotransmission

Unlike neurons, astrocytes are not electrically but chemically excitable (Oberheim *et al.*, 2012). Accumulating evidence shows that astrocytes play direct roles in synaptic transmission, via the controlled release of neuroactive substances such as glutamate, ATP, adenosine, GABA and D-serine (Halassa *et al*., 2007; Nedergaard *et al.*, 2003; Sofroniew and Vinters, 2010). The release of these agents is dependent upon the changes in neuronal synaptic activity, involving astrocyte excitability as reflected by increases in astrocyte intracellular calcium concentration $[Ca^{2+}]i$ (Halassa *et al.*, 2007; Nedergaard *et al.*, 2003; Sofroniew and Vinters, 2010). Astrocytes express a large range of receptors, including G-protein coupled-ionotrophic, growth factor, chemokine, steroid and Toll-like receptors (Wang and Bordey, 2008). The glutamate and GABA ionotrophic receptors allow astrocytes to take up and metabolize these major excitatory and inhibitory transmitters respectively (Kimelberg and Nedergaard, 2010; Wang and Bordey, 2008). This ability of astrocytes shows its involvement in synaptic transmission, where the release of these and other gliotransmitters (Figure 4) occur in response to changes in neuronal synaptic activity (Sofroniew and Vinters, 2010). The regulation of both GABA and glutamate transmitters suggests that astrocytes limits the spillover of glutamate and GABA from the synaptic cleft, thereby preventing inappropriate overstimulation of postsynaptic receptors (Wang and Bordey, 2008). This evidence suggests that astrocytes play direct and interactive roles with neurons during synaptic activity, which is essential in the processing of information by neural circuits (Araque *et al.*, 1999; Halassa *et al.*, 2007; Sofroniew and Vinters, 2010).

1.4.3 Astrocytes and CNS blood circulation

The foot processes of astrocytes make extensive contact and have multiple bidirectional interactions with blood vessels, including the regulation of CNS blood flow (Figure 4) (Kimelberg and Nedergaard, 2010;

Sofroniew and Vinters, 2010). Recent findings show that astrocytes produce as well as release prostaglandins, nitric oxide and arachidonic acid, which are mediators that can increase or decrease CNS blood vessel diameter and blood flow in a coordinated manner (Gordon *et al.*, 2007; Iadecola and Nedergaard, 2007; Sofroniew and Vinters, 2010). Changes in the CNS blood flow can consequently be aligned in response to changes in neuronal activity (Koehler *et al.*, 2009; Sofroniew and Vinters, 2010). Astrocytes express a high level of water channels or aquaporins, namely aquaporin 4 (AQP4) (Figure 4), which are clustered along astrocytic processes (Sofroniew and Vinters, 2010). The connections these processes have with blood vessels and synapses, are central to the ability of astrocytes to regulate blood flow in relation to levels of synaptic activity (Sofroniew and Vinters, 2010).

1.4.4 Astrocytes and anti-oxidation

The CNS is continually exposed to the damaging effects of reactive oxygen species (ROS) due to its high rate of metabolic activity and fatty acid contents in myelin and other membranes (Kimelberg and Nedergaard, 2010; Sofroniew and Vinters, 2010). These ROS need to be neutralized by antioxidants and enzymes, however during pathological conditions, these protective mechanisms are overwhelmed and ROS-induced damage becomes the cause of cellular injury (Kimelberg and Nedergaard, 2010). Astrocytes contain a high number of several key proteins involved in neutralizing ROS, though these enzymes are not specifically enriched in astrocytes (Kimelberg and Nedergaard, 2010; Magistretti and Ransom, 2002). Because of this anti-oxidative property of astrocytes, manipulation of these cells was at one time considered a feasible strategy for reducing injury in several neurological diseases (Kimelberg and Nedergaard, 2010; Magistretti and Ransom, 2002).

1.4.5 Astrocytes and CNS metabolism

Astrocytes make major contributions to the metabolism of the CNS (Brown and Ransom, 2007; Sofroniew and Vinters, 2010). The end-feet processes are well positioned to take up glucose from blood vessels and supply energy metabolites to several neural elements in the gray and white matter (Figure 4) (Sofroniew and Vinters, 2010). Glycogen storage in the CNS occurs principally in astrocytes, and convincing evidence now shows that these glycogen granules serve as an energy source for neurons during hypoglycemia, when neuronal activity is high (Brown and Ransom, 2007). In the CNS, astrocytes are a major source of adhesion molecules, extracellular matrix (ECM) proteins and growth-promoting molecules (Wang and Bordey, 2008). Astrocytes are also the source of proteolytic enzymes, like metalloproteinases, which play a role in ECM degradation and remodeling (Wang and Bordey, 2008; Yong *et al.*, 1998). These cells also release growth factors including nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and fibroblast growth factor, all of which control neuronal maturation and survival (Blondel *et al*., 2000; Ojeda *et al.*, 2000; Wang and Bordey, 2008).

1.4.6 Astrocytes and molecular markers

Molecular markers are essential tools in the identification and characterization of cells (Sofroniew and Vinters, 2010). Astrocytes contain intermediate filaments of 9nm diameter that are composed of glial fibrillary acidic protein (GFAP) (Magistretti and Ransom, 2002), a protein which is thought to be specific for astrocytes in the CNS (Wang and Bordey, 2008). GFAP is in fact a family of intermediate filament proteins that include vimentin, nestin, and others. In general, these proteins serve cyto-architectural functions (Sofroniew and Vinters, 2010). Expression of GFAP is considered a marker for the immunohistochemical identification of astrocytes (Molofsky *et al.*, 2012; Sofroniew and Vinters, 2010) and has been shown to label reactive astrocytes that are responding to CNS injuries and infection (Molofsky *et al.*, 2012; Sofroniew and Vinters, 2010). However, GFAP is not an absolute marker of all non-reactive astrocytes and is often not immunohistochemically detectable in astrocytes in healthy CNS tissue (Sofroniew and Vinters, 2010). Studies in transgenic mice have shown that the expression of GFAP is not essential for the normal appearance and function of astrocytes in the CNS, but essential for the process of reactive astrogliosis and glial scar formation (Pekny and Pekna, 2004; Sofroniew and Vinters, 2010).

1.4.7 Astrocyte and neuron signaling

Neurons have been known to signal to each other by a range of transmitter substances (Hansson and Ronnback, 2003). However, recent studies revealed that glial cell activity is most probably determined by neuronal activity, and these cells can signal to each other and back to neurons (Hansson and Ronnback, 2003). The majority of the glial cells in the CNS are astrocytes, and as described previously, they are thought to play vital roles in the regulation of extracellular concentrations of ions, neurotransmitters and metabolites, and support neuronal and synaptic function (Hansson and Ronnback, 2003). Astrocytes perform neuron supportive functions including the capture of neurotransmitters from the synaptic cleft and the release of energy-rich substrates essential to metabolically sustain high synaptic activities (Fellin and Carmignoto, 2004; Magistretti and Pellerin, 1996). The position of these cells favor the conduction of signals between different cells in the nervous system (Araque *et al.*, 2001). For instance, an astrocyte can be in contact with a neuron and a capillary simultaneously, thereby having the potential to shuffle nutrients and metabolites between the blood supply and the active neuron (Araque *et al.*, 2001). Astrocytes can also transfer information between neighboring neurons because a single astrocyte can have contacts with multiple neurons (Araque *et al.*, 2001; Halassa and Haydon, 2010). At an ultrastructural level, astrocytes have been shown to be closely associated with the neuronal synapse (Figure 4) (Araque *et al.*, 2001) .

Astrocyte calcium (Ca^{2+}) signaling is one of the mechanisms by which CNS cells communicate with and modulate the activity of adjacent cells (Hansson and Ronnback, 2003; Pasti *et al.*, 1997). Intracellular calcium concentration $[Ca^{2+}]i$ in astrocytes mediate the release of glutamate which triggers ionotrophic

glutamate receptor-mediated Ca^{2+} release in surrounding neurons (Fellin and Carmignoto, 2004; Parpura *et al.*, 1994). The regulated increase in $[Ca^{2+}]i$ represents a form of astrocyte excitability (Charles *et al.*, 1991; Sofroniew and Vinters, 2010). Regulated increases in astrocyte $[Ca^{2+}]i$ are of functional significance in astrocyte-astrocyte and astrocyte-neuron intercellular communication (Sofroniew and Vinters, 2010). Astrocyte $[Ca^{2+}]i$ elevations can occur as intrinsic oscillations from Ca^{2+} released from intracellular spaces and can be triggered by transmitters released during neuronal activity (Halassa and Haydon, 2010 ; Perea *et al.*, 2009; Sofroniew and Vinters, 2010; Volterra and Meldolesi, 2005). The elevations in $[Ca^{2+}]i$ may also elicit transmitter release from the astrocyte into extracellular spaces, which triggers receptor mediated currents in neurons and can be propagated to neighboring astrocytes (Halassa and Haydon, 2010 ; Perea *et al.*, 2009; Sofroniew and Vinters, 2010; Volterra and Meldolesi, 2005). It is this Ca^{2+} signaling that enables astrocytes to play a direct role in synaptic transmission (Sofroniew and Vinters, 2010).

Astrocytes express neurotransmitter receptors and transporters that have the potential to be activated by synaptically released neurotransmitters (Araque *et al.*, 2001; Mennerick *et al.*, 1996). Astrocytes possess neurotransmitter receptors coupled to intracellular calcium mobilization (Araque *et al.*, 2001). Therefore, due to the ability of neurotransmitters to mobilize astrocytic Ca^{2+}, neuronal activity could regulate astrocytic Ca^{2+} levels (Araque *et al.*, 2001). Thus, there is bi-directional signaling communication between neurons and astrocytes, where astrocytes not only integrate neurotransmitter inputs, but also release their own secretagogues that act on neighboring neurons (Araque *et al.*, 2001).

The release of glutamate by astrocytes may encode for two qualitatively distinct messages with diverse functional consequences (Fellin and Carmignoto, 2004). Glutamate release which is triggered by $[Ca^{2+}]i$ rise at the astrocyte processes, symbolizes a feedback, in the form of a short distance signal which influences synaptic transmission locally (Fellin and Carmignoto, 2004). Low synaptic activity triggers $[Ca^{2+}]i$ rise which is restricted to activated astrocyte processes (Fellin and Carmignoto, 2004). This short distance message evokes a Ca^{2+}-dependent release of neuroactive substances in astrocytes, and this release works as a feedback mechanism locally affecting neuronal transmission (Fellin and Carmignoto, 2004). The release of glutamate and other compounds far away from the site of initial activation symbolizes a feed forward, long distance signal which can be implicated in the regulation of distinct processes (Fellin and Carmignoto, 2004). High synaptic activity sets off a Ca^{2+} signal that propagates to other processes and is oscillatory in nature (Fellin and Carmignoto, 2004). This long distance message works as a feed forward mechanism, transferring information on neuronal activity to other cells distant from the initial site of activation at the astrocyte process (Fellin and Carmignoto, 2004).

Astrocyte–neuron signaling symbolizes an essential multifunctional unit contributing to the abundant diversity in neuronal transmission, which is a fundamental feature of CNS function (Fellin and Carmignoto, 2004).

1.5 Role of astrocytes in Neurological Diseases

Astrocytes preserve a barrier between blood and the brain, providing neuronal sustenance and functions (Chauhan *et al.*, 2003). Due to their vital functions, abnormal astrocyte activity may induce neuronal damage by loss of neuronal support functions or release of cellular products that are harmful to neurons (Chauhan *et al.*, 2003). Astrocytes may also serve as a reservoir for viruses such as HIV, or release viral products that have deleterious effects on neurons. It is because of this latter reason that infected astrocytes were thought to play a role in the development of cognitive impairments associated with HIV/AIDS (Chauhan *et al.*, 2003).

Astrocytes become reactive/activated in response to a number of brain insults, including trauma, infection, epilepsy and neurodegeneration (Halassa and Haydon, 2010). During infection, this response is apparent by a series of morphological and functional changes that are aimed at the damaged area, producing immune/inflammatory responses against the invading pathogens (Rodríguez and Verkhratsky, 2010). A major feature of reactive astrocytes is the upregulation of GFAP, which is associated with the thickening of the main processes of the astrocyte (Halassa and Haydon, 2010). Reactive astrocytes are also characterized by hypertrophy of cell bodies and processes, altered gene expression, increases in the calcium binding protein S100β, and proliferation that may occur in some neurodegenerative diseases (Barreto *et al.*, 2011).

There are various intercellular signaling molecules that trigger reactive astrogliosis, that are released from surrounding astrocytes or other cell types of the CNS, including neurons, microglia, endothelia and pericytes (Sofroniew and Vinters, 2010). The stimulated astrocytes then cause detrimental effects such as cytokine production, intensification of inflammation, production of neurotoxic levels of ROS, release of increased excitotoxic glutamate and morphological changes that compromise blood brain barrier function (Brambilla *et al.*, 2005; Sofroniew and Vinters, 2010; Swanson *et al.*, 2004). Astrocyte activation can be detrimental to regenerative events following CNS injury, or beneficial in restoring the integrity of the CNS microenvironment (Ridet *et al.*, 1997; Zhou *et al.*, 2004). A balance of these two processes is deterministic to the development of neurologic disease or not (Ridet *et al.*, 1997; Zhou *et al.*, 2004).

The pathogenesis of HIV-associated dementia (HAD) involves complex interactions between viral proteins and cytokines, leading to neuronal dysfunction and cell death (Nath *et al.*, 1999). As mentioned earlier, HIV-1 Tat protein is the potential cause of HAD (Price *et al.*, 1988). Astrocytes are susceptible to infection by HIV and neurons are rarely infected (Zhou *et al.*, 2004). It is believed that neuronal loss in the brain of HIV-infected individuals result from indirect mechanisms such as the release of soluble factors including the HIV-1 Tat protein, from infected macrophages, microglia and/or astrocytes (Zhou *et al.*, 2004). Astrocytes are of particular interest since they are non-productively infected (Brabers and Nottet, 2006). In these cells, viral structural protein translation is defective, and only regulatory proteins including HIV-

1 Tat protein, are produced (Brabers and Nottet, 2006). One of the effects of Tat protein is to activate uninfected astrocytes to cause the release of cytokines (Nath, 2002).

Studies have confirmed that activated astrocytes release inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in response to certain bacterial and viral infections (Lau and Yu, 2001). Under physiological conditions these cytokines have pleiotropic and overlapping functions and play vital roles in the induction and regulation of the immune and inflammatory response (Arai *et al.*, 1990; Lau and Yu, 2001). TNF-α may have neuroprotective properties, however, it has multiple damaging effects in the cells of the CNS including neuronal cell apoptosis and myelin damage (Chen *et al.*, 1997). IL-6 has beneficial effects in the CNS because of its neurotrophic properties, however when over-expressed it can be detrimental, adding to the pathophysiology associated with CNS disorders (Van Wagoner *et al.*, 1999). It has been shown that Tat protein stimulates the release of both IL-6 and TNF-α from astrocytes *in vitro* (Chen *et al.*, 1997).

1.6 Apoptosis

Cell death plays an imperative role in physiological processes; thus it is a natural part in the development and health of multicellular organisms (Sonker *et al.*, 2010). Apoptosis was initially explained by Kerr *et al.* (1972) and was distinguished from necrotic cell death by detailed morphological and biochemical differences in dying cells (Ouyang *et al.*, 2012). It has been recognized as a vital form of programmed cell death which involves the genetically determined elimination of cells (Elmore, 2007). Apoptosis is an important factor in normal cell turnover, proper development, function of the immune system, multiplication of mutated chromosomes, hormone-dependent atrophy, normal embryonic development, elimination of disinclined cells and maintenance of cell homeostasis (Rastogi *et al.*, 2009; Reed and Tomaselli, 2000). An imbalance in apoptosis regulation has been linked to many diseases (Elmore, 2007). Some diseases involve insufficient apoptosis, such as cancer, and others excessive apoptosis, such as neurodegeneration, AIDS and autoimmune disorders (Elmore, 2007). Apoptosis therefore plays a significant role in proper health, but also in the onset of disease (Elmore, 2007).

Apoptosis is achieved by 2 different signaling pathways; the intrinsic and extrinsic signaling pathways (Elmore, 2007). Damage of DNA, via ultraviolet radiation, ionizing radiation, viral infections, oxidative stress, replication or recombination errors and from environmental therapeutic genotoxins are the principle triggers of the intrinsic apoptotic pathway (Batista *et al.*, 2009; Nagata, 1997; Rastogi *et al.*, 2009), all of which compromise the mitochondrial membrane potential (Elmore, 2007). The extrinsic pathway can be induced by the binding of the death ligand to its appropriate receptor, which include members of the TNF-α gene family (Locksley *et al.*, 2001). An additional pathway called the perforin-granzyme pathway induces cell death via cytotoxic T-cell killing, which is initiated by single stranded DNA damage (Elmore, 2007). Perforin is a pore forming molecule recruited by T-cells to form a pore in the target cell (Trapani and Smyth, 2002). Through this pore, apoptosis inducing cytoplasmic granules called granzyme A and granzyme B, are released (Trapani and Smyth, 2002). The regulated control of apoptotic mitochondrial events is carried out by members of the B-cell lymphoma-2 (Bcl-2) family of proteins (Cory and Adams, 2002), which can either be anti-apoptotic or pro-apoptotic (Elmore, 2007). These proteins being pro-apoptotic or anti-apoptotic, will determine whether a cell commits to apoptosis or aborts the process (Rastogi *et al.*, 2009). Apoptosis is an energy dependent process involving a group of cysteine proteases called caspases. It is these enzymes that link the initial stimuli to cell death (Elmore, 2007). Caspases are expressed in an inactive pro-enzyme form in most cells, and once activated they initiate the protease cascade, by activating other pro-caspases (Ouyang et al., 2012; Elmore, 2007).

Extrinsic, death signal pathway: The tumor necrosis factor receptor (TNFR) family has a cytoplasmic domain of 80 amino acids called the death domain. This domain is essential in transmitting the death signal from the cell surface to the intracellular signaling pathway (Ashkenazi and Dixit, 1998). Examples of death

ligand receptor systems are TNF-α/TNFR1 and Fas ligand/Fas receptor (Rubio-Moscardo *et al.*, 2005). Once the ligand binds to its receptor, cytoplasmic adapter proteins (e.g. TNFR type 1-associated death domain protein (TRADD)), are recruited (Ouyang et al., 2012; Elmore, 2007). The adaptor proteins have their own death domains and once they are recruited, they interact with the activated receptor death domains, forming the death inducing signaling complex (DISC) (Elmore, 2007; Sonker *et al.*, 2010). This results in the auto-catalytic activation of pro-caspase-8 (Elmore, 2007; Kischkel *et al.*, 1995) (Figure 5).

Intrinsic, mitochondrial, non-receptor pathway: This pathway is induced by death stimuli which produce intracellular signals, directly affecting targets within the cell (Elmore, 2007). Viral infection, toxins and radiation amongst many more, cause changes in the mitochondrial membrane potential and the opening of the mitochondrial permeability transition pore (MPTP) (Saelens *et al.*, 2004). This results in the release of the pro-apoptotic proteins such as cytochrome c (Saelens *et al.*, 2004). This leads to the formation of an apoptosome by the interaction of cytochrome c with apoptotic protease activating factor 1 (Apaf-1) molecules (Romani *et al.*, 2010). The apoptosome converts the initiator protein pro-casapase-9 to caspase-9 (Romani *et al.*, 2010) (Figure 5).

Perforin-granzyme pathway: This pathway involves the release of perforin from cytotoxic T-lymphocytes to activate granzyme B and A (Elmore, 2007). Granzyme B converts pro-caspase-10 to caspase-10 which subsequently cleaves the initiator protein pro-caspase-3 to form caspase-3 (Elmore, 2007). Granzyme A activates caspase-independent pathways and cleaves the nucleosome assembly protein (SET) which initiates DNA cleavage (Elmore, 2007) (Figure 5).

The intrinsic, extrinsic and the granzyme B dependent pathways all terminate on the same execution pathway, which starts with pro-caspase-3 (Ouyang et al., 2012). Once caspase-3, the most vital execution protein, is activated by initiator caspases-8, 9 or 10, the final phase of apoptosis begins (Elmore, 2007). Cellular disruption involves a sequence of events which include endonuclease activation, DNA fragmentation, activation of proteases that degrade nuclear proteins, formation of apoptotic bodies, cytoplasmic blebbing and lastly, the phagocytosis of the blebbed contents by neighboring macrophages and other phagocytic cells (Rastogi *et al.*, 2009).

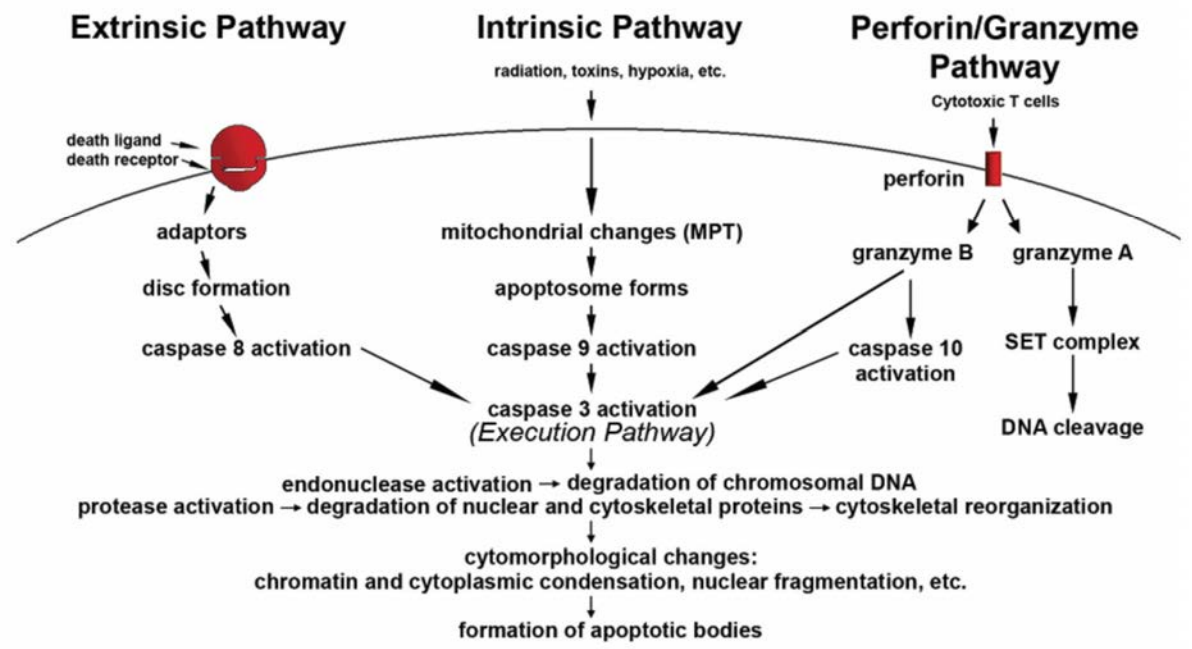


Figure 5. Schematic representation of apoptotic events (Elmore, 2007).

1.6.1. TNF-α and IL-6 induced neuronal apoptosis

As mentioned previously, HIV-1 appears to act via indirect mechanisms to induce neuronal cell death (Saha and Pahan, 2003). HIV-1 leads to the loss of CD4 cells resulting in immunodeficiency, whilst the immune profile in the brain suggests an immune activation involving mediators produced by macrophages and astrocytes (Mattson *et al.*, 2005; Wesselingh *et al.*, 1994). Studies have shown the increased production of TNF-α, IL-6, IL-1β and other chemokines in the brains of individuals with HIV infection (Chen *et al.*, 1997; Conant *et al.*, 1998; Wesselingh *et al.*, 1993b).

Pro-inflammatory cytokines have both beneficial and detrimental effects in the CNS depending on the circumstances under which they are secreted (Sheng *et al.*, 2005). These cytokines are involved in the immune response to infection and directly influence neuronal function (Hagberg and Mallard, 2005; Nimmervoll *et al.*, 2012). The major pro-inflammatory cytokine, TNF-α and its receptors are expressed at nominal levels in all types of brain cells under normal conditions, supporting its physiological role in brain functioning (Perry *et al.*, 2002; Saha and Pahan, 2003). However, studies have shown strong evidence of TNF-α and its receptors in the pathogenesis of HIV related cognitive abnormalities and other neurodegenerative disorders (Saha and Pahan, 2003). TNF-α can elicit both autocrine and paracrine effects on surrounding cells which in turn can lead to a positive feedback loop, leading to additional cytokine and chemokine production (Hanisch, 2002; Sheng *et al.*, 2005). Not only can this cytokine elicit inflammation, it can also induce apoptosis via its receptor and intracellular signaling pathways (Muppidi *et al.*, 2004; Sheng *et al.*, 2005; Wajant *et al.*, 2003). The homeostatic role of TNF-α is to control the equilibrium between neuronal excitation and inhibition (Nimmervoll *et al.*, 2012; Stellwagen and Malenka, 2006).

Studies have shown that TNF-α is also implicated in neuronal cell death (Araki *et al.*, 2001; Block *et al.*, 2004; Nimmervoll *et al.*, 2012), and therefore, the interaction between neuronal activity and TNF-α may control cell death and survival processes in developing neuronal networks (Nimmervoll *et al.*, 2012).

Studies have shown that TNF-α causes cell death mainly via the extrinsic apoptotic pathway, exerting its effects via 2 receptor subtypes situated on the target cell membranes: TNFR1 and TNFR2 (Cabal-Hierro and Lazo, 2012; Guadagno *et al.*, 2013). The association of Fas-Associated protein with death domain (FADD) to TNFR1 bound Tumor necrosis factor receptor type 1-associated death domain protein (TRADD) recruits pro-caspase-8 by protein-protein interaction via homologous death effector domain (DED) (Saha and Pahan, 2003). The death inducing signaling complex (DISC) is formed during which pro-caspase-8 is autolytically cleaved, liberating caspase-8 from DISC (Saha and Pahan, 2003). Following this the caspase cascade is triggered which includes the activation of effector caspases-3, 6 and 7 (Saha and Pahan, 2003). This leads to the death of the cell (Fotin-Mleczek *et al.*, 2002; Saha and Pahan, 2003).

Interleukin-6 is a pleiotropic cytokine having various biological activities in immune regulation, hematopoiesis, inflammation and oncogenesis (Kishimoto, 2010). This cytokine regulates various physiological events including cell proliferation, differentiation, survival and apoptosis (Figure 6) (Guzmán *et al.*, 2010). In general, it is regarded as a pro-inflammatory cytokine, but it also possesses anti-inflammatory properties (Scheller *et al.*, 2011). IL-6 provides survival to neurons, astrocytes and oligodendrocytes in cases of brain injury and disease (Rincon, 2012). While this cytokine has beneficial effects, it may also generate unwanted consequences causing the pathophysiology associated with CNS disorders (Van Wagoner *et al.*, 1999). Studies have shown the pathological roles of IL-6 in various disease conditions, like inflammatory, autoimmune, neurological and malignant diseases (Kishimoto, 2010; Kishimoto and Ishizaka, 1971; Rincon, 2012). Increased levels of IL-6 in the CNS have been demonstrated in several neurological diseases and in HIV infection (Qiu *et al.*, 1998). Despite these numerous studies, its exact role in the progression of these diseases remains unclear (Rincon, 2012). It has been suggested that elevated levels of IL-6 in the CNS in neurological disorders may be a contributing factor to histopathological, pathophysiological, and cognitive defects related to these disorders (Guzmán *et al.*, 2010). Hence, chronic exposure to IL-6 seems able to interrupt normal functioning of the CNS.

Astrocytes are a major source of IL-6 in the CNS (Van Wagoner *et al.*, 1999). These cells produce large quantities of IL-6 when stimulated (Lee *et al.*, 1993) and may therefore be important sources of IL-6 in CNS disease (Qiu *et al.*, 1998). As mentioned earlier, CNS neurons and astrocytes are closely linked, and neurons are likely to be exposed to high concentrations of IL-6 under pathological conditions (Qiu *et al.*, 1998). The N-methyl-D-aspartate receptor (NMDAR) is a highly Ca^{2+}-permeable, ligand gated ion channel found in neurons and is a member of the ionotropic glutamate receptor family (Kaul and Lipton, 2000). Chronic exposure of neurons to IL-6 enhances NMDA receptor mediated intracellular calcium signals and

increases resting calcium levels (Holliday *et al.*, 1995; Qiu *et al.*, 1995; Qiu *et al.*, 1998). The results of these studies implicate NMDAR and intracellular calcium as important mechanisms through which IL-6 alters neuronal function (Qiu *et al.*, 1998). Excessive Ca^{2+} accumulation after NMDAR stimulation leads to depolarization of the mitochondrial membrane potential and reduces the cellular ATP concentration (Kaul and Lipton, 2000). If the initial insult is severe, cells do not restore the ATP levels and subsequently die a necrotic death due to the loss of ionic homeostasis (Kaul and Lipton, 2000). When the initial insults are mild, ATP levels can be recovered to some extent, but these cells often undergo apoptosis (Kaul and Lipton, 2000).

Studies have shown both that IL-6 possesses pro-inflammatory, anti-inflammatory, neuroprotective and neurotoxic effects (Guzmán *et al.*, 2010; Rincon, 2012; Scheller *et al.*, 2011). These contrasting views reveal the ambiguous function of IL-6 in the CNS.

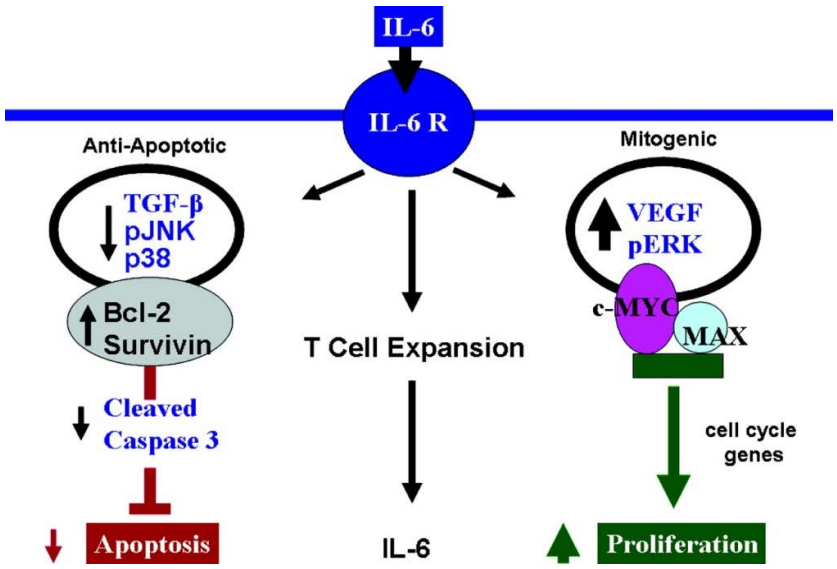


Figure 6. Schematic representation of IL-6 function in apoptosis and proliferation (Steiner, 2009).

1.7. Anti-retroviral therapy

AIDS is by far the most dreadful endemic experienced by mankind to date (Karn, 1999). HIV, the virus causing AIDS, results in a chronic infection for which there is no cure (Arts and Hazuda, 2012). This

creates great challenges for sustaining lifetime patient therapy (Arts and Hazuda, 2012). Original antiretroviral (ARV) therapy consists of reverse transcriptase inhibitors and/or protease inhibitors (Arts and Hazuda, 2012). These drugs target two of the essential enzymes required for the replication of HIV (Arts and Hazuda, 2012; Karn, 1999). A combined treatment including both these agents therefore suppresses viral replication and drops the plasma viral load to levels that are lower than the confines of detection by the most sensitive clinical assays (Arts and Hazuda, 2012). While combined therapy (also referred to as highly active antiretroviral therapy (HAART)), can suppress viral replication and increase a patient's life-span, it is unable to completely eliminate the HIV-1 infection (Arts and Hazuda, 2012). Subsequently clinicians managing people infected with HIV are constantly faced with the problem of a residual reservoir of the virus.

Besides the residual viral load problem, treatment with ARV's can also be impaired by non-adherence, poor drug tolerability and interactions between other medication and ARV agents (Arts and Hazuda, 2012; Bhattacharya and Osman, 2009). Factors such as these have been proposed to contribute to the evolution of drug resistance in these patients (Arts and Hazuda, 2012; Bhattacharya and Osman, 2009). Drug resistance has led to extensive research in novel managements for HIV-1 infection (Bhattacharya and Osman, 2009). There have been new targets approved for HIV-1 treatment, including chemokine receptor antagonists, nucleoside analogs, integrase inhibitors and maturation inhibitors (Arts and Hazuda, 2012; Bhattacharya and Osman, 2009). Agents acting on the HIV-1 proviral DNA, Tat-TAR interaction inhibitors and HIV-1 envelope oxidizers are targets amongst others which are in various clinical stages of development (Bhattacharya and Osman, 2009). Given this range of treatment, it is hoped that patients can be more successfully treated (Arts and Hazuda, 2012). Despite these therapeutic advances, the virus manages to evolve and evade the most effective therapy, essentially forcing the scientific community to continue their search for new HIV-1 treatments (Arts and Hazuda, 2012). At present novel ARV drugs are not considered a substitute for the original ARV's, since basic ARV treatment remains the chief support in managing HIV-1 (Bhattacharya and Osman, 2009).

There are also other challenges that persons living with HIV face. HAART does not offer complete protection against HIV/AIDS induced neurological damage (Palacio *et al.*, 2012). The availability of HAART has decreased the occurrence of HAD and the clinical manifestations are less severe (Mattson *et al.*, 2005). However, due to increased rates of patient survival, the rate of HAD incidence continue to rise (Mattson *et al.*, 2005). Control of HIV-1 in the CNS is still imperative because in some patients the control of the virus in the periphery is exceptional whilst cognitive decline persists (Mattson *et al.*, 2005). Increased viral loads can be identified in the cerebrospinal fluid of infected patients due to poor penetration of ARV's across the blood-brain barrier and viral advancement in the brain (Mattson *et al.*, 2005). Available ARV's have no effect on the production of non-structural proteins like Tat, which continues production and release from infected cells like astrocytes (Mattson *et al.*, 2005). Since HAART is insufficient in the treatment of

HAD, other targets include both structural and regulatory viral proteins, and the various steps involved in the pathogenesis of HAD (Nath, 2002).

Although HAART improves the lifespan of an infected individual, the effect of the drugs itself as well as the secondary complications from chronic infections, is causing the quality of life to decline (Bagashev and Sawaya, 2013). Therefore, there is plenty more to discover in the mission of controlling HIV (Bagashev and Sawaya, 2013). For this it is important to understand, at a molecular level, how the virus and its proteins influence cells (Bagashev and Sawaya, 2013). This will offer the opportunity to create additional therapeutic strategies that may facilitate the attenuation of HIV and HIV associated pathologies (Bagashev and Sawaya, 2013).

1.8 Hypothesis

Since astrocytes are the main target of HIV infection in the CNS and because of the intimate functional interaction between astrocytes and neurons, we hypothesized that

- HIV-1 Tat protein alone is sufficient to activate astrocytes,
- Once astrocytes become activated, they release TNF-α and IL-6,
- Released TNF-α and IL-6 may be harmful to neurons, and initiate neuronal cell death via apoptosis.

1.9 Aims

In order to prove our hypothesis, we formulated the following aims:

- To determine whether commercial Tat is biologically active.
- To determine whether Tat activates astrocytes.
- To establish whether astrocytes exposed to Tat, results in the release of IL-6 and TNF-α.
- To assess whether IL-6 and TNF-α can induce apoptosis of neuronal cells.

CHAPTER 2

METHODOLOGY

2.1 Introduction

To test our hypothesis that Tat stimulates astrocytes to release IL-6 and TNF-α and that these cytokines induce apoptosis in neurons, we asked the following questions:

1. Do astrocytes release IL-6 and TNF-α upon stimulation by Lipopolysaccharide (LPS) (that served as a positive control) and Tat?
2. Can IL-6 and TNF-α released in the medium in which the astrocytes were grown, induce apoptosis in neurons?

2.2 Cell lines

Cell lines, CRL 2137 (Human Neuroblastoma), U87.CD4.CCR5 (Human Astroglioma) and reporter CEM-GFP (Human T-cell, CD4+ Lymphoblastiod) cell lines were generous gifts from Dr D. Ramsuran of KwaZulu-Natal Research Institute for TB and HIV (K-RITH).

2.2.1 Culturing CEM-GFP cells

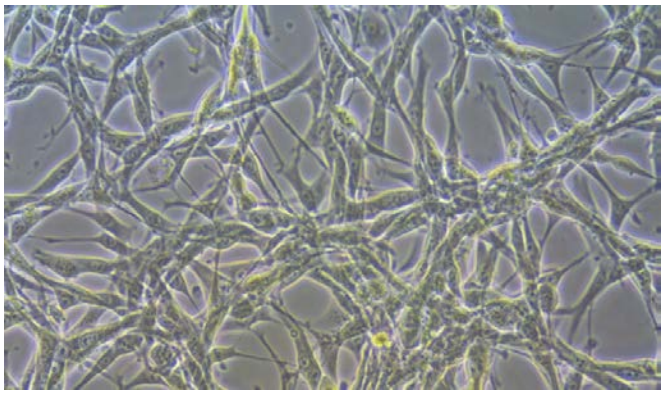
CEM-GFP cell lines were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (Biochrom, Merck Millipore, Darmstadt, Germany), penicillin and streptomycin (100 mg/ml) and L-Glutamine (PAA, Pasching, Austria); this constituted complete culture medium. The cells were incubated in a CO_2 water jacketed incubator (Shel Lab, Oregon, U.S.), at 37°C in a 95% humidified atmosphere containing 5% CO_2.

2.2.2 Culturing CRL and U87 cells

CRL and U87cells were grown in Dulbecco's Modified Minimal Essential Medium (DMEM) medium (Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (Biochrom, Merck Millipore, Darmstadt, Germany), penicillin and streptomycin (100 mg/ml) and L-glutamine (PAA, Pasching, Austria); this constituted complete culture medium for these cells. The cells were grown in a CO_2 water jacketed incubator (Shel Lab, Oregon, U.S.), at 37°C in a 95% humidified atmosphere containing 5% CO_2.

Trypan Blue (Bio-Rad, Hercules, CA) was used as an exclusion dye for viable cell counting. All cell lines were counted using an Automated Cell Counter (Bio-Rad, Hercules, CA). Cells were cultured in 25 and

75 cm^3 tissue culture flasks (TPP, Trasadingen, Switzerland), and 24-well tissue culture plates (Corning, Tewksburg, USA).



Light micrograph of U87 cell line at a 20X magnification

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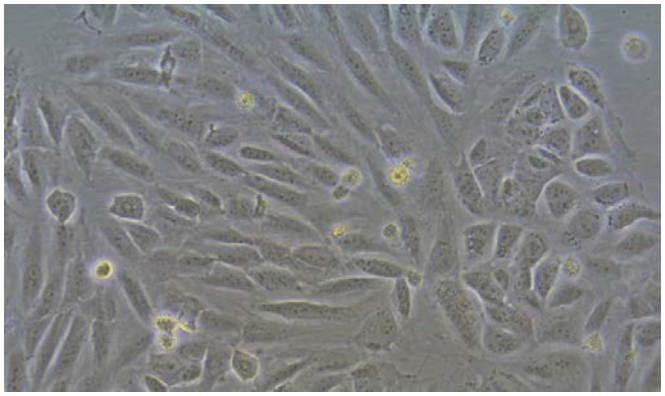


Light micrograph of CRL cell line at a 20X magnification

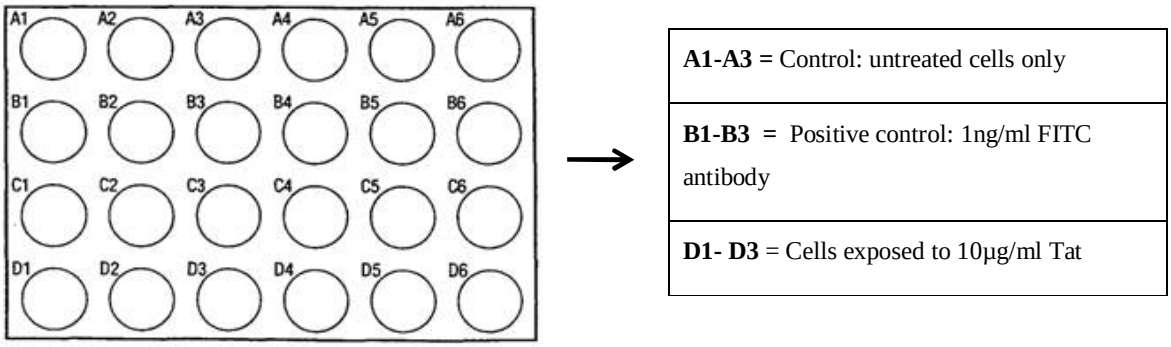
2.3. Biological activity of commercially procured HIV-1 Tat protein

BioPORTER reagent transfection of HIV-1 clade B Tat protein (Tat) into CEM-GFP reporter cell lines were done to determine whether Tat was biologically active. Here 10µg/ml of Tat (Diatheva, Fano, Italy) (Appendix 1) was delivered into CEM-GFP cells after formulation with the BioPORTER reagent (Gene Therapy Systems, San Diego, USA). The summarized protocol for the transfection is shown in Table 2 (as per BioPORTER reagent instruction manual).

Table 2. BioPORTER reagent transfection procedure.

Process	Method
Preparation of BioPORTER reagent	250µl methanol was added to the BioPORTER reagent.
	Vortexed at top speed for 20secs.
	2.5µl was aliquoted into 1.5ml Eppendorf tubes.
	9 of the Eppendorf tubes with BioPORTER reagent was left under a laminar flow hood for 2-4 hours (remaining tubes were stored away at -20°C).
Preparation of BioPORTER/ protein complexes	HIV-1 Tat protein was resuspended in 200µl PBS (Sigma, Missouri, USA) (Appendix 2).
	25µl diluted Tat protein solution was added to 3 of 9 BioPORTER reagent Eppendorf tubes.
	Tubes with BioPORTER/protein complex were mixed 3-5 times by pipetting.
	The tubes were left to stand at room temperature for 5mins and then vortexed gently for 3-5 secs.
	225µl serum free medium was added to the BioPORTER/protein complex, bringing the final volume to 250µl.
Preparation of BioPORTER/ Fluorescent Antibody –Positive Control	10µl (1ng/ml) Fluorescein isothiocyanate (FITC) antibody (provided by kit) was added to 3 of the 6 remaining BioPORTER reagent Eppendorf tubes.
	Tubes with BioPORTER/FITC antibody complex were mixed 3-5 times by pipetting.

	The tubes were left to stand at room temperature for 5mins and then vortexed gently for 3-5 secs.
	240µl serum free medium was added to the BioPORTER/protein complex, bringing the final volume to 250µl.
Cell culture preparation	The CEM-GFP cell suspension was centrifuged (Beckman Coulter, Brea California) at 1200rpm for 5mins and resuspended in serum free medium.
	Cells were counted and $1x10^6$cellswere transferred to the final delivery mix of each of the 9 BioPORTER reaction tubes.
BioPORTER/negative control	The remaining 3 tubes contained cells and BioPORTER reagent only
Transfer and incubation	Each reaction was transferred to a 24 well plate (Figure 7)
	The plate containing the reactions was incubated for 3-4 hours at 37°C.
	1ml serum containing medium was added to each reaction after 3-4 hours.
	The plate containing the reactions was incubated overnight.



A1-A3 = Control: untreated cells only
B1-B3 = Positive control: 1ng/ml FITC antibody
D1- D3 = Cells exposed to 10µg/ml Tat

Figure 7. A 24-well tissue culture plate seeded with CEM-GFP and transfected CEM-GFP cells for Tat biological activity testing.

2.3.1 Microscopic analysis

The emission of green fluorescence by CEM-GFP cells exposed to Tat was viewed as a wet preparation under a Zeiss Fluorescent Microscope, LSM 880 (Oberkochen, Germany).

2.4 Tat stimulation of astrocytes

Western blot assays were used to determine whether U87 cells were activated upon exposure to HIV-1 B Tat. Here U87 cell lines were seeded onto 24-well tissue culture plates (1 x 10^6 cells per well with 500µl medium) and exposed to either 1µg/ml Lipopolysaccharide (LPS) (Sigma, Missouri, US) (Appendix 3) or 10µg/ml Tat (Diatheva, Fano, Italy), as illustrated in Figure 8 and incubated overnight.

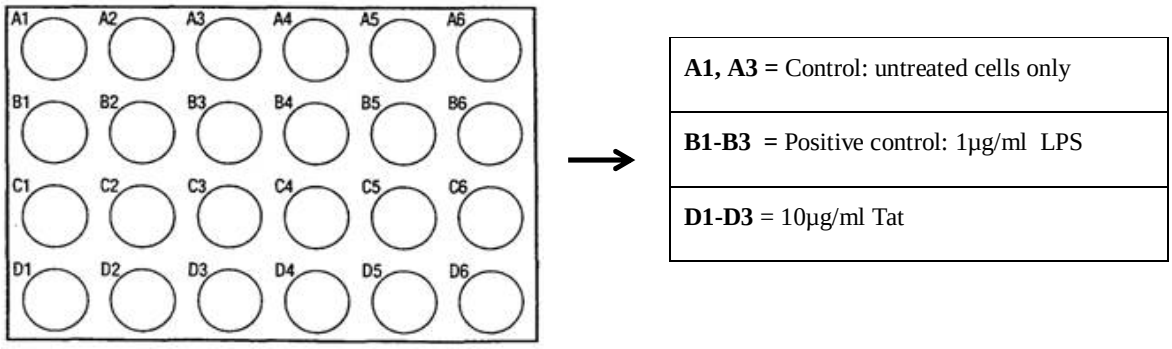


Figure 8. A 24-well tissue culture plate seeded with U87 and exposed to 1µg/ml LPS or 10µg/ml Tat for western blot analysis of GFAP.

Following overnight incubation, cells were detached using Trypsin EDTA (PAA, Pasching, Austria), collected in 1.5ml Eppendorf tubes (BD Biosciences, San Jose, CA), and spun down at 1500rpm for 5mins in a Microfuge 16 Centrifuge (Beckman Coulter, Brea, CA). The supernatant was discarded and pellets were lysed using 150µl 1% sodium dodecyl sulphate (SDS) (Sigma, Missouri, US), samples were shaken on a sunflower mini shaker (Grant Instruments, Cambridge, UK) at low speed for 10mins. Laemmli Sample Buffer (Bio-Rad, Hercules, CA) was added to cell lysates (Table 3).

Table 3. Sample buffer and cell lysate volumes.

Sample	Sample volume (µl)	Laemmli Volume (µl)
Untreated U87 cells	7	4
U87 cells exposed to LPS	8	4.5
U87 cells exposed to Tat	15	7

U87 cell lysate with Laemmli was placed in a Thermomixer (Eppendorf, Hamburg, Germany), for 10mins at 95°C. The cells were then centrifuged at 16 000 rpm for 1min.

2.4.1 Preparing and running of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels.

The Tris-Glycine extended (TGX) and TGX stain-free "fast cast" Acrylamide kit (Bio-Rad, Hercules, CA) was used to cast the Sodium Dodecyl Sulfate Polyacrylamide (SDS-PAGE) gel.

SDS-PAGE gel. The resolving and stacking gels were made as shown in Table 4.

Table 4. Preparation of SDS-PAGE Resolving and Stacking Gel.

Resolving gel (7.5%)	
Constituent	**Volume**
Resolver A	3ml
Resolver B	3ml
Tetramethylethylenediamine (TEMED)	3µl
10 % Ammonium Persulfate (APS) (Appendix 20)	30µl
Stacking gel (4%)	
Stacker A	1ml
Stacker B	1ml
TEMED	2µl
10% APS	10µl

The resolving gel was pipetted into a glass gel holder and the stacking gel was carefully pipetted on top of the resolving gel. A 10 well comb was carefully placed on top of the stacking gel and the gel was left to polymerize for 45mins. Once the gel polymerized, the comb was removed and the gel was firmly placed onto a running module (Bio-Rad, Hercules, CA), with the buffer dam (Bio-Rad, Hercules, CA) on the opposite side and subsequently placed in the buffer tank (Bio-Rad, Hercules, CA). The tank was filled with 1X Tris-Glycine-SDS running buffer (Bio-Rad, Hercules, CA) (Appendix 4) to the "2 blot" point. The wells were washed twice with buffer. In the first lane, 4µl of Precision Plus Protein Dual Xtra (Bio-Rad, Hercules, CA) molecular marker was added. The samples prepared as shown in Table 3, were added to the wells. The gel ran at 150V and 3Amps for 45mins.

Following the run an image of the pre-transfer gel (Figure 9) was captured using the ChemiDoc MP system (Bio-Rad, Hercules, CA).

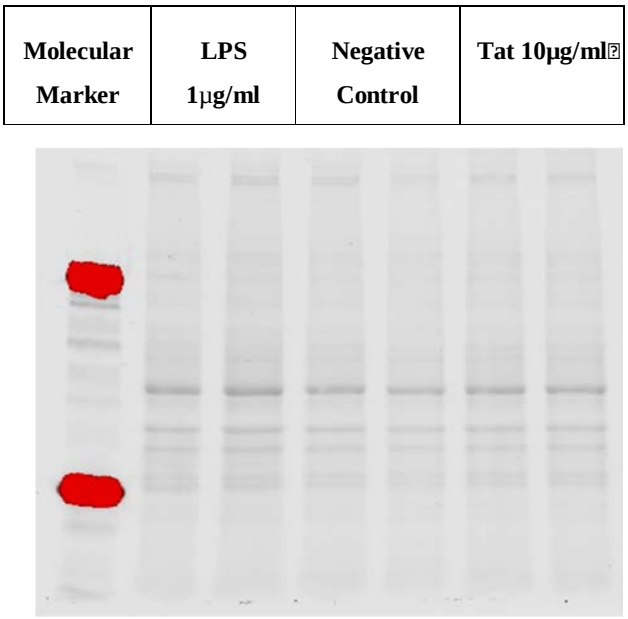


Figure 9. Pre-transfer gel stain-free image captured as image A on the ChemiDoc MP system (Image A was used to assess the sample integrity and separation quality).

2.4.2 Transfer of SDS-PAGE gel to nitrocellulose membrane and antibody treatments

Following the run, the gel was removed from the glass gel holder. Two separate blotting papers and a nitrocellulose membrane (Bio-Rad, Hercules, CA) were soaked in 1X Tris-Glycine (TG) transfer buffer (Bio-Rad, Hercules, CA) (Appendix 5) for 10mins. The first blotting paper was placed onto the tray of the Trans-Blot Turbo (Bio-Rad, Hercules, CA) and the membrane was placed over it. The gel was placed onto a membrane and covered with the second blotting paper and ran for 10mins at 2.5A and 25V. After the transfer the gel and blot was placed in deionized water. The post-transfer stain-free gel (Figure 10) and blot (Figure 11) was captured using the ChemiDoc MP system.

Molecular Marker	LPS 1µg/ml	Negative Control	Tat 10µg/ml

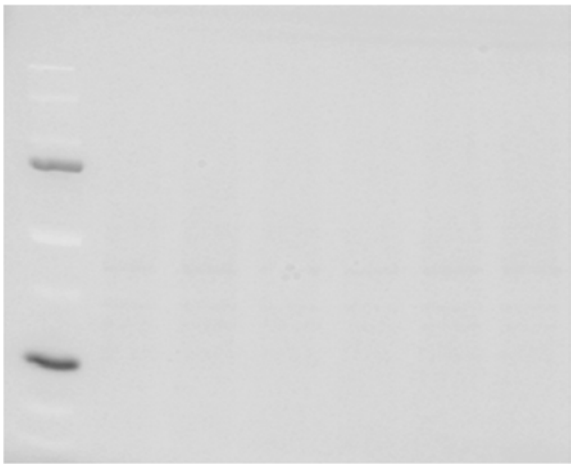


Figure 10. Post- transfer gel stain-free image captured as image B on the ChemiDoc MP system (Image B was used to measure the transfer efficiency of the samples).

Molecular Marker	LPS 1µg/ml	Negative Control	Tat 10µg/ml

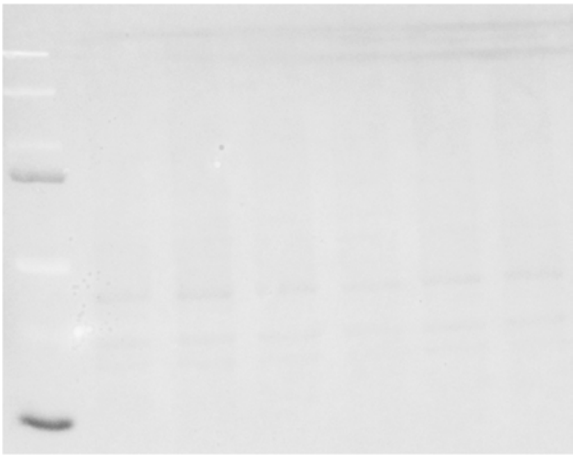


Figure 11. Post-transfer blot stain-free image captured as image C on the ChemiDoc MP system. Image C was used as the loading control.

After the transfer, the procedure in Table 5 was followed.

Table 5. Antibody, blocking treatments and washes.

Process	Method
Wash	The blot was washed 3X for 10mins on a sunflower mini shaker with 1X Tris-Buffered Saline (TBS) (Bio-Rad, Hercules, CA) wash buffer (Appendix 6).
Blocking	20ml of 5% Bovine Serum Albumin (BSA) (Roche Diagnostics, Basel, Switzerland) blocking buffer was added to the blot (Appendix 7), and left on shaker for 2 hours.
Primary Antibody	After calibration, the suitable Primary Anti-GFAP Antibody (Abcam, Cambridge, UK) solution was prepared at a dilution of 1:714 (Appendix 8), added to the blot and incubated at 4°C overnight.
Wash	The antibody solution was removed and the blot was washed 3X for 10mins on a shaker with TBS.
Secondary Antibody	After calibration, the suitable Antibody (Abcam, Cambridge, UK) solution was prepared at a dilution of 1:11000 (Appendix 9), added to the blot and incubated for an hour on a shaker.
Wash	The blot was washed 3X for 10mins on a shaker with TBS.

The Clarity Western ECL Substrate includes Clarity western peroxide reagent and Clarity western lumino/enhancer reagent (Bio-Rad, Hercules, CA). 6ml of each reagent was mixed and added to the blot. The blot was left to soak in the reagents for 5mins and then the chemiluminescent blot was captured using the ChemiDoc MP system.

2.4.3 Loading control and protein normalization

The normalization factor and normalized volume were performed automatically by the ChemiDoc MP software using the chemiluminescent and the post-transfer stain-free blot images. The target protein band intensity values were adjusted for any variations in the protein load, allowing for accurate comparison of target protein among the samples.

2.5 Release of IL-6 and TNF-α by astrocytes

ELISA assays were used to assess whether U87 cells release IL-6 and TNF-α into the medium upon exposure to LPS or Tat.

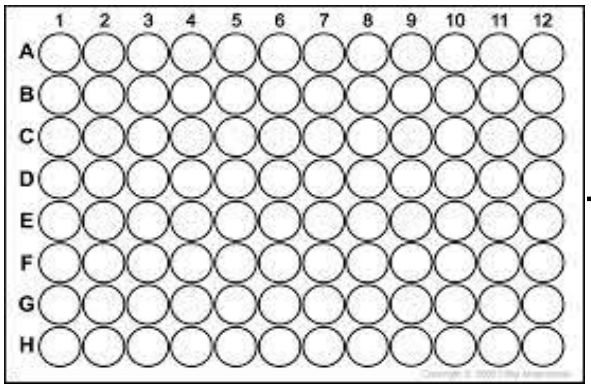
2.5.1 TNF-α detection

U87 cells were seeded onto a 24-well tissue culture plate (1 x 10^6 cells per well with 500µl medium). 2 hours prior to LPS or HIV-1 B Tat exposure, medium from each well was discarded and serum free medium was added. 2 hours later, U87 cells were treated as in Figure 8. Following a 1 hour incubation period (Chen e*t al.,* 1997), the medium from each sample was pooled, spun down and the supernatant was processed according to Human TNF-α ELISA Max Deluxe package inserts (BioLegend, San Diego, CA), protocol summarized in Table 6.

Table 6. ELISA protocol

Procedure	Method
• All the reagents were brought to room temperature before use. • Plates were washed in an Immunowash Microplate Washer (Bio-Rad, Hercules, CA). • Plates were shaken on a Rocker Shaker PMR 100 (Grant Instruments, Cambridge, UK) at 200rpm.	
Coating Plates	The 5X Coating Buffer was diluted (Appendix 10)
	The Capture Antibody was diluted with 1X coating buffer (Appendix 11)
	The plate was coated with 100µl of Capture Antibody and incubated for 18 hours at 4 °C
Wash	The plate was washed 4X with 300µl wash buffer (Appendix 12).
Blocking	The 5X Assay Diluent was diluted (Appendix 13) and 200µl of 1X Diluent Assay was added to each well of the plate.
	The plate was incubated for 1 hour at room temperature on a shaker.
Wash	The plate was washed 4X with 300µl wash buffer
	Six 2-fold dilutions of standard and samples were carried out (Appendix14)

Standards and Samples	100µl of standards and samples were added to the appropriate wells (Figure 12) and incubated for 2 hours at room temperature on a shaker.
Wash	The plate was washed 4X with 300µl wash buffer
Detection Antibody	The Detection Antibody was diluted (Appendix 15) and 100µl was added to each well
	The plate was incubated at room temperature for 1 hour on a shaker
Wash	The plate was washed 4X with 300µl wash buffer
Avidin-HRP	Avidin-HRP was diluted (Appendix 16) and 100µl was added to each well
	The plate was incubated at room temperature for 30mins on a shaker
Wash	Washed plate 5X with 300µl wash buffer, plate washer
TMB Substrate	Fresh Substrate was prepared (Appendix 17) and 100µl was added to each well
	The plate was incubated at room temperature, in the dark for 15mins
Stop Reaction	100µl 2N H_2SO_4 (Sigma, Missouri, US) Stop Solution was added to each well



Blank	A1-A7
Standard (Included in kit)	1B-1H, 2B-2H
Negative control (Cells only)	3B-3H, 4B-4H, 5B-5H
Positive control (100ng/ml LPS)	6B-6H, 7B-7H, 8B-8H
Experiment (10 µg/ml HIV-1 Tat)	9B-9H, 10B-10H, 11B-11H

Figure 12. A 96-well tissue culture plate containing standards and U87 cell culture medium for ELISA analysis.

After the reaction was stopped by the addition of the stop solution the absorbance values were read within 30mins at 450nm, using the BioTek plate reader and Gen5 2.0 software (BioTek, Winooski, US). Gen5 2.0 software generated the optical density values from which a standard curve was formulated to calculate the concentrations of TNF-α in the samples.

2.5.2 IL-6 detection

U87 cell lines were seeded onto a 24-well tissue culture plate (1 x 10^6 cells per well with 500µl medium). 2 hours prior to LPS or Tat exposure, medium from each well was discarded and serum free medium was added. 2 hours later, U87 cells were treated as in Figure 8. Following a 1 hour incubation period (Nath *et al.*, 1999), the medium from each sample was collected and the supernatant was processed according to Human IL-6 ELISA Max Deluxe package inserts (BioLegend, San Diego, CA), protocol summarized in Table 6.

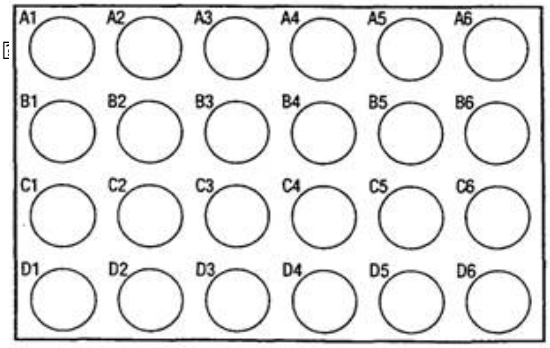
Viewing and plate analysis was conducted in a similar manner as to that of TNF-α determinations.

2.6 IL6 and TNF-α induced apoptosis in neurons

Flow cytometry including Annexin V and caspase-3 assays were used to assess whether exogenous IL6 and/or TNF-α could induce apoptosis in neurons.

2.6.1 Fluorescein isothiocyanate (FITC) Annexin V assay

CRL cells lines were seeded onto 24-well tissue culture plates (1 x 10^6 cells per well with 500µl medium). 2 hours prior to IL-6 and/or TNF-α (BioVision, Milpitas, CA) (Appendix 21) exposure, medium from each well was discarded and 500µl serum free medium was added. 2 hours later, cells were treated as in Figure 13. 10µM Camptothecin (Sigma, Missouri, U.S) (Appendix 18) was used as a positive control for apoptosis. The positive control was created over 8 wells for the purpose of electronic compensation. Cells were incubated overnight.



A1-A5 = Control: untreated cells only
A6-B6 = Positive control: 10µM Camptothecin
C1-C3 = 100ng/ml Recombinant TNF-α
C4-C6 = 100ng/ml Recombinant IL-6
D1-D3 = 100ng/ml Recombinant IL-6 and TNF-α

Figure 13. A 24-well tissue culture plate seeded with CRL cells for FITC Annexin V analysis.

2.6.1.1 Cell staining with FITC Annexin V

Apoptosis induction by cytokines IL-6 and TNF-α in CRL cells were evaluated using the FITC Annexin V Apoptosis Detection Kit (BioLegend, San Diego, CA). Following an overnight incubation period, CRL cells were detached using trypsin and washed twice with cold Cell Staining Buffer (BioLegend, San Diego, CA). Cells were then resuspended in 100µl Annexin V binding buffer (included in the kit), transferred to FACS tubes (Becton Dickenson, San Jose, CA) and stained according to the package insert, summarized in Table 7.

Table 7. Staining cells using FITC Annexin V kit.

Cells	Stain	No. of samples
Control: Untreated cells	Unstained	2
	5 µl FITC Annexin + 10 µl Propidium Iodide (PI)	3
Positive Control : 10µM Camptothecin	5 µl FITC Annexin + 10 µl PI	3
	5 µl FITC Annexin	2
	10 µl PI	2
100ng/ml Exogenous TNF-α	5 µl FITC Annexin + 10 µl PI	3
100ng/ml Exogenous IL-6	5 µl FITC Annexin + 10 µl PI	3
100ng/ml Exogenous IL-6 and TNF-α	5 µl FITC Annexin + 10 µl PI	3

Cells were gently vortexed and incubated for 15 minutes in the dark, at room temperature.

2.6.1.2 Flow cytometric data acquisition and data analysis

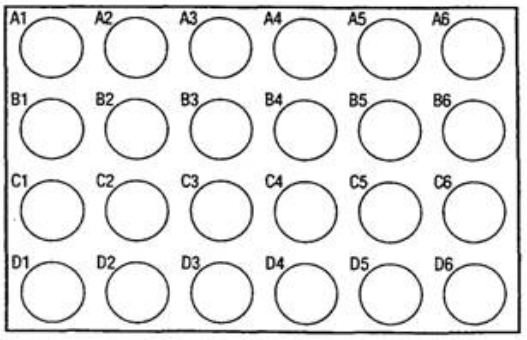
After incubation, 400µl Annexin V Binding Buffer was added to each sample. Cells were then analyzed on a LSR Fortessa SORP Flow Cytometer (Becton Dickenson, San Jose, CA) using a 488nm excitation and 530/30nm band pass filter for fluorochrome FITC detection, and 561nm laser excitation and band pass filter 610/20 for fluorochrome Propidium Iodide (PI) detection. Electronic compensation of the instrument was performed using single stain samples to exclude overlapping of the two emission spectra.

Acquisition of data was followed by analysis which was performed using FACS Diva v7.0 (Becton Dickenson, San Jose, CA). For each concentration, 50 000 cells were analyzed for the detection of apoptosis.

2.6.2 Phycoerythrin (PE) active caspase-3 assay

CRL cells were seeded onto a 24-well tissue culture plate (1 x 10^6 cells per well with 500µl medium). 2 hours prior to IL-6 and/or TNF-α exposure, medium from each well of cells were discarded and 500µl

serum free medium was added. 2 hours later, cells were treated as in Figure 14. 10µM Camptothecin (Appendix 18), was used as a positive control for apoptosis. Cells were incubated overnight.



A1-A5 = Control: untreated cells only
B1-B3 = Positive control: 10µM Camptothecin
C1-C3 = 100ng/ml Recombinant TNF-α
C4-C6 = 100ng/ml Recombinant IL-6
D1-D3 = 100ng/ml Recombinant IL-6 and TNF-α

Figure 14. A 24-well tissue culture plate seeded with CRL cells for caspase-3 analysis.

2.6.2.1 Cell staining for caspase-3 detection

Apoptosis induction by cytokines IL-6 and TNF-α in CRL cells was evaluated using the pycoerythrin (PE) Active Caspase-3 Apoptosis Kit (Becton Dickenson, San Jose, CA USA). Following 4 hours of incubation cells were detached using trypsin and stained according to manufacturer's procedure. A summarized protocol is shown in Table 8.

Table 8. Staining cells using PE active Caspase-3 apoptosis kit.

Process	Method
Wash 2X (PBS)	After detachment, cold PBS was added to cells and cells were centrifuged at 1500rpm for 5mins.
	The cells were resuspended in 500µl BD Cytofix/Cytoperm and incubated on ice for 20mins.
Wash 2X (BD Perm/Wash Buffer)	The cells were then centrifuged at 1500rpm for 5mins
	The supernatant was removed and the pellet was resuspended in 0.5ml 1X Perm/Wash Buffer (Appendix 19).
	The cells were centrifuged again at 1500rpm for 5mins

Staining (PE Anti-Active caspase-3 antibody)	The cells were then resuspended in 100µl BD Perm/Wash Buffer
	20µl PE rabbit anti-active caspase-3 antibody was added to each sample excluding the control group and incubated for 30mins at room temperature
Wash (BD Perm/Wash Buffer)	1ml BD Perm/Wash Buffer was added to each test
	The cells were centrifuged at 1500rpm for 5mins
	The cells were resuspended in 500µl BD Perm/Wash Buffer

2.6.2.2 Flow cytometric data acquisition and data analysis

Cells were then analyzed on a LSR Fortessa SORP Flow Cytometer using 561nm filter excitation and 610/20nm band pass filter for fluorochrome PE-A detection. Electronic compensation of the instrument was performed using single stain samples to exclude overlapping of the two emission spectra.

Acquisition of data was followed by analysis which was performed using FACS Diva v7.0. For each concentration, 50 000 cells were analyzed for the detection of apoptosis.

2.7 Induction of apoptosis in neurons by Tat stimulated astrocytes

Flow cytometry including Annexin V and caspase-3 assays were used to assess whether the medium of U87 cells treated with Tat induced apoptosis in CRL cells.

2.7.1 Fluorescein isothiocyanate (FITC) Annexin V assay

U87 and CRL cells were grown to confluence and seeded onto 2 separate 24-well tissue culture plates (1 x 10^6 cells per well with 500µl medium) (Figure 15 and 16). 2 hours prior to HIV-1 B Tat or LPS exposure, medium from each well of U87 cells were discarded and 250µl serum free medium was added. 2 hours later, U87 cells were treated as in Figure 15. Following a 1 hour incubation period (similar to the incubation period mentioned in 2.5.1 and 2.5.2) the U87 cell medium was collected and centrifuged at 1500rpm for 5mins. CRL cell medium was discarded. Medium from U87 cells were pooled and added to the CRL cell lines (Figure 16). 10µM Camptothecin (Appendix 18) was used as a positive control for apoptosis (Figure 16). The positive control was created over 7 wells for the purpose of electronic compensation. CRL cells were incubated overnight.

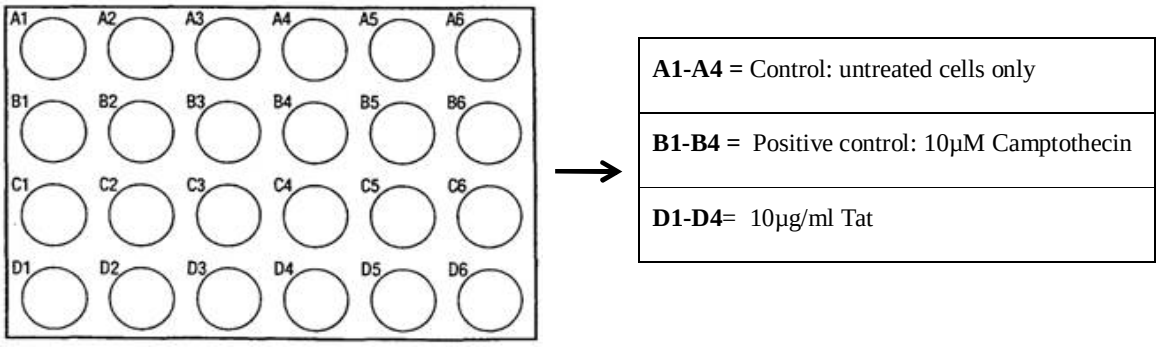
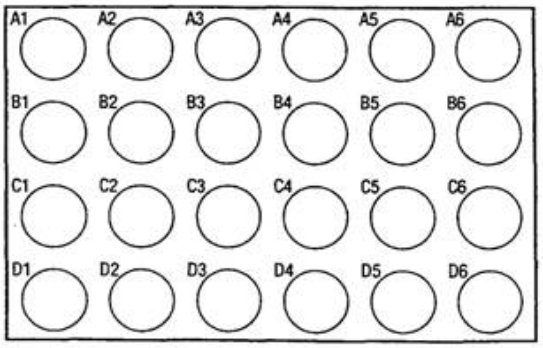


Figure 15. A 24-well tissue culture plate seeded with U87 cells exposed to HIV-1 Tat



A1-A5 = Control: untreated cells only
B1-B6, C1= Positive control: 10µM Camptothecin
D1-D3 = Medium from 10µg/ml Tat exposed U87 cells

Figure 16. A 24-well tissue culture plate seeded with CRL cell lines for FITC Annexin V analysis.

2.7.1.1 Cell staining with FITC Annexin V

Apoptosis induction in CRL cell lines was evaluated using the FITC Annexin V Apoptosis Detection Kit. Following overnight incubation CRL cells were detached using Trypsin EDTA and washed twice with cold Cell Staining Buffer. Cells were then resuspended in 100µl Annexin V binding buffer, transferred to FACS tubes and stained according to the package insert, summarized in Table 9.

Table 9. Staining cells using FITC Annexin V kit.

Cells	Stain	No. of samples
Control: Untreated cells	Unstained	2
	5 µl FITC Annexin + 10 µl PI	3
Positive Control : 10µM Camptothecin	5 µl FITC Annexin + 10 µl PI	3
	5 µl FITC Annexin	2
	10 µl PI	2
10µg/ml Tat	5 µl FITC Annexin + 10 µl PI	3

Cells were gently vortexed and incubated for 15mins in the dark, at room temperature.

Flow cytometric data acquisition and analysis were conducted in a similar manner as to that described for the FITC Annexin V assay in the exogenous IL-6 and TNF-α exposure.

2.7.2 Phycoerithrin (PE) active caspase-3 assay

U87 and CRL cells were grown to confluence and seeded onto 2 separate 24-well tissue culture plates (1 x 10^6 cells per well with 500µl medium) (Figure 15 and 17). 2 hours prior to Tat or LPS exposure, medium from each well of U87 cells were discarded and 250µl serum free medium was added. 2 hours later, U87 cells were treated as in Figure 16. Following a 4 hour incubation period the U87 cell medium was collected and centrifuged at 1500rpm for 5mins. CRL cell medium was discarded and U87 medium was added to the CRL cell lines (Figure 16). 10µM Camptothecin was used as a positive control for apoptosis (Figure 17). Cells were incubated overnight.

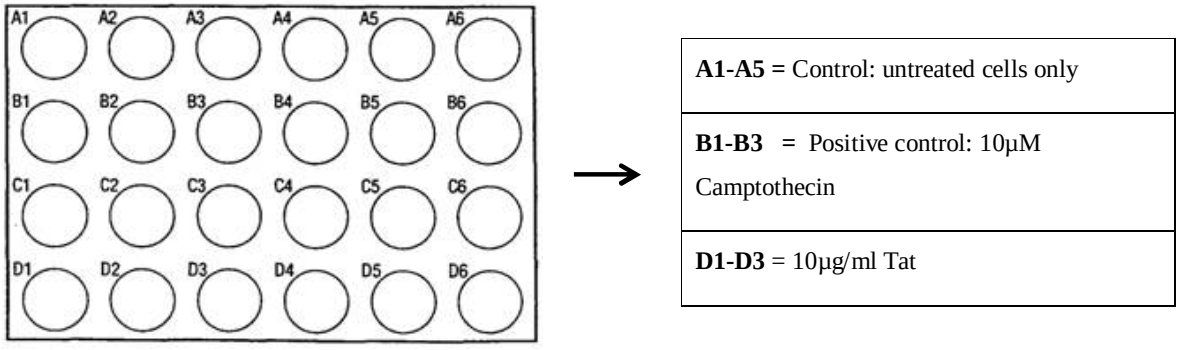


Figure 17. A 24-well tissue culture plate seeded with CRL cell lines for caspase-3 analysis.

2.7.2.1 Cell staining for caspase-3 detection

Apoptosis induction by cytokines IL-6 and TNF-α in CRL cell lines was evaluated using the PE Active Caspase-3 apoptosis Kit. Following the overnight incubation period cells were detached using Trypsin and stained according to the manufacturer's protocol. A summarized of the protocol is shown in Table 8.

Flow cytometric data acquisition and analysis were conducted in a similar manner as to that described for the PE active caspase-3 assay in the exogenous IL-6 and TNF-α exposure.

2.8 Statistical analyses

All data generated was analyzed using GraphPad Prism 6 software. ANOVA followed by Bonferroni's multiple comparison test was used to identify significant differences between groups. A p value of <0.05 was considered significant. Results are expressed as mean ± SEM as indicated.

CHAPTER 3

RESULTS

3.1 Commercially procured HIV-1 Tat is biologically active

3.1.1 Microscopic analysis

HIV-1 B Tat protein viability testing was used to determine the viability of commercially procured Tat. The BioPORTER reagent assay was used to transfect CEM-GFP cell lines with Tat protein. CEM cells contain the GFP gene under the control of HIV-1 subtype-B long terminal repeat (LTR). Biologically active Tat binds to a trans-activating region within the LTR and this binding leads to the activation of the GFP gene. Cells that were exposed to Tat protein will therefore only fluoresce green if Tat is indeed biologically active. The following images were captured using fluorescent microscopy (Zeiss Fluorescent Microscope, LSM 880) at a 10X magnification. Figure 18 A and B, show the negative control group of untreated CEM-GFP cells where no fluorescence was evidenced. Figure 19 A and B show the positive control group where the cells were exposed to 1ng/ml FITC Antibody. Here a large population of the cells fluoresced green. In Figure 20 A and B, the experimental group of cells transfected with 10µg/ml Tat protein, showed similar visuals as compared to the positive control (Figure 19), i.e. cells fluoresced green.

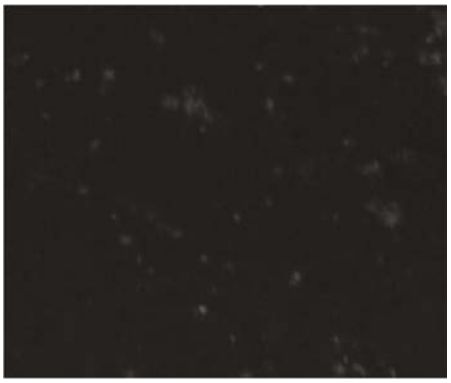
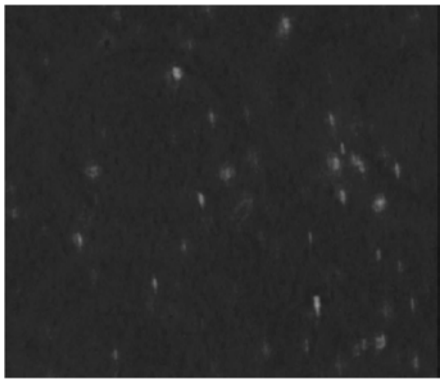
A  **B** 

Figure 18. A and B showing CEM-GFP cells, containing a stably integrated GFP gene under the control of HIV-1 subtype- B LTR, the control groups included untreated CEM-GFP cells. Cells were grown in 24-well plates and incubated overnight. Images were captured using fluorescent microscopy (Zeiss Fluorescent Microscope, LSM 880) at a 10X magnification.

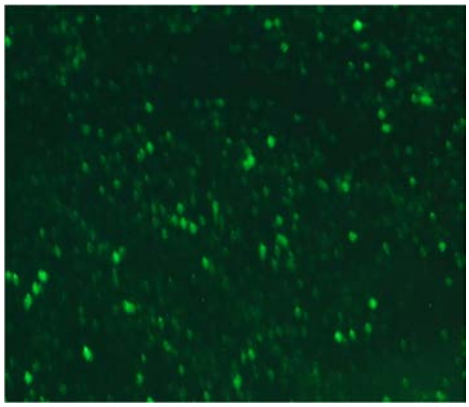
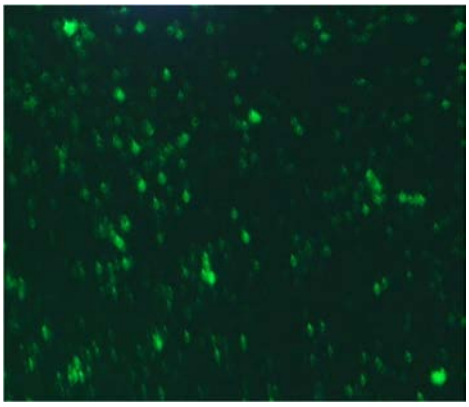
A  B 

Figure 19. A and B showing CEM-GFP cells, containing a stably integrated GFP gene under the control of HIV-1 subtype- B LTR, the positive control groups were exposed to FITC-Antibody. Cells were grown in 24-well plates, exposed to 1ng/ml FITC-Antibody and incubated overnight. Images were captured using fluorescent microscopy (Zeiss Fluorescent Microscope, LSM 880) at a 10X magnification.

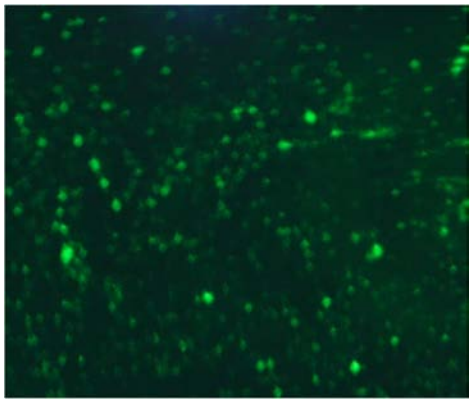
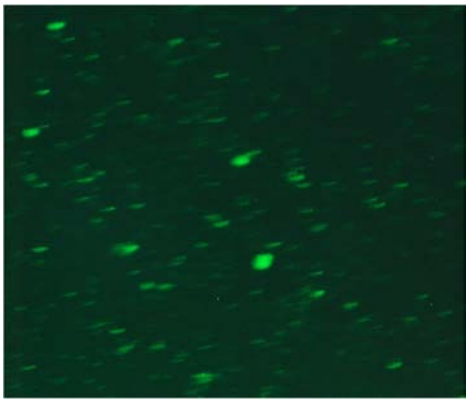
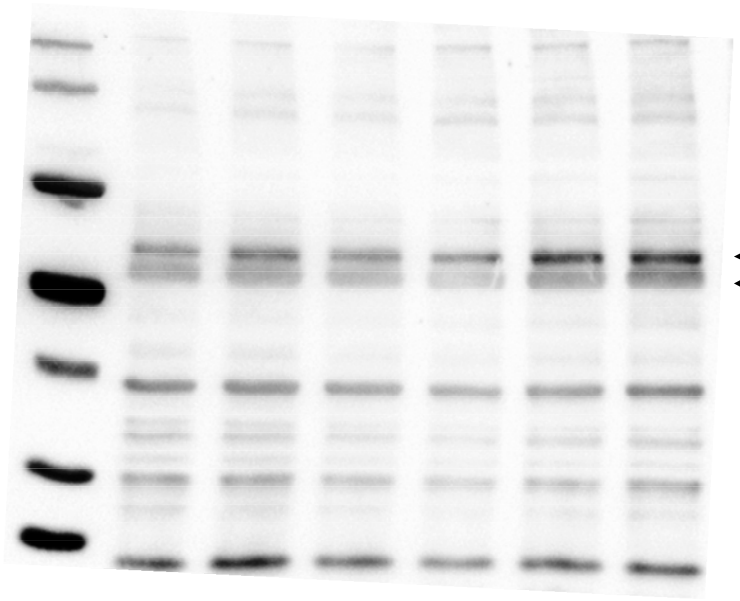
A  B 

Figure 20. A and B showing CEM-GFP cells, containing a stably integrated GFP gene under the control of HIV-1 subtype- B LTR, these groups were transfected with HIV-1 B Tat protein. Cells were grown in 24-well plates, exposed to 10µg/ml Tat and incubated overnight. Images were captured using fluorescent microscopy (Zeiss Fluorescent Microscope, LSM 880) at a 10X magnification.

3.2 HIV-1 Tat stimulates astrocyte activation

U87 cell lines were exposed to either 1µg/ml LPS or 10µg/ml HIV-1 B Tat, and the expression of glial fibrillary acidic protein (GFAP) was subsequently assessed as an indicator of astrocyte activation. Western blot analysis indicated higher expression levels of GFAP in LPS treated cells when compared to negative controls, while cells exposed to Tat protein had the highest expression levels (Figure 21 A). Band volumes and normalization calculations were generated via the ChemiDoc MP software. U87 cells exposed to LPS showed significantly greater expression levels than negative controls, with cells exposed to Tat protein showing significantly higher expression levels than both LPS treated and control cells (Figure 21 B).

Molecular Marker	LPS 1µg/ml	Negative Control	Tat 10µg/ml

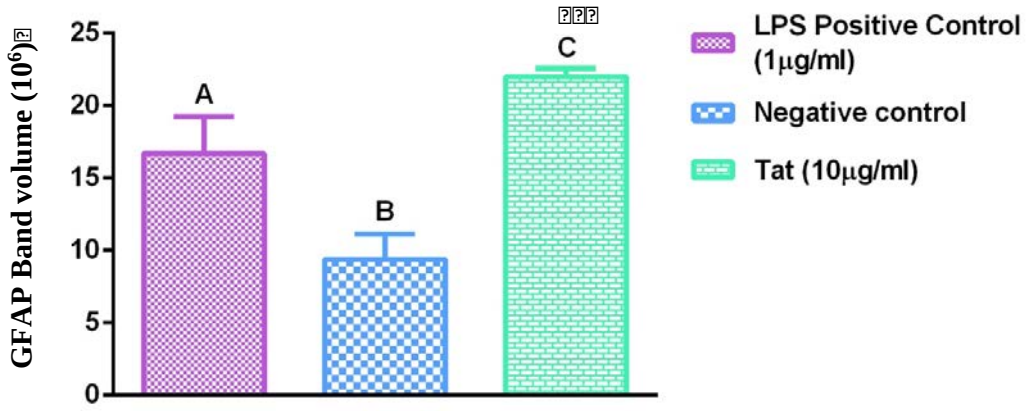


GFAP 50kDa

Degraded GFAP 45 kDa

A

GFAP release induced by HIV-1 Tat Protein



B

Figure 21. Western blot analysis of GFAP in U87 cell lines was used to determine whether HIV-1 B Tat protein causes astrocyte activation. The band intensities were normalized using stain-free technology and the ChemiDoc MP system. Precision Plus Protein Dual Xtra standard was used as the molecular marker. U87 cells were grown in 24-well plates and exposed to 10µg/ml HIV-1 B Tat or 1µg/ml LPS and western blot analysis was performed as described in section 2.4. GFAP protein can be seen at 50kDA and degraded GFAP at 45kDa. **(B)** GFAP band intensities (band volumes determined by ChemiDoc MP System) were expressed as mean ± SD of the experiment, $p<0.05$ were significantly different from other treatment groups, with *** being most significant and * being least significant (A vs C ns, A vs B ns and B vs C **). ANOVA followed by the Bonferroni's multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

3.3 IL-6 and TNF-α release by HIV-1 Tat exposed astrocytes

Enzyme-linked immunosorbent assays (ELISAs) were used to determine the release of IL-6 and TNF-α into the cell culture medium from Tat or LPS stimulated U87 cells.

3.3.1 TNF- α release

U87 cells were exposed to LPS or Tat, incubated for 2 hours and processed according to the manufacturer's instructions of the Human TNF-α ELISA Max Deluxe ELISA kit. Figure 22 represents the standard that was generated to determine the unknown values of TNF-α release per experimental condition. There was no significant difference in TNF-α levels in the culture medium of U87 cells exposed to LPS when compared to controls (1.14 ± 0.44pg/ml vs 0.40 ± 0.44pg/ml). However the culture medium of cells exposed to Tat had a significantly higher concentration of TNF-α when compared to both controls and LPS treated cells (2.52 ± 0.44pg/ml vs 0.40 ± 0.44pg/ml and 2.52 ± 0.44pg/ml vs 1.14 ± 0.44pg/ml) respectively (Figure 23).

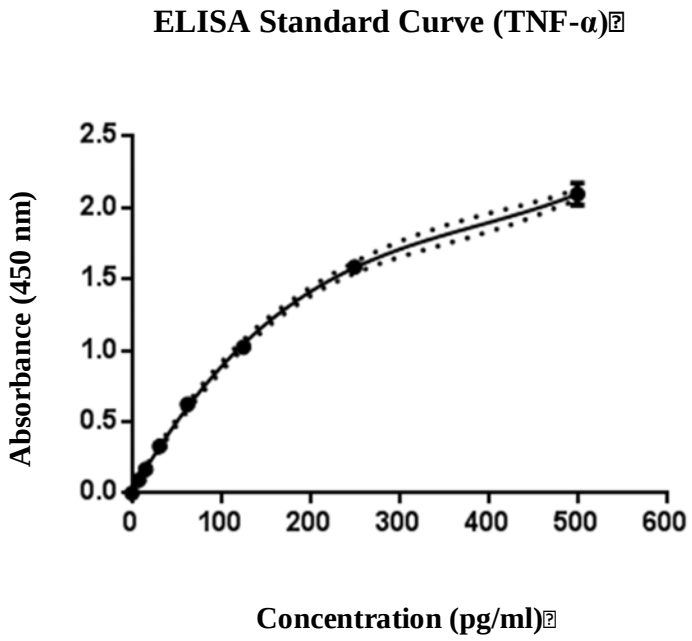


Figure 22. Standard curve for TNF-α. Curve was generated using Graph Pad Prism 6, with an R square value of 0.99.

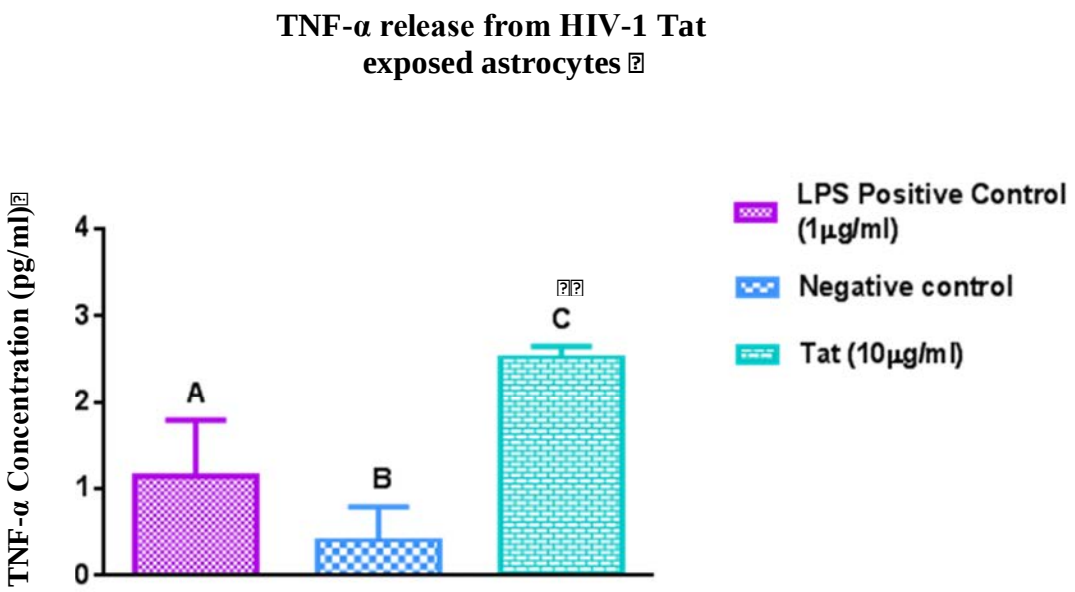


Figure 23. ELISA was used to determine the release of TNF-α from U87 cells exposed to HIV-1 B Tat protein or lipopolysaccharide (LPS). U87 cells were grown in 24-well plates and exposed to 10µg/ml Tat or 1µg/ml LPS. Following 1 hour of incubation, cell supernatant was collected and the ELISA assay was performed according to section 2.5.1. Results were expressed as mean ± SD of the experiment, $p<0.05$ were significantly different from other treatment groups, with *** being most significant and * being least significant (A vs C ns, A vs B ns and B vs C *). ANOVA followed by the Bonferroni`s multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

3.3.2 IL-6 release

The same culture medium used to determine the levels of TNF-α above, was used to measure the content of IL-6. Figure 24 is a representative standard curve of the assay which was used to determine the concentration of IL-6 in each sample. Similar to TNF-α, there was no significant difference in IL-6 levels in the culture medium of LPS exposed and control cells (Figure 25). However the culture medium of Tat treated cells had significantly higher IL-6 levels than both LPS and control cells (Figure 25). The levels of IL-6 per group were 11.68 ± 1.05pg/ml for the Tat (10µg/ml) stimulated cells, 5.72 ± 1.05pg/ml for the negative control and 8.08 ± 1.05pg/ml for the LPS (1µg/ml) stimulated cells, respectively.

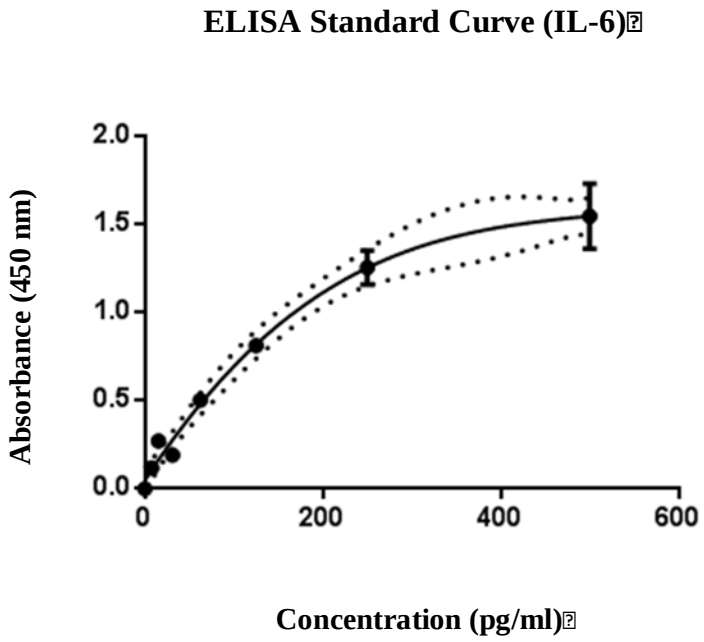


Figure 24. Standard curve for IL-6. Curve was generated using Graph Pad Prism 6, with an R square value of 0.98.

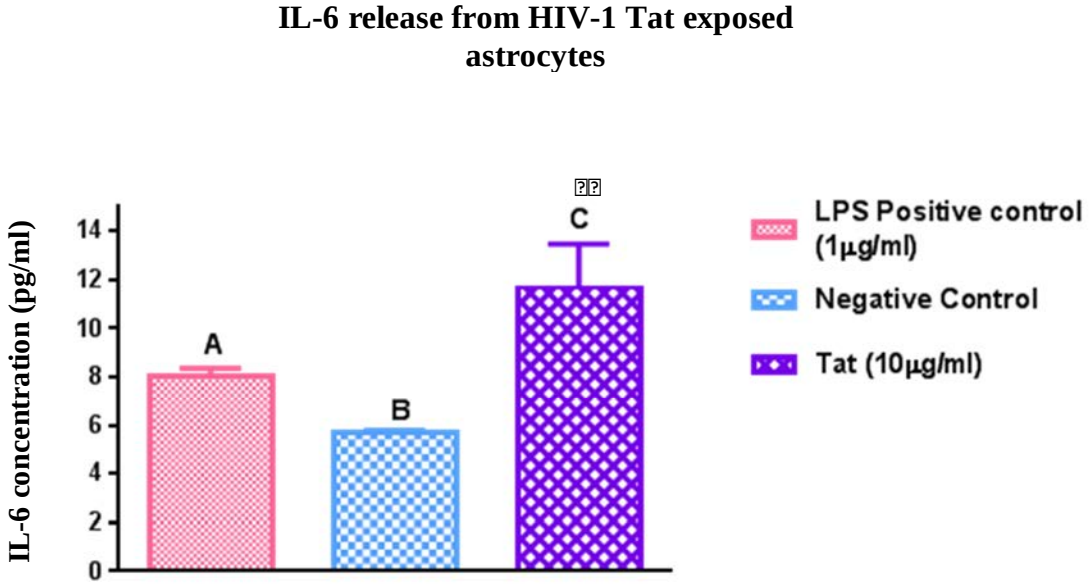


Figure 25. ELISA was used to determine the release of IL-6 from U87 cells exposed to HIV-1 B Tat or lipopolysaccharide (LPS). U87 cells were grown in 24-well plates and exposed to 10µg/ml Tat or 1µg/ml LPS. Untreated U87 cells served as the negative control. Following 1 hour of incubation, cell supernatant was collected and the ELISA assay was performed according to section 2.5.2. Results were expressed as mean ± SD of the experiment. A value of $p<0.05$ was significantly different from other treatment groups, with *** being most significant and * being least significant (A vs C ns, A vs B ns and B vs C *). ANOVA followed by the Bonferroni`s multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

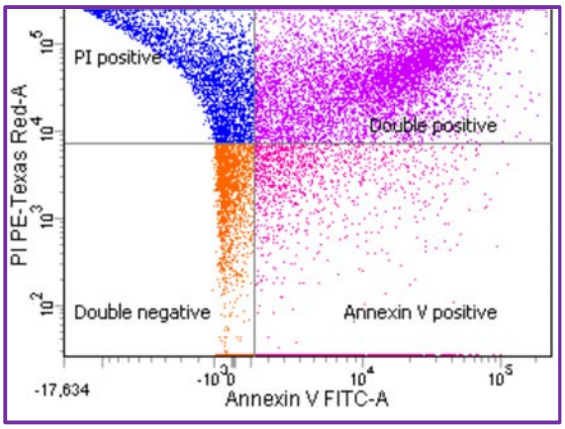
3.4 IL6 and TNF-α induces apoptosis in neurons

To assess whether IL-6 and TNF-α were toxic to neuronal cells, we exposed CRL cells to these cytokines and determined the presence of apoptotic cell death. Annexin V and caspase-3 expression levels were measured by flow cytometry methods in order to gauge to the extent of apoptosis.

3.4.1 Apoptosis detection using the FITC Annexin V assay

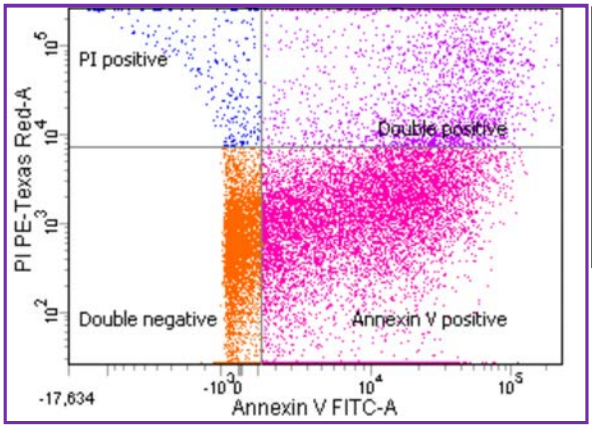
CRL cell lines were exposed to commercially obtained recombinant TNF-α (100ng/ml), IL-6 (100ng/ml) or a combination of TNF-α and IL-6. The cells were then incubated with Annexin V, a phospholipid binding protein conjugated to the FITC fluorochrome and subjected to flow cytometry. 10µM camptothecin was used as a positive control.

Figures 26, 27, 28, 29 and 30 are representative flow cytometric scatter graphs of negative control cells (untreated CRL cells, Figure 26), the positive control (Figure 27) and the treated CRL cells (Figure 28: 100ng/ml TNF-α; Figure 29: 100ng/ml IL-6; Figure 30: TNF-α + IL-6). The negative control cells showed an apoptotic percentage of 8.1% (Figure 26), the positive control 38.2% (Figure 27), cells exposed to TNF-α 27.7% (Figure 28), cells exposed to IL-6 14.0% (Figure 29) and cells exposed to both TNF-α and IL-6 14.8% (Figure 30). These percentages were the average of 3 experiments. All treated cells including the positive control showed significantly higher levels of Annexin V staining when compared to untreated CRL control cells (Figure 31), with camptothecin giving the highest level followed closely by TNF-α. Although IL-6 and the combination of TNF-α and IL-6 also yielded significantly high levels of apoptosis, these were not to the same level as TNF-α (Figure 28). All tests were done in triplicate and each Figure represents one such test.



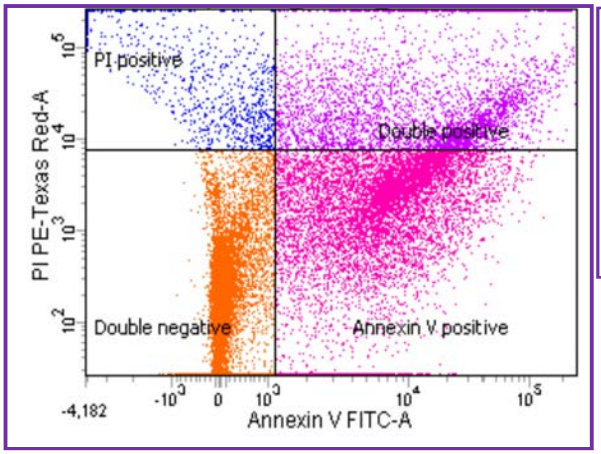
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	43,015	86.0	86.0
Singlet	21,083	49.0	42.2
PI positive	4,221	20.0	8.4
Double positive	9,702	46.0	19.4
Double negative	5,119	24.3	10.2
Annexin V positive	2,041	9.7	4.1

Figure 26. Flow cytometric analysis illustrating the negative control population of untreated CRL cells.



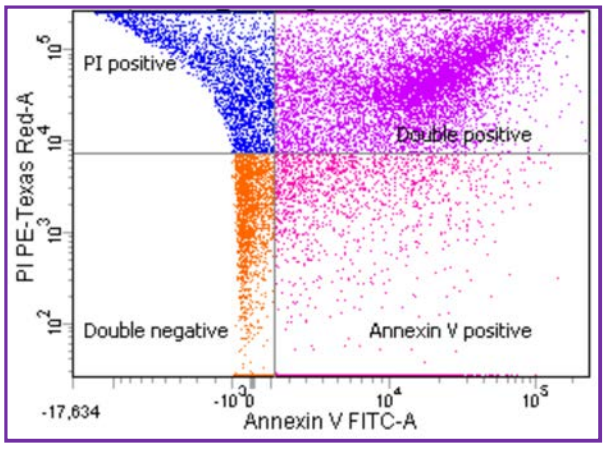
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	43,333	86.7	86.7
Singlet	21,053	48.6	42.1
PI positive	298	1.4	0.6
Double positive	2,118	10.1	4.2
Double negative	8,251	39.2	16.5
Annexin V positive	10,386	49.3	20.8

Figure 27. Flow cytometric analysis illustrating the positive control (10µM camptothecin) population of CRL cells.



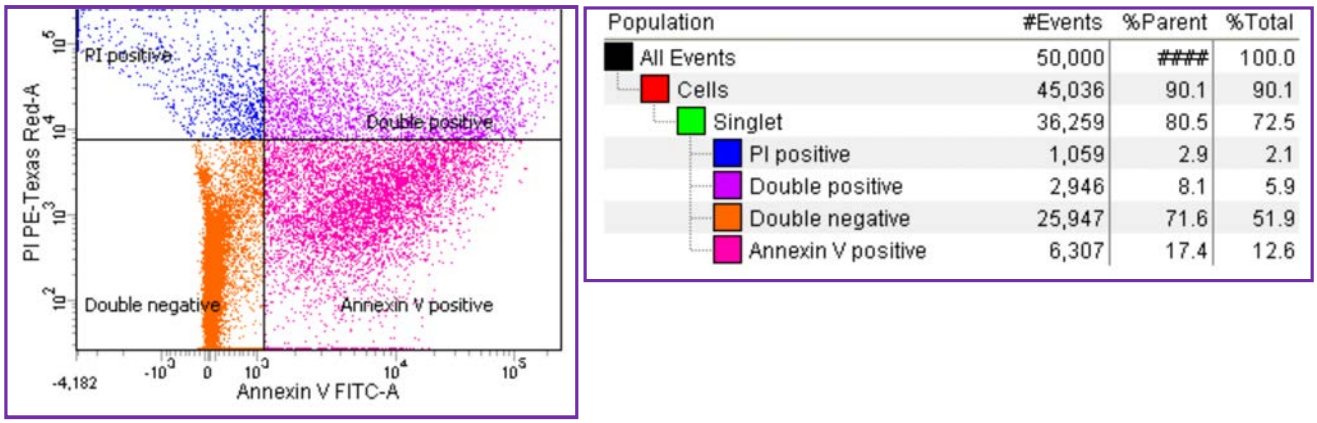
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	39,190	78.4	78.4
Singlet	30,269	77.2	60.5
PI positive	996	3.3	2.0
Double positive	3,915	12.9	7.8
Double negative	16,838	55.6	33.7
Annexin V positive	8,520	28.1	17.0

Figure 28. Flow cytometric analysis illustrating CRL cells exposed to recombinant 100ng/ml TNF-α.



Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	42,212	84.4	84.4
Singlet	20,929	49.6	41.9
PI positive	3,284	15.7	6.6
Double positive	9,925	47.4	19.8
Double negative	5,466	26.1	10.9
Annexin V positive	2,254	10.8	4.5

Figure 29. Flow cytometric analysis illustrating CRL cells exposed to 100ng/ml recombinant IL-6.



Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	45,036	90.1	90.1
Singlet	36,259	80.5	72.5
PI positive	1,059	2.9	2.1
Double positive	2,946	8.1	5.9
Double negative	25,947	71.6	51.9
Annexin V positive	6,307	17.4	12.6

Figure 30. Flow cytometric analysis illustrating CRL cells exposed to 100ng/ml recombinant TNF-α and IL-6.

3.4.1.1 Statistical Analysis

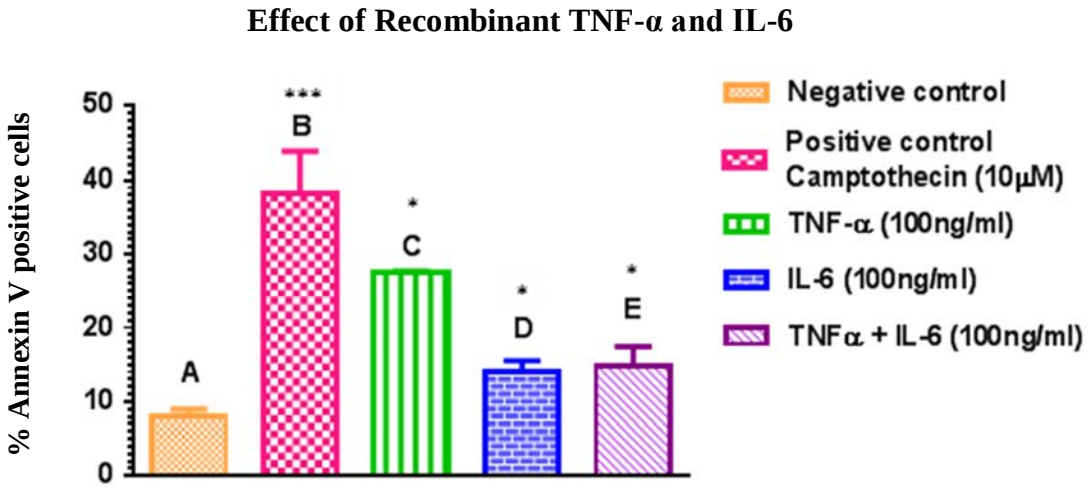
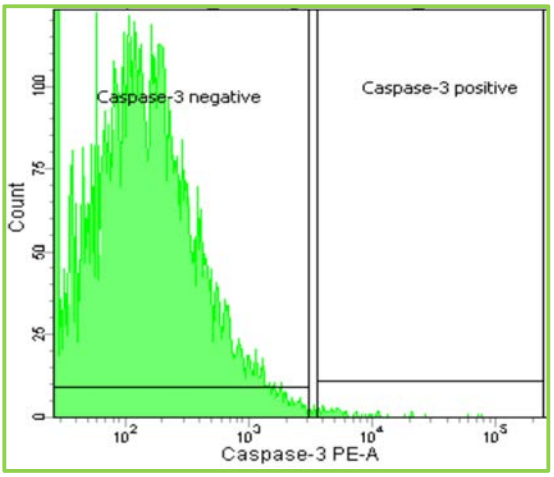


Figure 31. Annexin V, a phospholipid binding protein conjugated to the Fluorescein isothiocyanate (FITC) fluorochrome, and flow cytometry was used to determine the apoptotic inducing effect of recombinant TNF-α and IL-6 on CRL cells. Cells were grown in 24-well plates and exposed to 100ng/ml recombinant IL-6 or 100ng/ml of recombinant TNF-α, or a combination of recombinant IL-6 and TNF-α. The positive control included CRL cells exposed to 10μM camptothecin and untreated CRL cells were used as the negative control. Cultures were incubated overnight and analyzed using the LSR Fortessa SORP flow cytometer. Results were expressed as mean ± SEM of 3 experiments. A value of $p<0.05$ was considered to indicate significant differences between treatment groups, with *** being most significant and * being least significant (A vs B ***, A vs C *, A vs D *, A vs E *, B vs C ***, B vs D ***, B vs E **, C vs D ns and C vs E ns). ANOVA followed by the Bonferroni`s multiple comparison tests were done on the data. All statistical analysis was carried out using GraphPad Prism 6.

3.4.2 Apoptosis detection using caspase-3 assay

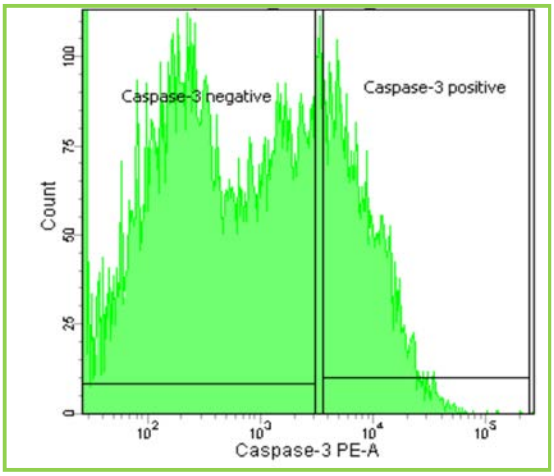
Similar to the Annexin V experiments, another batch of CRL cell lines were exposed to recombinant TNF-α, IL-6 or a combination of TNF- α and IL-6 in order to investigate the apoptotic effect of these cytokines on CRL cells using caspase-3 expression as indicator. An intracellular staining application using phycoerythrin (PE) rabbit anti-active caspase-3 antibody was used to determine caspase-3 expression on the flow cytometer. CRL cells were incubated with 100ng/ml TNF-α, 100ng/ml IL-6, or a combination of 100ng/ml TNF- α and 100ng/ml IL-6 and incubated overnight. A positive control of 10µM camptothecin was again used. Negative control (Figure 32) cells (untreated CRL cells) showed an average apoptotic percentage of 0.4%, positive control (10µM camptothecin) 17.1% (Figure 33), TNF-α treated cells 17% (Figure 34), IL-6 treated cells 8.1% (Figure 35) and, the combination of TNF-α and IL-6 treatments 10.9% (Figure 36). All tests were done in triplicate and each Figure represents one such test.

Statistical analysis of the flow cytometry data showed that all treatments induced a significantly high expression of caspase-3 when compared to the untreated control cells (Figure 37). Camptothecin and TNF-α induced the highest level of caspase-3 expression, with IL-6 and the combination of IL-6 and TNF-α resulting in caspase-3 levels that were nearly half the level of that of TNF-α (Figure 37).



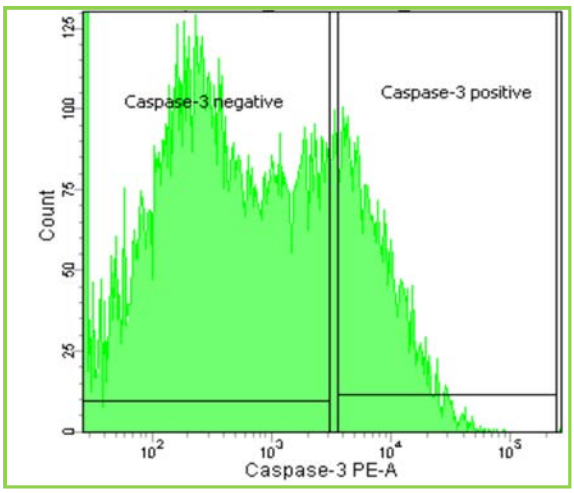
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	49,393	98.8	98.8
Singlet	10,760	21.8	21.5
Caspase-3 negative	10,698	99.4	21.4
Caspase-3 positive	54	0.5	0.1

Figure 32. Flow cytometric analysis illustrating the negative control population of untreated CRL cells.



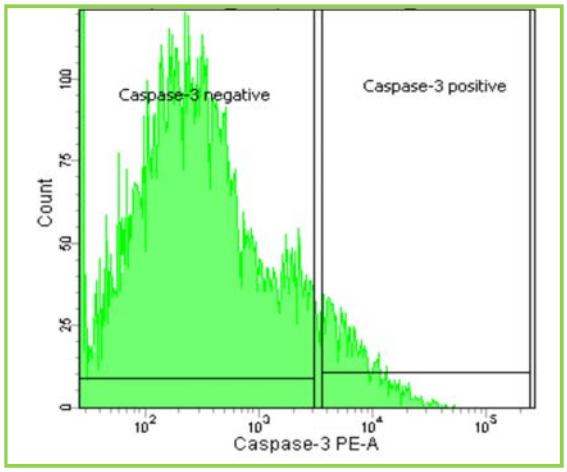
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	49,346	98.7	98.7
Singlet	15,402	31.2	30.8
Caspase-3 negative	11,894	77.2	23.8
Caspase-3 positive	3,082	20.0	6.2

Figure 33. Flow cytometric analysis illustrating the positive control population (10µM camptothecin) of CRL cells.



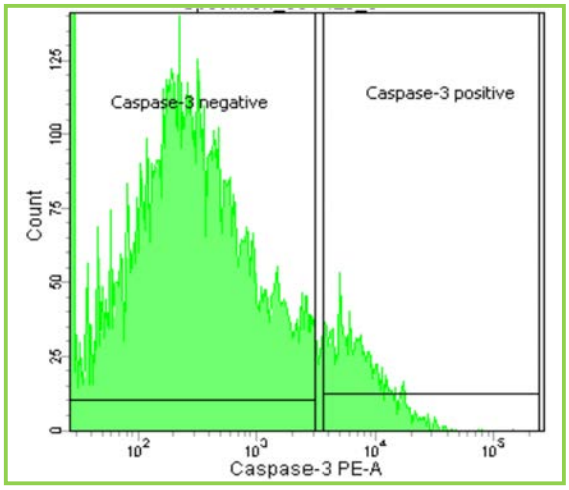
Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	49,237	98.5	98.5
Singlet	16,512	33.5	33.0
Caspase-3 negative	13,135	79.5	26.3
Caspase-3 positive	2,977	18.0	6.0

Figure 34. Flow cytometric analysis illustrating CRL cells exposed to 100ng/ml recombinant TNF-α.



Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	49,450	98.9	98.9
Singlet	12,320	24.9	24.6
Caspase-3 negative	11,348	92.1	22.7
Caspase-3 positive	850	6.9	1.7

Figure 35. Flow cytometric analysis illustrating CRL cells exposed to 100ng/ml recombinant IL-6.



Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
Cells	49,541	99.1	99.1
Singlet	13,098	26.4	26.2
Caspase-3 negative	11,794	90.0	23.6
Caspase-3 positive	1,174	9.0	2.3

Figure 36. Flow cytometric analysis illustrating CRL cell exposed to 100ng/ml recombinant TNF-α and IL-6.

3.4.2.1. Statistical Analysis

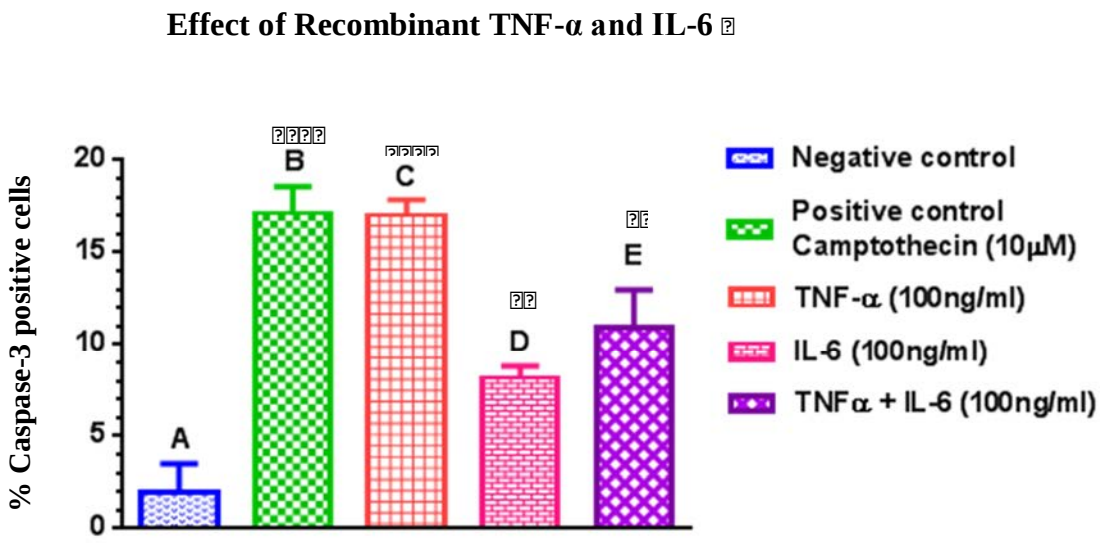


Figure 37. An intracellular staining application using phycoerythrin (PE) rabbit anti-active caspase-3 antibody, and flow cytometry to determine the apoptotic inducing effect of recombinant TNF-α and IL-6 on CRL cells. Cells were grown in 24 well plates and exposed to 100ng/ml recombinant IL-6 or TNF-α, or a combination of recombinant IL-6 and TNF-α. The positive control included CRL cells exposed to 10μM camptothecin and untreated CRL cells served as the negative control. Cultures were incubated overnight and analysis was done using the LSR Fortessa SORP flow cytometer. Results were expressed as mean ± SEM of the 3 experiments. A value of $p<0.05$ was significantly different from other treatment groups, with *** being most significant and * being least significant (A vs B ***, A vs C ***, A vs D *, A vs E *** B vs C *, B vs D * and B vs E *). ANOVA followed by the Bonferroni`s multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

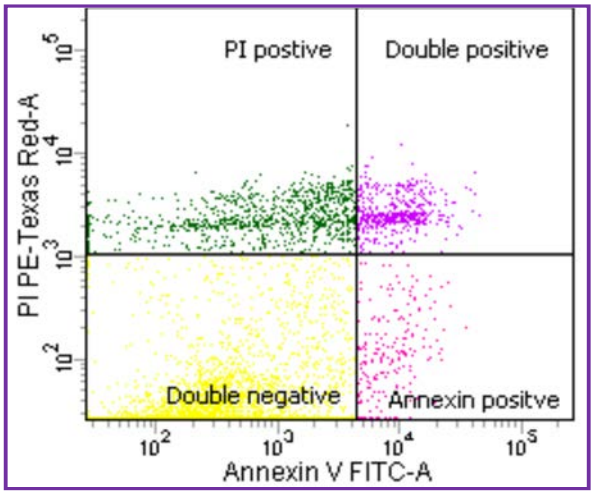
3.5 The culture medium of HIV-1 Tat exposed astrocytes induce apoptosis in neurons

The culture medium of astrocytes that were exposed to Tat were collected and added to the growth medium of the neuron culture. This was done to see if the culture medium of Tat-exposed astrocytes could induce apoptotic cell death of neurons.

3.5.1 Apoptosis detection using the FITC Annexin V assay

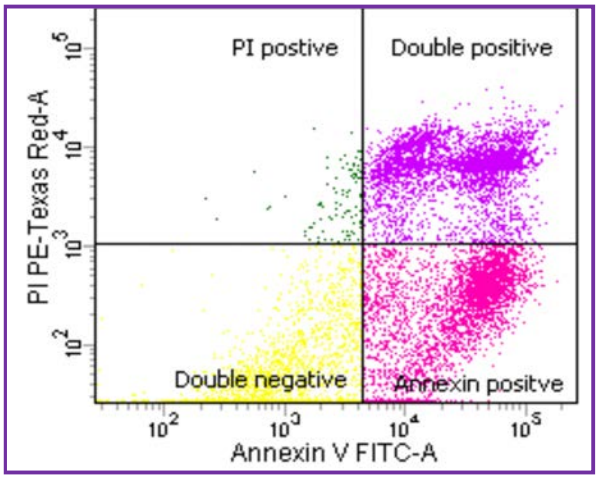
CRL cell lines were exposed to cell culture supernatant of U87 cells that were previously treated with HIV-1 B Tat. Staining the CRL cells with Annexin V subsequently assessed evidence of apoptosis. U87 cells were incubated with Tat for 1 hour, after which the medium was collected, pooled and centrifuged. This medium was then added to CRL cells and incubated overnight. Negative control (untreated CRL cells) showed an average apoptotic percentage of 2.2% (Figure 38), positive control (10µm camptothecin exposed CRL cells) 36.2% (Figure 39), and the Tat treated astrocyte medium exposed CRL cells 9.4 % (Figure 40). All tests were done in triplicate and each Figure represents one such test.

Statistical analysis showed that both camptothecin and the Tat treated astrocyte culture medium increased Annexin V staining significantly when compared to the negative control cells (Figure 41). The increase in Annexin V staining induced by camptothecin was substantially more than the Tat treated astrocyte medium (Figure 41).



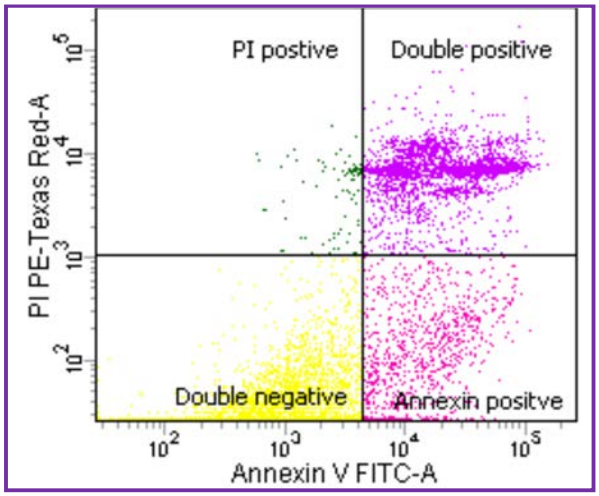
Population	#Events	%Parent	%Total
All Events	10,865	####	100.0
P1	8,908	82.0	82.0
P2	8,715	97.8	80.2
PI postive	848	9.7	7.8
Double positive	560	6.4	5.2
Double negative	7,061	81.0	65.0
Annexin positve	246	2.8	2.3

Figure 38. Flow cytometric analysis illustrating the negative control population of untreated CRL cells.



Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	8,854	88.5	88.5
P2	8,778	99.1	87.8
PI postive	108	1.2	1.1
Double positive	3,637	41.4	36.4
Double negative	1,795	20.4	18.0
Annexin positve	3,238	36.9	32.4

Figure 39. Flow cytometric analysis illustrating the positive control (10µm camptothecin) population of CRL cells.



Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	8,456	84.6	84.6
P2	8,294	98.1	82.9
PI postive	85	1.0	0.9
Double positive	2,723	32.8	27.2
Double negative	4,526	54.6	45.3
Annexin positve	960	11.6	9.6

Figure 40. Flow cytometric analysis illustrating CRL cells exposed to the collected cell culture supernatant from U87 cells that were treated with 10µg/ml HIV-1 B Tat.

3.5.1.1 Statistical Analysis

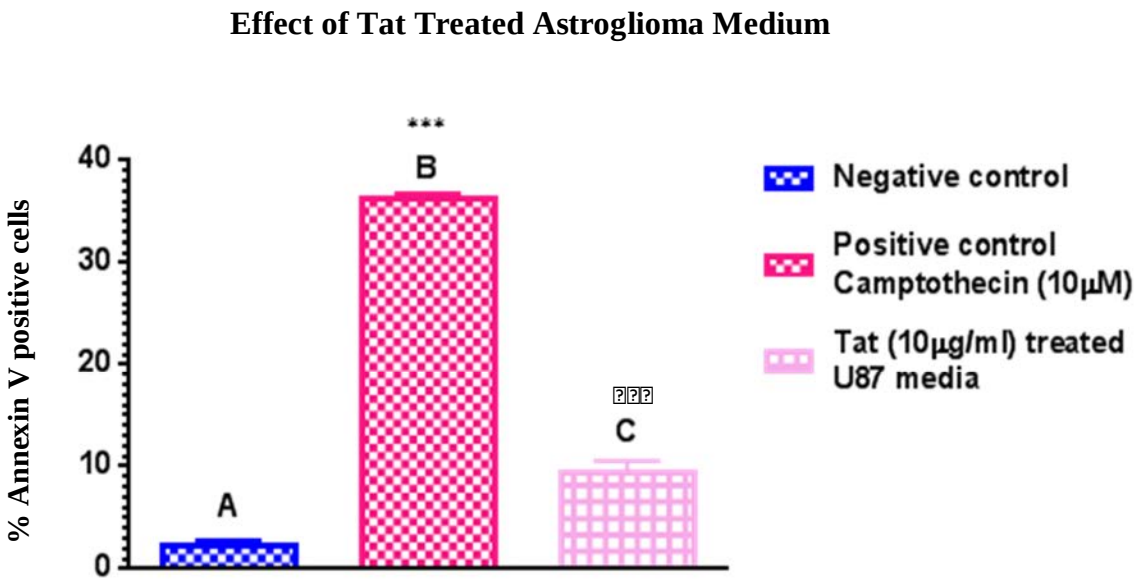


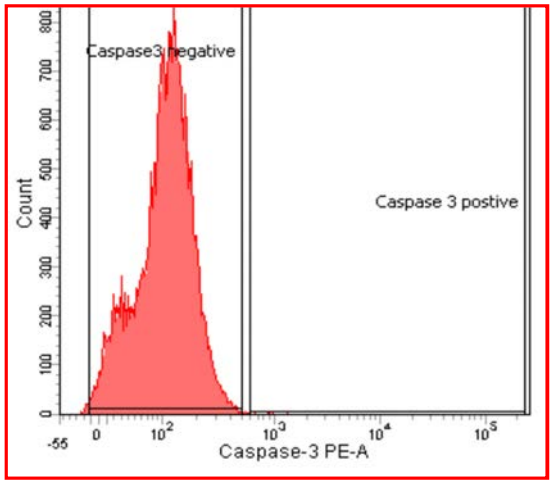
Figure 41. Annexin V, a phospholipid binding protein conjugated to the fluorescein isothiocyanate (FITC) fluorochrome was used to determine the apoptotic inducing effect in CRL cells exposed to the cell culture medium collected from U87 cells that were treated with 10µg/ml HIV-1 B Tat. Cells were grown in 24 well plates and incubated overnight with either 10µm camptothecin (positive control) or the cell culture supernatant of U87 cells exposed to Tat. The negative control group was untreated CRL cells. The analysis was done using the LSR Fortessa SORP flow cytometer. Results were expressed as mean ± SEM of 3 experiments. A value of $p<0.05$ was significantly different from other treatment groups, with *** being most significant and * being least significant (A vs B ***, A vs C ** and B vs C ***). ANOVA followed by the Bonferroni`s multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

3.5.2 Apoptosis detection using the Active PE caspase-3 assay

Similar to the Annexin V experiment, CRL cell lines were once again exposed to either camptothecin or Tat treated U87 cell supernatant to investigate the effect of this supernatant on the expression levels of caspase-3. The PE active Caspase-3 apoptosis kit was used to establish apoptotic cell death.

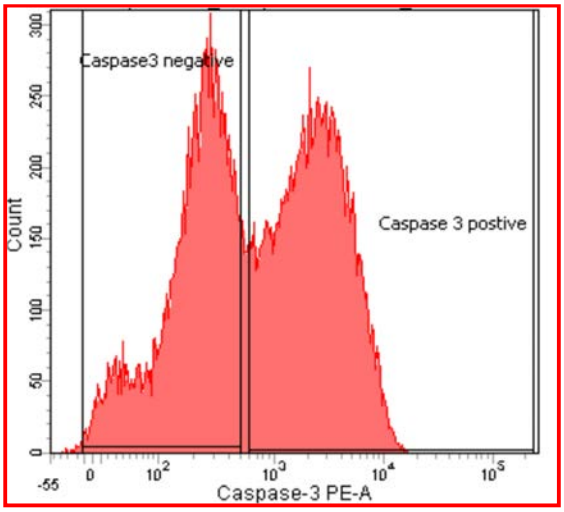
Negative control (untreated CRL cells) (Figure 42) showed an average apoptotic percentage of 0.27%, positive control (10µm camptothecin) 54.9% (Figure 43), and the Tat treated U87 cell supernatant 1.6 % (Figure 44). All tests were done in triplicate and the Figure represents one such test.

Statistical analysis of the data showed that although camptothecin induced a significant increase in caspase-3 expression in CRL cells, exposure of these cells to the cell culture supernatant of U87 cells that were previously exposed to 10µg/ml Tat, was unable to do so (Figure 45).



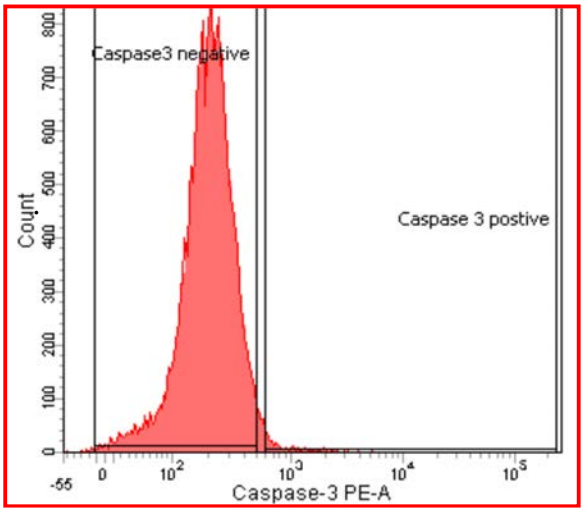
Population	#Events	%Parent	%Total
All Events	25,146	####	100.0
total cell population	24,626	97.9	97.9
Singlets	24,369	99.0	96.9
Caspase3 negative	24,160	99.1	96.1
Caspase 3 postive	62	0.3	0.2

Figure 42. Flow cytometric analysis of the negative control population of untreated CRL cells.



Population	#Events	%Parent	%Total
All Events	25,162	####	100.0
total cell population	23,793	94.6	94.6
Singlets	23,472	98.7	93.3
Caspase3 negative	10,606	45.2	42.2
Caspase 3 postive	12,101	51.6	48.1

Figure 43. Flow cytometric analysis of the positive control (10µm camptothecin) population of CRL cells.



Population	#Events	%Parent	%Total
All Events	25,000	####	100.0
total cell population	24,061	96.2	96.2
Singlets	23,705	98.5	94.8
Caspase3 negative	22,997	97.0	92.0
Caspase 3 postive	394	1.7	1.6

Figure 44. Flow cytometric analysis of the CRL cells exposed to the cell culture supernatant of U87 cells that were previously exposed to 10µg/ml HIV-1 B Tat.

3.5.2.1 Statistical Analysis

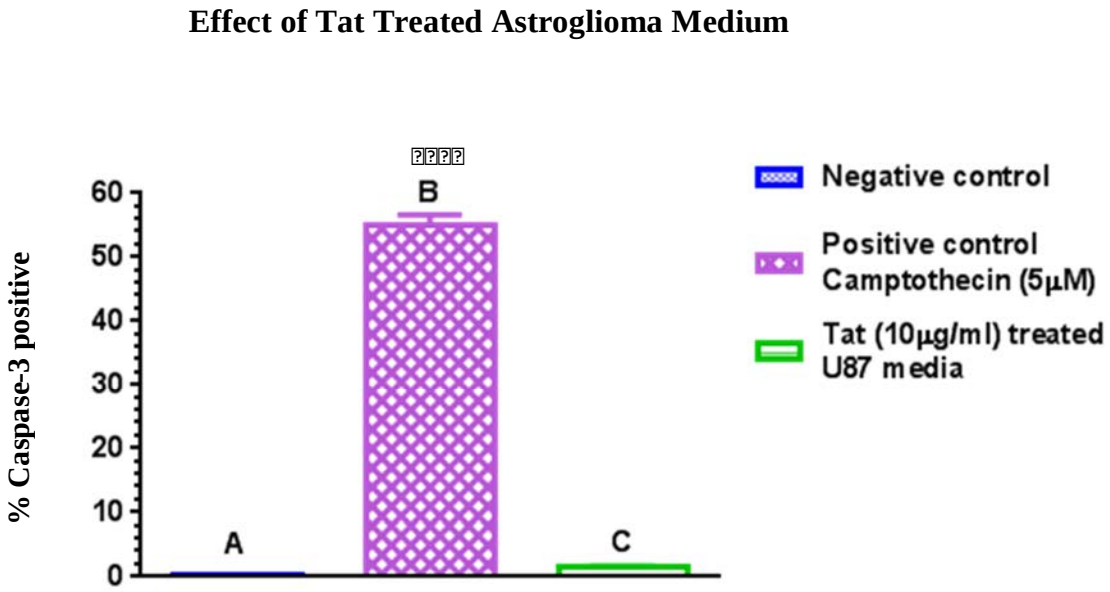


Figure 45. An intracellular staining application using phycoerythrin (PE) rabbit anti-active caspase-3 antibody, and flow cytometry to determine the apoptotic inducing effect in CRL cells exposed to the cell culture medium collected from U87 cells that were treated with 10µg/ml HIV-1 B Tat. Cells were grown in 24 well plates and incubated overnight with either 10µm camptothecin (positive control) or the cell culture supernatant of U87 cells exposed to Tat. The negative control group was untreated CRL cells. The analysis was done using the LSR Fortessa SORP flow cytometer. Results were expressed as mean ± SEM of 3 experiments. A value of $p<0.05$ was significantly different from other treatment groups, with *** being most significant and * being least significant (A vs B ***, A vs C ns and B vs C ***). ANOVA followed by the Bonferroni`s multiple comparison test were done. All statistical analysis was carried out using GraphPad Prism 6.

CHAPTER 4

DISCUSSION

HIV is most well-known for its negative effects on the immune system and the resulting development of AIDS, however it also has severe damaging effects on the central nervous system (CNS) (Palacio *et al*., 2012). Many infected individuals exhibit neuropsychological and behavioral dysfunctions which are collectively referred to as HIV-associated dementia (HAD) (Bhattacharya and Osman, 2009). One of the worrying aspects of HAD is the fact that current anti-retroviral therapy, while being effective in managing the onslaughts of HIV on the immune system, is less efficient in addressing the impact of HIV on the CNS. One of the reasons for this undesirable aspect of the management of HIV, is the lack of in-depth knowledge of the etiology of HAD and subsequently a shortage of targeted therapeutic approaches to deal with it effectively.

A number of proposals have attempted to explain the development of HAD (Brabers and Nottet, 2006; Nielsen *et al.*, 1984; Price *et al.*, 1988) indicating both host and viral proteins as potential causative candidates (Kaul *et al.*, 2001; Li *et al.*, 2009; Rappaport *et al.*, 1999; Shi *et al.*, 1998). As such, Tat protein, one of the regulatory proteins of HIV, has been identified as a possible etiological factor of HAD (Price *et al.*, 1988). Normal functions of Tat includes the augmentation of viral transcription, assisting in efficient replication and survival of the virus (Bagashev and Sawaya, 2013). However, Tat can be extremely harmful to host cells due to some of the exceptional abilities it possesses. For example, Tat can enter and exit cells with ease, travelling between cells, infecting uninfected bystander cells and disrupting essential pathways within cells (Bagashev and Sawaya, 2013). There are no effective treatment regimens that block Tat activity, and even in the presence of ARV's, Tat is persistently being produced by infected cells (Li *et al*., 2009). It is obvious that the mechanisms Tat implements to exert its effects are multifaceted and therefore require further investigation.

Neurotoxicity caused by HIV-1 is an indirect effect since the virus is unable to infect neurons directly (Gendelman *et al.*, 1994). In lieu of this we hypothesized that HIV infects non-neuronal cells in the CNS that leads to their activation, resulting in the release of cytokines that are detrimental to neurons. To test our hypothesis we exposed astrocytes to Tat and assessed whether this leads to the secretion of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). We subsequently determined the ability of astrocyte-secreted TNF-α and IL-6 to induce apoptotic cell death in neurons.

Is commercially procured HIV-1 Tat protein biologically active?

In order for us to successfully perform the experiments, we had to assess whether the Tat that we procured from commercial sources was biologically active. The viability of our clade B Tat protein was determined by transfecting CEM-GFP cells with Tat protein. In these cells GFP is under the control of HIV-1 subtype-B LTR, with the result that only biologically active Tat can drive the fluorescence of GFP in CEM-GFP cells. According to our obtained results, microscopic analysis of CEM-GFP cells exposed to Tat fluoresced green, demonstrating that commercially procured Tat protein used in this study was indeed biologically active. These observations were therefore similar to a previous study in our laboratory (Ramautar *et al.*, 2012) and in accordance with the findings of Siddappa *et al.* (2006). We were therefore confident that the Tat used in subsequent experiments would generate results that could give insights into the *in vivo* situation where individuals are infected with HIV.

Does HIV-1 Tat stimulate astrocyte activation?

After we have established the biological activity of Tat, we investigated the ability of this protein to stimulate astrocytes. We focused on glial fibrillary acidic protein (GFAP) not only because it is a known molecular marker specific for astrocytes, but also because it has been shown to label most, if not all, reactive astrocytes that are responding to CNS injuries and infection (Molofsky *et al.*, 2012; Sofroniew and Vinters, 2010). Western blot analysis showed that astrocytes exposed to Tat yielded a significantly greater GFAP band intensity when compared to the band intensity of the control group of untreated astrocytes. This finding suggested that treating astrocytes with Tat results in their activation. Zhou *et al.* (2004) reported a similar observation previously.

Astrocytes have various critical functions which pose a huge impact on the form and function of the CNS (Wang and Bordey, 2008). Astrocytes are of particular interest since they are non-productively infected (Brabers and Nottet, 2006). In these cells, viral structural protein translation is defective and, only regulatory proteins including Tat, are produced (Brabers and Nottet, 2006). One of the effects of Tat is the activation of uninfected astrocytes. Activated astrocytes cause toxic effects such as cytokine production to intensify inflammation (Nath, 2002), production of neurotoxic levels of ROS, and many other toxic effects (Brambilla *et al.*, 2005; Sofroniew and Vinters, 2010; Swanson *et al.*, 2004). Studies have identified TNF-α and IL-6 to be among the cytokines released from activated astrocytes in response to certain bacterial and viral infections including HIV-1 (Lau and Yu, 2001). In view of these findings, we set out to determine whether astrocytes in our experiments were also releasing these two cytokines following exposure to Tat. Our interest in TNF-α stems from reports showing TNF-α to be a major pro-inflammatory cytokine that plays a vital role in the pathogenesis of HIV-related cognitive abnormalities and other neurodegenerative disorders (Saha and Pahan, 2003). IL-6 on the other hand, has been shown to possess

pro-inflammatory, anti-inflammatory, neuroprotective and neurotoxic effects. Its function with respect to HIV infection however, remains unclear (Guzmán *et al.*, 2010; Rincon, 2012; Scheller *et al.*, 2011).

Do activated astrocytes release IL-6 and TNF-α?

The concentration of TNF-α and IL-6 were measured in the culture medium in which astrocytes treated with Tat were grown. ELISA assays showed that the culture medium of astrocytes exposed to Tat had significantly higher levels of TNF-α and IL-6 than non-treated controls. Interestingly, the level of TNF-α released from activated astrocytes was not as pronounced as that of IL-6. Tat treated cells released more than double the amount of IL-6 than the control group of untreated astrocytes. Our data therefore showed that IL-6 was over-expressed in cells that were exposed to Tat. This finding was not unexpected as astrocytes are known to be a major source of IL-6 in the CNS (Van Wagoner *et al.*, 1999). Previous studies using a 10-fold higher concentration of Tat protein, and being administered at varying time intervals, obtained levels of TNF-α and IL-6 much higher than in our experiments (Chen *et al.*, 1997; Nath *et al.*, 1999). The concentration of Tat used in our study was the minimum concentration which has been shown to elicit neurotoxic effects (Ramautar *et al.*, 2012; Siddappa *et al.*, 2006). Despite these discrepancies, our data was in alignment with the findings of others who demonstrated Tat to cause significant upregulation in the release of the pro-inflammatory cytokines, TNF-α and IL-6 from astrocytes (Gandhi *et al.*, 2009). The release of these cytokines therefore appears to be a robust response of astrocytes following their exposure to Tat.

Are IL-6 and TNF-α toxic to neurons?

Under physiological conditions TNF-α and IL-6 have pleiotropic and overlapping functions and are important in the induction and regulation of the immune response following infection (Arai *et al.*, 1990; Lau and Yu, 2001). In order to determine the effects of these cytokines on neurons, we initially exposed our neuronal cells to exogenous recombinant IL-6 and TNF-α, and followed this experiment with the exposure of our neurons to the culture medium of astrocytes treated with Tat. Flow cytometry (Annexin V and caspase-3 staining) was used to determine the apoptotic inducing effect of these cytokines individually and in combination.

Compared to the control group the TNF-α and IL-6 treated neuronal cells showed a significant percentage of apoptosis. Interestingly, the combination of TNF-α and IL-6 did not show an additive effect, but rather the apoptotic percentage of this combination was lower than that of TNF-α alone. There was therefore no synergistic effect. Similar results were seen for both Annexin V and caspase-3 assays, thereby providing confidence in our results that indicated the level of apoptosis.

TNF-α showed a greater potency to induce apoptosis than IL-6. The ability of TNF-α to induce apoptosis is supported by evidence showing involvement of this cytokine and its receptors in the pathogenesis of HIV-related cognitive abnormalities and other neurodegenerative disorders (Saha and Pahan, 2003). For instance, studies have shown TNF-α to elicit toxic autocrine and paracrine effects on cells of the CNS (Hanisch, 2002; Sheng *et al.*, 2005), that include the induction of neuronal apoptosis via the binding to its receptor and the subsequent activation of death signaling pathways (Muppidi *et al.*, 2004; Sheng *et al.*, 2005; Wajant *et al.*, 2003).

The exact role of IL-6 in inflammation is controversial. For example, it has been shown to have beneficial effects because of its neurotrophic properties, however over-expression has been shown to be detrimental to the CNS (Van Wagoner *et al.*, 1999). The decreased potency of the combined cytokine effect may be a result of IL-6 and its neuroprotective, anti-inflammatory effects that acted against the pro-inflammatory effects of TNF-α. This explanation is plausible since researchers have demonstrated that IL-6 offer protection against neurotoxicity, as well as having anti-inflammatory properties (Guzmán *et al.*, 2010; Rincon, 2012; Scheller *et al.*, 2011). However, others have reported that increased levels of IL-6 in the CNS have been demonstrated in several neurological diseases and in HIV infection (Qiu *et al.*, 1998). These authors showed that IL-6 can be detrimental to the functioning of the CNS. The overall effect of IL-6 therefore seems to be ambiguous, thereby requiring further investigation.

Is the culture medium of HIV-1 Tat exposed astrocytes toxic to neurons?

Since exogenous recombinant TNF-α and IL-6 caused a significant percentage of apoptosis of neuronal cells, the culture medium of astrocytes exposed to Tat was collected and added to the growth medium of the neuron culture. This was done to determine whether the culture medium of Tat-exposed astrocytes could induce IL-6 and TNF-αdirected apoptotic cell death of neurons. The Annexin assay showed a significant percentage of apoptotic cell death of neurons exposed to Tat-treated astrocyte culture medium, compared to the control group. In contrast, the caspase-3 assay showed no such significance. These results led us to believe that although the apoptotic pathway was initiated, as supported by the Annexin assay data, the final executioning part of the pathway was not active, as reflected by the caspase-3 assay.

As mentioned above IL-6 may have offered, to some extent, neuroprotective effects toward the neurons exposed to the insult of TNF-α, thus inhibiting caspase-3 activation and downstream execution of apoptosis. This can be due to the possibility of TNF-α and IL-6 meeting at the same execution point which is caspase-3 activation, therefore dampening a synergistic effect. Alternatively, it may also be possible that the triggers of caspase-3 activation have been inadequate to elicit a strong enough signal that would drive the pathway to completion. The concentration of the recombinant TNF-α and IL-6 used to evaluate the effects of exogenous cytokines on neurons, were in the nanogram range, while the levels of these cytokines

measured in the culture medium of Tat-exposed astrocytes, were only in the picogram range. Previous studies show that cell death occurred at TNF-α levels between 5 and 20ng/ml (Deghmane *et al.*, 2009; Sheng *et al.*, 2005). It is therefore possible that the lower levels of cytokines were unable to stimulate the entire apoptotic pathway to a sufficient extent that may lead to programmed cell death. In order for cells to undergo apoptosis, both the initiation and execution parts of the apoptotic pathway need to be activated (Riedl and Salvesen, 2007). The initiation part of the pathway can be activated by both the intrinsic or extrinsic pathway, and ends with the activation of caspase-9 and caspase-8 respectively. These caspases subsequently activate caspases-3 and 7, marking the beginning of the execution part that eventually results in apoptotic cell death. Recently there has been reports that caspase-8 may function as a switch between autophagy and apoptosis (Wu *et al.*, 2014). Autophagy refers to the process whereby damaged intracellular proteins and organelles are hydrolyzed by lysosomial proteases for re-use by the cell. The autophagic process is therefore part of the cell's attempts to survive under conditions of stress. It is likely that in our experiments a similar switching took place where apoptosis was initiated but not executed through the function of caspase-8.

CHAPTER 5

CONCLUSION

There has been much progress in the management of HIV-1, leading to a significant increase in the life span of the HIV-infected individual. Despite the advances, some serious issues remain unresolved. For instance, to date current therapeutic approaches are still unable to eradicate the virus completely. The existence of residual reservoirs continues to be one of the major challenges facing scientists and clinicians working in this field of infectious diseases.

Another area of concern revolves around the effects of HIV on the central nervous system. While antiretroviral therapy has been promising in extending an infected individuals' life span by slowing down the rate of viral replication, the treatment of HIV-induced neurotoxicity appears to be problematic. One of the reasons for this inadequacy stems from the limited understanding of how the virus interacts with host cells, and in this case, cells of the nervous system. Our study focused on the engagement of HIV with astrocytes, and investigated how this interaction may lead to neuronal cell death. This study therefore attempted to provide a molecular basis that may explain the neuronal death seen in individuals diagnosed with HIV-associated dementia (HAD).

We hypothesized that HIV infects astrocytes which results in the release of pro-inflammatory cytokines that are toxic to neurons. To prove our hypothesis we exposed astrocytes to the viral protein Tat and assessed whether such exposure would stimulate the secretion of TNF-α and IL-6 into the cell culture medium. We then subjected this culture medium to neurons and determined the level of apoptotic cell death.

Our data showed that HIV-1 Tat protein exposure resulted in astrocyte activation and consequently the release of cytokines IL-6 and TNF-α into the culture medium. Interestingly, these cytokines individually, by exogenous administration, resulted in a significant amount of neuron death. However, in combination, they did not show an additive synergistic effect. This finding suggested that the two cytokines very likely utilize a common pathway to induce apoptotic cell death. Further exposure of neurons to these cytokines in the astrocyte-grown culture medium did not yield the same extent of neuronal apoptosis. Huge differences between the concentrations of exogenous cytokines and that present in the culture medium, can possibly explain this discrepancy. Because of the rather low levels of cytokines, exposure of neurons to the culture medium resulted only in the initiation of neuronal apoptosis and not its final execution. A possible explanation for this observation may be that under conditions of exposure to low levels of pro-inflammatory cytokines, cells have the opportunity to resort to autophagy as a survival mechanism, thus escaping the final stages of apoptotic induced cell death. This finding suggested that the concentration of pro-inflammatory cytokines might be important in determining the eventual fate of cells.

In summary our results suggest that HIV may activate astrocytes via Tat protein leading to the secretion of cytokines that may be toxic to neurons and cause their demise. These findings are helpful as it indicates that in addition to the use of existing antiretroviral therapy that mainly aims to reduce viral entry and replication, drugs that target inflammatory processes should also be considered in the management of people living with HIV/AIDS, and in particular those who are showing signs of neurocognitive decline.

CHAPTER 6

RECOMMENDATIONS

Our study generated a number of new ideas for future research. It would be useful to determine whether viral proteins other than Tat, produce similar effects, as we know that Tat is not the only protein secreted by HIV. Also it would be interesting to increase the time to which astrocytes are exposed to low levels of Tat. Such an approach may help us understand the impact of the residual reservoir of HIV on brain function. Since the results obtained in the present study were confined to i*n vitro* studies, it would be ideal to repeat the experiments using an i*n vivo* model. This may be important to confirm the *in vitro* generated data. Finally, it would be interesting to see whether an intervention aimed at reducing pro-inflammatory cytokines such as TNF-α and IL-6, would be able to decrease the level of apoptotic neuronal cell death, thereby providing a lead in the development of therapeutic strategies to combat Tat induced neurotoxicity and HAD. Since we did not measure the Tat concentration in the medium of Tat-exposed astrocytes, this medium collected and used for subsequent experiments could have contained residual Tat protein that may have been responsible for some of the effects observed in the neuronal cells.

CHAPTER 7

REFERENCES

Adle-Biassette, H., Chretien, F., Wingertsmann, L., Hery, C., Ereau, T., Scaravilli, F., Tardieu, M. & Gray, F. 1999. Neuronal apoptosis does not correlate with dementia in hiv infection but is related to microglial activation and axonal damage. *Neuropathology and Applied Neurobiology,* 25**,** 123–133.

Alaeus, A. 2000. Significance of hiv-1 genetic subtypes. *Scandinavian Journal of Infectious Diseases,* 32**,** 455-463.

Arai, K. I., Lee, F. & Miyajima, A. 1990. Cytokines: Coordinators of immune and inflammatory responses. *Annu Rev. Biochem,* 59**,** 783-836.

Araki, E., Forster, C., Dubinsky, J., M, , Ross, M., E, & Ladecola, C. 2001. Cyclooxygenase-2 inhibitor ns-398 protects neuronal cultures from lipopolysaccharide-induced neurotoxicity. *Stroke,* 32**,** 2370-2375

Araque, A., Carmignoto, G. & Haydon, P. 2001. Dynamic signalling between astrocytes and neurons. *Annual Reviews of Physiology,* 63**,** 795-813.

Araque, A., Parpura, V., Sanzgir, R., P, & Haydon, P., G 1999. Tripartite synapses: Glia, the unacknowledged partner. *Trends in Neuroscience,* 22**,** 208-215.

Arts, E., J, & Hazuda, D., J 2012. Hiv-1 antiretroviral drug therapy. *Cold Spring Harbor Perspectives in Medicine,* 2**,** 1-23.

Ashkenazi, A. & Dixit, V., M 1998. Death receptors: Signaling and modulation. *Science,* 281**,** 1305-1308.

Bagashev, A. & Sawaya, B., E 2013. Roles and functions of hiv-1 tat protein in the cns: An overview. *Virology Journal,* 10**,** 1-20.

Barreto, G. E., Gonzalez, J., Capani, F. & Morales, L. 2011. Role of astrocytes in neurodegenerative diseases. *Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring, Dr Raymond Chuen-Chung Chang (Ed.).*

Batista, L., F, Z, , Kaina, B., Meneghini, R. & Menck, C., F, M 2009. How DNA lesions are turned into powerful killing structures: Insights from uv-induced apoptosis. *Mutation Research/Reviews in Mutation Research,* 681**,** 197-208.

Bhattacharya, S. & Osman, H. 2009. Novel targets for anti-retroviral therapy. *Journal of Infection,* 59**,** 377-386.

Block, M., L, , Wu, X., Pei, Z., Li, G., Wang, T., Qin, L., Wilson, B., Yang, J., Hong, J., S, & Veronesi, B. 2004. Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: The role of microglia, phagocytosis, and nadph oxidase. *Federation of American Societies for Experimental Biology,* 18**,** 1618-1640.

Blondel, O., Collin, C., Mccarran, W. J., Zhu, S., Zamostiano, R., Gozes, I., Brenneman, D. E. & Mckay, R. D. 2000. A gliaderived signal regulating neuronal differentiation. *J Neurosci* 20**,** 8012-8020.

Boykins, R. A., R. Mahieux, U. T. Shankavaram, Y. S. Gho, F. Lee, I. Hewlett, L. M. Wahl, H. K. Kleinman, Brady J. N, K. M. Yamada and S. Dhawan (1999). Cutting edge: A short polypeptide domain of HIV-1-Tat protein mediates pathogenesis. *The Journal of Immunology***,** 163, 15-20. Brabers, N. A. C. H. & Nottet, H. S. L. M. 2006. Role of the pro-inflammatory cytokines TNF-α and IL-1β in HIV-associated dementia. *European Journal of Clinical Investigation,* 36**,** 447-458.

Bradford, A., Navia, M., D, , Barry, D., Jordan, M., D, & Price, R., W 1986. The aids dementia complex: I. Clinical features. *Annals of Neurology,* 19**,** 517-524.

Brambilla, R., Bracchi-Ricard, V., Hu, W., Frydel, B., Bramwell, A., Karmally, S., Green, E. J. & Bethea, J. R. 2005. Inhibition of astroglial nuclear factor kappab reduces inflammation and improves functional recovery after spinal cord injury. *J Exp Med,* 202**,** 145-156.

Brigati, C., Giacca, M., Noonan, D. M. & Albini, A. 2003. Hiv tat, its targets and the control of viral gene expression. *FEMS Microbiol Lett,* 220**,** 57–65.

Brown, A., M, & Ransom, B., R 2007. Astrocyte glycogen and brain energy metabolism. *Glia,* 55**,** 1263-1271.

Cabal-Hierro, L. & Lazo, P., S 2012. Signal transduction by tumor necrosis factor receptors. *Cell Signal,* 24**,** 1297-1305.

Campbell , G., R & Loret , E., P 2009. What does the structure-function relationship of the hiv-1 tat protein teach us about developing an aids vaccine? *Retrovirology,* 6**,** 1-13.

Chang, H., C, , Samaniego, F. & Nair, B., C 1997. Hiv-1 tat protein exits from cells via a leaderless secretory pathway. *AIDS,* 11**,** 1421–1431.

Charles, A., C,, Merrill, J., E, , Dirksen, E., R, & Sanderson, M., J 1991. Intercellular signaling in glial cells: Calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron,* 6**,** 983–992.

Chauhan, A., Turchan, J., Pocernich, C., Bruce-Keller, A., Roth, S., Butterfield, D. A., Major, E. O. & Nath, A. 2003. Intracellular human immunodeficiency virus tat expression in astrocytes promotes astrocyte survival but induces potent neurotoxicity at distant sites via axonal transport. *J Biol Chem,* 278**,** 13512–13519.

Chen, P., Mayne, M., Power, C. & Nath, A. 1997. The tat protein of hiv-1 induces tumor necrosis factor-a production. *The Journal of Biological Chemistry,* 272**,** 22385-22388.

Claycomb, K., I,, Johnson, K., M,, Winokur, P., N,, Sacino, A., V, & Crocker, S., J, 2013. Astrocyte regulation of cns inflammation and remyelination. *Brain Science,* 3**,** 1109-1127.

Collins, D., R, & Collins, K., L 2014. Hiv-1 accessory proteins adapt cellular adaptors to facilitate immune evasion. *PLOS Pathogens,* 10**,** 1-4.

Conant, K., Garzino-Demo, A., Nath, A., Mcarthur, J., C, , Halliday, W., Power, C., Gallo, R., C, & Major, E., O 1998. Induction of monocyte chemoattractant protein-1 in hiv-1 tat-stimulated astrocytes and elevation in aids dementia. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 3117-3121.

Cory, S. & Adams, J., M 2002. The bcl2 family: Regulators of the cellular life-or-death switch. *Nature Reviews Cancer,* 2**,** 647-656.

Debaisieux, S., Rayne, F., Yezid, H. & Beaumelle, B. 2012. The ins and outs of hiv-1 tat. *Traffic,* 13**,** 355-363.

Deghmane, A. E., Veckerle´, C., Giorgini, D., Hong, E., Ruckly, C. & Taha, M. K. 2009. Differential modulation of tnf-a–induced apoptosis by neisseria meningitidis. *PLOS Pathogens,* 5**,** 1-17.

Elmore, S. 2007. Apoptosis: A review of programmed cell death. *Toxicologic Pathology,* 35**,** 495-516.

Fauci, A. S. 2003. Hiv and aids: 20 years of science. *Nature Medicine,* 9**,** 839-841.

Fellin, T. & Carmignoto, G. 2004. Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *Journal of Physiology,* 559**,** 3-15.

Fittipaldi, A., Ferrari, A., Zoppe, M., Arcangeli, C., Pellegrini, V., Beltram, F. & Giacca, M. 2003. Cell membrane lipid rafts mediate caveolar endocytosis of hiv-1 tat fusion proteins. *Journal of Biological Chemistry,* 278**,** 34141–34149.

Fotin-Mleczek, M., Henkler, F., Samel, D., Reichwein, M., Hausser, A., Parmryd, I., Scheurich, P., Schmid, J., A, & Wajant, H. 2002. Apoptotic crosstalk of tnf receptors: Tnf-r2 induces depletion of traf2 and iap proteins and accelerates tnf-r1 dependent activation of caspase-8. *Journal of Cell Science,* 115**,** 2757-2770.

Frankel, A. & Young, J. 1998. Hiv-1: Fifteen proteins and an rna. *Annu. Rev. Biochem.,* 67**,** 1-25.

Freed, E. O. 1998. Hiv-1 gag proteins: Diverse functions in the virus life cycle. *VIROLOGY,* 251**,** 1-15.

Gandhi, N., Saiyed, Z., Thangavel, S., Rodriguez, J., Rao, K., V, K, & Nair, M., P, N 2009. Differential effects of hiv type 1 clade b and clade c tat protein on expression of proinflammatory and antiinflammatory cytokines by primary monocytes. *AIDS Research and Human Retroviruses,* 25**,** 691-699.

Gavioli, R., Gallerani, E., Fortini, C., Marina Fabris, M., Bottoni, A., Canella, A., Bonaccorsi, A., Marastoni, M., Micheletti, F., Cafaro, A., Rimessi, P., Caputo, A. & Ensoli, B. 2002. Human immunodeficiency virus type 1 nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis. *Journal of Virology,* 76**,** 2692-2702.

Gendelman, H., E,, Lipton, S., A,, Tardieu, M., Bukrinsky, M., I, & Nottett, S., L, M 1994. The neuropathogenesis of hiv-1 infection. *Journal of Leukocyte Biology,* 56**,** 389-398.

Ghafouri, M., Amini, S., Khalili, K. & Sawaya, B., E, 2006. Hiv-1 associated dementia: Symptoms and causes. *Retrovirology,* 3**,** 1-11.

Gordon, G., R, , Mulligan, S., J, & Macvicar, B., A. 2007. Astrocyte control of the cerebrovasculature. *Glia,* 55**,** 1214-1221.

Guadagno, J., Xu, X., Karajgikar, M., Brown, A. & Cregan, S., P 2013. Microglia-derived tnfa induces apoptosis in neural precursor cells via transcriptional activation of the bcl-2 family member puma. *Cell Death and Disease,* 4**,** 1-10.

Guzmán, C., Hallal-Calleros, C., López, G. L. & Morales, M. J. 2010. Interleukin-6: A cytokine with a pleiotropic role in the neuroimmunoendocrine network. *The Open Neuroendocrinology Journal,* 3**,** 152-160.

Hagberg, H. & Mallard, C. 2005. Effect of inflammation on central nervous system development and vulnerability. *Current Opinion in Neurology,* 18**,** 117-123.

Halassa, M. & Haydon, P. 2010 Integrated brain circuits: Astrocytic networks modulate neuronal activity and behavior. *Annu Rev Physiol.* , 72**,** 335-355.

Halassa, M., M, , Fellin, T. & Haydon, P., G 2007. The tripartite synapse: Roles for gliotransmission in health and disease. . *Trends in Molecluar Medicine,* 13**,** 54-63.

Hanisch, U., K 2002. Microglia as a source and target of cytokines. *Glia,* 40**,** 140-155.

Hansson, E. & Ronnback, L. 2003. Glial neuronal signaling in the central nervous system. *Federation of American Societies for Experimental Biology,* 17**,** 341-348.

Harrich, D., Ulich, C., Garcia-Martinez, L., F, & Gaynor, R., B 1997. Tat is required for efficient hiv-1 reverse transcription. *European Molecular Biology Organization Journal,* 16**,** 1224-1235.

Herce, H. D. & Garcia, A. E. 2007. Molecular dynamics simulations suggest a mechanism for translocation of the hiv-1 tat peptide across lipid membranes. *Proc. Natl. Acad. Sci. USA.,* 104**,** 20805-208010.

Holliday, J., Parsons, K., Curry, J., Lee, S., Y, & Gruol, D., L 1995. Cerebellar granule neurons develop elevated calcium responses when treated with interleukin-6 in culture. . *Brain Res,* 673**,** 141-148.

Iadecola, C. & Nedergaard, M. 2007. Glial regulation of the cerebral microvasculature. *Nature Neuroscience,* 10**,** 1369–1376.

Jeang, K.-T. 1996. Hiv-1 tat: Structure and function. *Molecular Virology Section***,** 3-18.

Karn, J. 1999. Tackling tat. *Journal of Molecular Biology,* 293**,** 235-254.

Kaul, M., Garden, G. A. & Lipton, S. A. 2001. Pathways to neuronal injury and apoptosis in hiv-associated dementia. *Nature,* 410**,** 998-994.

Kaul, M. & Lipton, S., A 2000. The nmda receptor- its role in neuronal apoptosis and hiv - associated dementia. *NeuroAids,* 3**,** 1-11.

Kim, T.-A., Avraham, H., Karsenty, , Koh, Y.-H., Jiang, S., Park, I.-W. & Avraham, S. 2003. Hiv-1 tat-mediated apoptosis in human brain microvascular endothelial cells. *The Journal of Immunology,* 170**,** 2629–2637.

Kimelberg, H., K, & Nedergaard, M. 2010. Functions of astrocytes and their potential as therapeutic targets. *Neurotherapeutics,* 7**,** 338–353.

Kischkel, F., C, , Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P., H, & Peter, M., E 1995. Cytotoxicitydependent apo-1 (fas/cd95)- associated proteins form a death-inducing signaling complex (disc) with the receptor. *The European Molecular Biology Organization Journal,* 14**,** 5579-5588.

Kishimoto, T. 2010. Il-6: From its discovery to clinical applications. *International Immunology,* 22**,** 347-352.

Kishimoto, T. & Ishizaka, K. 1971. Regulation of antibody response in vitro. Suppression of secondary response by antiimmunoglobulin heavy chains. . *Journal of Immunology,* 107**,** 1567.

Koehler, R. C., Roman, R. J. & Harder, D. R. 2009. Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci,* 32**,** 160-169.

Lane, H. 2010. Pathogenesis of hiv infection. *Topics in HIV Medicine,* 18**,** 2-6.

Lau, L. T. & Yu, A. C.-H. 2001. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *Journal of Neurotrauma,* 18**,** 351-359.

Lee, S., C, , Liu, W., Dickson, D., W, , Brosnan, C., F, & Berman, J., W 1993. Cytokine production by human fetal microglia and astrocytes. *Journal of Immunology,* 150**,** 2659 -2667.

Li, W., Li, G., Steiner, J. & Nath, A. 2009. Role of tat protein in hiv neuropathogenesis. *Neurotoxicity Research,* 16**,** 205-220.

Locksley, R. M., Killeen, N. & Lenardo, M. J. 2001. The tnf and tnf receptor superfamilies: Integrating mammilian biology. *Cell,* 104**,** 487-501.

Magistretti, P., J, & Pellerin, L. 1996. Cellular basis of brain energy metabolism and their relevance to functional brain imaging: Evidence for a prominent role of astrocytes. *Cerebral Cortex,* 6**,** 50-61.

Magistretti, P. J. & Ransom, B. R. 2002. Astrocytes. *Neuropsychopharmacology,* 5**,** 133-145.

Mattson, M., P,, Haughey, N., J, & Nath, A. 2005. Cell death in hiv dementia. *Cell Death and Differentiation,* 12**,** 893–904.

Mennerick , S., Benz , A. & Zorumski, C., F 1996. Components of glial responses to exogenous and synaptic glutamate in rat hippocampal microcultures. *Journal of Neuroscience,* 16**,** 55-64.

Molofsky, A., V,, Krenick, R. & Ullian, E. 2012. Astrocytes and disease: A neurodevelopmental perspective. *Genes and Development*, 26**,** 891–907.

Muppidi, J., R, , Tschopp, J. & Siegel, R., M 2004. Life and death decisions: Secondary complexes and lipid rafts in tnf receptor family signal transduction. *Immunity,* 21**,** 461-465.

Nagata, S. 1997. Apoptosis by death factor. *Cell Death and Differentiation,* 88**,** 355-365.

Nath, A. 2002. Human immunodeficiency virus (hiv) proteins in neuropathogenesis of hiv dementia. *The Journal of Infectious Diseases,* 186**,** 193-198.

Nath, A., Conant, K., Cheni, P., Scotti, C. & Major, E. 1999. Transient exposure to hiv-1 tat protein results in cytokine production in macrophages and astrocytes. *The Journal Of Biological Chemistry,* 274**,** 17098-17102.

Nath, A., Geiger, J., D,, Mattson, M., P, , Magnuson, D., S,K, , Jones, M. & Berger, J., R 1998. Role of viral proteins in hiv-1 neuropathogenesis with emphasis on tat. *NeuroAids,* 1**,** 1-9.

Nedergaard, M., Ransom, B. & Goldman, S., A 2003. New roles for astrocytes: Redefining the functional architecture of the brain. *Trends in Neuroscience,* 26**,** 523-530.

Nielsen, S. L., Petito, C. D. & Urmacher, C. D. 1984. Subacute encephalitis in acquired immune deficiency syndrome : A postmortem study. *Journal of Clinical Pathology,* 82**,** 678-682.

Nimmervoll, B., White, R., Yang, J.-W., An, S., Henn, C., Sun, J.-J. & Luhmann, H., J 2012. Lps-induced microglial secretion of tnfa increases activity-dependent neuronal apoptosis in the neonatal cerebral cortex. *Cerebral Cortex,* 1-14.

Oberheim, N. A., Goldman, S. A. & Nedergaard, M. 2012. Heterogeneity of astrocytic form and function. *Methods Mol Biol,* 814**,** 23-45.

Ojeda, S. R., Ma, Y. J., Lee, B. J. & Prevot, V. 2000. Glia-to-neuron signaling and the neuroendocrine control of female puberty. *Recent Prog Horm Res* 55**,** 197-223.

Ott, M., Dorr, A., Hetzer-Egger, C., Kaehlcke K, Schnolzer M, Henklein, P., Cole P, Zhou, M., M, & Verdin, E. 2004. Tat acetylation: A regulatory switch between early and late phases in hiv transcription elongation. *Novartis Foundation Symposia,* 259**,** 182-193.

Ouyang, L., Shi, Z., Zhao, S., Wang, F., T,, Zhou, T., T,, Liu, B. & Bao, J., K 2012. Programmed cell death pathways in cancer: A review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation,* 45**,** 487-498.

Palacio, M., D, , Álvarez, S. & Muñoz-Fernández, Á. 2012. Hiv-1 infection and neurocognitive impairment in the current era. *Reviews in Medical Virology,* 22**,** 33-45.

Parpura, V., Basarsky, T., A,, Lui, F., Jeftinija, K. & Haydon, P., G 1994. Glutamate-mediated astrocyte-neuron signalling. *Nature,* 369**,** 744-747.

Pasti, L., Volterra, A., Pozzan, T. & Carmignoto, G. 1997. Intracellular calcium oscillations in astrocytes: A highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *The Journal of Neuroscience,* 17**,** 7817-7830.

Pekny, M. & Pekna, M. 2004. Astrocyte intermediate filaments in cns pathologies and regeneration. *J Pathol,* 204**,** 428-437.

Perea, G., Navarrete, M. & Araque, A. 2009. Tripartite synapses: Astrocytes process and control synaptic information. *Trends in Neuroscience,* 32**,** 421-431.

Perry, S., W, , Dewhurst, S., Bellizzi, M., J, & Gelbard, H., A 2002. Conflicting effects of tnfa in normal and diseased brain: Intraneural receptor crosstalk as one of the mechanism for explaining the paradox. *Journal of Neurovirology,* 8**,** 611-624.

Petito, C., K, , Vecchio, D. & Chen, Y., T 1994. Hiv antigen and DNA in aids spinal cords correlate with macrophage infiltration but not with vacuolar myelopathy. *Journal of Neuropathology Experimental Neurology,* 53**,** 86–94.

Pocernicha, C., B, , Sultanaa, R., Mohmmad-Abdula, Hafiz, , Nath, A. & Butterfielda, D., Allan 2005. Hiv-dementia, tat-induced oxidative stress, and antioxidanttherapeutic considerations. *Brain Research Reviews,* 50**,** 14 – 26.

Pomerantz, R., J, & Horn, D., L 2003. Twenty years of therapy for hiv-1 infection. *Nature Medicine,* 9**,** 867-873.

Price, R. W., Brew, B., Sidtis, J., Rosenblum, M., Scheck, A. C. & Cleary, P. 1988. The brain in aids: Central nervous system hiv-1 infection and aids dementia complex. *Science,* 239**,** 586-592.

Pugliese , A., Vidotto , V., Beltramo , T., Petrini , S. & Torre, D. 2005. A review of hiv-1 tat protein biological effects. *Cell Biochemistry and Function,* 23**,** 223-227.

Qiu, Z., Parsons, K., L, & Gruol, D., L 1995. Interleukin-6 selectively enhances the intracellular calcium response to nmda in developing cns neurons. *Journal of Neuroscience,* 15**,** 6688-6699.

Qiu, Z., Sweeney, D., D, , Netzeband, J., G, & Gruol, D., L 1998. Chronic interleukin-6 alters nmda receptor-mediated membrane responses and enhances neurotoxicity in developing cns neurons. *The Journal of Neuroscience,* 18**,** 10445–10456.

Ramautar, A., Mabandla, M., Blackburn, J. & Daniels, W. M. U. 2012. Inhibition of hiv-1 tat-induced transactivation and apoptosis by the divalent metal chelators, fusaric acid and picolinic acid—implications for hiv-1 dementia. *Neuroscience Research*.

Ranga, U., Shankarappa, R., Siddappa, B., N, , Ramakrishna, L., Nagendran, R. & Mahalitigam, M. 2004. Tat protein of human immunodeficiency virus type 1 subtype c strains is a defective chemokine. . *Journal of Virology,* 7**,** 2586-2590.

Rappaport, J., Joseph, J., Croul, S., Alexander, G., Valle, L. D., Amini, S. & Khalili, K. 1999. Molecular pathway involved in hiv-1-induced cns pathology: Role of viral regulatory protein, tat. *Journal of Leukocyte Biology,* 65**,** 458-465.

Rastogi, R., P, , Sinha, R. & Sinha, R., P 2009. Apoptosis : Molecular mechanisms and pathogenicity. *EXCLI Journal,* 8**,** 155-181.

Reed, J., C, & Tomaselli, K., J 2000. Drug discovery opportunities from apoptosis research. *Current Opinions in Biotechnology,* 11**,** 586-592.

Ridet, J. L., Malhotra, S. K., Privat, A. & Gage, F. H. 1997. Reactive astrocytes: Cellular and molecular cues to biological function. *Trends Neurosci.* , 20**,** 570–577.

Riedl, S. J. & Salvesen, G. S. 2007. The apoptosome: Signaling platform for cell death. *Nature Reviews Molecular Cell Biology,* 8**,** 405-413.

Rincon, M. 2012. Special issue on interleukin-6 (il-6). *International Journal of Biological Sciences,* 8**,** 1225-1226.

Robinson, L., Harriet 2002. New hope for an aids vaccine. *Nature Reviews Immunology,* 2**,** 239-250.

Rodríguez, J. J. & Verkhratsky, A. 2010. Neuroglial roots of neurodegenerative diseases? *Mol Neurobiol***,** 1-10.

Romani, B., Engelbrecht, S. & Glashoff, R., H 2010. Functions of tat: The versatile protein of human immunodeficiency virus type 1. *Journal of General Virology,* 91**,** 1-12.

Rosen, C., A, , Terwilliger, E., Dayton, A., Sodroski, J., G, & Haseltine, W., A 1988. Intragenic cis-acting art gene-responsive sequences of the human immunodeficiency virus. *Proceedings of the National Academy of Sciences of USA,* 85**,** 2071-2075.

Ross, T., M 2001. Using death to one's advantage : Hiv modulation of apoptosis *Leukemia,* 15**,** 332-341.

Rubio-Moscardo, F., Blesa, D., Mestre, C., Siebert, R., Balasas, T., Benito A, Rosenwald, A., Climent, J., Martinez, J., I, , Schilhabel, M. & Karran, E., L, 2005. Characterization of 8p21.3 chromosomal deletions in bcell lymphoma: Trail-r1 and trail-r2 as candidate dosage-dependent tumor suppressor genes. *Blood,* 106**,** 3214-3222.

Saelens, X., Festjens, N., Vande Walle, L. & Van Gurp, M. 2004. Toxic proteins released from mitochondria in cell death. *Oncogene,* 23**,** 2861-2874.

Saha, R., N, & Pahan, K. 2003. Tumor necrosis factor-a at the crossroads of neuronal life and death during hiv-associated dementia. *Journal of Neurochemistry,* 86**,** 1057-1071.

Scheller, J., Chalaris, A., Schmidt, A. D. & Rose, J. S. 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta,* 1813**,** 878-888.

Schwartz, S., Felber, B., K, , Benko, D., M, , Fenyo, E., M, & Pavlakis, G., N 1990. Cloning and functional analysis of multiply spliced mrna species of human immunodeficiency virus type 1. *Journal of Virology,* 64**,** 2519-2529.

Seifert, G., Schilling, K. & Steinhäuser, C. 2006. Astrocyte dysfunction in neurological disorders: A molecular perspective. *Nature Reviews Neuroscience,* 7**,** 194-206.

Sheng, W. S., Hu, S., Ni, H. T., Rowen, T. N., Lokensgard, J. R. & Peterson, P. K. 2005. Tnf-a induced chemokine production and apoptosis in human neural precursor cells. *Journal of Leukocyte Biology,* 78**,** 1233-1241.

Shi, B., Raina, J., Lorenzo, A., Busciglio, J. & Gabuzda, D. 1998. Neuronal apoptosis induced by hiv-1 tat protein and tnf-a: Potentiation of neurotoxicity mediated by oxidative stress and implications for hiv-1 dementia. *Journal of Neurovirology,* 4**,** 281-290.

Siddappa, N., Byrareddy, , Venkatramanan, M., Venkatesh, P., Janki, M., Vijayamma, , Jayasuryan, N., Desai, A., Ravi, V. & Ranga, U. 2006. Transactivation and signaling functions of tat are not

correlated: Biological and immunological characterization of hiv-1 subtype-c tat protein. *Retrovirology,* 3**,** 1-20.

Sierra, S., Kupfer, B. & Kaiser, R. 2005. Basics of the virology of hiv-1 and its replication. *Journal of Clinical Virology,* 34**,** 233-244.

Sofroniew, M., V, & Vinters, H., V 2010. Astrocytes: Biology and pathology. *Acta Neuropathol* 119**,** 7-35.

Sonker, M., Chib, R. & Hooda, A. 2010. Apoptosis: An overview. *Journal of Pharmacy Research,* 3**,** 2293-2295.

Steiner, M., K, 2009. Circulation research. *American Heart Association,* 104**,** 236-244.

Stellwagen, D. & Malenka, R., C 2006. Synaptic scaling mediated by glial tnf-alpha. *Nature,* 440**,** 1054-1059.

Stevenson, M. 2003. Hiv-1 pathogenesis. *Nature Medicine,* 9**,** 853-860.

Swanson, R. A., Ying, W. & Kauppinen, T. M. 2004. Astrocyte influences on ischemic neuronal death. *Curr Mol Med,* 4**,** 193-205.

Tiwari, S., Nair, P. N. & Saxena, K. 2012. Rendezvous with tat during human immunodeficiency virus pathogenesis. *American Journal of Infectious Dieases,* 8**,** 79-91.

Trapani, J., A, & Smyth, M., J 2002. Functional significance of the perforin/granzyme cell death pathway. *Nature Reviews Immunology,* 2**,** 735-747.

Van Wagoner, N. J., Oh, J. W., Repovic, P. & Benveniste, E., N. 1999. Interleukin-6 (il-6) production by astrocytes: Autocrine regulation by il-6 and the soluble il-6 receptor. *The Journal of Neuroscience,* 19**,** 5236-5244.

Vendeville, A., Rayne, F., Bonhoure, A., Bettache, N., Montcourrier, P. & Beaumelle, B. 2004. Hiv-1 tat enters t cells using coated pits before translocating from acidified endosomes and eliciting biological responses. *Molecular Biology of the Cell,* 15**,** 2347-2360.

Volterra, A. & Meldolesi, J. 2005. Astrocytes, from brain glue to communication elements: The revolution continues. *Nature Reviews Neuroscience,* 6**,** 626-640.

Wajant, H., Pfizenmaier, K. & Scheurich, P. 2003. Tumor necrosis factor signaling. *Cell Death and Differentiation,* 10**,** 45-65.

Wallace, D. R. 2006. Hiv neurotoxicity: Potential therapeutic interventions. *Journal of Biomedicine and Biotechnology*, 1-10.

Wang, D. & Bordey, A. 2008. The astrocyte odyssey. *Progress in Neurobiology,* 86**,** 342-367.

Wesselingh, S., L, , Glass, J., D, , Mcarthur, J., C, , Griffinl, J., W, & Griffinl, D., E 1994. Cytokine dysregulation in hiv-associated neurological disease. *Advanced Neuroimmunology,* 4**,** 199-206.

Wesselingh, S., L, , Power, C., Glass, J., D, , Tyor, W., R, , Mcarthur, J., C, , Farber, J., M, , Griffin, J., W, & Griffin, D., E 1993a. Intracerebral cytokine messenger rna expression in acquired immunodeficiency syndrome dementia. *Ann Neurol,* 33**,** 576-582.

Wesselingh, S., L, , Power, C., Glass, J., D, , Tyor, W., R, , Mcarthur, J., C, , Farber, J., M,, Griffin, J., W, & Griffin, D., E 1993b. Intracerebral cytokine messenger rna expression in acquired immunodeficiency syndrome dementia. *Annals of Neurology,* 33**,** 576-582.

Wu, H., Che, X., Zheng, Q., Wu, A., Pan, K., Shao, A., Wu, Q., Zhang, J. & Hong, Y. 2014. Caspases: A molecular switch node in the crosstalk between autophagy and apoptosis. *International Journal of Biological Sciences,* 10**,** 1072-1083.

Yedavalli, V., S, , Benkirane, M. & Jeang, K., T 2003. Tat and transactivation-responsive (tar) rna-independent induction of hiv-1 long terminal repeat by human and murine cyclin t1 requires sp1. *Journal of Biological Chemistry,* 278**,** 6404–6410.

Yong, V. W., Krekoski, C. A., Forsyth, P. A., Bell, R. & Edwards, D. R. 1998. Matrix metalloproteinases and diseases of the cns. *Trends Neurosci,* 21**,** 75-80.

Zhou, B., Y,, Liu, Y., Kim, B., Xiao, Y. & He, J., J 2004. Astrocyte activation and dysfunction and neuron death by hiv-1 tat expression in astrocytes. *Mol. Cell. Neurosci.*, 27**,** 296-305.

CHAPTER 7

APPENDIX

Appendix 1

HIV-1 Tat Protein

Full length 86 amino acid HIV-1 Tat (clade B, 100µg) was resuspended in 200µl PBS:

C1 V1 = C2 V2

C2 = C1 x V1 / V2

=100µg X 1ml = C2 X 0.2ml

= 500µg/ml

Therefore resuspending Tat in 200ul PBS gave a concentration of 500µg/ml.

C1 (Tat total = 500µg/ml)

V1 (?)

C2 (Tat treatment = 10µg/ml)

V2 (Medium in each treated well = 0.5ml)

} **C1V1 = C2V2**

V1 = C2 x V2 / C1

= 10µg/ml x 0.5ml / 500µg/ml

= 0.01ml

= 10µl

The volume from resuspended Tat that gave a final concentration of 10µg/ml was 10µl.

Appendix 2

Phosphate Saline Buffer (PBS)

4 PBS tablets were dissolved in 1L distilled water. PBS solution was autoclaved in a Steridium Microdigital Autoclave (Steridium, Queensland, Australia) at 121kPa for 10mins, cooled and aliquoted in 50ml aliquots prior to use.

Appendix 3

Lipopolysaccharide (LPS)

5mg/ml solution of LPS was prepared as per package insert by the addition of 1ml endotoxin free water (included in package) to 5mg LPS. The recommended working concentration of LPS is 1ng/ml - 10µg/ml. A 1µg/ml concentration was used. From the 5mg/ml solution, 0.01ml was removed and added to 990µl PBS to make up a stock solution of 0.05mg/ml.

C1 (LPS stock = 0.05mg/ml = 50µg/ml)

V1 (?)

C2 (LPS treatment = 1µg/ml)

V2 (Medium in each treated well = 0.5ml)

C1V1 = C2V2

V1 = C2 X V2 / C1

= 1µg/ml x 0.5ml / (50µg/ml)

= 0.01ml

= 10µl

The volume from the 0.05mg/ml stock solution that gave a final concentration of 1µg/ml when added to 0.5ml medium was 10µl.

Appendix 4

1X Tris-Glycine - sodium dodecyl sulphate (TGS)

100ml 10X TGS was added to 900ml distilled water resulting in a 1X TGS solution.

Appendix 5

1X Tris-Glycine buffer (TG)

100 ml 10X TG was added to 900ml distilled water resulting in a 1X TG solution.

Appendix 6

1X Tris-Buffered Saline (TBS)

100ml 10X TG was added to 900ml distilled water resulting in a 1X TG solution to which 1ml 0.01% Tween20 (Sigma, Missouri, US) was added.

Appendix 7

5% Bovine Serum Albumin (BSA)

5g BSA was added to 100ml 1X TBS (Appendix 6), mix well.

Appendix 8

Primary Antibody

Anti-GFAP Antibody (Rabbit-polyclonal to GFAP) was diluted according to the ratio 1:714, after various rounds of optimization. 21µl antibody was diluted in 15ml 5% BSA

Appendix 9

Secondary Antibody

After various rounds of optimization the suitable dilution of Secondary Antibody (Anti-Rabbit) was obtained, (1:11000). 1.8µl antibody was diluted in 20ml 5% BSA and 0.8µl HRP was added to the solution.

Appendix 10

1X Coating Buffer

All reagents from Appendix 10 – 17 were included in the ELISA max deluxe kits. The preparation in the above-mentioned appendices was done separately for the IL-6 and TNF-α experiments. All dilutions were done for one 96 MicroWell plate.

2.4ml 5X Coating Buffer was added to 9.6ml de-ionized water.

Appendix 11

1:200 Capture Antibody

60µl Capture Antibody was added to 11.94ml 1X coating buffer.

Appendix 12

Wash Buffer

0.05% Tween 20 (0.5ml) was added to 1L PBS.

Appendix 13

1X Assay Diluent

10 ml 5X assay diluent was added to 40ml PBS.

Appendix 14

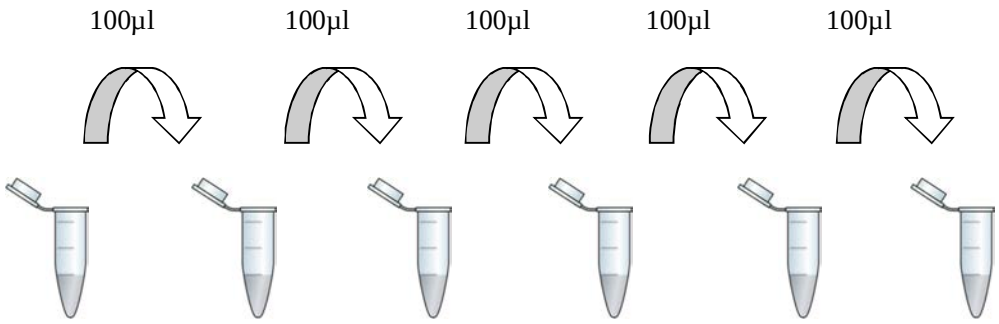
Standard and Sample Dilution

Standard Dilutions

Standard was prepared and diluted as per package insert, where six 2 fold serial dilutions were made (500, 250, 125, 62.5, 31.3, 15.6, 7.8 pg/ml) using 1X Assay Diluent.

Sample Dilutions

Six 2 fold dilutions were prepared with the samples similar to the standard preparation. 100µl 1X Assay Diluent was added to tubes 2-6.



200µl sample

Dilution	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**

Appendix 15

1:200 Detection Antibody

60µl Detection Antibody was added to 11.94ml 1X Coating Buffer.

Appendix 16

1:1000 Avidin-HRP

6µl Avidin-HRP was added to 11.88ml 1X Assay Diluent

Appendix 17

TMB Substrate Solution

6ml Substrate Solution A was added to 6ml Substrate Solution B

Appendix 18

10µM Camptothecin

1mM stock solution of Camptothecin was prepared:

0.9mg was weighed out (0.0009g) to which 1ml dimethyl sulfoxide (DMSO) (Sigma, Missouri, US) was added.

N (moles) = m (mass) /m (molar mass)

= 0.0009g / 348.4g/mol

= 0.000002583mol

C (concentration) = n (moles) / v (volume)

= 0.000002583mol / 0.001L

= 0.002583mol/l

= 2.583mM

C1 (2583µm)
V1 (?)
C2 (100µm)
V2 (1ml)

C1V1 = C2V2

V1 = C2 X V2 /C1

=1mM x 1ml / 2.583mM

= 0.387ml

= 387µl

This means that from the initial 1ml solution of Camptothecin, 387µl was removed and added to a new tube containing 913µl DMSO, which formed the 1mM stock solution.

C1 (100µm)
V1 (?)
C2 (5µm)
V2 (0.5ml)

C1V1 = C2V2

V1 = C2 X V2 /C1

= 10µm x 0.5ml / 1000µm

= 0.005ml

= 5µl

The volume from stock that gave a final concentration of 10µM Camptothecin was 5µl.

Appendix 19

1 X BD Perm/Wash

If 8 tests were carried out, 13ml of 1 X BD Perm/Wash was needed therefore:

1.3ml 10X BD Perm/Wash was added to 8.7ml distilled water.

Appendix 20

10% Ammonium persulphate

0.1g APS was added to 1 ml distilled H20

Appendix 21

Recombinant TNF-α and IL-6

Recombinant TNF-α

10µg lyophilized TNF-α was purchased and the reconstitution concentration was 0.1-1mg/ml. TNF-α was reconstituted in100µl distilled water giving a 0.1mg/ml concentration:

10µg (0.01mg) in 1000µl and 0.1mg/ml in X

X = 0.01mg x 1000µl / 0.1mg/ml

X = 100µl

From the 100µl solution of 0.1mg/ml, 100ng/ml in a specific volume was required:

C1 (Stock 0.1mg/ml)

V1 (?)

C2 (Treatment 100ng/ml = 0.0001mg/ml)

V2 (Medium per well 0.5ml)

C1V1 = C2V2

V1 = C2V2 /C1 = 0.0001mg/ml x 0.5ml / 0.1mg/ml

= 0.0005ml

= 0.5µl

The volume from the stock that gave a 100ng/ml concentration of TNF-α was 0.5µl.

Recombinant IL-6

20µg lyophilized IL-6 was purchased and the reconstitution concentration was 0.1-0.5mg/ml. IL-6 was reconstituted in 200µl distilled water giving a 0.1mg/ml concentration:

20µg (0.02mg) in 1000µl and 0.1mg/ml in X

X = 0.02mg x 1000µl / 0.1mg/ml

X = 200µl

From the 200µl solution of 0.1mg/ml, 100ng/ml in a specific volume was required:

C1 (Stock 0.1mg/ml)

V1 (?)

C2 (Treatment 100ng/ml = 0.0001mg/ml)

V2 (Medium per well 0.5ml)

C1V1 = C2V2

V1 = C2V2 / C1 = 0.0001mg/ml x 0.5ml / 0.1mg/ml

= 0.0005ml

= 0.5µl

The volume from the stock that gave a 100ng/ml concentration of IL-6 was 0.5µl.