

**HIV-ENCEPHALITIS: MECHANISMS FOR CXCL10 INDUCTION IN  
ASTROCYTES**

By

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Submitted to the graduate degree program in Molecular & Integrative Physiology  
and the Graduate Faculty of the University of Kansas  
in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy.

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ASTROCYTES**

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## **Dedication**

This work is dedicated to my parents, Bill and Jean Williams, for all their loving support.

### **Acknowledgments**

I would like to acknowledge the members of my lab, Dr. Honghong Yao, Dr. Fuwang Peng, Shannon Callen, Crystal Bethel-Brown, and especially Navneet Dhillon for all their help and guidance through the years.

I would like to thank my friends and fellow IGPBS colleagues, Argenia Doss, Laurie Shannon, and Katryn Allen for the friendship and support they have provided throughout.

I would also like to acknowledge my committee members, Dr. Norberto Gonzalez, Dr. Kee Jun Kim, Dr. Kathy Newell, and Dr. Thomas Yankee and my mentor Dr. Shilpa Buch.



## **Abstract**

With the prevalence of HIV-associated neurocognitive disorders (HAND) increasing, understanding the mechanisms by which HIV induces neuro-inflammation and subsequent neuronal damage is of paramount importance. We hypothesized that HIV-1 and IFN- $\gamma$ /TNF- $\alpha$  co-operation could increase CXCL10 expression in astrocytes through redox sensitive pathways. Our initial studies focused on determining which signaling pathways were involved in CXCL10 induction in HIV-1 and cytokine treated astrocytes. The next studies were aimed at determining which HIV-1 protein was co-operating with IFN- $\gamma$  and TNF- $\alpha$  to cause this effect. Additionally, to verify if an oxidative burst was impacting CXCL10 regulation through redox sensitive pathways we utilized apocynin, an inhibitor of NADPH oxidase. Apocynin was also able to diminish Jnk, Erk1/2, and Akt pathway activation, decrease NF- $\kappa$ B activation and decrease CXCL10 expression, improving neuronal survival. This data has implications for the development of therapeutic strategies aimed at reducing the release of pro-inflammatory agents to prevent HIV-1 neuropathogenesis.

## Table of Contents

Title page.....	i
Acceptance page.....	ii
Dedication.....	iii
Acknowledgments.....	iv
Abstract.....	v
Table of Contents.....	vii
List of Figures.....	viii
List of Abbreviations.....	x
Chapter 1: Introduction.....	1
HIV-associated Dementia (HAD).....	2
Brain inflammation and HAD.....	2
Critical role for astrocytes in HAD.....	3
Role of TNF- $\alpha$ and IFN- $\gamma$ in HAD.....	4
CXCL10 involvement in HAD.....	6
Detrimental effects of oxidative stress (OS) in HAD.....	7
Role of NADPH oxidase in HAD.....	8
Significance.....	9
Chapter 2: Pro-inflammatory cytokines and HIV-1 synergistically enhance CXCL10 expression in human astrocytes.....	13
Abstract.....	14
Introduction.....	15
Materials and Methods.....	17
Results.....	21
Discussion.....	27
Figures.....	33
Chapter 3: Dissecting out which HIV-1 protein(s) is responsible for the co-operative induction of CXCL10 in IFN- $\gamma$ and TNF- $\alpha$ stimulated astrocytes.....	43
Introduction.....	44
Materials and Methods.....	45

Results & Discussion.....	46
Figures.....	50
 Chapter 4: HIV-1 Tat co-operates with IFN- $\gamma$ and TNF- $\alpha$ to increase CXCL10 in human astrocytes.....	53
Abstract.....	54
Introduction.....	55
Materials and Methods.....	57
Results.....	60
Discussion.....	64
Figures.....	71
 Chapter 5: Co-operative induction of CXCL10 involves NADPH Oxidase: Implications for HIV Dementia.....	80
Abstract.....	81
Introduction.....	82
Materials and Methods.....	85
Results.....	89
Discussion.....	95
Figures.....	103
 Chapter 6: Conclusions.....	116
 Chapter 7: Future Directions.....	122
 References .....	126

## **List of Figures**

### **Chapter 2**

<b>Figure 1.....</b>	<b>33</b>
<b>Figure 2.....</b>	<b>34</b>
<b>Figure 3.....</b>	<b>36</b>
<b>Figure 4.....</b>	<b>37</b>
<b>Figure 5.....</b>	<b>38</b>
<b>Figure 6.....</b>	<b>40</b>
<b>Figure 7.....</b>	<b>42</b>

### **Chapter 3**

<b>Figure 1.....</b>	<b>50</b>
<b>Figure 2.....</b>	<b>51</b>
<b>Figure 3.....</b>	<b>52</b>

### **Chapter 4**

<b>Figure 1.....</b>	<b>71</b>
<b>Figure 2.....</b>	<b>73</b>
<b>Figure 3.....</b>	<b>75</b>
<b>Figure 4.....</b>	<b>77</b>
<b>Figure 5.....</b>	<b>79</b>

### **Chapter 5**

<b>Figure 1.....</b>	<b>103</b>
<b>Figure 2.....</b>	<b>105</b>

**Figure 3.....107**

**Figure 4.....109**

**Figure 5.....111**

**Figure 6.....112**

**Figure 7.....114**

## **List of Abbreviations**

HIV.....	Human Immunodeficiency Virus
AIDS.....	Acquired Immunodeficiency Syndrome
HAD.....	HIV-associated Dementia
HAND.....	HIV-associated Neurocognitive Disorders
BBB.....	Blood Brain Barrier
MAPK.....	Mitogen Activated Protein Kinases
OS.....	Oxidative Stress
ROS.....	Reactive Oxygen Species

## **Chapter 1**

### **Introduction**

### **HIV-associated Dementia (HAD)**

There are 40 million people world wide infected with Human Immunodeficiency Virus-1 (HIV-1) [1]. Shortly after infection, HIV-1 penetrates the brain eventually resulting in HIV-1 associated CNS disease [2]. Studies show that 60% of HIV-1 carriers have some form of neuropsychiatric impairment diagnosed by behavioral, cognitive, and motor abnormalities broadly termed HIV-associated neurocognitive disorders (HAND) [3]. HIV associated dementia (HAD), the most severe form of HAND [4], is clinically characterized by motor and behavioral dysfunctions leading to seizures, coma, and death within six months of onset [5]. Before the era of combinational antiretroviral therapy (cART) 20-30% of adults and 50% of children infected with HIV-1 would develop HAD [6]. Even with cART, 60% of individuals are affected by HAND and 9-11% of HIV-1 infected individuals still develop HAD [6]. HIV encephalitis (HIVE), the pathologic correlate of HAD reveals a broad spectrum of pathological changes including widespread reactive astrogliosis, multifocal and sub-acute encephalitis, focal accumulation of macrophages & multinucleated giant cells, cerebral cortical atrophy, loss of specific neuronal subpopulations, and diffuse white matter pallor [7-11].

### **Brain inflammation and HAD**

The CNS has been thought to be an immunologically privileged organ. However, increasing evidence suggests that inflammation is actively involved in the



pathogenesis of HAD [2, 6, 12, 13]. In the CNS of patients with HAD, astrocytes serve as mediators for inflammatory events, thereby controlling the cascade of actions contributing to the disruption of neuronal homeostasis and development of neurological abnormalities. HIVE is characterized by widespread astrogliosis, cytokine/chemokine dysregulation, oxidative stress, and neuronal degeneration [5, 6, 14]. Since the severity of HAD/HIVE correlates with the presence of activated astroglial and microglial cells rather than with the presence and amount of HIV-infected cells in the brain, the current thinking about the disease is that CNS injury is mainly caused by the release of neurotoxic factors by immune-activated glial cells [6, 14]. Activation of astroglia, through direct infection or their interactions with the host factors from infected cells or viral products thus contributes to HIV-associated neuropathology.

### **Critical role for astrocytes in HAD**

Since the severity of HAD/HIVE correlates with the presence of activated glial cells rather than with the viral load in the brain [6, 14] it is critical to understand the immune capabilities and consequences of immune activation within these cells. Astrocytes, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators in response to HIV-1 infiltration into the brain [14-16]. One of the main functions of astrocytes is to protect neurons from injury by metabolizing neurotoxins and regulating their homeostatic environment with the release of neurotropic factors [17]. However, once activated by

insult/injury astrocytes undergo astrogliosis characterized by proliferation and release of several different cytokines and chemokines, along with amplifying neurotoxic signals from activated microglia [18, 19]. Although HIV-1 does not productively infect astrocytes, the viral proteins Tat, Rev, and Nef are expressed in astrocytes in brain tissue derived from HAD patients [17, 20]. Tat and Nef, along with gp120, have been shown to activate several signaling pathways that lead to the dysregulation of cytokine/chemokine release and oxidative stress in astrocytes [17, 21]. This increase in cytokine/chemokine dysregulation and oxidative stress can lead to less effective astrocyte function and perhaps death. Studies show that astrocyte apoptosis can be directly correlated with the neurological symptoms of HAD [16]. Brain tissue derived from patients with HAD have significantly more apoptotic astrocytes than brain tissues from HIV-1 positive individuals who do not develop HAD [16]. These findings further demonstrate that normally functioning astrocytes are needed to maintain neuronal health and their loss results in neurological disease.

### **Role of TNF- $\alpha$ and IFN- $\gamma$ in HAD**

The neuronal apoptosis that occurs in HAD is thought to occur by indirect toxicity from the increased secretion of pro-inflammatory cytokines by activated astroglia and microglia. These pro-inflammatory cytokines play a critical role in the pathogenesis of several neurodegenerative diseases, including HAD [19, 22-24].

TNF- $\alpha$  is a pro-inflammatory cytokine produced by activated astrocytes and microglia cells in response to HIV-1 [19, 25]. Levels of this cytokine are positively

correlated with HAD disease pathogenesis with studies showing that brain tissue derived from patients afflicted with HAD demonstrate significantly higher levels of TNF- $\alpha$  mRNA than control tissues [19, 25-29]. Underscoring the importance of this pro-inflammatory cytokine in the pathogenesis of HAD is that the inhibition of TNF- $\alpha$  is able to profoundly reduce the inflammation in the brain and decrease neuronal loss in a murine model of HAD [19, 30]. TNF- $\alpha$  is able to exert its neurotoxic effects in several different ways. This cytokine can elicit increased expression of iNOS in astrocytes to increase amounts of nitric oxide, resulting in oxidative stress that can be detrimental to neuronal health [19, 25, 31]. During HIV-1 infection TNF- $\alpha$  can synergize with the HIV-1 viral proteins gp120 [32] and Tat [33] to cause oxidative stress in neurons, leading to neuronal apoptosis [19, 25, 32-34]. Furthermore, TNF- $\alpha$  can increase chemokine expression not only by itself [25, 35], but can also synergize with different host factors to increase the toxicity and inflammation in the surrounding environment [19, 25, 36-39]. One of the host cellular factors that has been shown to synergistically interact with TNF- $\alpha$  in respect to CXCL10 release is the pro-inflammatory cytokine IFN- $\gamma$  [37, 39].

IFN- $\gamma$  has been shown to be markedly increased in CNS tissues during HIV-1 infection in the brain and has been implicated in the pathophysiology of HAD [40]. IFN- $\gamma$  can exert wide ranging effects on several cell types within the brain, in particular, astrocytes and neurons. This cytokine is a known inducer for the expression of CXCL10 in several cell types including astrocytes [37, 41, 42]. This pro-inflammatory cytokine can also affect astrocytes by causing an up-regulation of

CXCR4 and CCR5, the co-receptors for HIV-1 [43] while at the same time augmenting the restricted viral replication that occurs in these cells [44, 45]. In neurons, IFN- $\gamma$  can act in concert with HIV-1 viral proteins gp120 and Tat to enhance neuronal damage by taking advantage of the dysregulation of the JAK/STAT pathway [3, 46]. This signaling pathway is critical to the regulation of proinflammatory and apoptotic signals during the inflammatory processes within the brain [3, 46]. Both IFN- $\gamma$  and TNF- $\alpha$  utilize the JAK/STAT pathway offering a mechanism by which not only the two cytokines can synergize with each other, but also with HIV-1 viral proteins to cause increased inflammation and apoptosis within the HIV-1 infected brain.

#### **CXCL10 involvement in HAD**

Chemokines in the brain have been recognized as essential elements in neurodegenerative disease and related neuroinflammation. Chemokines and their receptors are expressed by a wide variety of cells, including those intrinsic to the CNS. These proteins can regulate inflammatory responses by recruiting lymphocytes and monocytes/macrophages to areas of inflammation within the brain [47, 48]. Chemokines also contribute to injury and eventual loss of neurons [49, 50]. Cerebral expression of various chemokines and their receptors is increased in HIVE. CXCL10 (interferon  $\gamma$ -inducible peptide, or IP-10) was first identified as an early response gene induced after IFN- $\gamma$  treatment in a variety of cells [41, 42]. Interactions of soluble host factors, such as those between IFN- $\gamma$  and TNF- $\alpha$ , have been shown to

synergistically induce the expression of this chemokine [37, 51]. In addition to its induction by host factors, CXCL10 can also be induced by the HIV-1 viral proteins gp120, Tat, and Nef [52-54].

Increased levels of CXCL10 have been detected in the CSF and plasma of individuals with HIV-1 infection [55]. Additionally, brain tissue derived from patients with HAD also reveal increased expression of mRNA for CXCL10 [54, 56, 57] and this expression can be localized to astrocytes [58-60]. Levels of this neurotoxic chemokine are positively correlated with HAD disease progression [55]. It has been previously demonstrated that in SHIV-infected macaque brains with lentiviral lesion, CXCL10 is significantly up-regulated and apoptotic to neurons [57]. Treatment of fetal neuronal cultures with exogenous CXCL10 produced elevations in intracellular  $\text{Ca}^{2+}$  and this effect was modulated via the CXCL10 receptor, CXCR3 [56]. Furthermore, the increased  $\text{Ca}^{2+}$  was associated with mitochondrial membrane permeabilization and cytochrome c release, followed by activation of initiator caspase-9 and effector caspase-3, ultimately resulting in apoptosis [56]. More recently, occupation of the CXCR3 receptor by the proteolytically cleaved chemokine SDF-1 $\alpha$  has also been shown to be apoptotic for neurons [61], thereby underscoring the role of CXCL10 and its homologs in neurodegeneration. Our recent preliminary studies suggest a synergistic induction of CXCL10 in astrocytes exposed to HIV-1, IFN- $\gamma$ , and TNF- $\alpha$ , which can lead to increased inflammation and enhanced neuronal death.

### **Detrimental effects of oxidative stress (OS) in HAD**

One of the main characteristics of HIVE, and a hallmark feature of several other neurodegenerative diseases, is extensive OS in the brain [14, 62]. Oxidative stress is defined as an accumulation of reactive oxygen species (ROS), oxidized proteins and DNA, lipid peroxidation, and a depletion of reduced thiols [14, 63, 64]. In HIVE, immunohistochemistry against markers of OS reveals widespread effects concentrated in the glial cells and neurons [63]. Several studies indicate that the HIV-1 viral proteins Tat and gp120 can cause OS in astrocytes and neurons [63, 65-68]. Increasing evidence suggests that OS mediated by HIV-1 or its protein products leads to the apoptosis of astrocytes [69, 70]. Additionally, exposure of neurons to HIV-1 gp120, and/or the ROS generated from HIV-1 activated glial cells, triggers stress-induced neuronal apoptosis [66-68]. Another mechanism by which OS can mediate its effect is through intracellular signaling pathways that culminate in the activation of transcription factors impacting inflammatory cytokine/chemokine regulation [71-73]. A recent study has shown that HIV-1 induced OS in astrocytes can regulate certain genes under the control of NF- $\kappa$ B [74].

NF- $\kappa$ B is one of the crucial transcription factors activated by OS stress signaling pathways [71-73]. There is evidence that OS in astrocytes can influence the expression of the NF- $\kappa$ B regulated chemokine, MCP-1 [73]. Therefore, it is probable that CXCL10 expression, which is under the control of NF- $\kappa$ B as well, is also modulated by OS occurring within the cell. Since astrocytes are responsible for maintaining the sensitive neuronal environment, such a disruption in chemokine

release could tip the balance towards neuronal degeneration, thus presenting another mechanism for enhanced progression of HAD.

### **Role of NADPH oxidase in HAD**

Recently, the role of oxidative stress in the regulation of cytokine and chemokine expression has garnered increases awareness. One mechanism by which oxidative stress can mediate its effect is through intracellular signaling pathways that culminate in the activation of critical transcription factors [71-73]. Interestingly, immunohistochemistry against markers for oxidative stress reveal widespread effects concentrated in the glial cells and neurons in HIVE [63]. Several studies indicate that the HIV-1 Tat can cause oxidative stress in astrocytes [63, 65-68] possibly leading to cell death [66, 69, 70]. Song et. al. has shown that HIV-1 induced oxidative stress in astrocytes can regulate certain genes under the control of NF- $\kappa$ B, one of the essential transcription factors responsible for CXCL10 induction [74].

One mechanism by which oxidative stress is able to impact signaling pathways and their corresponding transcription factors is through a respiratory burst orchestrated by the activation of NADPH oxidase [71-73, 75, 76]. NADPH oxidase, a multi-subunit membrane associated enzyme, is capable of producing superoxide [77-80]. This enzyme consists of two membrane associated subunits, gp91phox and p22phox, and the cytosolic components p67phox, p47phox, p40phox, and the small GTPase Rac1/2 [77-79, 81-83]. In the active state p47phox is phosphorylated, leading to the recruitment of itself, p67phox, p40phox, and activated Rac1/2 to the

membrane bound components [78-80]. Once the cytosolic subunits have docked with the membrane associated subunits, the interaction between p67phox and gp91phox results in the transfer of electrons from NADPH oxidase to molecular oxygen, resulting in the production of superoxide [78-80, 83]. This superoxide is later converted to hydrogen peroxide, a critical redox signaling intermediate [77, 80, 84].

Due to recent studies linking NADPH oxidase activity to cytokine and chemokine production in microglia, macrophages, and astrocytes [73, 75] we became interested in whether NADPH oxidase could have a role in CXCL10 induction in human astrocytes stimulated with HIV-1 Tat and the cytokines IFN- $\gamma$  and TNF- $\alpha$ .

### **Significance**

HIV encephalitis (HIVE), the pathologic correlate of HAD is characterized by astrogliosis, cytokine/chemokine dysregulation, oxidative stress, and neuronal degeneration [5, 6, 14]. The severity of HAD/HIVE seems to correlate with the presence of activated glial cells rather than with the presence and amount of HIV-infected cells in the brain [6, 14, 85]. The current thinking about the disease is that CNS injury is mainly caused by the release of neurotoxic factors, such as CXCL10, by immune-activated glial cells [6, 14]. Alone, both HIV-1/HIV-1 Tat or the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  can induce CXCL10 release from astrocytes. However, the interplay between HIV-1/HIV-1 Tat and host factors greatly exacerbates the effect on CXCL10 production, thus contributing to HIV-associated neuropathology. Along with marked cytokine/chemokine dysregulation, another



main characteristic of HIVE, and a hallmark feature of several other neurodegenerative diseases, is extensive OS in the brain [14, 62].

One mechanism by which OS can mediate its effect is through intracellular signaling pathways that culminate in the activation of transcription factors impacting inflammatory cytokine/chemokine regulation [71-73]. NF- $\kappa$ B, a known regulator of CXCL10 transcription, is one of the transcription factors affected by OS mediated signaling, presenting another mechanism by which the synergistic induction of CXCL10 in virus and host factor stimulated astrocytes may occur [74].

Studies outlined in this thesis, designed to understand the molecular mechanisms involved in the induction of CXCL10 by viral and host factors, will therefore provide a key to developing therapeutic strategies against HAD. One approach for developing such a strategy is the use of the antioxidant and NADPH oxidase inhibitor, apocynin, as a therapy to reduce the amount of CXCL10 and neuronal loss in the brain. The ultimate goal of this thesis is to test the hypothesis that an antioxidant therapy can lower the neurotoxic levels of CXCL10 and reduce neuronal damage/loss. If this form of intervention is successful this methodology could be used in conjunction with the currently available antiretroviral drugs, which although successful in treating the periphery, are often ineffective in the CNS. This approach could also be applicable as a therapy for other types of chronic inflammatory processes in the brain.

This hypothesis was tested by exploring the following questions:

**Question 1:** What is the molecular mechanism(s) by which HIV-1 co-operates with

IFN- $\gamma$  and TNF- $\alpha$  to induce CXCL10 expression in astrocytes? This question is addressed in Chapter 2.

**Question 2:** Which HIV-1 viral protein(s) is co-operating with IFN- $\gamma$  and TNF- $\alpha$  to enhance CXCL10 release from astrocytes and by what mechanism? This question is addressed in Chapters 3 and 4.

**Question 3:** What is the role of oxidative stress in the induction of CXCL10, and can the

NADPH oxidase inhibitor, apocynin, be used as a therapy for reducing CXCL10 release and concomitant neuronal damage? This question is addressed in Chapter 5.

## **Chapter 2**

### **Pro-inflammatory cytokines and HIV-1 synergistically enhance CXCL10 expression in human astrocytes**

## **Abstract**

HIV encephalitis (HIVE), the pathologic correlate of HIV-associated dementia (HAD) is characterized by astrogliosis, cytokine/chemokine dysregulation and neuronal degeneration. Increasing evidence suggests that inflammation is actively involved in the pathogenesis of HAD. In fact, the severity of HAD/HIVE correlates more closely with the presence of activated glial cells than with the presence and amount of HIV-infected cells in the brain. Astrocytes, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators, including interferon- $\gamma$  inducible peptide-10 (CXCL10), a neurotoxin and a chemoattractant, implicated in the pathophysiology of HAD. Additionally, the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , are also markedly increased in CNS tissues during HIV-1 infection. In the present study we hypothesized that the interplay of host cytokines and HIV-1 could lead to enhanced expression of the toxic chemokine, CXCL10. Our findings demonstrate a synergistic induction of CXCL10 mRNA and protein in human astrocytes exposed to HIV-1 and the pro-inflammatory cytokines. Signaling molecules, including JAK, STATs, MAPK (via activation of Erk1/2, AKT, and p38), and NF- $\kappa$ B were identified as instrumental in the synergistic induction of CXCL10. Understanding the mechanisms involved in HIV-1 and cytokine mediated up-regulation of CXCL10 could aid in the development of therapeutic modalities for HAD.

## Introduction

There are more than 40 million people infected with human immunodeficiency virus (HIV)-1 worldwide. Approximately 10-15% of HIV-1 infected individuals suffer from CNS pathologies including HIV-associated encephalitis (HIVE) and HIV-associated dementia (HAD), collectively termed NeuroAIDS [2, 5, 86]. HIVE, the pathologic correlate of HAD is characterized by increased astrogliosis, microglial activation, enhanced expression of inflammatory mediators and neuronal dysfunction/death [5, 6, 14]. While the exact mechanism by which HIV-1 causes these neuropathologies is not completely understood, increasing evidence suggests neuronal damage results in part from microglial and astroglial mediated inflammation [6, 14, 87]. In fact, the severity of HIVE/HAD seems to correlate better with the presence of activated glial cells than with the presence and number of HIV-infected cells in the brain [6, 14, 85].

Brain tissue derived from patients with HAD reveals increased expression of mRNA for the chemokine, CXCL10, which is both a neurotoxin and a chemoattractant [54, 57]. Astrocytes, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators, including CXCL10 [14-16]. Additionally, the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , are markedly increased in CNS tissues during HIV-1 infection in the brain and are implicated in the pathophysiology of HAD [26, 40]. While both the cellular (IFN- $\gamma$  and TNF- $\alpha$ ) [37, 39, 88, 89] and viral factors (Tat and gp120) [52, 53] induce

CXCL10, it remains unclear how the interplay of host factors and virus modulate chemokine expression.

Because the severity of HAD/HIVE correlates with the presence of activated glial cells rather than with the viral load in the brain [6, 14], the current thinking about the disease is that CNS injury is mainly caused by the release of neurotoxic factors from immune-activated glial cells. These secreted host immune factors are capable of exerting toxicity on their own or in concert with HIV-1/HIV-1 proteins to increase the pathogenic effects on the neurons [6, 14].

Chemokines in the brain have been recognized as essential elements in neurodegenerative disease and related neuroinflammation through their regulation of inflammatory responses [41, 47, 48] thereby contributing to injury and eventual loss of neurons [49, 50]. Cerebral expression of various chemokines, including CXCL10 (interferon  $\gamma$ -inducible peptide, or IP-10), and their receptors are increased in HIV-E [55, 58-60]. Increased levels of CXCL10 have been detected in the CSF and plasma of individuals with HIV-1 infection [55] and in the brains of individuals with HAD [58-60]. Importantly, CXCL10 levels in the CNS of HIV-1 infected individuals correlated positively with disease progression (28). There is also evidence that CXCL10 participates in the neuropathogenesis of SHIV-infected macaques [90, 91] by contributing to the degeneration of neurons possibly through activation of a calcium dependent apoptotic pathway [56, 57]. Increased CXCL10 levels were critical for the increased migration of inflammatory cells into the CNS, a hallmark feature of HAD [5, 8].

Additionally, the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , are also markedly increased in CNS tissues during HIV-1 infection and have been implicated in the pathophysiology of HAD [19, 26, 40]. Furthermore, IFN- $\gamma$  and TNF- $\alpha$ , interact to synergistically up-regulate CXCL10 expression [37, 51]. Besides its induction by host factors, CXCL10 can also be induced by the HIV-1/HIV-1 proteins [52-54]. Due to the potentially neurotoxic function of CXCL10 in disease states, it is crucial to analyze how combinatorial interactions of the virus and host factors can lead to an increased pool of this toxic chemokine in the CNS. There is a paucity of information on the combined effects of IFN- $\gamma$ /TNF- $\alpha$  with HIV-1 on CXCL10 expression. In the current study we hypothesized that IFN- $\gamma$  and TNF- $\alpha$  not only synergize with each other, but also have the potential to synergize with HIV-1 to induce CXCL10 expression in astrocytes. To our knowledge, this is the first published report characterizing the synergistic enhancement of astroglial CXCL10 expression by IFN- $\gamma$ /TNF- $\alpha$  and HIV-1. Furthermore, our findings demonstrate that this synergistic increase in CXCL10 expression involves the signaling cascades of the JAK/STAT and MAPK pathways. Together, these insights may be instrumental in the development of therapeutic strategies aimed at treating or preventing HIV-1 neuropathogenesis.

## **MATERIALS AND METHODS**

**Astrocyte cell culture and treatments:** Primary human astrocytes (cat# HA1800; ScienCell Research Laboratories, Carlsbad, CA) were plated on poly-L-lysine coated

plates ( $2\mu\text{g}/\text{cm}^2$ ) at a density of  $5,000\text{ cells}/\text{cm}^2$  in Astrocyte Medium (cat.# 1801) containing 2% FBS, growth supplement (cat.# 1852), and penicillin/streptomycin solution (cat.# 0503) as described by the supplier. Primary human astrocytes were allowed to grow for two weeks in order to reach 90% confluence. The human astrocytic cell line, A172 (ATCC #CRL-1620; American Type Culture Collection, Manassas, VA), were grown to confluency in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 50 U/ml penicillin, 0.05 mg/ml streptomycin and  $2\mu\text{g}/\text{ml}$  amphotericin B. All cultures were maintained in a humidified incubator at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  and 95% air and culture medium replenished every 48-72 hours. Cells were serum-starved for 24 hours prior to treatment. Cells (triplicate or quadruplicate wells) were treated for 6-12 hrs (A172) or 24 hours (primary astrocytes), with: 1) a combination of the cytokines IFN- $\gamma$  (100ng/ml) and TNF- $\alpha$  (30ng/ml), 2) HIV-1 NL4.3 (multiplicity of infection of 0.01) [92], or 3) HIV-1 NL4.3 and cytokines. The rationale for using CXCR4 (X4)-tropic NL4.3 in these studies is based on published reports indicating increased activation of astrocytes by X4 viruses [18, 93]. In addition, X4 viral strains are capable of penetrating the blood brain barrier during the late stages of infection and have a greater impact on intracellular signaling and apoptosis in astrocytes and neurons than their R5 counterparts [94].

The following specific pharmacological inhibitors were used at the final concentration specified: PI3-K Inhibitor LY294002, PLC inhibitor U73122, MEK inhibitor U0126, JAK inhibitor I, JNK inhibitor II, P38 inhibitor SB203580, and the



PLC inhibitor U73122 (all at 20 $\mu$ M, Calbiochem, Gibbstown, NJ), and NF- $\kappa$ B inhibitor N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) (2 $\mu$ M, Sigma, St. Louis, MO). Inhibitor concentrations chosen for this study were based on published reports demonstrating their effectiveness in inhibiting the respective signaling molecules [95-99].

**CXCL10 mRNA analysis:** RNA was extracted from A172 astrocytes that were either untreated or treated with HIV-1 NL4.3 and/or the cytokines IFN- $\gamma$  and TNF- $\alpha$  using TRIzol reagent following the 6 hour treatment period (Invitrogen Life Technologies). Quantitative analysis of CXCL10 mRNA was done by Real-Time RT-PCR using the SYBR Green detection method. RT2 PCR primer pair set for CXCL10 was obtained from SuperArray Bioscience and amplification of CXCL10 from first strand cDNA was performed as described earlier [100] using ABI Prism 7700 sequence detector. Data were normalized using Ct values for the house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) in each sample. To calculate relative amounts of CXCL10, the average Ct value of the HPRT was subtracted from that for each target gene to provide changes in Ct value. The fold change in gene expression (differences in changes in Ct value) was then determined as log<sub>2</sub> relative units.

**CXCL10 protein analysis by ELISA:** Supernatants collected from both primary human astrocytes and A172 astrocytes that were either untreated or treated with HIV-

1 and/or cytokines, were examined for secreted CXCL10 protein levels using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

**Western Blot Analysis:** Treated A172 cells were lysed using the Mammalian Cell Lysis kit (Sigma, St. Louis, MO) and the NE-PER Nuclear and Cytoplasmic Extraction kits (Pierce, Rockford, IL). Equal amounts of the corresponding proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (12%) in reducing conditions followed by transfer to PVDF membranes. The blots were blocked with 5% non fat dry milk in phosphate buffered saline. Western blots were then probed with antibodies recognizing the phosphorylated forms of Erk1/2, AKT, P38, p706S (Cell Signaling, Danvers, MA 1:200), PI3-K (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100), Stat1- $\alpha$  and Stat-3 (Cell Signaling, 1:500), NF- $\kappa$ B p65 (Cell Signaling, 1:1000), and  $\beta$ -actin (Sigma, St. Louis, MO, 1:4000). The secondary antibodies were alkaline phosphatase conjugated to goat anti mouse/rabbit IgG (1:5000). Signals were detected by chemiluminescence (CDP-star; Tropix, Bedford, MA).

**Immunocytochemistry:** Immunocytochemical analysis for NF- $\kappa$ B activation was performed on A172 astrocytes cultured on coverslips and treated with HIV-NL4.3 and cytokines for 60 minutes. Following treatment cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature followed by permeabilization with 0.5% Triton X 100 in PBS. Cells were then incubated with a blocking buffer

containing 5% BSA in PBS for 1 hour at room temperature. Following blocking, anti-human NF- $\kappa$ B p65 rabbit polyclonal antibody (1: 500, Cell Signaling) was added to each coverslip and incubated for 2 hours at room temperature. Finally the secondary antibody, AlexaFluor 488 goat anti-rabbit IgG, was used at a 1:1000 dilution for 2 hours to view NF- $\kappa$ B activation in cells. DAPI was used to stain the cell nuclei. Fluorescent digital images were obtained using a Zeiss LSM510 confocal microscope equipped with an Argon/2 laser (25 mW) for the excitation (488 nm) and detection (band pass 505–530 nm filter; BP505–530) of the Alexa Fluor 488. Images were acquired in Multitrack channel mode (sequential excitation/emmission) with LSM510 (version 3.2) software and a Plan-Apochromat objective with a zoom factor of 1 or 2 and frame size of 1024 x 1024 pixels. Detector gain was set initially to cover the full range of all of the samples and background corrected by setting the amplifier gain, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. Control coverslips comprised of: 1) cells without any secondary antibody treatment and, 2) cells treated with secondary antibody only.

## **Results**

**Synergistic induction of CXCL10 in astrocytes exposed to HIV-1 and pro-inflammatory cytokines.** Astrocytes, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators in response to HIV-1 infiltration into the brain [14-16]. Several studies have shown that IFN- $\gamma$  and TNF- $\alpha$  can synergistically induce CXCL10 in several cell types, including astrocytes [43, 101]. Other studies have shown that HIV-1/HIV-1 proteins can also

induce CXCL10 expression [52-54]. The objective of this study was to determine whether HIV-1 in conjunction with the pro-inflammatory cytokines IFN- $\gamma$ , and TNF- $\alpha$  can synergistically impact the expression of CXCL10 in astrocytes. Serum-starved A172 cells were treated with CXCR4 tropic HIV-1 NL4.3 and/or the cytokine mix (IFN- $\gamma$  and TNF- $\alpha$ ) for six hours, following which the cells were lysed in Trizol for RNA extraction and Real Time RT-PCR analysis for CXCL10. As shown in Fig. 1 there was a dramatic induction of CXCL10 RNA (about 80,000 fold) in cells treated with both HIV-1 and the cytokine mix compared with astrocytes treated with either the cytokine mix or virus alone.

The next step was to determine whether such a dramatic increase in CXCL10 mRNA also translated into a concomitant increase in CXCL10 protein levels. Serum-starved A172 cells were treated with HIV-1 NL4.3 in presence or absence of the cytokine mix for 12 hrs and CXCL10 release in the supernatant was quantified by CXCL10 ELISA. As shown in Figure 2A, virus and cytokine treatment of the astrocyte cell line resulted in a dramatic up-regulation of CXCL10 protein expression. The phenomenon of CXCL10 protein induction by combination of HIV and cytokines was also reproduced in primary normal human astrocytes (Fig.2B). Astrocytes exposed to the cytokines and inactivated virus (UV and heat treated), on the other hand, failed to demonstrate virus-mediated synergy of CXCL10 induction. Conversely, UV-inactivated virus was able to synergize with the cytokines with respect to CXCL10 release (data not shown), indicating thereby that it is not the infectious virus, rather it is the viral protein(s) that could be mediating the effect. Heat

treatment of the virus would render the denaturation of proteins and thus negatively impact the synergy. This could explain the lack of synergy observed with cytokines and heat-inactivated virus. These data confirmed the importance of HIV-1 in the synergistic enhancement of astroglial CXCL10 expression.

### **Signaling pathways involved in HIV-1 and cytokine-mediated synergistic induction of CXCL10.**

#### **Role of the JAK-STAT pathway.**

Since IFN- $\gamma$  and TNF- $\alpha$  in conjunction with HIV-1 synergistically induced expression of CXCL10, and because IFN- $\gamma$  is known to mediate its effects primarily via the JAK/STAT1 signaling pathways [3, 43, 101, 102] we chose to assess the role of this signaling pathway in stimulated astrocytes. STAT proteins are a family of transcription factors that are present in many cell types and function as major signal transduction pathway in IFN- $\gamma$  signaling. Following the binding of ligands to their receptors, JAKs are activated and, in turn, phosphorylate STAT-1 $\alpha$  and/or STAT 3 proteins. Phospho-STATs dimerize and translocate into the nucleus, binding to the interferon stimulated response element (ISRE) on the promoter regions of target genes, such as CXCL10 [103]. Since IFN- $\gamma$  is a major inducer of CXCL10 expression and efficiently induces its transcription without intervening protein synthesis, we rationalized that augmented induction of CXCL10 by HIV-1 in co-operation with IFN- $\gamma$ /TNF- $\alpha$ , must involve increased activation of the JAK/STAT1 signaling system in astrocytes.

Western blot analysis of the nuclear extracts from astrocytes treated with the virus and cytokines showed time-dependent activation of both STAT-1 $\alpha$  and STAT-3 (Fig. 3A). As shown in Fig. 3B, pre-treatment of A172 astrocytes with the JAK inhibitor followed by stimulation with HIV-1 and cytokines resulted in a substantial decrease in CXCL10 protein expression to a level lower than that achieved with cytokines alone. These findings suggested a crucial role for JAK/STAT signaling in the synergistic induction of CXCL10 in HIV-1 and IFN- $\gamma$ /TNF- $\alpha$  treated astrocytes.

### **Role of Mitogen activated protein kinase (MAPK) signaling pathways**

Mitogen activated protein kinase (MAPK) activation is critical in regulating inflammatory responses, such as cytokine/chemokine secretion in response to multiple stimuli [104-107]. Therefore, we next explored the involvement of Erk1/2 and p38 MAP kinases in the regulation of CXCL10 induction by HIV-1 and cytokine mix. As shown in Figure 4A, A172 cells stimulated with HIV-1 and the cytokine mix demonstrated a time-dependent activation of Erk1/2. Confirmation of the specificity of Erk1/2 in the synergistic induction of CXCL10 was further carried out by examining the chemokine expression by ELISA in the supernatants collected from virus and cytokine-stimulated A172 cells in the presence of the pharmacological inhibitor of MEK signaling, U0126. As shown in Figure 4B, levels of CXCL10 were significantly decreased in the presence of the inhibitor, thus confirming the role of Erk1/2 MAPK signaling pathway in the induction of CXCL10.

We next assessed the role of another MAPK signaling pathway, p38 in the synergistic induction of CXCL10 in astrocytes. A172 cells stimulated with the virus and cytokines led to the activation of p38 protein as early as 10 min following stimulation with peak activation attained at 30 min (Fig. 4A). To determine the functional role of p38 pathways in CXCL10 regulation, cells were treated with SB203580, a pharmacological inhibitor of p38 MAPK and assessed for CXCL10 release in the supernatant by ELISA (Fig. 4B). In the presence of the inhibitor synergistic increase of CXCL10 with HIV-1 and cytokine mix was significantly decreased thereby indicating the importance of p38 signaling pathway in the induction of the chemokine.

#### **Role of Phospho-inositol 3-kinase (PI3-Kinase) signaling pathway**

Phospho-inositol 3-kinase (PI3-K) is a signaling protein that can be activated by various stimuli and is critical in the regulation of extracellular signals along with modulation MAPK activity [108]. To dissect the role of the PI3-K pathway in HIV-1 and cytokine-mediated synergy of CXCL10 expression, cell lysates from A172 stimulated cells were examined for phosphorylation of PI3-Kinase and its downstream effectors, Akt and p70S6 kinase. As shown in Figure 5A, there was a temporal activation of the signaling proteins of the PI3-Kinase pathway in cells stimulated with the virus and cytokines. PI3-K, which is directly upstream of Akt, was activated as early as 10-15 min, followed by activation of Akt at around 30-120 min and this, in turn, was followed by phosphorylation of p70S6 at 60-120 min (Fig. 5A). To address the functional role of PI3-K in the synergistic induction of CXCL10,

A172 cells were pretreated with the PI3-K inhibitor, LY294002, and with the PLC inhibitor U73122 as a negative control, followed by stimulation of the cells with HIV-1 and the cytokines, then subsequently monitoring the supernatants for CXCL10 expression. As shown in Fig. 5B, pretreatment of A71 cells with the PI3-K inhibitor led to a significant decrease in the synergistic induction of CXCL10 expression, while inhibition of PLC did not significantly decrease CXCL10 levels.

### **NF- $\kappa$ B Signaling**

The transcription factor NF- $\kappa$ B plays a pivotal role in inflammatory and immune responses [3, 109]. This family of transcription factors is present in the cytosol in an inactive state complexed with the inhibitory I $\kappa$ B proteins. Activation occurs via the phosphorylation of the inhibitory protein and the subsequent release of active p65 subunit of NF- $\kappa$ B [110]. Both TNF- $\alpha$  and IFN- $\gamma$  have been shown to synergistically increase CXCL10 expression through the transcription factor NF- $\kappa$ B [37, 39, 88, 89]. We next examined the role of NF- $\kappa$ B in the synergistic induction of CXCL10 by HIV-1 and cytokines. Nuclear lysates were isolated from A172 cells stimulated with the virus and host factors for different time intervals and probed for the release of p65 subunit of NF- $\kappa$ B in the nuclear fraction by Western blot analysis (Fig. 6A). Stimulation of astrocytes with the virus and cytokines induced time-dependent nuclear translocation of NF- $\kappa$ B p65, which was observed by 10 min and peaked by 60 min as indicated by Western blot analysis (Fig. 6A).



Immunocytochemistry was used to further visualize the nuclear translocation of NF- $\kappa$ B. Figure 6B clearly shows the nuclear translocation of NF- $\kappa$ B p65 in virus and cytokine treated cells versus control untreated cells, where the NF- $\kappa$ B is seen localized to the cytoplasm. To further confirm the role of NF- $\kappa$ B in the synergistic induction of CXCL10 in HIV-1 and cytokine stimulated astrocytes, we pre-treated the cells with TPCK, an inhibitor of NF- $\kappa$ B followed by stimulation of cells. Cell supernatants were then monitored for CXCL10 expression by ELISA. There was a significant decrease in the amount of CXCL10 released from astrocytes stimulated with the virus and cytokine mixture in the presence of TPCK (Fig. 6C) compared with cells not pre-treated with the inhibitor. Clearly, NF- $\kappa$ B plays a pivotal role in the synergistic induction of CXCL10 in astrocytes activated by the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$  in conjunction with HIV-1.

**Discussion:** Despite the use of combinational antiretroviral therapy (cART) HIV-associated cognitive impairments afflict 60% of the HIV-1 infected population [3, 101]. HAD, the most severe form of these impairments [4, 102], is pathologically characterized by astrogliosis, cytokine/chemokine imbalance, and neuronal degeneration [5, 6, 14]. Because of the crucial role astrocytes play in maintaining neuronal homeostasis, astrocyte hyper-activation can severely impact the neuronal environment, resulting in disease pathogenesis [103, 111].

During HIV-1 infection there is an imbalance of the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , which have been shown to be markedly increased in CNS tissues

[26, 40]. In addition to cytokine dysregulation, enhanced expression of chemokines, such as CXCL10 and MCP-1, is also known to correlate with the severity of HAD [112]. Functionally, astroglial CXCL10 recruits inflammatory cells into the CNS, and has also been demonstrated to exhibit potent neurotoxic effects [56, 57]. Consequently, it is critical to comprehend the regulation of the CXCL10 expression in the inflamed brain. Therefore, the objective of this study was to understand how these dysregulated host cytokines can interact with HIV-1 in astrocytes to amplify CXCL10 expression in the CNS.

Due to the ability of CXCL10 expression to be regulated by diverse stimuli and studies demonstrating the synergistic effects of IFN- $\gamma$  and TNF- $\alpha$  on CXCL10 in multiple cell types [104-106, 113-115], we evaluated whether these two cytokines in combination with HIV-1 could further increase CXCL10 expression in astrocytes. Based on our findings, it was evident that HIV-1 in combination with the pro-inflammatory cytokines, synergistically enhanced CXCL10 mRNA and protein expression in both the human A172 astroglia and primary human astrocytes. These findings have implications for increased neuronal toxicity in HIV-1-infected individuals and underscore the importance of host-virus interactions in the pathogenesis of HAD.

Having determined the co-operative interaction of HIV-1 with the pro-inflammatory cytokines, it was of interest to explore the signaling pathways involved in this synergy. IFN- $\gamma$  activates the JAK/STAT pathway through the  $\alpha$ - and  $\beta$ -subunits [103, 107, 108, 116] leading to activation of JAK1 and JAK2 kinases

followed by tyrosine phosphorylation of STAT1 [103, 107, 109, 110, 117, 118]. Complete activation of STAT 1 by IFN- $\gamma$  requires phosphorylation of serine 727 in addition to tyrosine 710 [4, 111, 119, 120]. Herein, we found nuclear translocation and time-dependent phosphorylation of both STAT1 and STAT 3, with activation as early as 15 min following stimulation of cells. Inhibition of JAK, an upstream effector of STAT1/3, through a specific pharmacological inhibitor drastically reduced the amount of CXCL10 expressed by stimulated astrocytes. These findings suggested that JAK/STAT pathway played a crucial role in the cooperative induction of CXCL10 in IFN- $\gamma$ , TNF- $\alpha$ , and HIV-1 stimulated astrocytes. These findings are in agreement with those reported by Dhillon *et. al.* on the synergistic induction of CXCL10 by IFN- $\gamma$  and PDGF in macrophages [99, 113].

Since HIV-1/viral proteins, TNF- $\alpha$ , and IFN- $\gamma$  all have the ability to activate MAPK signaling cascades [52, 53, 114, 121, 122], we next investigated the role of these pathways in the induction of CXCL10 in stimulated astrocytes. In congruence with the findings by Lee *et. al.* on gp120-stimulated macrophages [108, 115], we also found that both the Erk1/2 and p38 MAPK pathways were strongly activated following stimulation of astrocytes with HIV-1 and cytokines. Both of these pathways have also been implicated in the induction of proinflammatory genes [99, 113] and such activation is responsible for the transcriptional stabilization of the target proinflammatory genes [99, 113]. Thus it is possible that these pathways are critical for the autocrine feed forward loop involved in the maintenance of the pro-inflammatory state associated with HAD [108, 115].

Furthermore, we also demonstrated temporal activation of the PI3-K pathway, involving sequential phosphorylation of Akt and its downstream p70S6 kinase. Confirmation of the role of this pathway in CXCL10 expression was examined by pre-treating the cells with the PI3-K inhibitor, LY294002. A significant decrease in the amount of CXCL10 was observed in stimulated cells pre-treated with the inhibitor compared with the cells not exposed to the inhibitor. Interestingly, a similar pathway has been reported in the enhanced expression of TNF- $\alpha$  in gp120-treated macrophages [108, 123]. Furthermore, Western Blot analysis demonstrated clear evidence of Akt activation, a critical survival factor [124-126] and a downstream target of PI3-K. Taken together, these results indicate a role for the PI3-K pathway in CXCL10 induction in virus and cytokine stimulated astrocytes.

Several studies have shown that astrocytes activated by HIV-1/viral proteins have increased nuclear translocation and activation of the transcription factor NF- $\kappa$ B [112, 120, 121, 127, 128], which, in turn, can regulate CXCL10 expression. Since activation of the Erk1/2, p38, and PI3-K signaling pathways can converge on a common transcription factor such as NF- $\kappa$ B, and since the CXCL10 promoter has NF- $\kappa$ B binding sites [37, 39, 123, 129], we next examined the activation and translocation of NF- $\kappa$ B in stimulated astrocytes. Our findings showed dramatic and sustained activation of NF- $\kappa$ B in the nucleus of stimulated astrocytes. These findings are consonant with other reports implicating the role of NF- $\kappa$ B in the regulation of various chemokines and cytokines, such as MCP-1 and IL-6, in astrocytes [112, 120, 124, 130]. The role of NF- $\kappa$ B was further confirmed by pre-treating the cells with

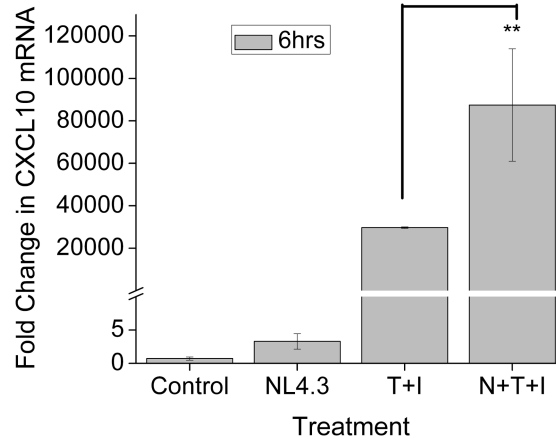
TPCK, a NF- $\kappa$ B inhibitor, which resulted in significant decrease in expression of CXCL10. These findings underscore the role of this transcription factor in the synergistic induction of CXCL10.

As mentioned earlier, the CXCL10 promoter region has two NF- $\kappa$ B sites and one ISRE site. The NF- $\kappa$ B site,  $\kappa$ B2, in conjunction with the ISRE site are necessary for the synergistic induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  simulated astrocytes [88]. Both IFN- $\gamma$  and TNF- $\alpha$  have been shown to activate the JAK/STAT and MAPK/NF- $\kappa$ B pathways. HIV-1/viral proteins also have the ability to activate the MAPK pathways Erk1/2, JNK and p38 [53] in astrocytes, and viral gp120-mediated induction of CXCL10 is independent of the STAT1 pathway [52, 122].

Thus, while activation of MAPK and NF- $\kappa$ B can be attributed to all the three stimuli in astrocytes, STAT 1 activation is unique to IFN- $\gamma$  and thus IFN- $\gamma$  plus TNF- $\alpha$ . It has been well documented that activation of the MAPK pathways by HIV-1/viral proteins can lead to the activation and nuclear translocation of NF- $\kappa$ B [112, 128] potentially increasing the amount of NF- $\kappa$ B available to the  $\kappa$ B2 site essential for synergistically up-regulating CXCL10. Therefore, while IFN- $\gamma$  is activating the JAK/STAT pathway providing for the binding of the ISRE site on the CXCL10 promoter, all three stimuli can impact the amount of NF- $\kappa$ B needed for the binding of the two NF- $\kappa$ B binding sites. In addition, none of the inhibitors tested were able to totally abolish the release of CXCL10 in our study thereby indicating the involvement of more than one pathway in the synergy. This does not however, preclude cross talk between the pathways.

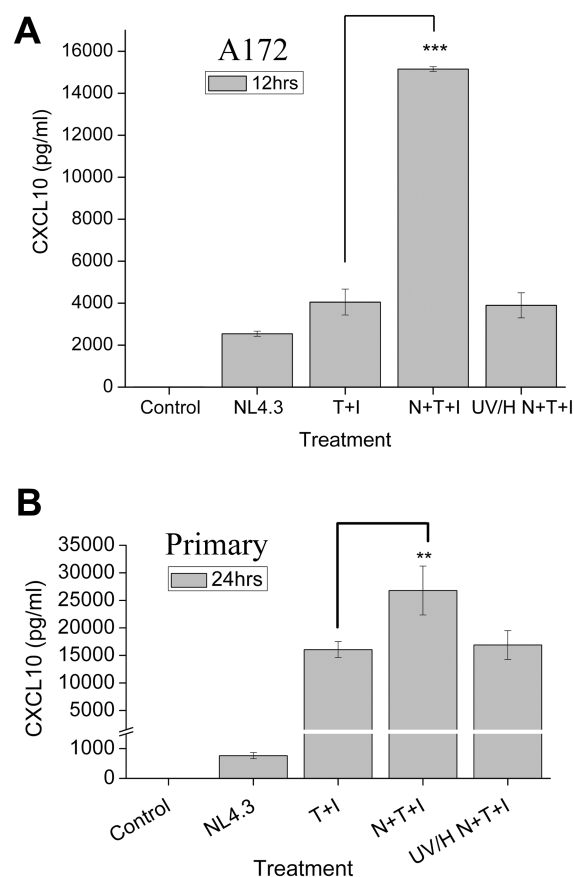
In conclusion, we have shown that HIV-1 NL4.3 in co-operation with the cytokines IFN- $\gamma$  and TNF- $\alpha$  is capable of synergistically inducing CXCL10 in human astrocytes at both the RNA and protein levels. This induction is likely due to the activation of the JAK/STAT and PI3-K signaling pathways, along with the MAPK pathways, Erk1/2 and p38 (Fig. 7). The significance of the potential synergistic interactions between HIV-1 and soluble host factors and the implications of this type of complex interplay on the progression of HAD is progressively garnering more attention. Our studies demonstrating the signal transduction pathways activated by HIV-1 and the pro-inflammatory cytokines in the enhancement of CXCL10 expression, define a precarious proinflammatory mechanism that exacerbate the pathogenesis of HAD. Due to the neurotoxic potential of CXCL10 these findings lend to important implication in the progression of AIDS-associated dementia. The consequences of CXCL10 over expression may include amplified neuronal dysfunction and death, as well as an enhanced influx of inflammatory cells into the CNS, a combination that creates elevated toxic, pathological responses characteristic of end-stage HAD. These findings have implications in the development of therapeutic strategies aimed at inhibiting glial cell activation to prevent HIV-1 neuropathogenesis.

**Figure 1**



**Figure 1:** Real Time PCR analysis showing a significant increase in CXCL10 RNA in virus and cytokine treated A172 astrocytes. Cells were stimulated with HIV-1 NL4.3 (moi of 0.01), the cytokines IFN- $\gamma$  (100ng/ml) and TNF- $\alpha$  (30ng/ml), or both for 6 hrs followed by total cell lysis and RNA extraction. The graph shows an 80,000 fold induction of CXCL10 RNA in the virus and cytokine stimulated astrocytes over the untreated astrocytes. The average Ct value of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was subtracted from that of the CXCL10 gene to give changes in Ct (dCt). The fold change in gene induction (differences in dCt, or ddCt) was then determined as  $\log_2$  relative units. The data represents the mean  $\pm$  SD from three independent experiments (\*\*,  $p < 0.01$ ).

**Figure 2**

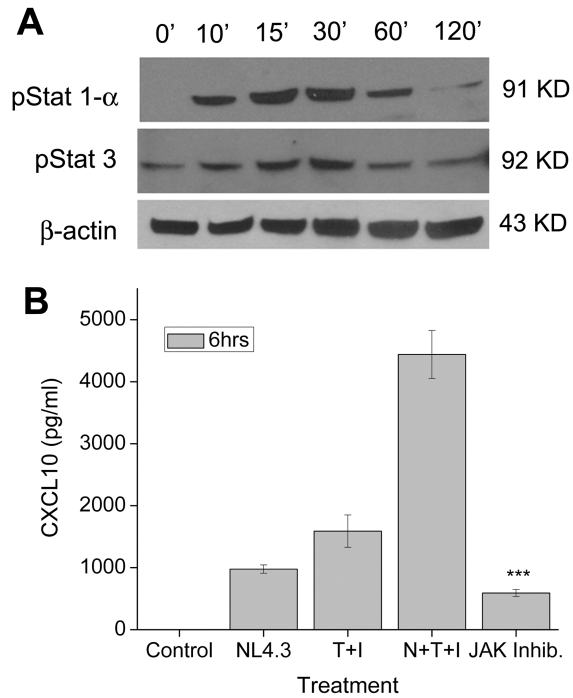


**Figure 2:** Synergistic induction of CXCL10 protein in astrocytes treated with virus and cytokines. Supernatants from cells treated with either virus or cytokine or both virus and cytokines were collected at **A)** 12hrs for A172 cells and **B)** 24hrs for primary human astrocytes and analyzed for CXCL10 protein levels by ELISA. Both A172 and primary human astrocytes showed a significant increase in CXCL10 protein levels in the cells treated with virus in conjunction with the cytokines, then either treatment alone. The data represents the mean  $\pm$  SD from three independent



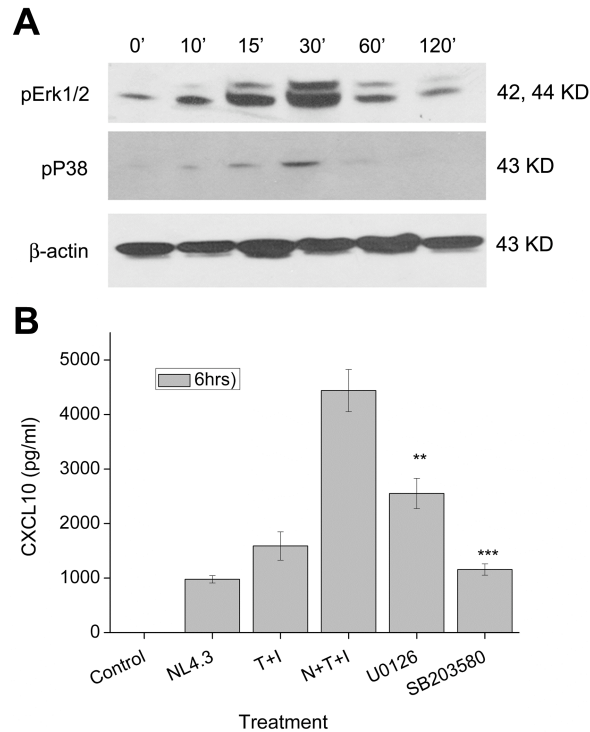
experiments. Statistical significance from independent experiments was calculated (\*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ ).

**Figure 3**



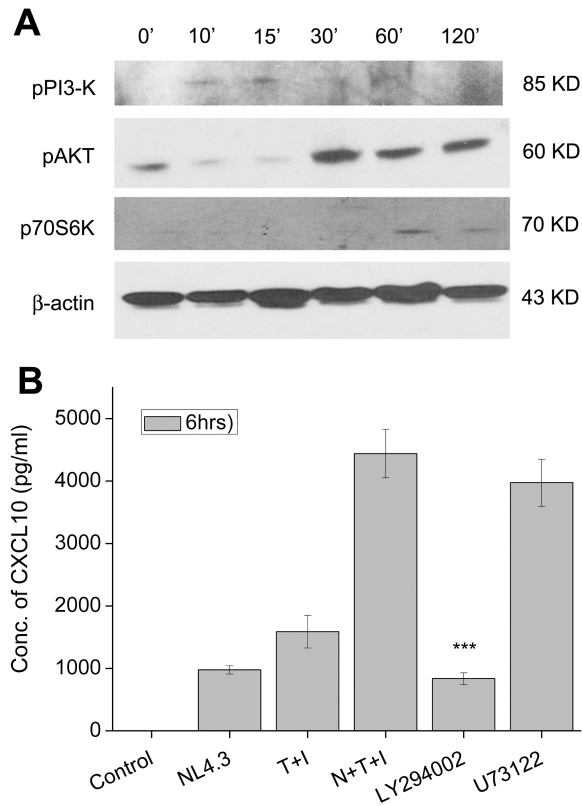
**Figure 3:** The JAK/STAT pathway is a critical player in the regulation of CXCL10 expression in virus and cytokine stimulated astrocytes. **A)** Western Blot analysis using phosphorylated antibodies against STAT1 $\alpha$  and STAT3 on the nuclear lysates from untreated or HIV-1 NL4.3 and cytokine (T+I) treated A172 cultures. Blots were stripped and reprobed with  $\beta$ -actin for normalization. **B)** CXCL10 ELISA on supernatants collected from A172 astrocytes pretreated with JAK inhibitor for 1 hour, then stimulated with HIV-1 (N) and the cytokines (T+I) for 6hrs before analysis. CXCL10 protein content in JAK inhibitor treated cells was compared to cells that receive virus and cytokine stimulation in the absence of the inhibitor. Values are mean  $\pm$  SD from three independent experiments (\*\*\*,  $p < 0.001$ ).

**Figure 4**



**Figure 4:** The Erk1/2 and p38 MAPK pathways mediate CXCL10 expression in stimulated astrocytes. **A)** Western Blot analysis of cytosolic lysates from untreated and virus and cytokine treated A172 astrocytes was conducted at various time points using antibodies against the phosphorylated forms of Erk1/2 and p38. An antibody against  $\beta$ -actin was used to reprobe the blots for normalization. **(B)** Activation of these pathways were shown to be involved in the synergistic increase in CXCL10 protein expression in these cells through inhibition of the Erk1/2 pathway by U0126 and of the p38 pathway by SB203580. Values are mean  $\pm$  SD from three independent experiments (\*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ ).

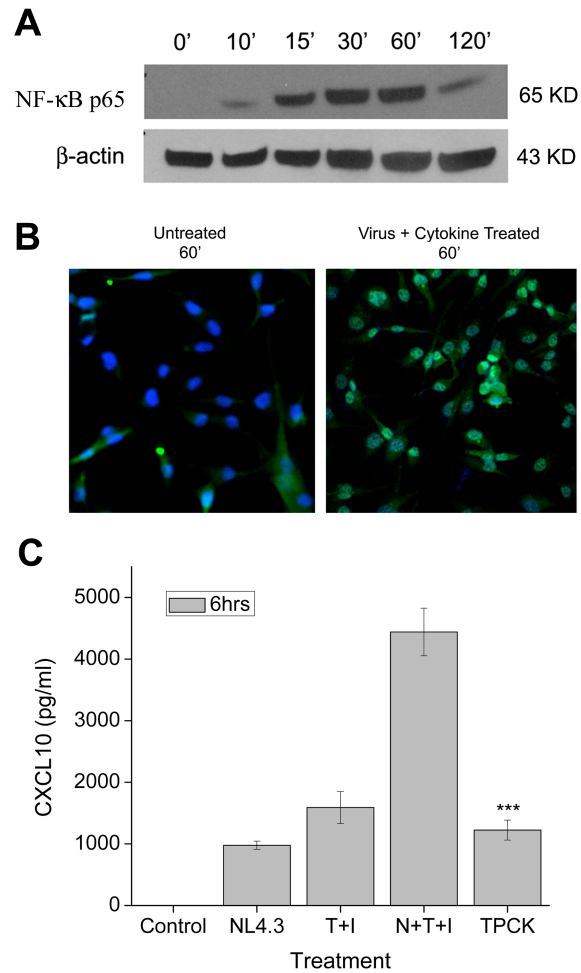
**Figure 5**



**Figure 5:** The PI3-Kinase pathway is involved in CXCL10 expression in virus and cytokine treated astrocytes. **A)** Western Blot analysis of cytosolic lysates from untreated and virus and cytokine treated A172 astrocytes was conducted at various time points using antibodies against the phosphorylated forms of PI3-K, and its downstream targets Akt and p706S-kinase. An antibody against  $\beta$ -actin was used to reprobe the blots for normalization. **(B)** Confirmation of the involvement of the PI3-K pathway in production of CXCL10 in virus and cytokine treated astrocytes was confirmed by pretreating the cells with an inhibitor of PI3-K, LY294002, and the

PLC inhibitor U73122 followed by CXCL10 ELISA. Values are mean  $\pm$  SD from three independent experiments (\*\*\*,  $p < 0.001$ ).

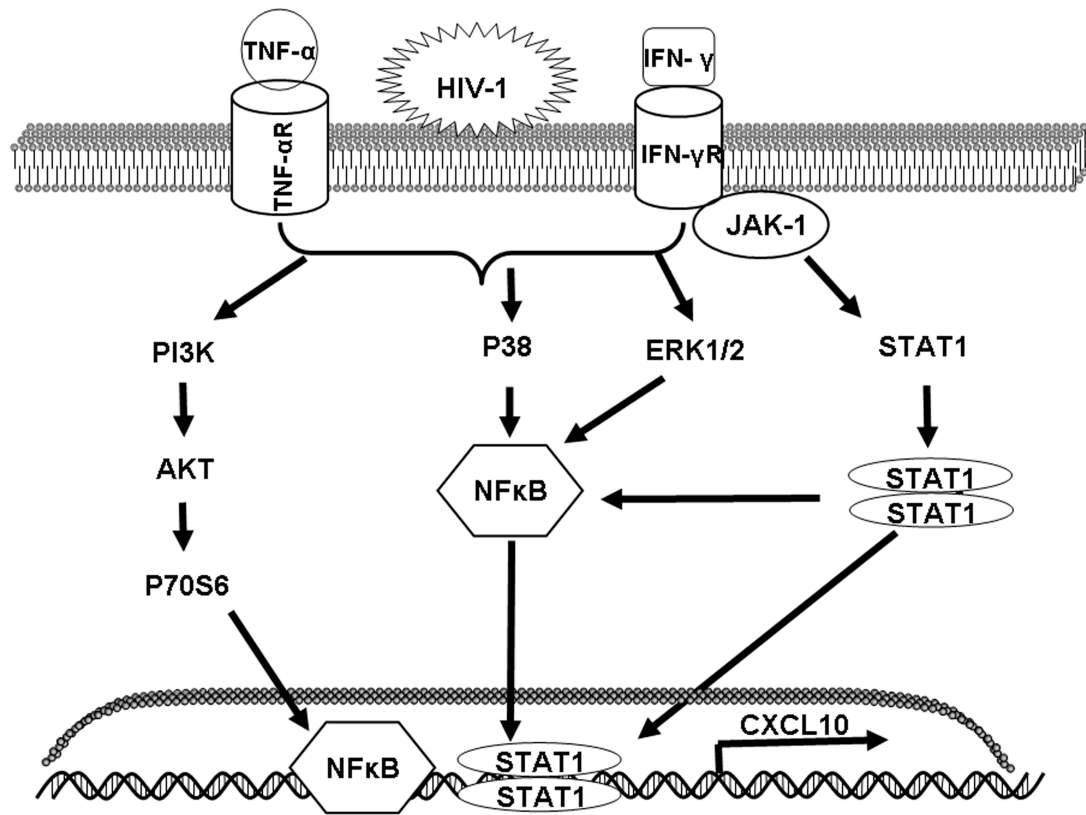
**Figure 6**



**Figure 6:** NF- $\kappa$ B plays a role in the synergistic induction of CXCL10 by HIV-1 and cytokines in astrocytes. **A)** Western Blot analysis of nuclear lysates from untreated and virus plus cytokine treated cells using an antibody against the p65 subunit of NF- $\kappa$ B. **B)** A172 astrocytes grown on coverslips were either untreated or treated with HIV-1 plus cytokines for 60 min and stained with an anti- NF- $\kappa$ B p65 antibody followed by treatment with an Alexa Flour 488-conjugated secondary antibody. Slides were mounted in Slow Fade antifade reagent (with DAPI, blue nuclear stain)

and images were captured by confocal microscopy (magnification X 250). After 60 min nuclear translocation of NF- $\kappa$ B is clearly evident in the treated cells, as shown by the green (NF- $\kappa$ B) and blue (DAPI) stains overlapping. **C)** To confirm the role of NF- $\kappa$ B in the synergistic induction of CXCL10 in stimulated astrocytes a CXCL10 ELISA utilizing the NF- $\kappa$ B inhibitor TPCK was conducted on stimulated cells untreated or treated with the inhibitor. Values are mean  $\pm$  SD from three independent experiments (\*\*\*,  $p < 0.001$ ).

**Figure 7**



**Figure 7:** Schematic of signaling pathways involved in the synergistic induction of CXCL10 in astrocytes stimulated with HIV-1 in conjunction with IFN- $\gamma$  and TNF- $\alpha$ . The major signaling pathways activated include JAK/STAT, MAPK, and PI3-K. The latter two are capable of converging on NF- $\kappa$ B, resulting in the transcription of CXCL10.



### **Chapter 3**

**Dissecting out which HIV-1 protein(s) is responsible for the co-operative  
induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  stimulated astrocytes**

## **Introduction**

Shortly after infection, HIV-1 penetrates the brain eventually resulting in HIV-1 associated CNS disease [2]. Studies show that 60% of HIV-1 carriers have some form of neuropsychiatric impairment diagnosed by behavioral, cognitive, and motor abnormalities [3]. HIV associated dementia (HAD), the most severe form of HIV-1 induced CNS impairment [4], is clinically characterized by motor and behavioral dysfunctions leading to seizures, coma, and death within six months of onset [5]. HIV-encephalitis (HIVE), the pathologic correlate of HAD, is characterized by widespread astrogliosis, cytokine/chemokine dysregulation, and neuronal degeneration [5, 6, 14]. Since the severity of HAD/HIVE correlates better with the presence of activated glial cells rather than with the viral load in the brain [6, 14] it is critical to understand the immune capabilities and consequences of immune activation within these cells.

Astrocytes, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators in response to HIV-1 infiltration into the brain [14-16]. Once activated by insult/injury astrocytes undergo astrogliosis characterized by proliferation and the release of several different cytokines and chemokines [18, 19]. Although HIV-1 does not productively infect astrocytes, the viral proteins Tat and Nef are expressed in astrocytes in brain tissue derived from HAD patients [17, 20]. Tat and Nef, along with gp120, activate several signaling pathways that lead to the dysregulation of cytokine/chemokine release in

astrocytes [17, 21]. This increase in cytokine/chemokine dysregulation can lead to less effective astrocyte function and concomitant neuronal damage.

Chemokines in the brain have been recognized as essential elements in neurodegenerative disease and related neuroinflammation. Cerebral expression of various chemokines and their receptors is increased in HIV. Increased levels of the chemokine CXCL10 have been detected in the CSF and plasma of individuals with HIV-1 infection [55]. Additionally, brain tissue derived from patients with HAD reveal increased expression of mRNA for CXCL10 [54, 56, 57] and this expression can be localized to astrocytes [58-60]. CXCL10 expression can be induced by the HIV-1 viral proteins gp120, Tat, and Nef, along with being synergistically enhanced by IFN- $\gamma$  and TNF- $\alpha$  [52-54].

Our recent preliminary studies suggested a synergistic induction of CXCL10 in astrocytes exposed to HIV-1, IFN- $\gamma$ , and TNF- $\alpha$ . However, the whole virus was used for those studies, leaving the issue of which HIV-1 protein was actually responsible for the synergistic induction of CXCL10 unanswered. In these studies the HIV-1 proteins Nef, gp120, and Tat have been examined for their ability to co-operate with IFN- $\gamma$  and TNF- $\alpha$  in the synergistic induction of CXCL10.

## **Materials and Methods**

**Astrocyte cell culture and treatments:** The human astrocytic cell line, A172 (ATCC #CRL-1620; American Type Culture Collection, Manassas, VA), were grown to confluency in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 50 U/ml penicillin, 0.05

mg/ml streptomycin and 2 µg/ml amphotericin B. All cultures were maintained in a humidified incubator at 37°C, 5% CO<sub>2</sub> and 95% air and culture medium replenished every 48-72 hours. Cells (triplicate or quadruplicate wells) were treated for 24 hours with either gp120 (LAV), Nef, Tat, HIV-1 NL4.3, or HIV-1 NL4.3 ΔNef individually or in combination with the cytokines IFN-γ (50ng/ml) and TNF-α (5ng/ml). In certain instances the gp120 neutralizing antibodies 1511 and 1510 (AIDS References and Reagent Program) were incubated with HIV-1 NL4.3 prior to the virus' addition to the cells.

**CXCL10 protein analysis by ELISA:** Supernatants collected from both primary human astrocytes and A172 astrocytes that were either untreated or treated with HIV-1 and/or cytokines, were examined for secreted CXCL10 protein levels using a commercially available ELISA kit (R&D Systems, Minneapolis, MN).

## **Results and Discussion**

### **HIV-1 Nef is not involved in the synergistic induction of CXCL10**

Nef was investigated for its ability to co-operate with IFN-γ and TNF-α in the synergistic induction of CXCL10 due to the fact that in HIVE Nef can be co-localized to astrocytes [17, 20]. Nef has also been demonstrated to increase CXCL10 expression in astrocytes [52-54]. Therefore two methods were used to determine Nef's potential role in the synergistic induction of CXCL10. First a replicate virus of HIV-1 NL4.3 was created with the *nef* gene deleted, named NL4.3ΔNef (AIDS Reference and Reagent Program). This virus was used to treat astrocytes along with the original NL4.3 virus with or without the cytokines before the supernatants

were collected and analyzed by ELISA (Fig. 1A). If Nef were to play a role in the synergistic induction of CXCL10 then the NL4.3 $\Delta$ Nef virus would not have shown an increase in CXCL10 in the presence of IFN- $\gamma$  and TNF- $\alpha$ . However, no difference was seen in CXCL10 expression between the NL4.3 and NL4.3 $\Delta$ Nef viruses in the presence of the cytokines. To further rule out the possibility of Nef involvement the astrocytes were treated with exogenous Nef protein with or without the cytokines before the supernatants were collected and analyzed by ELISA (Fig. 1B). Again, there was no significant difference between the cytokine treatment alone, and the cytokine treatment with Nef. This was solid evidence that Nef was not responsible for the synergistic induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  treated astrocytes.

### **Gp120 may be involved in the induction of CXCL10**

The role of gp120 in the synergistic induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  treated astrocytes was investigated due to gp120 inherent location on the HIV-1 virus. Gp120 is the coat protein for HIV, meaning that it is the first protein to come into contact with the cell membrane surface of the astrocytes. Not only is gp120 the first protein of HIV-1 to initiate contact between the host cell and the virus, but gp120 has been demonstrated to activate astrocytes and induce CXCL10 expression [52-54] . This made gp120 a prime suspect in the synergistic induction of CXCL10.

Gp120 neutralizing antibodies were utilized to assess the effect of viral bound gp120 on astrocytes. These neutralizing antibodies were incubated with the virus before the virus was added to the cells. Then the cellular supernatants were collected and analyzed for CXCL10 content by ELISA (Fig 2A). There was a significant

reduction in CXCL10 expression in astrocytes exposed to the gp120 neutralizing antibody treated virus. This indicated a role for gp120 in the synergistic induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  treated astrocytes. This trend was further investigated by the treatment of astrocytes with exogenous gp120 with or without the cytokines before the collection of the supernatants and analysis by CXCL10 ELISA (Fig. 2B). This method, however, did not reproduce the results demonstrated by the gp120 neutralizing antibodies. This could be due to the fact that the gp120 protein used to treat the cells was monomeric, whereas on the virus gp120 is a trimer, and this trimer is what the gp120 neutralizing antibodies bind to. Therefore, gp120 is probably involved in the induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  treated astrocytes, though its action is limited to its trimeric form.

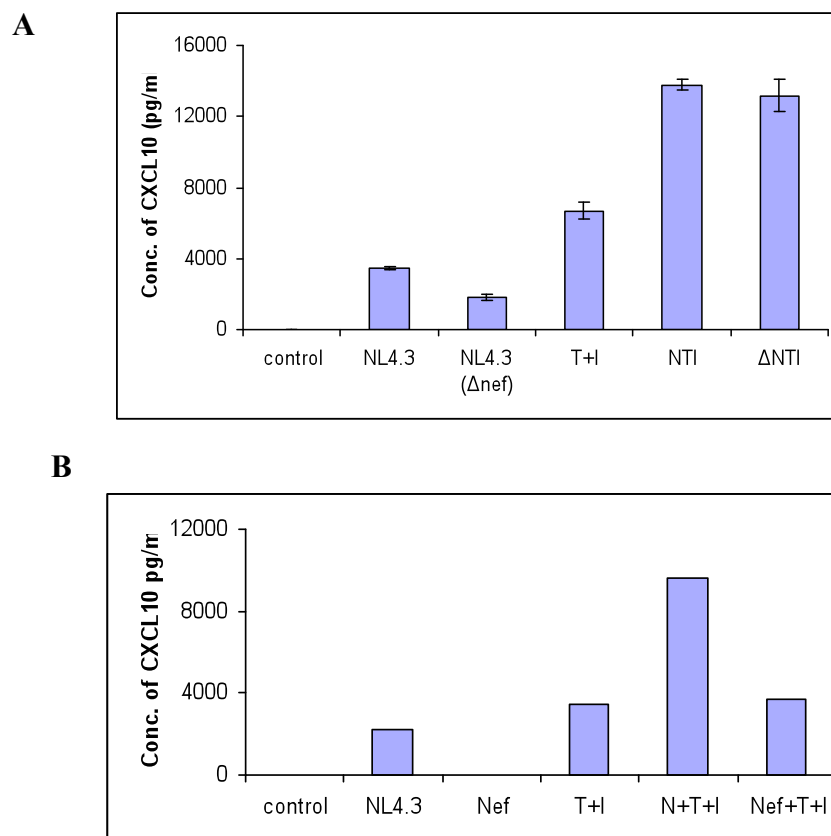
#### **Tat co-operates with IFN- $\gamma$ and TNF- $\alpha$ to increase CXCL10 expression in astrocytes**

HIV-1 Tat was investigated for its ability to co-operate with IFN- $\gamma$  and TNF- $\alpha$  in the synergistic induction of CXCL10 due to the fact that in HIV Tat can be co-localized to astrocytes [17, 20]. Tat has also been demonstrated to increase CXCL10 expression in astrocytes [52-54]. Therefore, astrocytes were treated with exogenous Tat with or without the cytokines before supernatants were collected and analyzed for CXCL10 content by ELISA (Fig. 3). The results indicated that Tat, in combination with the cytokines, IFN- $\gamma$  and TNF- $\alpha$ , significantly increased CXCL10 concentrations above those found for either treatment alone. Thus, Tat is the HIV-1 protein

responsible for the synergistic induction of CXCL10 in IFN- $\gamma$  and TNF- $\alpha$  stimulated astrocytes and will be used for future studies.

Of note, however, is the fact that Tat in combination with the cytokines did not increase CXCL10 levels to those reached by HIV-1 in combination with the cytokines. This leaves room for the possibility that more than one HIV-1 protein could be responsible for the synergistic induction of CXCL10. Furthermore, none of the HIV-1 proteins elicited an increase of CXCL10 by themselves, contrary to what previous literature had reported. Interestingly, while Tat did not increase CXCL10 by itself, it was able to potentiate CXCL10 expression in the presence of IFN- $\gamma$  and TNF- $\alpha$ , which neither gp120 nor Nef were able to do. Regardless of the inconsistencies with previous literature and the uncertainty of gp120's potential, the results with Tat were reproducible and significant, making Tat the HIV-1 protein of choice for continued studies.

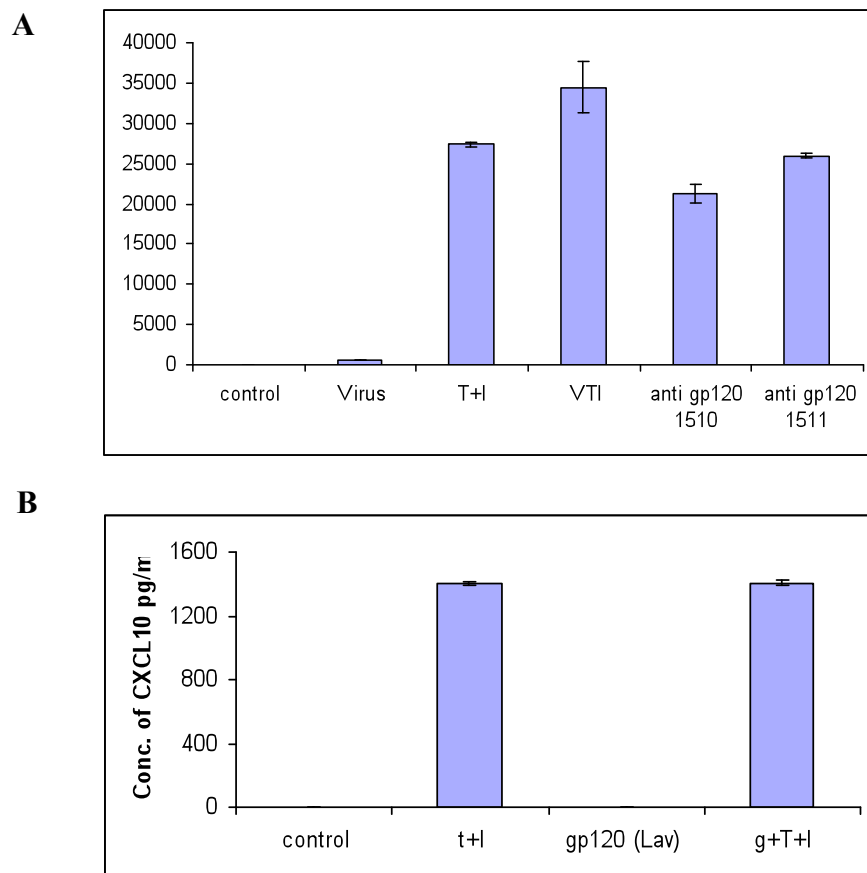
**Figure 1**



**Figure 1:** HIV-1 Nef is not involved in the synergistic induction of CXCL10 in astrocytes co-stimulated with IFN- $\gamma$  and TNF- $\alpha$ . **A)** A CXCL10 ELISA representing the HIV-1 laboratory strain NL4.3 and its counterpart  $\Delta$ NL4.3 in which the Nef gene is deleted. The deletion of Nef made no significant difference in CXCL10 expression. **B)** A CXCL10 ELISA showing that treatment of astrocytes with the Nef protein in combination with IFN- $\gamma$  and TNF- $\alpha$  does not increase CXCL10 expression.

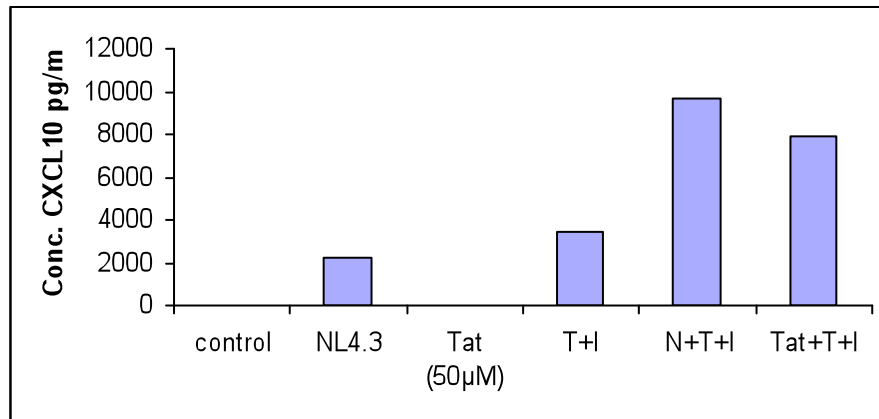


**Figure 2**



**Figure 2:** HIV-1 gp120 (LAV) may or may not be involved in the synergistic induction of CXCL10 in astrocytes co-stimulated with IFN- $\gamma$  and TNF- $\alpha$ . **A)** A CXCL10 ELISA showing that gp120 neutralizing antibodies were successful in mitigating CXCL10 expression in astrocytes treated with HIV-1 and the cytokines, IFN- $\gamma$  and TNF- $\alpha$ . **B)** A CXCL10 ELISA demonstrating that treatment of astrocytes with gp120 protein in combination with IFN- $\gamma$  and TNF- $\alpha$  does not increase CXCL10 expression.

**Figure 3**



**Figure 3:** HIV-1 Tat can significantly increase CXCL10 expression in astrocytes co-stimulated with IFN- $\gamma$  and TNF- $\alpha$ . **A)** A CXCL10 ELISA demonstrating Tat's ability, in the presence of the cytokines, to increase CXCL10 expression to levels over that achieved by either treatment alone.

## **Chapter 4**

### **HIV-1 Tat co-operates with IFN- $\gamma$ and TNF- $\alpha$ to increase CXCL10 in human astrocytes**

## **Abstract**

HIV-associated neurological disorders (HAND) are estimated to affect 60% of the HIV infected population. HIV-encephalitis (HIVE), the pathological correlate of the most severe form of HAND is often characterized by glial activation, cytokine/chemokine dysregulation, and neuronal damage and loss. However, the severity of HIVE correlates better with glial activation rather than viral load. One of the characteristic features of HIVE is the increased amount of the neurotoxic chemokine, CXCL10. This chemokine can be released from astroglia activated with the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ , in conjunction with HIV-1 Tat, all of which are elevated in HIVE. In an effort to understand the pathogenesis of HAND, this study was aimed at exploring the regulation of CXCL10 by cellular and viral factors during astrocyte activation. Specifically, the data herein demonstrate that the combined actions of HIV-1 Tat and the pro-inflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , result in the induction of CXCL10 at both the RNA and protein level. Furthermore, CXCL10 induction was found to be regulated transcriptionally by the activation of the p38, Jnk, and Akt signaling pathways and their downstream transcription factors, NF- $\kappa$ B and STAT-1 $\alpha$ . Since CXCL10 levels are linked to disease severity, understanding its regulation could aid in the development of therapeutic intervention strategies for HAND.

## **Introduction**

Despite the use of combinatorial anti-retroviral therapy (cART) HIV-associated dementia (HAD), a neurological complication in end stage AIDS, still afflicts 9-11% of the HIV infected population [2, 5, 86]. Even more disturbing is the fact that HIV-associated neurocognitive disorders (HAND), which includes HAD, Minor Cognitive Motor Disorders (MCMD), and other HIV related neuropsychiatric impairments, are estimated to affect almost 60% of HIV-1 patients [3, 131]. These patients are diagnosed by changes in behavior, and cognitive and motor abnormalities [3]. HAD, the most severe form of HIV-1 induced CNS impairment [4], is clinically characterized by motor and behavioral dysfunction that in the absence of therapy may lead to seizures, coma, and death within six months of onset [5].

HIV-1 is capable of penetrating the brain shortly after initial infection [2]. However, while cART is able to control the virus in the periphery, the drugs have inferior penetration across the blood brain barrier [62]. So while HIV-1 patients are living longer, they now have to deal with the long term effects of having HIV in the brain. With the increasing prevalence of HAND it is essential to understand the cellular and molecular mechanisms by which HIV exerts its detrimental effects on the CNS. However, since this virus does not infect neurons, the mechanism of neuronal damage and loss seen in HIVE, a pathological correlate of HAD, is not completely understood [6, 14]. Neuronal toxicity is thought to occur, in part, through glial activation and the release of cytotoxic chemokines/cytokines [6, 14], a hallmark feature of HIVE.

Astrocytes are a type of glial cell in the brain capable of releasing neurotoxic chemokines/cytokines after activation by either infection or injury. CXCL10, one of the neurotoxic chemokines released by stimulated astrocytes is up-regulated in the brains and CSF of patients with HIVE and is known to be positively correlated with disease progression [54-56]. Furthermore, two regulators of CXCL10 expression, IFN- $\gamma$  and TNF- $\alpha$ , are pro-inflammatory cytokines that are elevated in the brains of patients with HIVE and are also associated with neuropathogenesis [19, 25-27].

Another positive regulator of CXCL10 induction is the HIV-1 protein, Tat [17, 21, 53]. While astrocytes are not productively infected with HIV-1, the provirus in these cells is able to make the early HIV-1 genes, Tat, Rev and Nef [53, 54]. Tat is not only expressed in astrocytes and other productively infected cells like microglia, but it can also be released from these cells to activate other neighboring cells. Kutsch et. al. (2000) has reported that astrocytes activated with Tat have the ability to release CXCL10. Thus, with the increased expression of Tat and the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  in brains of patients with HIVE, there exists a perfect milieu for exaggerated induction of CXCL10 and the corresponding neuronal damage.

Increased levels of CXCL10 can be damaging to neurons both directly and indirectly [56, 57, 132]. CXCL10 has direct toxic effects by initiating the activation of a calcium-dependent apoptotic pathway in neurons [56, 57]. Indirectly, CXCL10 has the ability to create a chemotactic gradient between the brain and the periphery, allowing T-cells to infiltrate the brain, a hallmark feature of HAND [6, 132]. This T-

cell assault not only weakens the blood brain barrier, but increases local inflammation, which can be damaging to the neurons.

While both cellular (IFN- $\gamma$  and TNF- $\alpha$ ) and viral (Tat) mediators are known to induce CXCL10, it remains unclear how the interplay of these host and viral factors modulates chemokine expression in astrocytes. The data herein demonstrates the increased induction of CXCL10 at both the RNA and protein level in astrocytes activated with HIV-1 Tat, IFN- $\gamma$ , and TNF- $\alpha$ . The data also reveals that this increase is regulated transcriptionally by the activation of the p38, Jnk, and Akt signaling pathways and their downstream transcription factors, NF- $\kappa$ B and STAT-1 $\alpha$ . Since CXCL10 levels are linked to disease severity, understanding its regulation could lead to therapeutic intervention strategies for those suffering from HAND.

## **Materials and Methods**

**Astrocyte cell culture and treatments:** Primary human astrocytes (cat# HA1800; ScienCell Research Laboratories, Carlsbad, CA) were prepared as described by the supplier. The human astrocytic cell lines, U-87 and A172 (ATCC; American Type Culture Collection, Manassas, VA), were grown as described previously [133]. The cells (triplicate or quadruplicate wells) were treated for 6-12 hrs (U87/A172) or 24 hours (primary astrocytes), with: 1) HIV-1 Tat (1-72), 2) a combination of the cytokines IFN- $\gamma$  (50ng/ml) and TNF- $\alpha$  (5ng/ml) or 3) HIV-1 Tat and the cytokines. Control treatments included heat inactivated HIV-1 Tat and cells receiving no treatment. The concentration of Tat in the cerebral spinal fluid (CSF) has been reported at 16ng/ml [134]. However the concentration of Tat in the brain is unknown,

though expected to be much higher than in the CSF [135]. The Tat concentration utilized in this *in vitro* study is generally accepted [136-140].

The following specific pharmacological inhibitors were used at the final concentration specified: PI3-K Inhibitor LY294002, PLC inhibitor U73122, JAK inhibitor I, JNK inhibitor II, P38 inhibitor SB203580, (all at 10 $\mu$ M, Calbiochem, Gibbstown, NJ), and NF- $\kappa$ B inhibitor N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) (2 $\mu$ M, Sigma, St. Louis, MO) [95-99].

**CXCL10 mRNA analysis:** RNA was extracted from U-87 astrocytes that were either untreated or treated with HIV-1 Tat and/or the cytokines IFN- $\gamma$  and TNF- $\alpha$  using TRIzol reagent following the treatment periods (Invitrogen Life Technologies). Quantitative analysis of CXCL10 mRNA was done by quantitative Real-Time PCR using the SYBR Green detection method. RT2 PCR primer pair set for CXCL10 was obtained from SuperArray Bioscience and amplification of CXCL10 from first strand cDNA was performed as described earlier [100] using ABI Prism 7700 sequence detector. Data were normalized using Ct values for the house-keeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) in each sample. To calculate relative amounts of CXCL10, the average Ct value of the HPRT was subtracted from that for each target gene to provide changes in Ct value. The fold change in gene expression (differences in changes in Ct value) was then determined as log<sub>2</sub> relative units.

**Luciferase assay:** To determine the effects of HIV-1 Tat, IFN- $\gamma$ , and TNF- $\alpha$  on the transcriptional regulation of CXCL10, astrocytes were transfected with either the



Luciferase reporter plasmid, TGL-CXCL10 or a GFP plasmid (Amaya Biosystems, using a Nucleofector kit (Amaya Biosystems) and allowed to recover overnight [99]. The transfection efficiency of astrocytes was around 30% as determined by analyzing the number of GFP expressing cells in the GFP reporter plasmid transfected wells. Following recovery the astrocytes transfected with the TGL-CXCL10 plasmid were treated with HIV-1 Tat and the cytokines, IFN- $\gamma$  and TNF- $\alpha$ . After 12 hours the cells were lysed and Luciferase activity was measured using the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The resulting data was normalized by the protein content in each sample. The data represents results obtained from three independent experiments.

**CXCL10 protein analysis by ELISA:** Supernatants collected from primary human astrocytes or the astrocytes cell lines U-87 and A172 that were either untreated or treated with HIV-1 Tat and/or cytokines were examined for secreted CXCL10 protein levels using a commercially available CXCL10 ELISA kit (R&D Systems, Minneapolis, MN). The data represents results obtained from three independent experiments.

**Western Blot Analysis:** Treated U-87 cells were lysed using the NE-PER Nuclear and Cytoplasmic Extraction kits (Pierce, Rockford, IL). Equal amounts of the corresponding proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (12%) in reducing conditions followed by transfer to PVDF membranes. The blots were blocked with 5% non fat dry milk in phosphate buffered saline. Western blots were then probed with antibodies recognizing the

phosphorylated forms of Jnk, Akt, p38, (Cell Signaling, Danvers, MA 1:200), STAT1- $\alpha$  (Cell Signaling, 1:500), NF- $\kappa$ B p65 (Cell Signaling, 1:1000), and  $\beta$ -actin (Sigma, St. Louis, MO, 1:4000) The secondary antibodies were alkaline phosphatase conjugated to goat anti mouse/rabbit IgG (1:5000). Signals were detected by chemiluminescence (CDP-star; Tropix, Bedford, MA).

**Statistical Analysis:** All of the statistical analyses were performed by using a one-tail, independent, Student's *t*-test. The results were judged as statistically significant at *p* values  $\leq 0.05$ .

## **Results**

### **Up-regulation of CXCL10 mRNA in astrocytes treated with Tat, IFN- $\gamma$ and TNF- $\alpha$ .**

It has been previously shown that HIV-1 in conjunction with the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  can induce expression of CXCL10 in astrocytes [141]. In this study we wanted to dissect the contribution of HIV-1 Tat in this phenomenon. U-87 astrocytes treated with either the cytokines alone or with Tat plus the cytokines show a significant increase in CXCL10 mRNA as early as three hours by Real Time PCR (Fig. 1A). While Tat by itself did not induce CXCL10 mRNA transcription, when combined with IFN- $\gamma$  and TNF- $\alpha$ , it was capable of eliciting an increase in CXCL10 mRNA. As shown in Figure 1, cells treated with both Tat and the cytokines for just 6 hours demonstrated an almost 4000 fold increase in CXCL10 mRNA as

compared with the cells treated with the cytokines alone, thus underscoring the role of HIV-1 Tat in potentiating the transcriptional regulation of CXCL10.

To confirm the role of Tat in the transcriptional regulation of CXCL10, we performed luciferase reporter assays utilizing the TGL-CXCL10 plasmid [99]. Briefly, U-87 cells were transfected with a TGL-CXCL10 plasmid followed by a 12 hour treatment with HIV-1 Tat and the cytokines, IFN- $\gamma$  and TNF- $\alpha$ . There was a significant increase in luciferase expression in the cells exposed to both Tat and the cytokines, as compared with cells treated with the cytokines alone, thereby confirming a role for Tat in the transcription of CXCL10 (Fig. 1B). Furthermore, when the U-87 astrocytes were transfected with the TGL-CXCL10 plasmid containing a mutated IFN-stimulated response element (ISRE) or a mutated  $\kappa$ B binding site followed by treatment with Tat plus the cytokines, there was little to no luciferase detected above negative control levels. These data thus suggest that Tat in combination with the cytokines can strongly activate the CXCL10 gene, and this activation is highly dependent on the occupancy of the two key regulatory sequences, ISRE and  $\kappa$ B.

**CXCL10 protein expression is increased in the presence of Tat and the cytokines.**

To confirm that the increase in CXCL10 RNA correlated with an increase in CXCL10 protein, supernatants from U-87 astrocytes, A172 astrocytes, and primary human astrocytes were collected and analyzed for CXCL10 expression using an ELISA assay. As shown in Figure 2 and similar to the mRNA study, in both the

astrocyte cell lines, Tat by itself was unable to induce CXCL10 expression. Similarly to the RNA studies, Tat in combination with IFN- $\gamma$  and TNF- $\alpha$ , exerted a 2 fold increase in CXCL10 protein levels over that of levels from cells treated with only the cytokine mix. This phenomenon was confirmed in primary human astrocytes and, similar to findings in cell lines, Tat in conjunction with the cytokines resulted in a 2 fold increase in CXCL10 expression over that of the cytokines by themselves. Similar findings in both the cell lines as well as in primary human astrocytes lend credence to the important role of Tat in potentiating cytokine-mediated up-regulation of CXCL10.

**Tat enhances activation of IFN- $\gamma$ /TNF- $\alpha$  signaling pathways involved in CXCL10 regulation.**

Past studies have demonstrated that mitogen activated protein kinase (MAPK) activation is critical in regulating inflammatory responses such as cytokine/chemokine expression in response to multiple stimuli [104-107]. We next sought to explore whether HIV-1 Tat could potentiate the existing signaling pathways used by IFN- $\gamma$  and TNF- $\alpha$ . U-87 astrocytes were treated with Tat alone, the cytokines IFN- $\gamma$  and TNF- $\alpha$  alone, or a mixture of Tat and the cytokines for 30min. This time point was chosen based on the time-dependent activation of p38, Jnk, and Akt in astrocytes treated with Tat and the cytokines, where 30min post-treatment was the optimal time for enhanced phosphorylation as determined by Western blot analysis (data not shown). As shown in Figure 3A there was an increase in the activation of Jnk, p38, and Akt in cells treated with the combination of Tat and cytokines compared with cells exposed to either treatment alone. In each instance, as expected

each of the cytokine was able to induce phosphorylation of each of the pathways. While Tat was able to mediate only a modest activation of these pathways by itself, in the presence of cytokines, it potentiated a robust activation of these pathways as compared with the cytokines by themselves. These data demonstrate the ability of Tat to enhance signaling pathways activated by other pro-inflammatory molecules, thereby resulting in an increased expression of the target gene CXCL10.

The roles of Jnk, p38, and Akt activation in CXCL10 induction were further examined using a pharmacological approach. U-87 astrocytes were pretreated with either the Jnk II inhibitor, the P38 inhibitor SB203580, or the PI3-K Inhibitor LY294002, all at a concentration of 10 $\mu$ M, followed by stimulation of cells with Tat and the cytokine mix for 6 hrs (Fig. 3E). Supernatants from treated cells were collected and analyzed for CXCL10 content by ELISA. Pre-treatment of the astrocytes with the Jnk, p38, and Akt inhibitors followed by stimulation with Tat and cytokines resulted in significant reduction of CXCL10, thus underscoring the role of these signaling pathways in the induction of CXCL10.

#### **Role of transcription factors NF- $\kappa$ B and STAT-1 $\alpha$ in induction of CXCL10.**

The transcription factors NF- $\kappa$ B and STAT-1 $\alpha$  play key roles in the induction of CXCL10 [3, 37, 39, 43, 88, 89]. Both of these transcription factors mediate CXCL10 regulation by binding to specific regulatory sequences in the promoter region, the ISRE site for STAT-1 $\alpha$  and the  $\kappa$ B1 and  $\kappa$ B2 sites for NF- $\kappa$ B. Binding of both NF- $\kappa$ B and STAT-1 $\alpha$  is necessary for the synergistic increase in CXCL10 mRNA/protein. IFN- $\gamma$  and TNF- $\alpha$ , through their respective signaling pathways and

subsequent activation of NF- $\kappa$ B and STAT-1 $\alpha$ , are known to synergistically increase CXCL10 [37, 88]. Therefore, U8-7 cells were treated with either Tat or the cytokines, or Tat in conjunction with the cytokines for 60min followed by cell lysis and isolation of nuclear proteins. Nuclear extracts were subsequently analyzed for p65- NF- $\kappa$ B and pSTAT-1 $\alpha$  content by Western Blot analysis.

As demonstrated in Figure 4A the ability of Tat to enhance IFN- $\gamma$ /TNF- $\alpha$  signaling directly correlates with an increase in the phosphorylation/activation of NF- $\kappa$ B and STAT-1 $\alpha$ . To link the increased activation of NF- $\kappa$ B and STAT-1 $\alpha$  to CXCL10 regulation, U-87 cells were pretreated with the inhibitors specific for NF- $\kappa$ B (TPCK at 2 $\mu$ M) or JAK I (JAK I inhibitor at 10 $\mu$ M) followed by stimulation of cells with Tat and cytokine mix and analyzed for CXCL10 by ELISA. As shown in Fig. 4D inhibition of both NF- $\kappa$ B and STAT-1 resulted in a significant and remarkable reduction in CXCL10 expression in astrocytes, thereby confirming the role of these factors in viral and cytokine-mediated synergistic induction of CXCL10.

## **Discussion**

Up-regulated expression of chemokines in the brain has been recognized as an important correlate of various neurodegenerative diseases and related neuroinflammation. Chemokines and their receptors are expressed by a wide variety of cells, including those intrinsic to the CNS. These proteins can regulate inflammatory responses by recruiting lymphocytes and monocytes/macrophages to areas of inflammation within the brain contributing to the injury and eventual loss of neurons [49, 50]. Cerebral expression of various chemokines and their receptors is

increased in HIV. CXCL10 was first identified as an early response gene induced after IFN- $\gamma$  treatment in a variety of cells [41, 42]. Interactions of soluble host factors, such as those between IFN- $\gamma$  and TNF- $\alpha$ , have been shown to synergistically induce the expression of this chemokine [37]. In addition to its induction by host factors, CXCL10 can also be induced independently by the HIV-1 viral protein Tat [52-54].

Increased levels of CXCL10 have been detected in the CSF and plasma of individuals with HIV-1 infection [55]. Additionally, brain tissue derived from patients with HAD also reveal increased expression of mRNA for CXCL10 and this expression can be localized to astrocytes [54, 60]. Levels of this neurotoxic chemokine are positively correlated with HAD disease progression [55]. Additionally, up-regulated expression of CXCL10 and its neurotoxic role has also been previously demonstrated in SHIV-infected macaque brains with lentiviral lesions [56, 57].

In the current study we sought to explore the regulation of CXCL10 in human astrocytes stimulated with a mixture of IFN- $\gamma$ , TNF- $\alpha$ , and HIV-1 Tat. The rationale for using three different stimulants was based on the premise that several host immune and viral factors have the potential to interact during HAD, resulting in neuronal damage and loss. The pro-inflammatory cytokine TNF- $\alpha$  was chosen because it is a key cytokine produced by activated astrocytes and microglia in response to HIV-1 [19, 25]. Levels of this cytokine are also known to positively correlate with HAD pathogenesis [19, 25-29]. Furthermore, TNF- $\alpha$  has the ability of not only inducing CXCL10 expression by itself [25, 35], but also synergistically with

various other host factors, such as IFN- $\gamma$ , thus increasing the toxicity and inflammation in the surrounding milieu [19, 25, 36-39].

One of the well-studied host factors that has been shown to co-operatively interact with TNF- $\alpha$  to induce CXCL10 is the pro-inflammatory cytokine IFN- $\gamma$  [37, 39]. Therefore IFN- $\gamma$  was also chosen as an additional stimulant in the present study. IFN- $\gamma$  is a known inducer for the expression CXCL10 in several cell types, including astrocytes [37, 41, 42]. Additionally, IFN- $\gamma$  has been shown to be markedly increased in CNS tissues during HIV-1 infection in the brain and has been implicated in the pathophysiology of HAD [40].

HIV-1 in combination with IFN- $\gamma$  and TNF- $\alpha$  has been shown to synergistically up-regulate CXCL10 expression in human astrocytes. However, which viral protein contributes to this effect remains poorly understood. In our attempts to dissect the role of known viral toxins involved in cytokine-mediated induction of CXCL10, we initially examined the effects of HIV-1 Nef, the envelope protein gp120, and Tat in conjunction with the cytokine mix for their ability to up-regulate CXCL10 in U-87 astrocytes. The rationale for selecting these viral toxins stems from their expression in astrocytes and their ability to activate astrocytes [52-54]. Since HIV-1 Tat (Fig. 2A) but not gp120 or Nef (data not shown) demonstrated the most significant increase with the cytokines in inducing both CXCL10 RNA and protein (Figs 1A and 2A and B), we used HIV-1 Tat for our further studies.

Increased induction of CXCL10 RNA in astrocytes was mediated via transcriptional regulation as demonstrated by transfection of promoter constructs



linked to luciferase reporter gene. Using these assays it was demonstrated that combinatorial treatment of astrocytes with the viral and host factors resulted in increased transcription of the luciferase gene. Promoter constructs with mutations in the ISRE (STAT-1 $\alpha$ ) binding site or I $\kappa$ B (NF- $\kappa$ B) binding sites, the two major regulatory sequences in the CXCL10 promoter [39, 89, 103], however, resulted in abrogation of luciferase expression, thus underscoring the role of each of these binding sites in the induction of CXCL10.

Exploration of the signaling pathways critical for the increased induction of CXCL10 in astrocytes suggested the involvement of the Jnk, p38, and Akt pathways. These findings are in agreement with the synergistic induction of CXCL10 mediated by intact HIV-1 virus and the cytokine mix as reported earlier [141]. In each of the three pathways (Jnk, p38, and Akt) there was modest activation by Tat alone, and definitive activation by the cytokines themselves, however, in the presence of all the three stimulants there was a significant activation of each of these pathways, more so than with either treatment alone. Confirmation of these pathways using specific pharmacological inhibitors further indicated their involvement in the regulation of CXCL10 expression. Activation of the survival factor Akt [124-126] in the presence of viral and host factors leads us to speculate that this could be a mechanism by the virus to hijack the host machinery in order to maintain a long term reservoir of the neurotoxic inflammatory mediators, including CXCL10. It should be pointed out that inhibition of one specific signaling pathway or transcription factor never completely

abrogated the expression of CXCL10, thus indicating involvement of parallel signaling pathways and transcription factors that could be working in unison.

All three pathways (Jnk, p38 and Akt) once activated, can in turn activate the downstream transcription factor NF- $\kappa$ B, which has two separate, yet vital binding sites on the CXCL10 promoter [88]. Multiple studies have demonstrated that astrocytes activated by HIV-1/viral proteins have increased nuclear translocation and activation of the transcription factor NF- $\kappa$ B [112, 120, 121, 127, 128, 141], which, in turn, can regulate CXCL10 expression. In the present study we demonstrate that exposure of astrocytes to Tat and the cytokines results in increased activation and nuclear translocation of the p65 subunit of NF- $\kappa$ B compared with cells treated with either the viral or cellular stimuli. Since Jnk, p38, and Akt all have the ability to activate NF- $\kappa$ B, their convergence on NF- $\kappa$ B could lead to a dramatic increase in CXCL10 induction. In fact, when NF- $\kappa$ B was pharmacologically inhibited, there was a significant inhibition of CXCL10 expression.

While activation of NF- $\kappa$ B can be attributed to all the three stimuli in astrocytes, STAT-1 $\alpha$  activation is unique to IFN- $\gamma$  or IFN- $\gamma$  plus TNF- $\alpha$ . Intriguingly, our data indicated significant activation of STAT-1 $\alpha$  in astrocytes treated with all three stimuli and this was further confirmed using the JAK I specific inhibitor, resulting in a dramatic decrease also in CXCL10 expression. Since Tat does not bind to a known cell receptor, and instead acts by diffusing through the cell membrane, it should be noted that while Tat activation of MAPK signaling pathways and transcription factors has been reported [112], there is a paucity of information of how

Tat actually activates these proteins. Recent studies point to the role of Tat-mediated activation of NADPH oxidase, a membrane protein, as a key upstream player involved in the activation of MAPK signaling pathways [75]. It is thus likely that the activation of NADPH oxidase could explain the ability of Tat to activate Jnk, p38, Akt and their downstream transcription factors NF- $\kappa$ B and STAT-1 $\alpha$ . Further studies exploring the role of NADPH oxidase in this process are warranted.

In conclusion, we have provided evidence that HIV-1 Tat in conjunction with the cytokines, IFN- $\gamma$  and TNF- $\alpha$ , is capable of regulating CXCL10 expression in human astrocytes at both the RNA and protein levels. This regulation is likely due to the activation of the Jnk, p38, and Akt signaling pathways and activation of their downstream transcription factors NF- $\kappa$ B and STAT-1 as demonstrated in an overall schematic in Figure 5.

Given that the neuronal toxicity in HAD is thought to occur through glial activation and the release of cytotoxic chemokines/cytokines, dissecting the complex interplay between host factors and viral proteins can lead to a better understanding of disease pathogenesis. The ability of Tat to potentiate activation of signaling pathways stimulated by IFN- $\gamma$  and TNF- $\alpha$  is an ingenious approach by the virus to exploit the activated cells into generating reservoirs of pro-inflammatory factors, such as CXCL10. This in turn could aid in perpetuating activation, infection, and destruction of several other cell types in the CNS.

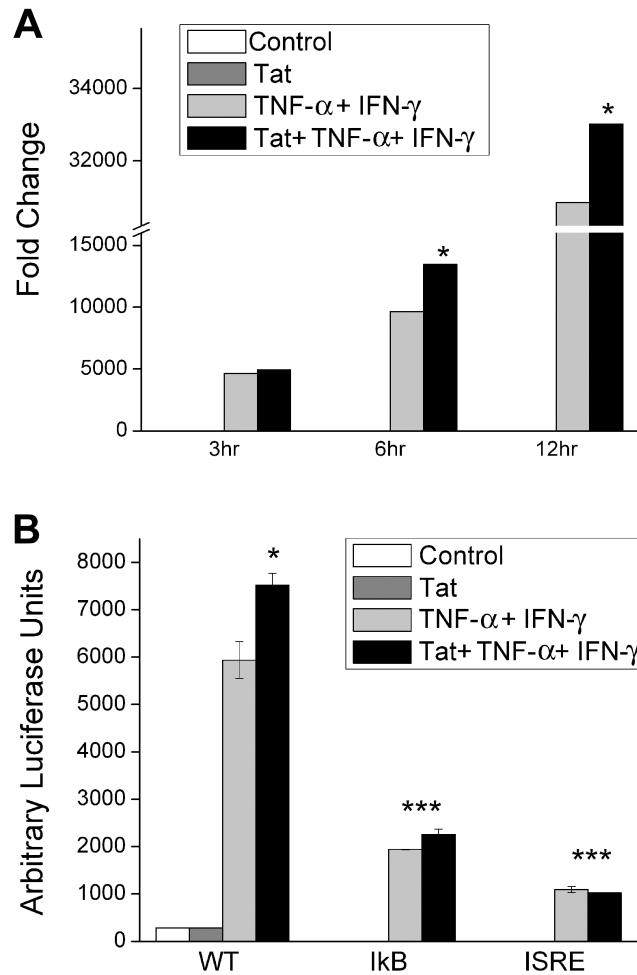
Since excessive amounts of CXCL10 can be neurotoxic, our findings lend further credence to the role of CXCL10 in progression of AIDS-associated dementia.

The consequences of CXCL10 over expression not only include enhanced influx of inflammatory cells into the CNS, but also amplification of neuronal dysfunction/death in end-stage HAD. Since CXCL10 levels are linked to disease severity, understanding its regulation could aid in the development of therapeutic intervention strategies for HAND.

### **Acknowledgments**

We would like to thank Dr. Avindra Nath (Department of Neurology, Johns Hopkins University for generously providing the HIV-1 Tat (1-72). We acknowledge the technical assistance of Shannon Callen in the preparation of this manuscript

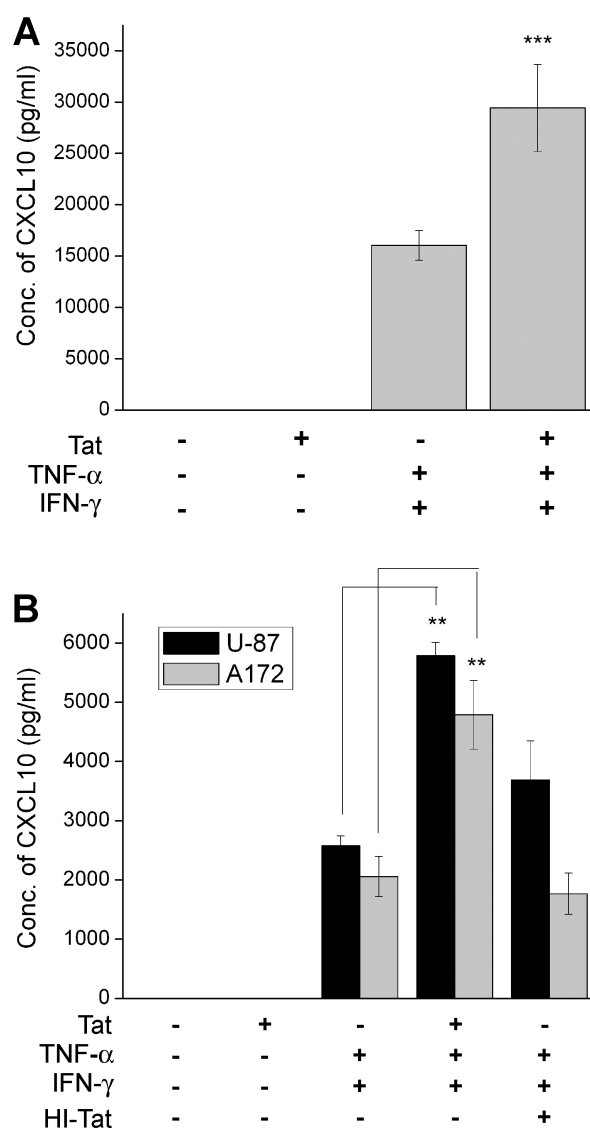
**Figure 1**



**Figure 1: (A)** Real Time PCR analysis showing a significant increase in CXCL10 RNA in U-87 astrocytes treated with a combination of Tat and cytokines. Cells were stimulated with either HIV-1 Tat alone, the cytokines IFN- $\gamma$  and TNF- $\alpha$ , or Tat and the cytokines together for 6 hour followed by total cell lysis and RNA extraction. **(B)** Luciferase assay demonstrating the ability of Tat in combination with the cytokines to transcriptionally regulate the CXCL10 gene. The induction of CXCL10 is dependent

upon the binding of the ISRE and I $\kappa$ B regulatory sequences. The data represents the mean  $\pm$  SD from three independent experiments (\*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ ).

**Figure 2**

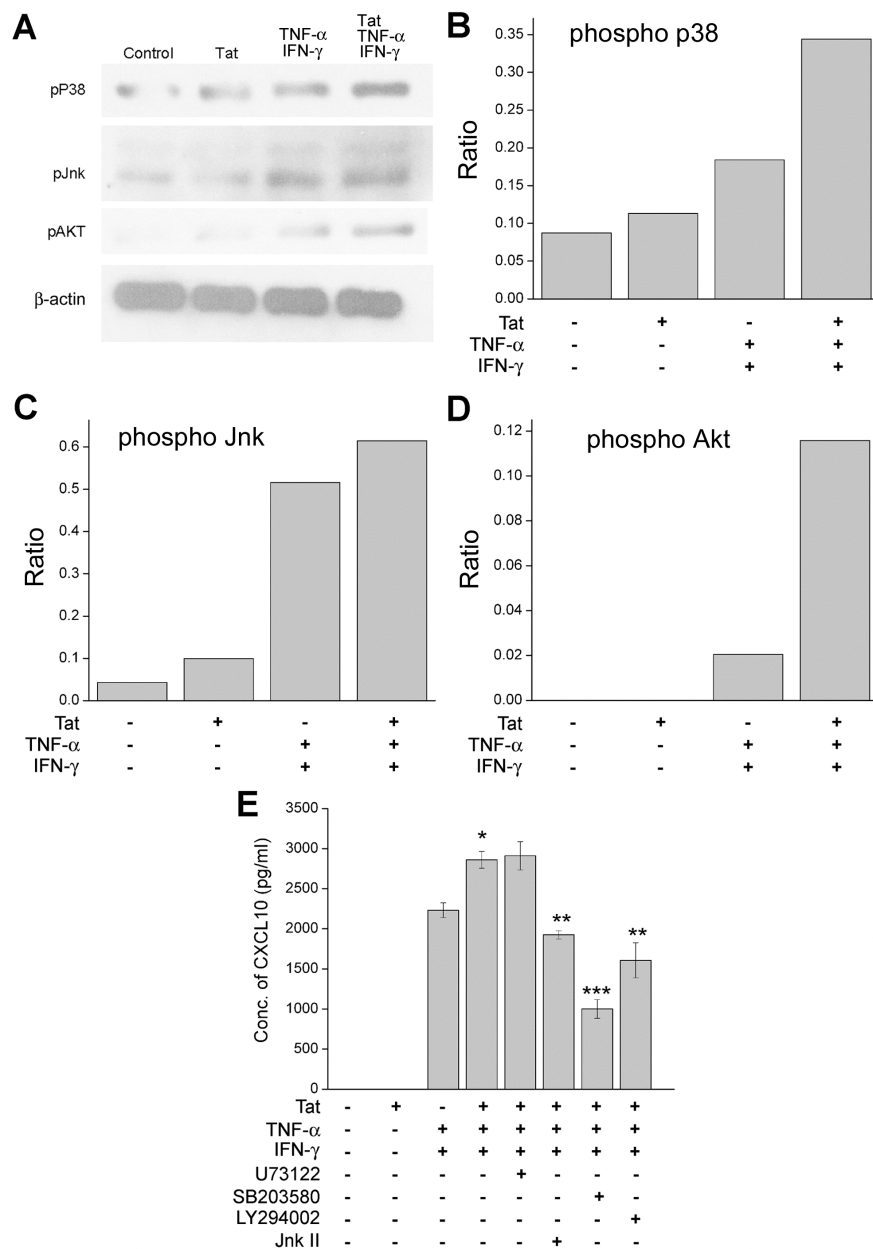


**Figure 2:** Increased CXCL10 protein expression in **(A)** primary human astrocytes or **(B)** U-87 and A172 astrocytes cell lines treated with HIV-1 Tat alone, the cytokines IFN- $\gamma$  and TNF- $\alpha$ , or Tat and the cytokines together for 24 and 12 hours respectively. Both primary and cell line astrocytes showed a significant increase in CXCL10 protein levels in the cells treated with HIV-1 Tat and the cytokines, than with either

treatment alone. Treatment of U-87 and A172 cells with heat inactivated (**HI**) Tat in conjunction with the cytokines did not lead to an increase in CXCL10 protein levels compared with cells treated with the cytokines alone. The data represents the mean  $\pm$  SD from three independent experiments (\*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).



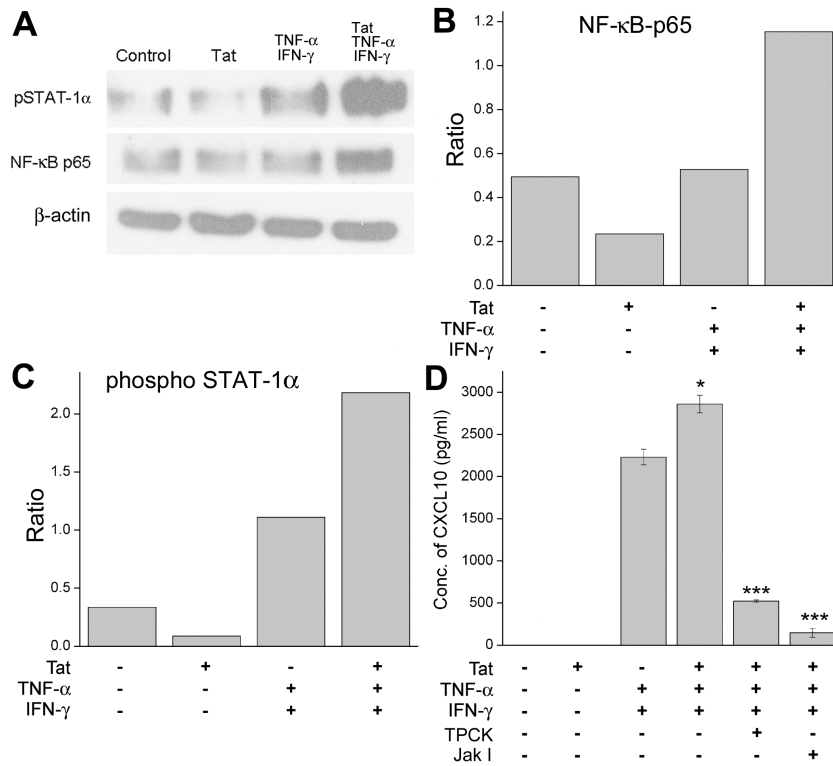
**Figure 3**



**Figure 3:** The p38, Jnk, and Akt signaling pathways mediate CXCL10 induction in stimulated U-87 astrocytes. **A)** Western Blot analysis of cytosolic lysates collected

from cells untreated, HIV-1 Tat treated, IFN- $\gamma$  and TNF- $\alpha$  treated, or treated with Tat in combination with the cytokines for 30 min. The blots were probed with antibodies against phospho-p38, phospho-Jnk, and phospho-Akt. An antibody against  $\beta$ -actin was used to reprobe the blots for normalization. **B), C) and D)** Densitometric scans illustrating the ratio of phospho-p38, Jnk, and Akt to  $\beta$ -actin levels. **E)** Activation of these pathways was shown to be involved in the increased expression of CXCL10 through inhibition of the p38 pathway by SB203580, the Jnk pathway by Jnk II inhibitor, and the Akt pathway by LY294002. Inhibition of the PLC- $\gamma$  pathway by U73122 had no effect on CXCL10 protein levels. The data represents the mean  $\pm$  SD from three independent experiments (\*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

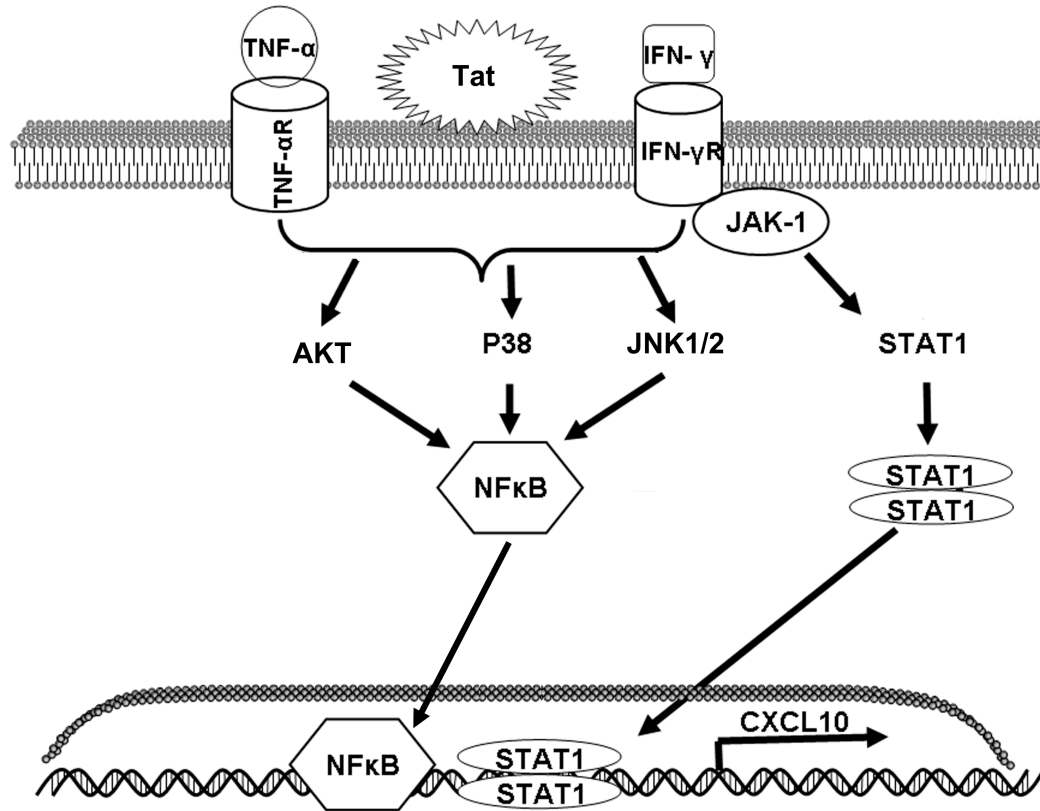
**Figure 4**



**Figure 4:** STAT-1 $\alpha$  and NF- $\kappa$ B play a role in the increased induction of CXCL10 by HIV-1 Tat and the cytokines in U-87 astrocytes. **A)** Western Blot analysis of nuclear lysates collected from cells untreated, HIV-1 Tat treated, IFN- $\gamma$  and TNF- $\alpha$  treated, or treated with Tat in combination with the cytokines for 60 min. The blots were probed with antibodies against phospho- NF- $\kappa$ B p65 and phospho-STAT-1 $\alpha$ . Antibodies against  $\beta$ -actin were used to reprobe the blots for normalization. **B), C)** Densitometric scans illustrating the ratio of phospho- NF- $\kappa$ B p65 and phospho-STAT-1 $\alpha$  to  $\beta$ -actin levels. **E)** Activation of these transcription factors was shown to be involved in the increased expression of CXCL10 through inhibition of the NF- $\kappa$ B by TPCK and the

inhibition of the Jak/STAT pathway by a Jak I inhibitor. The data represents the mean  $\pm$  SD from three independent experiments (\*\*\*,  $p < 0.001$ ).

**Figure 5**



**Figure 5:** Schematic of the signaling pathways involved in the increased induction of CXCL10 in astrocytes stimulated with HIV-1 Tat in conjunction with IFN- $\gamma$  and TNF- $\alpha$ . The major signaling pathways activated include p38, Jnk, and Akt, which are able to converge on NF- $\kappa$ B. The activation of NF- $\kappa$ B, along with the activation of STAT-1 $\alpha$ , results in the transcription of CXCL10.

## **Chapter 5**

### **Co-operative induction of CXCL10 involves NADPH Oxidase: Implications for HIV Dementia**

## **Abstract**

With the increasing prevalence of HIV-associated neurocognitive disorders (HAND), understanding the mechanisms by which HIV-1 induces neuro-inflammation and subsequent neuronal damage is of paramount importance. The hallmark features of HIV-encephalitis, the pathological correlate of HIV-associated Dementia (HAD), are gliosis, oxidative stress, chemokine dysregulation, and neuronal damage/death. Since neurons are not infected by HIV-1, the current thinking in the field is that these cells are damaged indirectly by pro-inflammatory chemokines released by activated glial cells. CXCL10 is a neurotoxic chemokine that is dramatically up-regulated in astroglia activated by HIV-1 Tat, IFN- $\gamma$ , and TNF- $\alpha$ . In this study we have demonstrated that HIV-1 Tat increases CXCL10 expression in IFN- $\gamma$  and TNF- $\alpha$  stimulated human astrocytes via NADPH oxidase. We have shown that the treatment of astrocytes with a mixture of Tat and the cytokines leads to a respiratory burst that is abrogated by apocynin, an NADPH oxidase inhibitor. Pre-treatment of Tat, IFN- $\gamma$ , and TNF- $\alpha$  stimulated astrocytes with apocynin also resulted in concomitant inhibition of CXCL10 expression. Additionally, apocynin was also able to reduce Tat and cytokine-mediated activation of the corresponding signaling molecules Erk1/2, Jnk, and Akt with a concomitant decrease in activation and nuclear translocation of NF- $\kappa$ B, all of which are important regulators of CXCL10 induction. Understanding the mechanisms involved in reducing both oxidative stress and the release of pro-inflammatory agents could lead to the development of therapeutics aimed at decreasing neuro-inflammation in patients suffering from HAD.

## Introduction

Shortly after infection, HIV-1 is able to penetrate the brain, eventually resulting in HIV-1 associated complications in the CNS [2]. Studies in the literature show that 60% of HIV-1 infected individuals have some form of neuropsychiatric impairment diagnosed by behavioral, cognitive, and motor abnormalities categorically classified as HIV-associated neurocognitive disorders [3]. HIV associated dementia (HAD), the most severe form of HAND [4], is clinically characterized by motor and behavioral dysfunctions leading to seizures, coma, and death within six months of onset [5]. HIV-encephalitis (HIVE), the pathological correlate of HAD, is characterized by widespread astrogliosis, oxidative stress, cytokine/chemokine dysregulation, and neuronal degeneration [5, 6, 14]. Since the severity of HAD/HIVE correlates better with the presence of activated glial cells rather than with the viral load in the brain, the current thinking about the disease is that the neuronal damage is an indirect consequence of pro-inflammatory cytokines and chemokines released by activated glial cells [6, 14].

Astroglia, the most numerous cell type within the brain, provide an important reservoir for the generation of inflammatory mediators in response to HIV-1 [14-16]. Once activated by the virus/viral proteins astrocytes undergo astrogliosis characterized by the release of several different cytokines and chemokines, including the neurotoxic chemokine, CXCL10. Increased levels of CXCL10 (interferon  $\gamma$ -inducible peptide, or IP-10) have been detected in the CSF and plasma of individuals with HIV-1 infection [55]. Additionally, brain tissue derived from patients with HAD



reveal increased expression of CXCL10 mRNA [54, 56, 57] and this expression can be localized to astrocytes [58-60]. Levels of this neurotoxic chemokine are positively correlated with HAD disease progression [55]. In SHIV-infected macaque brains with lentiviral lesions, CXCL10 has been shown to be significantly up-regulated and is apoptotic to neurons [57]. Furthermore, treatment of fetal neuronal cultures with exogenous CXCL10 induces neuronal apoptosis through the caspase-3 cascade [56]. In addition to its synergistic induction by the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ , CXCL10 can also be induced by the HIV-1 viral proteins gp120, Nef, and Tat [52-54].

Although HIV-1 does not productively infect astrocytes, Tat is expressed in astrocytes in brain tissue derived from HAD patients [17, 20]. Studies have demonstrated that Tat can activate several signaling pathways that lead to the dysregulation of cytokine/chemokine release in astrocytes [17, 21]. Furthermore, Tat, in the presence of IFN- $\gamma$  and TNF- $\alpha$ , is able to enhance the expression of CXCL10 compared the cytokines themselves. It has been proposed that Tat increases CXCL10 expression by potentiating both the activation of signaling pathways and transcription factors utilized by the IFN- $\gamma$  and TNF- $\alpha$  receptors through an unknown mechanism [142].

Recently, the role of oxidative stress in the regulation of cytokine and chemokine expression has garnered increased awareness. One possible mechanism by which oxidative stress can mediate its effect on protein expression is through intracellular signaling pathways that culminate in the activation of critical

transcription factors [71-73]. Interestingly, in HIVE oxidative stress markers have been found to co-localize with glial cells and neurons [63]. Several studies point to the effect of HIV-1 Tat in mediating oxidative stress in astrocytes [63, 65-68, 143] possibly leading to cell death [66, 69, 70]. Furthermore, it has also been demonstrated that HIV-1 induced oxidative stress in astrocytes can regulate target genes that are under the control of NF- $\kappa$ B, one of the essential transcription factors responsible for CXCL10 induction [74].

One mechanism by which oxidative stress is able to impact signaling pathways and their corresponding transcription factors is through a respiratory burst orchestrated by the activation of NADPH oxidase [71-73, 75, 76]. NADPH oxidase, a multi-subunit membrane associated enzyme, is capable of producing superoxide [77-80]. This enzyme consists of two membrane associated subunits, gp91<sup>phox</sup> and p22<sup>phox</sup>, and the cytosolic components p67<sup>phox</sup>, p47<sup>phox</sup>, p40<sup>phox</sup>, and the small GTPase Rac1/2 [77-79, 81-83]. Following its phosphorylation, p47<sup>phox</sup> forms a complex with p67<sup>phox</sup>, p40<sup>phox</sup>, and activated Rac1/2 before being recruited to the membrane bound components [78-80]. Once the cytosolic subunits have docked with the membrane associated subunits, the interaction between p67<sup>phox</sup> and gp91<sup>phox</sup> results in the transfer of electrons from NADPH to molecular oxygen, resulting in the production of superoxide [78-80, 83]. The superoxide is subsequently converted to hydrogen peroxide, a critical redox signaling intermediate [77, 80, 84].

Based on recent findings linking NADPH oxidase activity to cytokine and chemokine production in microglia, macrophages, and astrocytes [73, 75] we

hypothesized that NADPH oxidase could have a role in CXCL10 induction in human astrocytes stimulated with HIV-1 Tat and the cytokines IFN- $\gamma$  and TNF- $\alpha$ . In this study we have demonstrated that the treatment of astrocytes with a mixture of Tat and the cytokines leads to a respiratory burst, an effect that is abrogated by apocynin, an NADPH oxidase inhibitor. Treatment with apocynin also decreased CXCL10 expression in Tat, IFN- $\gamma$ , and TNF- $\alpha$  stimulated astrocytes. Western blot analysis of U-87 astrocytes treated with apocynin and the Tat/cytokine mixture demonstrated decreased activation of the signaling molecules Erk1/2, Jnk, and Akt, and subsequent decreased activation and nuclear translocation of NF- $\kappa$ B. Understanding the mechanisms involved in reducing both oxidative stress and the release of pro-inflammatory agents could aid in the development of therapies targeted at reduction of overall neuro-inflammation in patients affected by HAD.

## **Materials and Methods**

**Astrocyte cell culture and treatments:** The human astrocytic cell line, U-87 (ATCC; American Type Culture Collection, Manassas, VA), was grown as described previously [133]. Primary human fetal astrocytes were a generous gift from Dr. Anuja Ghorpade (University of North Texas Health Science Center, Fort Worth, Texas). The cells (triplicate or quadruplicate wells) were treated for 24 hours with: 1) HIV-1 Tat (1-72) (supplied by Philip Ray, University of Kentucky), 2) a combination of the cytokines IFN- $\gamma$  (50ng/ml) and TNF- $\alpha$  (5ng/ml) or 3) HIV-1 Tat and the cytokines. In the instances where the NADPH Oxidase inhibitor apocynin (Sigma, St. Louis, MO)

was utilized cells were pretreated for one hour with the inhibitor (50 $\mu$ M-1mM) before stimulation with Tat and cytokines.

**Measurement of oxidative stress:** U-87 astrocytes were untreated, treated with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture, or pre-treated with apocynin (250 $\mu$ M) for one hour followed by treatment with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture. Cells were then trypsinized and centrifugation and the resulting cell pellet was stained for 30min with 15 $\mu$ M of 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H<sub>2</sub>-DCF-DA) (Molecular Probes, Inc, Eugene, OR), to assess cytoplasmic reactive oxygen species (ROS) [144]. During the last five minutes of incubation Hoechst (1 $\mu$ M) was added to the cell suspension. After the 30min incubation period the cells were washed and resuspended in PBS containing 20mM glucose before being analyzed by a Tecan fluorescence plate reader at two separate excitation/emission settings. In the first setting the plate was read at an excitation of 485nm with an emission of 530nm for DCF, the second setting measured the Hoechst at an excitation of 355nm and emission of 460nm. The DCF fluorescent values were divided by their corresponding Hoechst fluorescent values for normalization. The data represents the mean  $\pm$  SD from three independent experiments (\*  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

**CXCL10 protein analysis by ELISA:** Supernatants collected from primary human astrocytes or the astrocytic cell line U-87 that were either untreated or treated with HIV-1 Tat and/or cytokines were examined for secreted CXCL10 protein levels using a commercially available CXCL10 ELISA kit (R&D Systems, Minneapolis, MN).

The data represents the mean  $\pm$  SD from three independent experiments (\*  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

**Gp91phox knock down:** U-87 astrocytes were incubated with the siRNA transfection reagent, Ribojuice (Novagen, Gibbstown, NJ), and 400nM of Accell Green Non-targeting siRNA, or a Human CYBB (gp91phox) siGENOME SMARTpool siRNA consisting of four different siRNA sequences (Dharmacon Inc, Chicago, IL). The sequences of the siRNA's are as follows 5'-GAAGACAACUGGACAGGAA-3', 5'-GGAACUGGGCUGUGAAUGA-3', 5'-GUGAAUGCCCGAGUCAUA-3', and 5'-GAAACUACCUAAGAUAGCG-3'. Forty eight hours after transfection the cells were lysed using TriZol reagent for RNA extraction and assessed for gp91<sup>phox</sup> knock down using RT-PCR analysis. Primers for gp91phox were as follows: forward primer 5'-CAAGATGCGTGGAACTACCTAAGAT-3' and reverse 5'-TCCCTGCTCCCCTAACATCA-3'. The RT-PCR set up consisted of 1 cycle of 50°C for 30min, a 1 cycle 95°C, 15min hot start, and 40 cycles of 94°C for 1min, 55°C for 1min, and 72°C for 1min followed by one extension cycle of 72°C for 10min. Following confirmation of the gp91<sup>phox</sup> knock down, U-87 cells were again transfected and 48 hours later were stimulated with either IFN- $\gamma$ /TNF- $\alpha$  or the combination of Tat/ IFN- $\gamma$ /TNF- $\alpha$  for 24 hours before supernatants were collected and analyzed for CXCL10 content by ELISA. The data represents the mean  $\pm$  SD from three independent experiments (\*  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

**Western Blot Analysis:** Treated U-87 cells were lysed using the NE-PER Nuclear and Cytoplasmic Extraction kits (Pierce, Rockford, IL). Equal amounts of the corresponding proteins were electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (12%) in reducing conditions followed by transfer to PVDF membranes. The blots were blocked with 5% non fat dry milk in phosphate buffered saline. Western blots were then probed with antibodies recognizing the phosphorylated forms of Erk1/2, Jnk, Akt, p38, (Cell Signaling, Danvers, MA 1:200), Stat1- $\alpha$  (Cell Signaling, 1:500), NF- $\kappa$ B p65 (Cell Signaling, 1:1000), and  $\beta$ -actin (Sigma, St. Louis, MO, 1:4000). The secondary antibodies were alkaline phosphatase conjugated to goat anti mouse/rabbit IgG (1:5000). Signals were detected by chemiluminescence (CDP-star; Tropix, Bedford, MA).

**MTT Assay:** Astrocytes were untreated, treated with Tat/IFN- $\gamma$ /TNF- $\alpha$ , or pre-treated for one hour with apocynin (250 $\mu$ M) then treated with Tat/IFN- $\gamma$ /TNF- $\alpha$  in serum free neuronal media. After 48 hours the supernatants were placed on primary rat cortical neuronal cultures prepared as previously described [145]. Half of the supernatants from the Tat/IFN- $\gamma$ /TNF- $\alpha$  treated astrocytes were incubated for one hour at room temperature with a CXCL10 neutralizing antibody as described earlier [146]. This was followed by an additional one hour incubation period with protein-G sepharose beads (Sigma, St. Louis, MO) to pull down the antibody/antigen complex by centrifugation. The resulting supernatant was added to the neurons. After 24 hours cell viability was measured by mitochondrial dehydrogenases [3(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (MTT) (Sigma, St. Louis, MO) assay as

described earlier [147]. The data represents the mean  $\pm$  SD from three independent experiments (\*  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

## Results

### **Tat/IFN- $\gamma$ /TNF- $\alpha$ induce oxidative stress in human astrocytes**

Oxidative stress is a hallmark feature of several neurodegenerative diseases, including HAD [63]. Since oxidative stress has been demonstrated to impact signaling pathways and cytokine/chemokine production, we sought to explore whether treatment of human astrocytes with Tat/IFN- $\gamma$ /TNF- $\alpha$  could cause an oxidative burst. U-87 astrocytes were treated with Tat/IFN- $\gamma$ /TNF- $\alpha$  over a period of 60min prior to staining with H<sub>2</sub>DCF-DA and assessed for the presence of reactive oxygen species by flourometer. Our findings demonstrated that in stimulated cells there was a time dependent increase in the formation of reactive oxygen species (ROS) with a peak at 30min post treatment, indicative of an oxidative burst (Fig. 1A).

Recent reports have shown both Tat exposure and TNF- $\alpha$  receptor engagement can activate NADPH oxidase, a membrane protein capable of producing oxidative burst [75, 148]. In order to examine whether NADPH oxidase was involved in the oxidative burst of Tat and cytokine stimulated of astrocytes, cells were pretreated for one hour with the NADPH oxidase inhibitor, apocynin (250 $\mu$ M) followed by stimulation and subsequent staining with H<sub>2</sub>DCF-DA. The data in Figure 1B demonstrated the ability of apocynin to abrogate the Tat and cytokine mix-mediated oxidative burst in U87 astrocytes. Taken together these data suggest that

Tat/IFN- $\gamma$ /TNF- $\alpha$  have the ability to activate NADPH oxidase, resulting in an oxidative burst.

### **Inhibition of NADPH oxidase resulted in decreased CXCL10 expression**

Based on our findings that treatment of astrocytes with Tat/IFN- $\gamma$ /TNF- $\alpha$  generated ROS through NADPH oxidase, it was of interest to examine first whether ROS played a role in the induction of CXCL10. We thus pre-treated U-87 astrocytes with various concentrations of apocynin (50 $\mu$ M-1mM) prior to stimulation with the Tat and cytokine mixture. Supernatants were collected after 24 hours and assessed for CXCL10 content by ELISA. As seen in Figure 2A, apocynin was able to dose dependently decrease CXCL10 levels, with the optimal inhibition occurring at a concentration of 250 $\mu$ M apocynin. All further experiments were thus conducted with apocynin at 250 $\mu$ M.

The next step was to identify whether the apocynin-mediated reduction of CXCL10 in astrocytes was attributable to treatment with Tat, the cytokine mix, or both. Therefore both the U-87 and primary human astrocytes were pre-treated with apocynin prior to stimulation with Tat alone, cytokines alone, or the Tat/cytokine mix followed by collection of the supernatants 24hrs later and assessment of CXCL10 content by ELISA. As shown in Figures 2B and 2C, apocynin treatment significantly decreased the amount of CXCL10 generated by both the cytokine mix alone and the Tat/cytokine mixture. However, the extent of apocynin-mediated reduction of CXCL10 expression was greater in the Tat/cytokine mix-treated cells compared with cells treated with only the cytokine mix, suggesting a role for NADPH



oxidase in Tat-mediated potentiation of CXCL10 expression. It should be pointed out that while apocynin was able to significantly decrease CXCL10 expression in both the Tat/cytokine and cytokine alone groups, it was unable to completely inhibit CXCL10 expression. Therefore, it is likely that while the ROS generated from NADPH oxidase activation is involved in the induction of CXCL10, there could be other pathways contributing to CXCL10 induction.

**siRNA knockdown of gp91<sup>phox</sup> resulted in decreased CXCL10 expression.**

In order to confirm the role of NADPH oxidase in the induction of CXCL10, we used the approach of a siRNA targeted knock down of gp91<sup>phox</sup>, the critical subunit of NADPH oxidase. The rationale for choosing gp91<sup>phox</sup> was based on its critical role in the activation of NADPH oxidase. In the absence of gp91<sup>phox</sup> the activated cytosolic subunits of NADPH oxidase are unable to dock with the membrane components (gp91<sup>phox</sup> and gp22<sup>phox</sup>), resulting in the failure of enzyme activation. It has also been demonstrated that gp91<sup>phox</sup> is up-regulated in activated astrocytes [149, 150], possibly through a positive feedback loop with the transcription factor NF- $\kappa$ B [151].

We thus sought to examine whether blocking gp91<sup>phox</sup> expression in U-87 astrocytes by siRNA transfection could result in the inhibition of CXCL10 expression. U-87 astrocytes were transfected with either the siRNA pool against gp91<sup>phox</sup> or with the scrambled siRNA conjugated with GFP. Effective transfection was confirmed at 48 hrs in the scrambled-GFP transfected cells as shown in Fig 3A. Knockdown of gp91<sup>phox</sup> was subsequently confirmed by RT-PCR analysis (Fig. 3B).

Having achieved the siRNA-mediated knockdown, we next stimulated U-87 astrocytes either with the Tat and cytokine mix or with the cytokine mix alone for 24 hours prior to collection of supernatants for assessment of CXCL10 content by ELISA. As shown in Figure 3C, there was a significant reduction of CXCL10 expression in cells transfected with gp91<sup>phox</sup> siRNA compared with cells transfected with scrambled siRNAs. These findings underscore the role of NADPH oxidase in Tat and cytokine-mediated induction of CXCL10.

### **NADPH oxidase impacts the activation of the Erk1/2, Jnk, and Akt signaling pathways**

NADPH oxidase activation can affect cell signaling by several different mechanisms. Its activation relies on the phosphorylation of Rac1 or Rac2, both of which, when activated can affect MAPK pathways [79, 84, 152]. Another mechanism of action for NADPH oxidase is the production of superoxide, which is then dismutated to hydrogen peroxide, a critical redox signaling intermediate [76, 77, 80, 84]. Increased levels of hydrogen peroxide can activate Ras, which in turn can activate MAPK pathways or the PI3-K-Akt pathway [152-154].

We next wanted to examine the downstream mediators of NADPH oxidase activation in stimulated astrocytes. Stimulated U-87 astrocytes in the presence or absence of apocynin (250 $\mu$ M) were lysed after 60 min (time based on our previous findings –unpublished results) and analyzed for MAPK activation by Western Blot analysis. As shown in Figure 4 Tat and cytokine mix-mediated activation of MAPK proteins, Jnk and Erk1/2, was inhibited in the presence of apocynin. Interestingly, the

Tat and cytokine mix-mediated activation of p38 was not affected by apocynin treatment, thus leading to the speculation that p38 activation was independent of NADPH oxidase activation.

Since Akt signaling has been shown to be critical in the induction of CXCL10 in stimulated astrocytes [141], we next wanted to examine the role of NADPH oxidase in this signaling pathway. Stimulated U-87 astrocytes in the presence or absence of apocynin (250 $\mu$ M) were lysed and analyzed for Akt activation by Western Blot analysis. As shown in Figure 5 the Tat and cytokine mix-mediated activation of Akt was inhibited in the presence of apocynin. Taken together these findings underpin the role of NADPH oxidase-mediated activation of Jnk, Erk1/2, and Akt in CXCL10 induction in astrocytes.

#### **Inhibition of NADPH oxidase decreased NF- $\kappa$ B activation and translocation**

The Jnk, Erk1/2, and Akt pathways are capable of converging on a common transcription factor, NF- $\kappa$ B. NF- $\kappa$ B is one of several transcription factors sensitive to redox related signaling, and has been shown to be affected by the generation of ROS [73, 74]. It has been previously reported that NADPH oxidase activation can be directly linked to NF- $\kappa$ B phosphorylation and nuclear translocation [84, 151, 155]. Taking into account the decreased activation of the Jnk, Erk1/2, and Akt pathways in response to apocynin and the fact that NF- $\kappa$ B has been previously linked to NADPH oxidase, the effect of apocynin on NF- $\kappa$ B activation and translocation in Tat/IFN- $\gamma$ /TNF- $\alpha$  stimulated U-87 cells was assessed by Western Blot analysis. As shown in Figure 6, treatment with apocynin was able to decrease the phosphorylation of I $\kappa$ B- $\alpha$ ,

which sequesters NF- $\kappa$ B in the cytosol until phosphorylation leads to its degradation, resulting in the activation and nuclear translocation of NF- $\kappa$ B. Since NF- $\kappa$ B is one of the critical transcription factors responsible for CXCL10 induction, its decreased activation could therefore negatively impact CXCL10 expression.

**Treatment with apocynin decreased the overall toxicity to neurons.**

Since CXCL10 has been demonstrated to be neurotoxic, it was of interest to examine whether blocking CXCL10 expression in astrocytes via the NADPH oxides inhibitor could result in decreased neuronal toxicity in conditioned-media experiments. In these studies, primary rat cortical neurons were cultured in the presence of conditioned media collected from stimulated astrocytes treated or untreated with apocynin. Additionally, stimulated astrocytes conditioned media was also incubated with a CXCL10 neutralizing antibody prior to exposure to neurons. Neuronal survival was assessed after 24 hrs using the MTT survival assay.

As demonstrated in Figure 7, astrocyte conditioned media from the untreated astrocytes had no effect on neuronal survival as compared with neurons incubated with neuronal media. However, the astrocyte conditioned media from the stimulated astrocytes significantly decreased neuronal survival, and this effect was reversed in the apocynin treated group. Since astrocytes are capable of releasing a plethora of cytokines and chemokines in response to the Tat/cytokine mixture, the CXCL10 neutralizing antibody was utilized to confirm the neurotoxic potential of CXCL10. Neurons treated with conditioned media exposed to the CXCL10 neutralizing antibody demonstrated significantly increased survival compared with the neurons

treated with conditioned media from stimulated cells. However, CXCL10 neutralizing antibody conditioned media did not exert complete reversal of cell toxicity, thus implying release of other neurotoxic factors in addition to CXCL10, in stimulated astrocytes.

## **Discussion**

Oxidative stress is a common denominator in several neurodegenerative diseases, including HAD, the most severe form of HAND. However, the exact cause and mechanism for its generation and the impact it has on disease pathogenesis is poorly understood. In this study we explored the mechanism by which HIV-1 Tat and the cytokines, IFN- $\gamma$  and TNF- $\alpha$ , induce oxidative stress in astrocytes and the implications of NADPH oxidase in inducing the respiratory burst involved in generation of CXCL10. Furthermore, we have also demonstrated the ability of the NADPH oxidase inhibitor, apocynin, to diminish this response, ultimately sustaining neuronal health.

The chemokine, CXCL10, was of interest in these studies because levels of this neurotoxic chemokine are positively correlated with HAD disease progression [55]. Moreover, in SHIV-infected macaque brains with lentiviral lesions, CXCL10 is significantly up-regulated and is apoptotic to neurons [57]. Additionally, brain tissue derived from patients with HAD revealed increased expression of CXCL10 mRNA [54, 56, 57] and this expression was localized to astrocytes [58-60].

CXCL10 also has the capacity to be both directly and indirectly neurotoxic [56, 57, 132]. CXCL10 has direct toxic effects by initiating the activation of a

calcium-dependent apoptotic pathway in neurons [56, 57]. Indirectly, CXCL10 has the ability to create a chemotactic gradient between the brain and the periphery, allowing cells from the periphery to infiltrate the brain, a hallmark feature of HAD, that increases local neuro-inflammation [6, 132].

We have previously demonstrated that HIV-1 Tat in combination with the cytokines, IFN- $\gamma$  and TNF- $\alpha$ , is able to potentiate the expression of CXCL10 compared with cells treated with only the cytokines [142]. The mechanism of Tat-mediated induction of CXCL10 involved enhanced activation of the Jnk, p38, and Akt pathways that were already triggered by the cytokines and their cognate receptors. This potentiation lead to the activation of NF- $\kappa$ B and STAT-1 $\alpha$ , critical transcription factors involved in the synergistic induction of CXCL10 by the cytokines [142].

In the current studies we sought to explore additional pathways by which HIV Tat mediated CXCL10 potentiation in the presence of cytokines. The membrane associated enzyme, NADPH oxidase, has been garnering increased attention for its ability to participate in signal transduction, ultimately impacting cytokine and chemokine production [75, 84, 149, 151]. A recent study by Turchan-Cholewo *et. al.* demonstrated the ability of Tat to activate NADPH oxidase, inducing the production of ROS that ultimately lead to the increased expression of TNF- $\alpha$ , IL-6, and MCP-1 in microglia and macrophages [75]. Furthermore, the chemokine MCP-1 has been shown in astrocytes to be regulated by the transcription factor, NF- $\kappa$ B, in response to

ROS [73]. Based on these findings we hypothesized that CXCL10 expression, which is also a target of NF- $\kappa$ B, is modulated by ROS and possibly NADPH oxidase.

We thus decided to investigate the role of NADPH oxidase in the regulation of CXCL10 in astrocytes treated with Tat, IFN- $\gamma$ , and TNF- $\alpha$ . First we confirmed that there was indeed the production of ROS in U-87 astrocytes treated with the Tat/cytokine mixture by way of DCF staining, which non-discriminatorily visualizes ROS in the cytoplasm. There was a time-dependent increase in DCF fluorescence with a peak at 30min following stimulation of astrocytes (Fig. 1A). To determine whether NADPH oxidase may be influencing the release of ROS in the stimulated cells, we pretreated the cells with the NADPH oxidase inhibitor, apocynin prior to stimulation of cells. The rationale for choosing apocynin was based on its specificity to block activation of NADPH oxidase, the proposed mechanism of action being its interference with the ability of p47<sup>phox</sup> subunit to associate with the membrane bound subunits [156]. In the presence of apocynin there was abrogation of the Tat and cytokine-mediated respiratory burst observed at 30 min of cell stimulation (Fig. 1B), thus underscoring the role of NADPH oxidase activity in generation of ROS.

Since NADPH oxidase activity has been reported to impact the expression of several different immunomodulatory proteins [75, 84, 149, 152], we next sought to examine whether it also played a role in Tat and cytokine-mediated induction of CXCL10 in astrocytes. U-87 cells were pre-treated with apocynin, followed by stimulation with Tat and the cytokines to determine if inhibiting NADPH oxidase activity could decrease the expression of CXCL10. Our findings demonstrated a

dose-dependent decrease in CXCL10 expression in the presence of apocynin in stimulated astrocytes (Fig. 2A), thereby confirming the role of NADPH oxidase in the induction of CXCL10.

In order to further confirm the role of NADPH oxidase in the induction of CXCL10 expression U-87 cells were transfected with either siRNAs against gp91<sup>phox</sup> or scrambled siRNA conjugated to GFP. This critical membrane bound subunit was chosen because in the absence of gp91<sup>phox</sup> the activated cytosolic subunits of NADPH oxidase are unable to dock with the membrane components (gp91<sup>phox</sup> and gp22<sup>phox</sup>), consequently leading to lack of enzymatic activity. It has also been demonstrated that gp91<sup>phox</sup> is up-regulated in activated astrocytes [149, 150], possibly through a positive feedback loop with the transcription factor NF-κB [151]. Additional support for selection of gp91<sup>phox</sup> as a target comes from the use of gp91<sup>phox</sup> knock out mice. The neuro-inflammation, and thus the neuronal toxicity, caused by cells in these mice is greatly reduced, indicating that therapies targeting NADPH oxidase could be beneficial [75, 149].

To assess the role of NADPH oxidase in CXCL10 induction, 48 hours following transfection with siRNA against gp91<sup>phox</sup> U-87 cells were stimulated for 24 hours before the supernatants were collected and analyzed for CXCL10 content. Similar to findings with apocynin pretreatment, knock down of the gp91<sup>phox</sup> subunit also resulted in a concomitant reduction of CXCL10 expression, thus underlining the role of NADPH oxidase in this process.



Given that NADPH oxidase can participate in signal transduction in a multitude of ways [84, 152-154], ultimately impacting transcriptional regulation, several signaling pathways were assessed for changes in the presence of apocynin. Since the Rac1/2 subunit of NADPH oxidase can effect MAPK signal pathways [84], and the dismutation of superoxide to hydrogen peroxide affects Ras activation leading to MAPK and Akt phosphorylation [152-154], these pathways were selected for further examination. Previously it has been shown that Tat in combination with the cytokines IFN- $\gamma$  and TNF- $\alpha$ , was able to increase the phosphorylation states of p38, Jnk, and Akt compared with cells stimulated with the cytokines alone. Interestingly, in our previous findings we have demonstrated that Tat-mediated potentiation of CXCL10 did not involve Erk1/2 phosphorylation, unlike the cytokine-mediated activation of CXCL10 that did involved Erk1/2 activation [141]. In our current findings we demonstrated that pre-treatment of astrocytes with apocynin followed by stimulation with the Tat/cytokine mix resulted in decreased phosphorylation of the MAPK proteins Jnk and Erk1/2, but not that of p38 (Fig. 4A). This data thus implicates that NADPH oxidase is involved, at least, in part, in the activation of Jnk and Erk1/2, but plays no role in Tat-mediated activation of p38.

In addition to diminished activation of MAPK, apocynin was also able to decrease the activation of the Akt survival pathway (Fig. 5A). One possible explanation for this could be the ability of superoxide (generated via NADPH oxidase) to impact Ras through its conversion to hydrogen peroxide, and Ras in turn, can result in activation of PI3K- Akt pathway [84, 152-154]. These findings this

suggest the pivotal role of NADPH oxidase in regulation of various signal transduction pathways at multiple levels.

It has been well documented that the Jnk, Erk1/2, and Akt pathways are capable of converging on a common transcription factor, NF- $\kappa$ B. NF- $\kappa$ B is sensitive to redox related signaling, and has been shown to be affected by the generation of ROS [73, 74]. It has been previously reported that NADPH oxidase activation can be directly linked to NF- $\kappa$ B phosphorylation and nuclear translocation [84, 151, 155]. Taking into account apocynin-mediated reduction of Jnk, Erk1/2, and Akt activation, and the fact that NF- $\kappa$ B has been previously linked to NADPH oxidase, we next examined the effect of apocynin on NF- $\kappa$ B activation and translocation in U-87 cells stimulated with the Tat/cytokine mix. Astrocytes pretreated with apocynin followed by stimulation with the Tat/cytokine mix resulted in the decreased phosphorylation of cytosolic I $\kappa$ B $\alpha$  (Fig. 6A). I $\kappa$ B $\alpha$  normally sequesters NF- $\kappa$ B in the cytosol, but upon phosphorylation I $\kappa$ B $\alpha$  is degraded, thereby releasing NF- $\kappa$ B which subsequently translocates into the nucleus.

In addition to decreased levels of phosphorylated I $\kappa$ B $\alpha$  in the cytosol, apocynin treated astrocytes also displayed a corresponding reduction of activated NF- $\kappa$ B p65 subunit in the nuclear extracts of treated cells. Decreased expression of CXCL10 in apocynin pretreated, stimulated astrocytes could therefore be explained by the inhibition of NF- $\kappa$ B activation, which has been implicated as a critical regulatory factor in the transcription of CXCL10.

Having determined the mechanism by which apocynin pre-treatment of stimulated astrocytes resulted in decreased CXCL10 expression, it was of interest to examine the functional implications of this process in CXCL10-mediated neurotoxicity. Neuronal survival was monitored in the presence of astrocyte conditioned media collected from stimulated astrocytes in the presence or absence of apocynin pre-treatment. The astrocyte conditioned media from the control astrocytes did not impact neuronal survival. As expected, astrocyte conditioned media from the Tat/cytokine mix stimulated cells was significantly toxic to the neurons. In contrast, apocynin pre-treated conditioned media from stimulated astrocytes was able to rescue neuronal toxicity. The role of CXCL10 as a player in astrocytes conditioned media was validated by blocking the CXCL10 effect using a CXCL10 neutralizing antibody. It was of interest, however, that blocking CXCL10 activity with a neutralizing antibody did not reverse the neuronal toxicity to control levels. This lead us to speculate that there must be other neurotoxic factors, such as MCP-1 and/or IL-6, that could be toxic to the neurons and are released by stimulated astrocytes. Both MCP-1 and IL-6 expression are regulated NADPH oxidase activation, and apocynin treatment has been shown to decrease their expression.

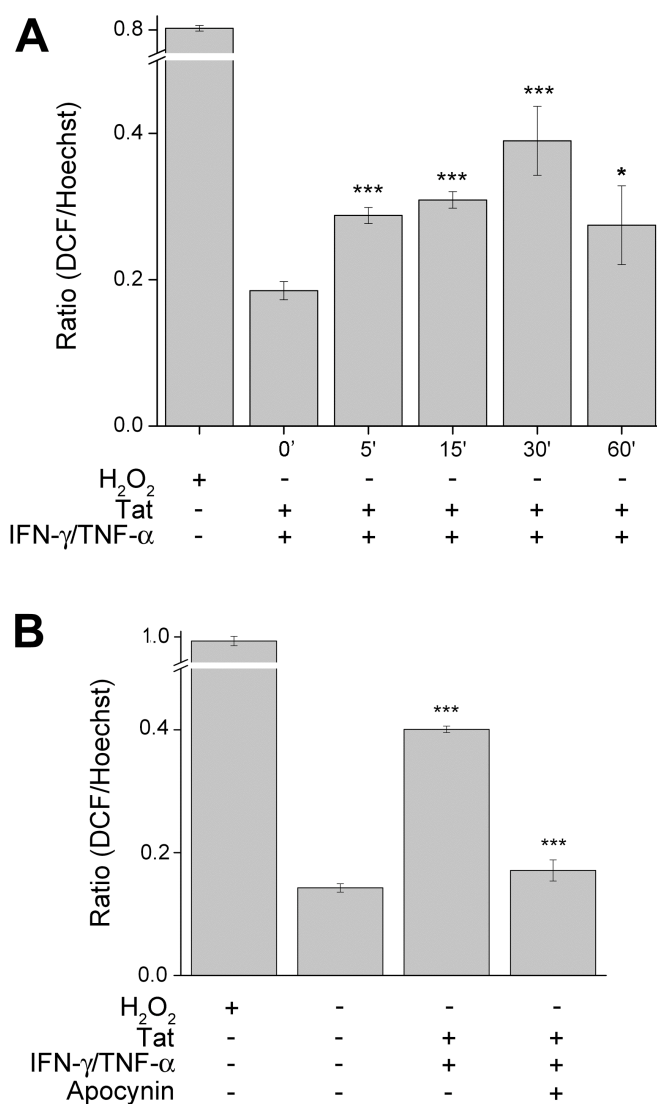
Taken together these data suggest the mechanism(s) by which Tat, IFN- $\gamma$ , and TNF- $\alpha$  can activate NADPH oxidase to augment CXCL10 induction in astrocytes. Using pharmacological and gene knock down approaches, we demonstrate that Tat and cytokine-mediated activation of astrocytes and induction of CXCL10 involves generation of ROS, activation of Jnk, Erk1/2, and Akt pathways and subsequent

activation of NF- $\kappa$ B leading to CXCL10 gene transcription. Released CXCL10 could in turn, be toxic for neurons, thereby enhancing neuropathogenesis. These findings have implications for patients affected by HAD in that it represents a mechanism whereby therapeutic reduction of both oxidative stress and the release of pro-inflammatory agents, can have beneficial effects for HIV-infected patients.

### **Acknowledgments**

We acknowledge the technical assistance of Shannon Callen in the preparation of this manuscript.

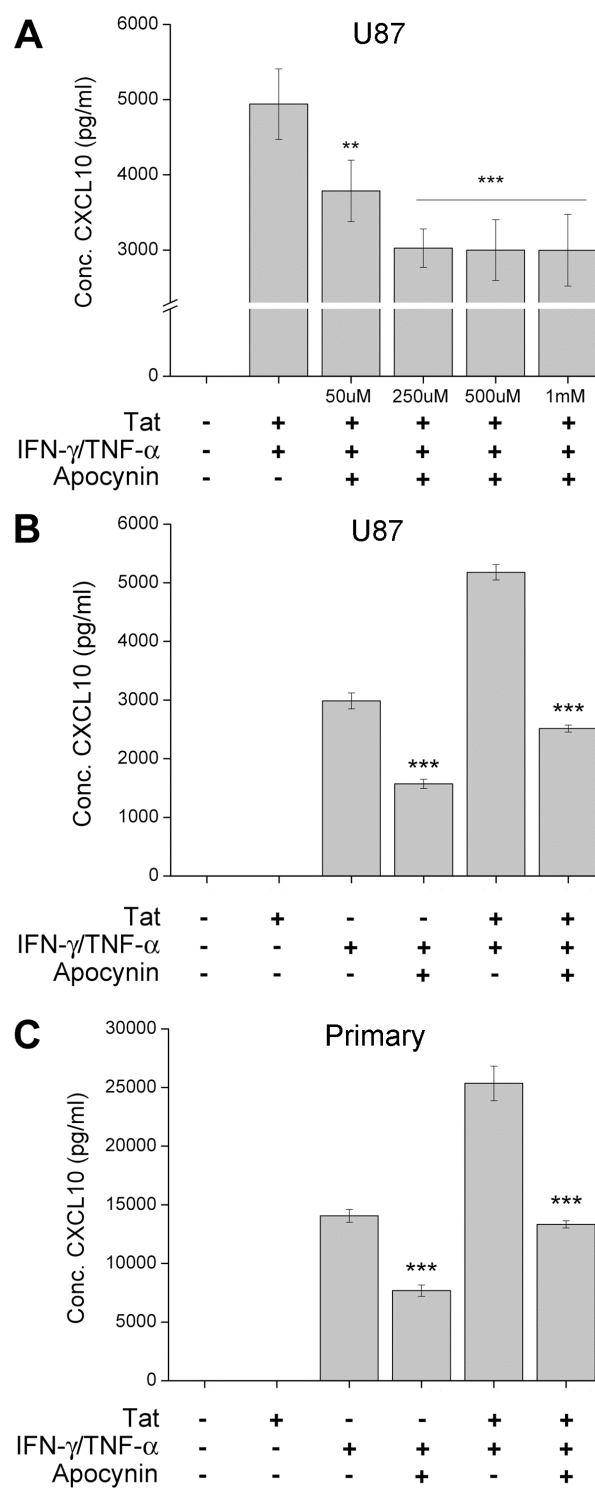
**Figure 1**



**Figure 1:** Measurement of oxidative stress. **(A)** U-87 astrocytes were either untreated or treated with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture for 0-60min, prior to incubation with carboxy-H<sub>2</sub>-DCF-DA and assessed for oxidative stress. Values are displayed as a ratio of the DCF fluorescent value/Hoechst (nuclear stain) fluorescent value. A respiratory burst culminates after 30 min of stimulation. **(B)** U-87 astrocytes were

untreated, treated with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture, or pre-treated with apocynin (250 $\mu$ M) followed by stimulation with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture for 30min. Apocynin pre-treatment was able to abrogate the respiratory burst observed in the Tat/cytokine treated astrocytes. The data represents the mean  $\pm$  SD from three independent experiments (\*,  $p < 0.05$ , \*\*\*,  $p < 0.001$ ).

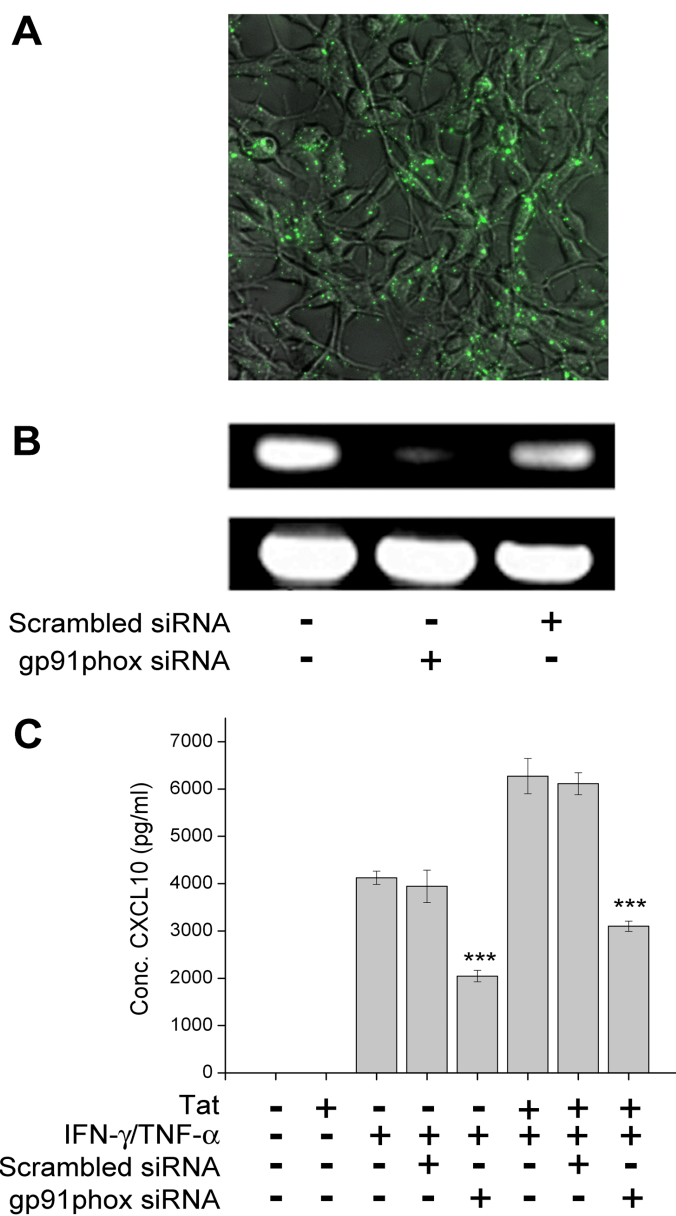
**Figure 2**



**Figure 2:** Apocynin decreases CXCL10 expression. **(A)** U-87 astrocytes were pretreated with various doses of apocynin (50 $\mu$ M-1mM) for one hour prior to stimulation with the Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture. After 24hours of incubation supernatant fluids were assessed for CXCL10 content by ELISA. Both U-87 **(B)** and primary human astrocytes **(C)** were treated either with the cytokines alone, the Tat/cytokine mixture, or pretreated with apocynin followed by stimulation with either the cytokines or the Tat/cytokine mixture. The data represents the mean  $\pm$  SD from three independent experiments (\*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).



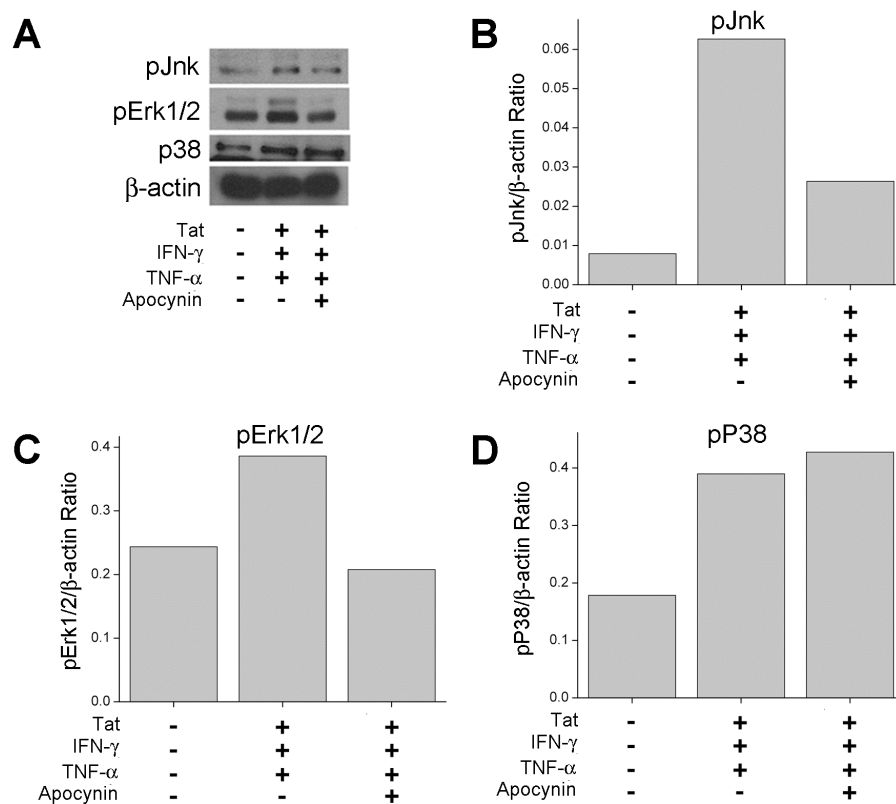
**Figure 3**



**Figure 3:** Knocking down the NADPH oxidase subunit gp91<sup>phox</sup> decreased CXCL10 expression. (A) U-87 cells demonstrating transfection of the scrambled siRNA

conjugated to GFP 24hours after transfection. **(B)** RT-PCR for gp91<sup>phox</sup> 48hours after mock transfection or transfection with gp91<sup>phox</sup> siRNA or scrambled siRNA. **(C)** CXCL10 ELISA demonstrating decreased CXCL10 in U-87 astrocytes transfected with gp91<sup>phox</sup> siRNA as compared to mock transfected or scrambled siRNA transfected cells. The data represents the mean  $\pm$  SD from three independent experiments (\*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

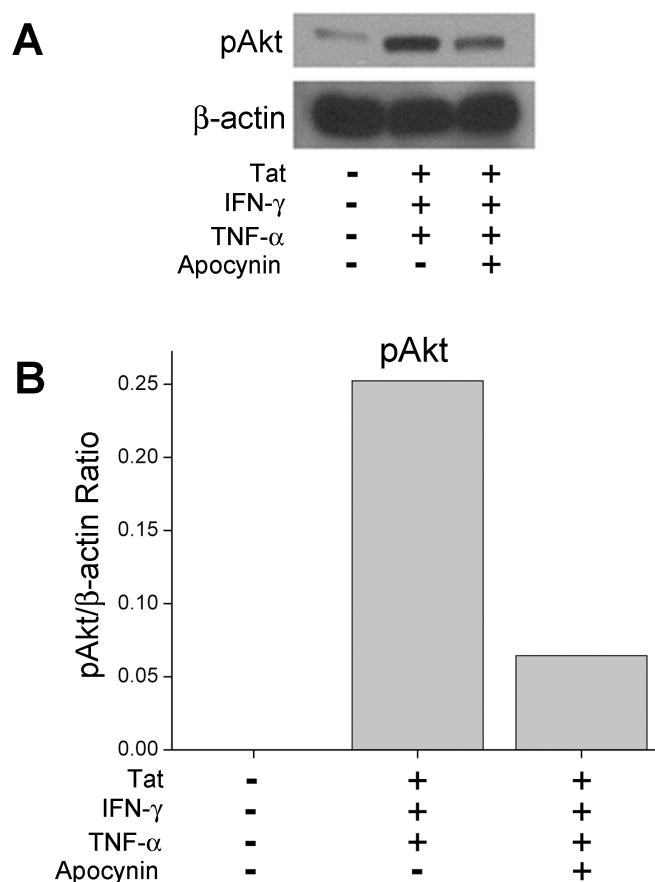
**Figure 4**



**Figure 4:** The Jnk and Erk1/2 signaling pathways, but not p38, are affected by apocynin treatment in stimulated U-87 astrocytes. **(A)** Western Blot analysis of cytosolic lysates collected from cells untreated, treated with Tat/IFN-γ/TNF-α, or pre-treated with apocynin prior to stimulation with Tat/IFN-γ/TNF-α for 60 min. The blots were probed with antibodies against phospho-p38, phospho-Jnk, and phospho-Akt. An antibody against β-actin was used to reprobe the blots for normalization. **(B,**

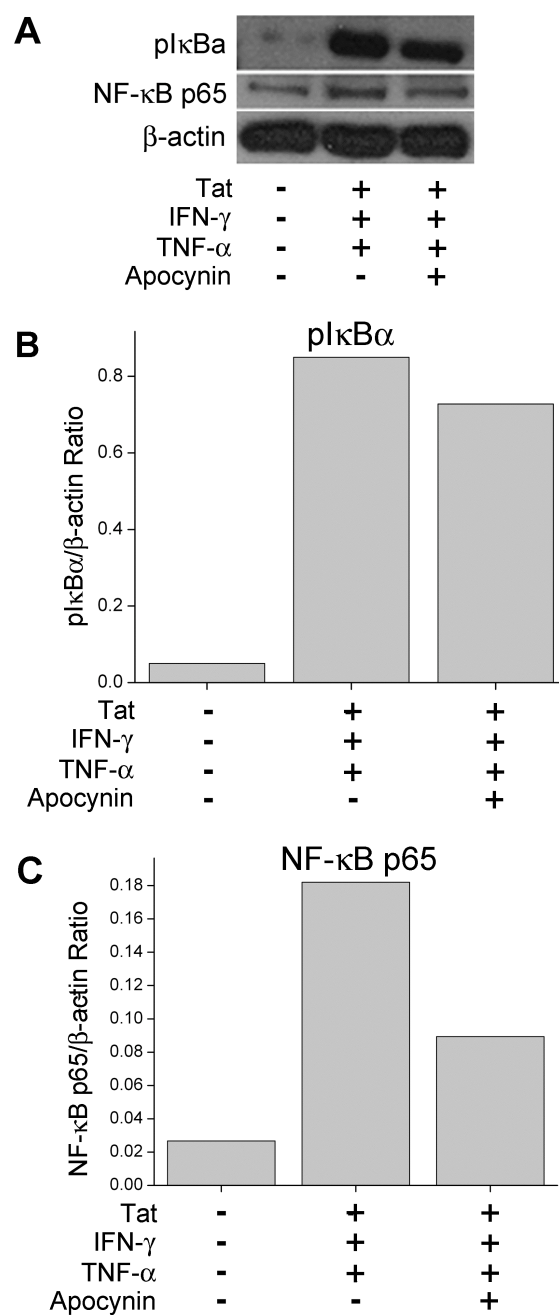
**C and D)** Densitometric scans illustrating the ratio of phospho-Jnk, Erk1/2 and p38 normalized to  $\beta$ -actin levels.

**Figure 5**



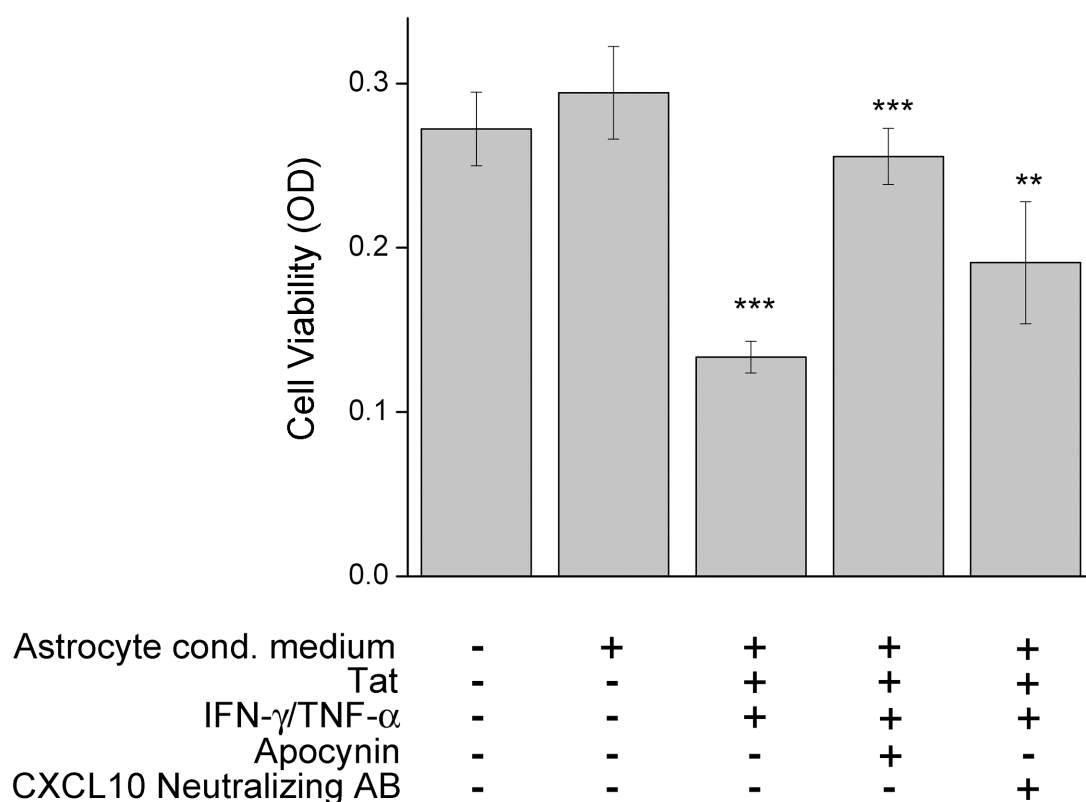
**Figure 5:** The Akt signaling pathway is affected by apocynin treatment in stimulated U-87 astrocytes. **(A)** Western Blot analysis of cytosolic lysates collected from cells untreated, treated with Tat/IFN- $\gamma$ /TNF- $\alpha$ , or pre-treated with apocynin prior to stimulation with Tat/IFN- $\gamma$ /TNF- $\alpha$  for 60 min. The blots were probed with antibodies against phospho-p38, phospho-Jnk, and phospho-Akt. An antibody against  $\beta$ -actin was used to reprobe the blots for normalization. **(B)** Densitometric scans illustrating the ratio of phospho-Akt to  $\beta$ -actin levels.

**Figure 6**



**Figure 6:** NF- $\kappa$ B activation and nuclear translocation was affected by apocynin pre-treatment of stimulated astrocytes. **(A)** Western Blot analysis of the cytosolic (pI $\kappa$ B $\alpha$ ) and nuclear (NF- $\kappa$ B p65) lysates collected from cells untreated, treated with Tat/IFN- $\gamma$ /TNF- $\alpha$ , or pre-treated with apocynin prior to stimulation with Tat/IFN- $\gamma$ /TNF- $\alpha$  for 60 min. The blots were probed with antibodies against phospho- I $\kappa$ B $\alpha$  and phospho- NF- $\kappa$ B p65. Antibody against  $\beta$ -actin was used to reprobe the blots for normalization. **(B, and C)** Densitometric scans illustrating the ratio of phospho- I $\kappa$ B $\alpha$  and phospho- NF- $\kappa$ B p65.

**Figure 7**



**Figure 7:** An MTT cell survival assay utilizing primary rat cortical neurons treated with U-87 astrocytes conditioned media. U-87 astrocytes were untreated, treated with Tat/IFN- $\gamma$ /TNF- $\alpha$ , or pre-treated with apocynin (250 $\mu$ M) before stimulation with Tat/IFN- $\gamma$ /TNF- $\alpha$  for 48 hours. The conditioned media collected from treated cells was then added onto primary rat cortical neurons for 24 hours prior to conducting the MTT assay. Portion of the Tat/IFN- $\gamma$ /TNF- $\alpha$  treated conditioned media from the astrocytes was also incubated with a CXCL10 neutralizing antibody prior to addition



to the neurons. The data represents the mean  $\pm$  SD from three independent experiments (\*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

## **Chapter 6**

### **Conclusions**

To this date there has been a paucity of information on the molecular mechanisms surrounding signal transduction in astrocytes during HIV and the impact these signaling pathways have had on the induction of cytokines and chemokines within these astrocytes. While several studies have reported on the ability of activated astrocytes to release specific cytokines and chemokines, such as CXCL10, MCP-1, IL-6, and TNF- $\alpha$ , these papers failed to navigate through the molecular mechanisms driving such production. Additionally, very little work on signal transduction in astrocytes activated as a result of multiple stimuli has been published regardless of the stimulating factor(s) or end product.

CXCL10 was the end product chosen for our studies due to its increased presence in HIV, that fact that it is positively correlated with HIV neuropathogenesis, and its innate ability to be both directly and indirectly neurotoxic. Astrocytes have been demonstrated to release CXCL10 upon treatment with several individual stimulants, including the pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ . These cytokines are two well studied molecules in their own right, but of more significance might be the fact that they can act synergistically on CXCL10 in several cell types, including astrocytes. Astrocytes also express CXCL10 in the presence of three HIV-1 proteins, Tat, Nef, and gp120, although at modest levels.

However, despite that fact that HIV/viral proteins and the cytokines had been demonstrated to affect CXCL10 expression individually, there were no reports using all three as a stimulus. Without a doubt it is challenging to assess the impact that three stimulants will have on a cell, yet the physiological relevance of using several

stimuli is explicit. During HIVE there are a plethora of inflammatory factors and other host factors, along with HIV/viral proteins interacting with cells of the CNS at the same time. Assessing the singular effect of one stimulant does not give an accurate picture of the stresses of a cells exposed to multiple stimulants. To this end we engaged in evaluating the effect that all three stimuli, IFN- $\gamma$ /TNF- $\alpha$ /HIV, had on CXCL10 induction in astrocytes by exploring the molecular and cellular mechanisms through which this induction occurred.

We started by asking the question, what is the molecular mechanism(s) by which HIV-1 co-operates with IFN- $\gamma$  and TNF- $\alpha$  to induce CXCL10 expression in astrocytes? What we found was that in the presence of HIV/IFN- $\gamma$ /TNF- $\alpha$  the RNA and protein levels of CXCL10 synergistically increased compared to either treatment alone. Delving into the cellular and molecular mechanism of this synergistic induction we found that astrocytes, in the presence of all three stimuli, under go massive signal transduction in the MAPK pathways Erk1/2 and p38, along with the Akt pathway, all of which were crucial for CXCL10 induction as determined by inhibitor assays. Furthermore, these pathways converged on the essential transcription factors in CXCL10 regulation, NF- $\kappa$ B and STAT-1 $\alpha$  as a means to increase CXCL10 expression. These results represented the first time that signal transduction was explored in astrocytes treated with three different stimuli in an effort to establish the pathways responsible for CXCL10 induction. Since there were so few reports on astrocyte signaling these results are helping to lay the groundwork on detailed molecular and cellular mechanisms of chemokine induction in astrocytes.

However, the signaling pathways examined in that study did not look at the effects of the individual stimuli on the pathways. Therefore, there was no way of knowing whether HIV-1 was actually acting on any of these pathways, or if the activation was a result of only the cytokine/receptor mediated events. This is a crucial piece of information since astrocytes lack CD4, the receptor for HIV-1. So if HIV-1 is causing changes in signal transduction, how is it doing so?

We addressed that question in Chapters 3 and 4 when we asked ourselves, which HIV-1 viral protein(s) is co-operating with IFN- $\gamma$  and TNF- $\alpha$  to enhance CXCL10 release from astrocytes and by what mechanism? Since the receptor for HIV-1 on astrocytes is unknown we decided to examine the individual viral proteins known to activate astrocytes and induce CXCL10 expression. These proteins were Nef, Tat, and gp120. While results for Nef and gp120 were less than inspiring, Tat in combination with IFN- $\gamma$  and TNF- $\alpha$  was able to increase CXCL10 expression significantly compared to either treatment alone. Therefore, Tat was the HIV-1 protein chosen for the continuation of the study. We found that although Tat had little effect on signal transduction on its own, when in the presence of the cytokines IFN- $\gamma$  and TNF- $\alpha$  it was able to potentiate the activation of those signals. In doing so Tat was also able to potentiate the activation and translocation of the transcription factors NF- $\kappa$ B and STAT-1 $\alpha$  to impact CXCL10 regulation.

These findings were novel on several fronts: 1) there was little to no literature available on the effect of Tat on astrocyte signaling; 2) the idea that Tat is able to potentiate the activation of signaling pathways being utilized by the IFN- $\gamma$  and TNF- $\alpha$

receptors is very intriguing and has not been reported elsewhere; and 3) the ability of Tat in combination with IFN- $\gamma$  and TNF- $\alpha$  to significantly increase CXCL10 expression in astrocytes, or any cell type for that matter, had not been published. Therefore these findings advanced the knowledge base of astrocyte signaling in the field. However, since Tat is able to diffuse through the cellular membrane the mechanism by which it was impacting signal transduction in astrocytes remained undetermined.

In an effort to elucidate the mechanism by which Tat was able to potentiate cellular signaling responsible for CXCL10 induction we examined the role of oxidative stress in Chapter 5. When utilizing the DCF assay to verify the presence of a respiratory burst we found that treatment of the astrocytes with IFN- $\gamma$ /TNF- $\alpha$ /Tat lead to a significant respiratory burst that was abrogated by the NADPH oxidase inhibitor, apocynin. Furthermore, treatment of the stimulated astrocytes with apocynin was able to decrease CXCL10 expression remarkably. Upon examining the cellular signaling pathways involved in CXCL10 regulation we found that apocynin was able to decrease the activation of these pathways, leading to a decrease in the activation and nuclear translocation of the transcription factor, NF- $\kappa$ B. Thus, we proposed that Tat was able to potentiate the effects of IFN- $\gamma$  and TNF- $\alpha$  by activating the membrane bound enzyme NADPH oxidase, resulting in increased CXCL10 expression.

With a possible mechanism teased out for Tat potentiation of CXCL10 expression in IFN- $\gamma$  and TNF- $\alpha$  treated astrocytes, we wanted to know if inactivating

this pathway would result in less expression of the neurotoxic CXCL10, and therefore increased neuronal survival. We found that inhibiting NADPH oxidase with apocynin not only decreased CXCL10, but significantly increased neuronal survival. Taken together these results represented the first time NADPH oxidase has been linked to CXCL10 regulation in non-phagocytic cells and the first implication that Tat may be able to interact with membrane proteins as it diffuses through the cell.

The information gathered from the studies conducted in this dissertation define a mechanism by which HIV-1 Tat can potentiate CXCL10 expression in IFN- $\gamma$  and TNF- $\alpha$  stimulated astrocytes. These results are critical to field of HIV neuropathogenesis because they represent a wealth of information on astrocyte signaling biology, of which there was little prior knowledge, and introduces a novel mechanism in which Tat exploits cellular NADPH oxidase to increase the neurotoxic chemokine, CXCL10. While these studies contributed significantly to the field, they also raised pivotal questions regarding the role of NADPH oxidase in non-phagocytic cells and what the exact mechanism by which Tat activates NADPH oxidase may be. With any luck, future studies will be able to identify these mechanisms utilizing the signaling foundation laid down by the research conducted for this dissertation.

## **Chapter 7**

### **Future Directions**



Recently, the role of oxidative stress in the regulation of cytokine and chemokine expression has garnered increased awareness. Recent studies have linked NADPH oxidase activity to cytokine and chemokine production in microglia, macrophages, and astrocytes [73, 75]. Indeed, our studies utilizing apocynin support the role for NADPH oxidase in the regulation of CXCL10 in Tat, IFN- $\gamma$ , and TNF- $\alpha$  stimulated astrocytes at the cellular level. Future studies will utilize gp91<sup>phox</sup> deficient mice to further explore the role of NADPH oxidase/the therapeutic benefit of apocynin in the regulation of CXCL10. A number of studies have utilized this mouse model to explore the impact of NADPH oxidase on neuro-inflammation [75, 149, 157]. In particular Turchan-Cholewo *et. al.* used these mice to demonstrate decreased ability of these mice to express the pro-inflammatory factors such as MCP-1, IL-6, and TNF- $\alpha$  in activated microglia and macrophages [75]. Furthermore, this decrease in MCP-1, IL-6, and TNF- $\alpha$  resulted in enhanced neuronal survival in neurons subjected to microglia/macrophage conditioned media from the gp91<sup>phox</sup> deficient mice as compared to the microglia/macrophage conditioned media from WT mice [75].

In our future studies we will utilize the gp91<sup>phox</sup> deficient mice at both the *in vitro* and *in vivo* level. For the *in vitro* studies we would harvest primary astrocytes and neurons [145] from WT and gp91<sup>phox</sup> deficient mice. The astrocytes from both groups would then be treated with Tat, IFN- $\gamma$ , and TNF- $\alpha$  as previously described [142] and assessed for CXCL10 content by ELISA. An additional experiment would be to take the supernatants from the stimulated WT and gp91<sup>phox</sup> deficient astrocytes,

and place them on WT neurons and assess the cells for neuronal damage using an MTT assay described previously [142]. Using an NADPH oxidase inhibitor (apocynin) as a therapeutic target to decrease both CXCL10 expression and the concomitant neuronal dysfunction/death could aid in the development of therapeutic intervention strategies for HAND.

For the *in vivo* study we would again utilize the gp91<sup>phox</sup> deficient mice along with the WT mice. In these studies the mice would be injected with either saline or a Tat/IFN- $\gamma$ /TNF- $\alpha$  mixture via the carotid artery. The carotid artery approach will be utilized in hopes of decreasing the amount of neuronal tissue damage caused by direct micro-injection into the brain. After injection the brains will be extracted and flash frozen for RNA and protein analysis. If our data from cell culture holds true for *in vivo* work, then the levels of the pro-inflammatory agents should be decreased in gp91<sup>phox</sup> deficient mice injected with the Tat/cytokine mixture as compared to the Tat/cytokine injected WT mice at both the RNA and the protein level and we should see increased neuronal survival.

Utilizing the gp91<sup>phox</sup> mouse model would determine whether NADPH oxidase plays a critical role in HIV-associated neuroinflammation. With that information it would be useful to see if apocynin, the specific pharmacological inhibitor of NADPH oxidase, can cross the BBB and be an effect anti-inflammatory drug for the CNS. To accomplish this apocynin could be injected I.P. or I.V. into a mouse and after a certain period the mouse brain would be analyzed of the presence of apocynin either by mass spectrometry or by tagging apocynin with a dye or

fluorescent molecule. Enzymatic activity of CNS derived NADPH oxidase could also be assessed.

If apocynin has the potential to cross the blood brain barrier and lower HIV-associated neuro-inflammation, it might also be a valid adjunct therapy for other causes of neuroinflammation, be it viral/bacteria or stroke/traumatic brain injury. In each of these cases neuroinflammation can spiral out of control, with the end result being detrimental instead of helpful. In the process of inflammation, cytokine and chemokines are released from activated cells and activate or recruit other cells to the area, but another aspect of inflammation is the release of growth factors and other molecules to help facilitate repair of the damage. Therefore, completely inhibiting inflammation would not solve the problem. Since apocynin selectively inhibits NADPH oxidase, it doesn't completely abrogate the release of inflammatory agents/growth factors. Thus, apocynin may represent a treatment option for those who still need a partially functioning immune system to fight off infection or heal neuronal damage. This hypothesis could be tested in animal models of viral/bacterial meningitis or in animal models of stroke/traumatic brain injury to see if it can reduce neuroinflammation while decreasing the recovery period.

Taken together, the data from these experiments could have implications for the use of apocynin as an anti-inflammatory not only for those suffering from HAND, but also for other causes of CNS inflammation.

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