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This dissertation explores the role of chemokine CXCL8 during human immune deficiency virus (HIV)-1 infection in the brain. Chemokine CXCL8 is an important neutrophil chemoattractant implicated in various neurodegenerative disorders. It is upregulated in the brains and cerebrospinal fluid of HIV-1 infected individuals suggesting its potential role in HIV-1 associated neuroinflammation. Astrocytes are known to be the major contributors to the CXCL8 pool. Interleukin (IL)-1β activated astrocytes exhibit significant upregulation of CXCL8.

In order to determine the signaling pathways involved in CXCL8 regulation in astrocytes, we employed pharmacological inhibitors for non-receptor Src homology-2 domain-containing protein tyrosine phosphatase (SHP) 2 and mitogen-activated protein kinases (MAPK) pathway and observed reduced expression of CXCL8 following IL-1β stimulation. Thus, our findings suggest an important role for SHP2 in CXCL8 expression in astrocytes during inflammation, as SHP2, directly or indirectly, modulates p38 and extracellular signal regulated kinase (ERK) MAPK in the signaling cascade leading to CXCL8 production.

In the post-antiretroviral therapy (ART) era, low level of productive replication of HIV-1 in brain is a critical component of neuropathogenesis regulation. HIV-1 replication is a complex mechanism involving both host and viral factors. The majority of viral replication in brain occurs in perivascular macrophages and/or

microglia. In this study, we investigated the effect of CXCL8 on productive infection of HIV-1 in human monocytes-derived macrophages (MDM) and primary human microglia. The results show that CXCL8 mediates productive infection of HIV-1 in MDM and microglia *via* receptors CXCR1 and CXCR2 and induces HIV-1 long terminal repeat (LTR) promoter activity.

Detailed understanding of astrocyte signaling and HIV-1 replication, as presented in the thesis, will be relevant to glial-neuronal interactions, which are central to neuroinflammation in HIV-1 and many other neurodegenerative conditions. Also, modulation of levels of CXCL8 can be a therapeutic strategy for control of productive HIV-1 replication in the brain.

CHEMOKINE CXCL8 MEDIATED INTERCELLULAR INTERACTIONS IN HIV-1 ASSOCIATED DEMENTIA

By

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LIST OF PUBLICATIONS

1. **Manmeet K Mamik,** Sugato Banerjee, Timothy F Walseth, Renee Hirte, Lin Tang, Kathleen Borgmann and Anuja Ghorpade **(2011)** HIV-1 and IL-1beta regulate astrocytic CD38 through mitogen- activated protein kinases and nuclear factor-kappa B signaling mechanisms. **Journal of Neuroinflammation**, 8:145
2. **Mamik MK**, Ghorpade A **(2012)** Src Homology-2 Domain-Containing Protein Tyrosine Phosphatase (SHP) 2 and p38 Regulate the Expression of Chemokine CXCL8 in Human Astrocytes. **PLoS ONE** 7(9): e45596. doi:10.1371/journal.pone.0045596
3. **Mamik MK**, Ghorpade A **(2013)** Chemokine CXCL8 promotes HIV-1 replication in human monocyte-derived macrophages and primary microglia *via* NF-κB pathway. (*Submitted to Retrovirology*)
4. **Mamik MK**, Ghorpade A **(2013)** Chemokine CXCL8 in neuroinflammatory diseases: implications in HIV-1 infection: A Review. (*In preparation for submission to Retrovirology*)

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ABBREVIATIONS

Aβ	Amyloid beta
ANOVA	Analysis of variance
AP-1	Activator protein
BBB	Blood brain barrier
Bcl2	B-cell lymphoma 2
Ca^{2+}	Calcium
cART	Combined Anti-retroviral therapies
CCL2	Chemokine C-C motif ligand 2
C/EBP	CCAAT enhancer binding protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acid protein
gp120	Glycoprotein 120
GPCR	G-protein coupled receptor
HAART	Highly active anti retroviral therapy
HAD	HIV-1-associated dementia
HAND	HIV-1-associated neurocognitive disorders
HCV	Human cytomegalovirus
HIV-1	Human immunodeficiency virus-1
HIVE	HIV encephalitis
IL	Interleukin
JNK	Jun N-terminal kinase
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinases
MGC	Multinucleated giant cell
MMP	Matrix metalloproteinase
MDM	Monocyte-derived macrophages
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na^{+}	Sodium
NF-κB	Nuclear factor-kappaB
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PTP	Protein tyrosine phosphatase
PVDF	Polyvinyldifluoride
SHP2	Src Homology-2 Domain-Containing Protein Tyrosine Phosphatase
Tat	HIV-1 trans-activator of transcription
TNF-α	Tumor necrosis factor-alpha
Vpr	Viral protein R

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INTRODUCTION

Chemokine CXCL8 in neuroinflammatory diseases: Implications in HIV-1 infection

Abstract

Chemokine CXCL8 is a low molecular weight neutrophil chemoattractant implicated in various neurodegenerative disorders including Alzheimer's disease and stroke. Increased expression of CXCL8 has been reported in serum, plasma and brain of human immunodeficiency virus (HIV)-1 infected individuals with neurocognitive impairment, indicating its role in neuroinflammation associated with HIV-1 infection of the brain. Since chemokines are critical in eliciting immune responses in the central nervous system, CXCL8 is of particular importance for being the first chemokine described in the brain. Activation of astrocytes and microglia by HIV-1 and virus associated proteins results in production of this chemokine in the brain microenvironment. Consequently, CXCL8 exerts its effect on target cells *via* G-protein coupled receptors CXCR1 and CXCR2. Neutrophils are the main target cells for CXCL8; however, microglia and neurons also express CXCR1/CXCR2 and therefore are important targets for CXCL8-mediated crosstalk. In this Chapter, we will focus on CXCL8 production, signaling and regulation in neuronal and glial cells in response to HIV-1 infection. We highlight the role of HIV-1 secreted proteins such as Tat, gp120, Nef and Vpr in the regulation of CXCL8. We discuss dual role of CXCL8 in neurodegeneration as well as neuroprotection in the CNS. In conclusion, CXCL8 can potentially be one of the next generation therapeutic targets for controlling neuroinflammation in context of HIV-1 infection.

1. Introduction

Chemokines, or chemotactic cytokines, are low molecular weight proteins (8-11 kDa) that have the ability to induce directional migration and attract leukocyte subsets to sites of inflammation. Depending upon position of conserved two N-terminal cysteine residues, chemokines are divided into four families; CXC, CC, C and CX_3C. More than 50 chemokines and 20 chemokine receptors have been identified. Interestingly, the signaling amongst the chemokines and their receptors is complex with one chemokine binding to various receptors and vice versa. Our molecule of interest, CXCL8, belonging to CXC family, is the first chemokine identified in human brain and has a key role in neutrophil recruitment and activation (188, 194). In the periphery, CXCL8 is produced by a variety of cells such as monocytes, T-cells, neutrophils, endothelial cells, fibroblasts, etc (150). Natural Killer (NK) cells produce CXCL8 during early stages of differentiation, which in turn contribute towards preferential expansion of NK cell lineage (147). However, CXCL8 is generally expressed in cells activated by certain stimuli like interleukin (IL)-1β and tumor necrosis factor (TNF)-α (142) and/or by bacterial or viral infections.

Chemokines have been implicated in wide range of neurological disorders like Parkinson's disease, Alzheimer's disease (AD), traumatic brain injury and others. Increased production of CXCL8 by peripheral blood monocytes in patients with multiple sclerosis (MS) has been reported (199). Positive correlation was found between amyloid-beta (Aβ) levels and CXCL8 (38). Brain pericytes play an important role in formation and function of blood brain barrier (BBB). They

regulate BBB by controlling gene expression in endothelial cells and astrocyte functions in neurovascular unit (6). Human cytomegalovirus (HCMV) infected pericytes secrete CXCL8 along with many neuroinflammatory cytokines like CCL5, TNF-α, IL-1β and IL-6 (1). Since CXCL8 is an important inflammatory mediator regulating neutrophils and T-cells, it is of prime importance in diseases that affect host's immune system such as HIV-1 infection. Levels of CXCL8 are upregulated in plasma, serum (140, 154) and cerebrospinal fluid (CSF) of HIV-1 infected individuals, on or off antiretroviral therapy (ART).

This chapter is aimed at providing regulation and function of CXCL8 in various cell types of central nervous system (CNS). We will discuss its role in neurodegenerative disorders with emphasis on HIV-1 infection of the brain. In addition to reporting the current progress in CXCL8-mediated interactions in the CNS, we will focus on CXCL8 as a potential therapeutic target in HIV-1 infection.

1.1 HIV-1 Epidemic and Neurodeneration

HIV-1 infection is pandemic, which is evident from the fact that at present an estimated 33 million individuals are infected worldwide with the predominant form of virus, HIV-1. There are currently 1.3 million people living with HIV-1 infection in the United States of America (UNAIDS report, 2012). Despite the advent of ART, HIV-1 associated neurocognitive disorders (HAND) still afflict 40-50% of the HIV infected population. The most severe form of HAND is described as HIV-1 associated dementia (HAD), which is defined as frontal-subcortical pattern of impairment (163). Treatment of HIV-1 infected individuals with ART considerably

reduced prevalence of opportunistic infections and improved survival rates (157). As HIV-1 infected individuals are living longer, the damaging effects of the virus and immune responses persist in the brain. Diminished coordination, reduced functioning and impaired memory are key features of HAD (84). The causes of HIV-1 associated neurodegeneration include chronically activated glial cells and oxidative stress. Secretion of proinflammatory cytokines and prolonged activation of glial cells results in inflammatory responses generated in the CNS, leading to long-term neuroinflammation and neuronal damage (79). Therefore, the study of cytokine/chemokine imbalances has emerged as an important area in the context of controlling neuroinflammation caused by HIV-1 infection.

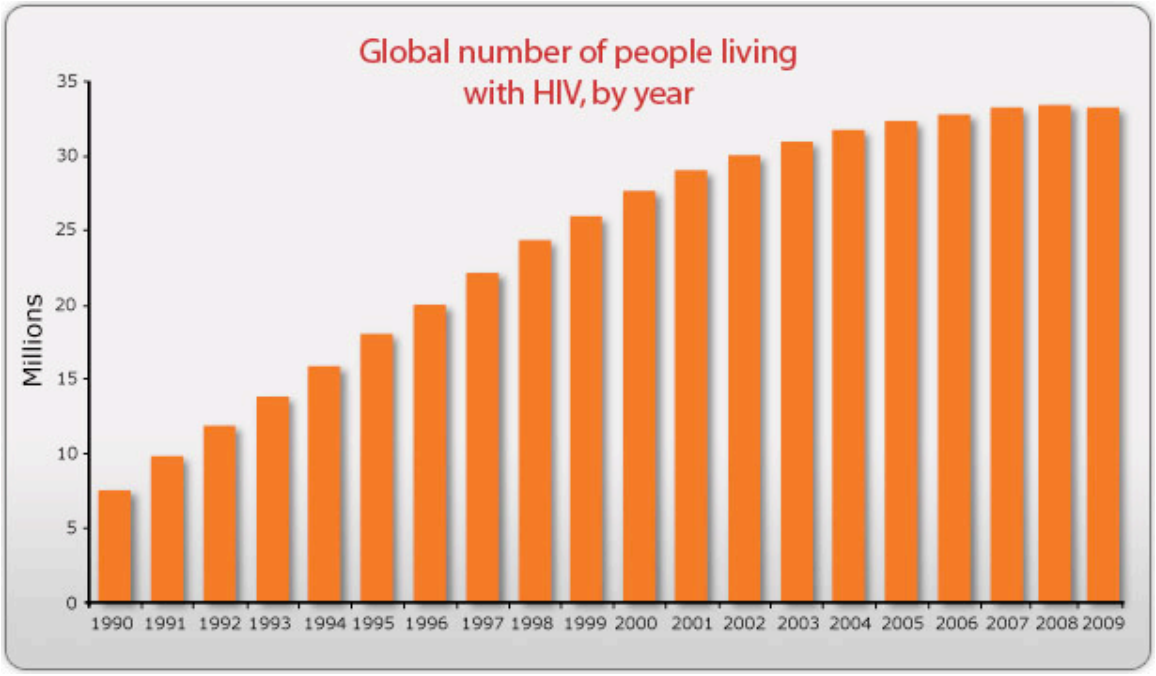


Figure 1.1 Graph showing number of people living globally with HIV. The number of people living with HIV increased from 8 million in 1990 to 34 million by the end of 2011. This may be attributed to both new infections as well as

increased longevity of infected persons due to ART (adapted from www.avert.org).

1.2 Chemokine CXCL8: Structure and Function

CXCL8 is a 99 amino acid precursor protein, which is then cleaved into residues containing 69 to 77 amino acids. The various isoforms differ in their neutrophil attracting properties with the shorter isoform being a stronger chemoattractant. *In vivo*, CXCL8 with 72 amino acid residues is the most predominant form (148). Neutrophil recruitment by CXCL8 depends upon chemical composition where both monomeric and dimeric forms are essential. The monomeric form of CXCL8 is the functional form (170); however, dimerization of CXCL8 induces recruitment of neutrophils, therefore a balance between the two biologically active forms is important for maintaining neutrophil recruitment in equilibrium and for proper inflammatory responses (44). The monomer and dimer differentially activate and regulate CXCR1/2 receptors. Monomeric CXCL8 increases intracellular calcium (Ca^{2+}) mobilization and chemotaxis. Phosphorylation, desensitization and internalization of CXCR1 is also enhanced by monomeric CXCL8 as compared to dimeric form. In contrast both monomeric and dimeric CXCL8 exert similar effects on CXCR2 (156). The CXC motif is a critical component in binding affinity of the chemokine with receptors CXCR1/2. However, receptor binding and activation are two different phenomena and the CXC motif primarily regulates the binding (105).

The CXCL8 promoter has binding sites for various transcription factors like nuclear factor (NF)-κB and activator protein-1 (AP-1). Inflammatory stimuli like IL-1β and TNF-α, viruses and viral proteins can promote nuclear localization of NF-κB and AP-1, leading to upregulation of CXCL8 in different cells (134, 153). Consequently, CXCL8 promotes adhesion of neutrophils to extracellular matrix proteins and endothelial monolayers. CXCL8 promotes neutrophil migration into tissues and across endothelium (150). Apart from neutrophils, CXCL8 can induce chemotaxis in basophils, B and T lymphocytes. Moreover, CXCL8 exerts angiogenic properties on endothelial cells expressing CXCR1/2 (88).

1.3 CXCL8 signaling in different cell types of CNS

Astrocytes release CXCL8 when activated by HIV-1 glycoprotein (gp) 120 *via* the NF-κB pathway (189). HIV-1 gp120 also induces CXCL8 expression in human brain microvascular endothelial cells *via* signal transducers and activators of transcription (STAT)1 activation. This suggests the involvement of this chemokine in HIV-1 gp120-induced inflammation and BBB dysfunction (225). In cells co-infected with human cytomegalovirus (HCV) and HIV, both microglia and astrocytes show upregulation of CXCL8, which is exacerbated due to coinfection (211). Soluble factors released by activated peripheral blood mononuclear cells (PBMCs) lead to upregulation of CXCL8 in astrocytoma cell lines (97). Microglia produce CXCL8, following CD40 ligation, *via* ERK1/2 dependent pathway. Transcription factors NF-κB and AP-1 localize to nucleus and are necessary for CD40 ligand-induced CXCL8 expression in microglia (42). CXCL8 is produced by multiple cell types including macrophages, monocytes, and endothelial cells (48,

133, 151, 167, 172, 213). CXCL8 colocalizes within reactive astrocytes, but is absent in the normal CNS (160). Moreover, CXCL8 levels in CSF and serum are elevated during clinical relapses in MS (15). Considering the increased presence of monocytes and macrophages in and around inflammation, it suggests that increased production of CXCL8 impacts the CNS microenvironment and plays an important role in disease pathology (133)

1.4 CXCL8 receptors CXCR1/CXCR2: expression and function in CNS

CXCL8 binds to rhodopsin like G-protein coupled receptors, CXCR1 and CXCR2, having important function in cellular signal transduction. Chemokines having glutamic acid-leucine-arginine (ELR) motif in their N-terminal domain bind to CXCR1/2 and attract neutrophils whereas non-ELR motif chemokines attract lymphocytes (188). However, due to limited structural information available CXCR1 mediated molecular mechanisms are not clearly understood. Recent demonstration of three-dimensional structure of CXCR1 (162) may facilitate the study of CXCL8 mediated CXCR1 activation. CXCR1 and CXCR2 are expressed by a variety of cells in the CNS. In fact, both CXCR1 and CXCR2 are expressed on astrocytes, neurons, oligodendrocytes and microglia (65, 80, 168) indicating possibility of CXCL8-mediated crosstalk amongst these cells. Despite their structural similarities, these receptors activate distinct signaling pathways in distinct cells (66). CXCR1 signaling primarily stimulates neutrophil migration through epithelial layers, whereas CXCR2 signaling promotes angiogenesis (198).

Both CXCL8 and the receptors are reported to be upregulated in MS and other neurological diseases (4, 160). CXCR1 and CXCR2 expressed on endothelial cell surface interact with HIV protein p17 and promote angiogenesis in HIV-related vascular diseases *via* activation of ERK and Akt pathway (21).

1.5 CXCL8: Role in neurodegenerative disorders

A. Alzheimer's disease and stroke

CXCL8 levels were elevated in patients with AD (38). Amyloid-beta induces CXCL8 production in human monocytes (222). CXCL8 is produced by microglia, astrocytes and neurons in response to beta amyloid and in turn protects human neurons from Aβ induced toxicity (7). Astrocytes release CXCL8 after exposure to thrombin. Thrombin is implicated in neuroinflammatory response in brain after stroke and CNS pathologies. (193). Brain injury may result from microvascular occlusion caused by activated neutrophils in cerebral ischemia. Since CXCL8 is a neutrophil chemokine, inhibiting its action is a potential therapy target. Reparixin, a CXCL8 receptor blocker reduces neutrophil infiltration and tissue damage in ischemic rat brains (68, 210).

B. CXCL8 and HIV-1 infection

The link between HIV-1 disease progression and chemokine CXCL8 was suggested very early in the study of HIV-1 infection when increased CXCL8 was reported in serum of infected individuals (140). Plasma CXCL8 levels are also reported to be elevated in HIV-infected children (24). HIV-1 associated dementia individuals have elevated levels of CXCL8 (231). We have also shown increased

expression of CXCL8 mRNA and protein in brain lysates, collected from frontal cortex, of HIV-infected patients (136).

CXCL8 is increased and colocalizes with CD68/CD40 positive microglial cells (15) and expressed by astrocytes (182) in HIV-1 encephalitic tissue. CXCL8 is upregulated in both an astrocytic cell line infected with HIV-1 (40) and astrocytes treated with the HIV-1 trans-activator protein (Tat) (117).

It may not be concluded that increased CXCL8 observed during HIV-1 infection is directly leading to neuropathogenesis because various reports suggest a positive role for the chemokine. Interestingly, CXCL8 drives immune responsiveness in HIV-1 infected individuals *via* CXCR1+ subset of cytotoxic CD8 T cells. This subset of CD8 T cells is reported to preferentially express functional CXCR1 in HIV-1 infected individuals who are able to control HIV-1 replication transiently, when taken off ART (92). It suggests CXCL8 drives antigen specific effector mechanism of cytotoxic $CD8^+$ T cells to fight HIV-1 infection. Also, CXCL8 mediates CCR5 receptor cross phosphorylation and internalization providing resistance to HIV-1 infectivity since CCR5 is an important co-receptor required for viral entry (176).

1.6 HIV-1 Protein Interaction and CXCL8 Regulation

Exogenously secreted HIV-1 proteins are found in the sera and brains of HIV-1 infected patients and cultured cells. The secreted proteins including HIV-1 gp120, Tat, negative factor protein (Nef) and viral protein R (Vpr) have the ability to induce neurotoxic effects. Also, these secreted proteins interact with cells of

CNS and activate glial cells to produce inflammatory cytokines and chemokines (101). CXCL8 is differentially regulated in response to various HIV-1 proteins.

a) HIV-1 Vpr

HIV-1 viral protein R (Vpr) is a 14 kDa regulatory protein of HIV-1 virus which modulates virus replication in host cells by controlling nuclear import of pre-integration complex or modulating gene expression by transactivating HIV-long terminal repeat (LTR) (reviewed in (179)). The HIV-1 Vpr protein as well as the transcript is present in brains of HIV-infected individuals, suggesting potential role in direct/indirect neurotoxicity (102). High levels of HIV-1 Vpr are present in CSF of patients with HIV-1-associated neurocognitive impairments. Indeed, HIV-1 Vpr colocalizes with macrophages and neurons in basal ganglia and frontal cortex of HIV encephalitis (HIVE) brains (216). Considering that HIV-1 predominantly resides and replicates in macrophages, it clearly suggests role of Vpr in HIV-1 replication in macrophages. HIV-1 Vpr increases CXCL8 expression in variety of cells including primary T-cells, macrophages, Jurkat cell line and monocytic cell line U937 by activating NF-κB and nuclear factor for IL-6 (NF-IL-6) (181). In addition, the HIV-1 Vpr driven nuclear translocation of transcription factors concomitantly activates HIV-LTR promoter, which is consistent with fact that HIV-LTR contains NF-κB binding sites (8, 154).

Recent report by a group showed that HIV-1 Vpr was important for monocyte-derived macrophages (MDM), infection and that deletion of HIV-1 Vpr reduced expression of CXCL8 in infected MDM, even in the presence of HIV-1 gp120 and Tat (85). Deletion of HIV-1 Vpr also results in reduced phosphorylation of p38

and Jun N-terminal kinase (JNK) in MDM. Since mitogen-activated protein kinases (MAPK) are involved in cytokine production, it shows HIV-1 Vpr activates the signaling cascade eventually leading to enhanced cytokine production. Furthermore, neutralizing CXCL8 in supernatants of HIV-1 infected MDM reduces neuronal apoptosis, which emphasizes that relationship between HIV-1 Vpr and neuronal apoptosis is indirectly mediated by CXCL8 (85). Several *in vivo* studies have also confirmed the relation between HIV-1 Vpr expression and neuronal apoptosis. Basal ganglia and cerebral cortex of transgenic mice preferentially expressed HIV-1 Vpr, as compared to hindbrain, and such mice exhibited impaired motor tasks and neuronal injury (102). HIV-1 Vpr transgenic mice exhibited neuropathological and neurobehavioral deficits that were augmented by interaction of HCV core protein (211). Therefore, HIV-1 Vpr causes direct and indirect cell death and neurotoxicity in HIV-infected patients and CXCL8 is evidently an important mediator contributing to neurological impairment caused by HIV-1 Vpr.

b) HIV-1 gp120

The protein HIV-1 gp120 is an important part of HIV-1 envelope as it binds to CD4 receptor, a step critical for viral entry. Gp120 is found in serum of HIV-1 infected individuals. HIVE patients exhibit high levels of Env mRNA that encodes gp120 (55, 217). It is well known that gp120 increases CXCL8 expression in human monocytes, astrocytes and brain endothelial cells (23, 189, 225). Neuroinflammation is a complex mechanism with various factors contributing towards it. For example, HIV-1 gp120 mediates increase in IL-1β in

neuroblastoma cells, leading to neuronal apoptosis. Several reports suggest IL-1β mediated increased CXCL8 expression, suggesting an indirect mechanism of HIV-1 gp120 induced CXCL8 increase (37, 232). Also, the observed neurotoxicity caused by HIV-1 gp120 is increased in the presence of other HIV-1 proteins like tat, and signaling chemicals like glutamate showing synergistic effect. (104, 126). Direct administration of HIV-1 gp120 into the striatum is highly neurotoxic and emphasizes independent contribution of HIV-1 gp120 towards development of HAND (13, 130).

HIV-1 gp120 transgenic mouse models have been developed lately since mice cannot be directly infected with HIV-1. Circulating HIV-1 gp120 acts as a neurotoxin and alters BBB permeability in transgenic mice (31). A recent study has shown HIV-1 gp120 to be directly toxic to brain endothelial cells. In addition, it increases MMP production, which may explain how it affects BBB permeability since MMP contribute to interactions between cells and matrix (130). Infiltration of lymphocytes across brain endothelial cells contributes to inflammatory responses in CNS with production of CXCL10, CCL2 and CXCL8 (18). Thus, HIV-1 gp120 causes neuronal cell death and leads to neuroinflammation during HAND, the possible mechanisms remain unclear and require further investigation.

c) HIV-1 Tat

HIV-1 Tat is important for viral replication. It is an 86–101 amino-acid protein encoded by two exons (reviewed in (103)). HIV-1 Tat binds to trans-activating response (TAR) element within the LTR of integrated viral genome and regulates

viral gene expression (114). HIV-1 Tat is released by infected cells and can interact with nearby cells in CNS (57). Treatment of monocytes with HIV-1 Tat enhances production of CXCL8 and other inflammatory mediators like IL-1β, IL-6 and TNF-α (118). Interaction of human microglia with viral protein HIV-1 Tat led to increased CXCL8 production along with elevated levels of chemokines like CCL2, CXCL10, CCL3, CCL4 and CCL5. Interestingly, each chemokine was differentially regulated with CCL2 and CCL4 by extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K) whereas CXCL8 and CCL3 were produced by activation of p38. HIV-1 Tat also stimulated CXCL8 expression in human astrocytes *via* MAPK pathway (117) and in human brain derived endothelial cells (94), both at mRNA and protein levels. Astrocytes treated with HIV-1 Tat express inducible nitric oxide synthase (iNOS) *via* ERK, NF-κB and C/EBPβ (128). Activation of ERK in human astrocytes is an important event in CXCL8 production, suggesting that interaction of astrocytes with HIV-1 Tat may activate MAPK leading to enhanced CXCL8 expression.

Another mechanism linking HIV-1 Tat and CXCL8 in brain may be through TNF-α. The release of CXCL8 may be indirectly controlled by HIV-1 Tat as it induces cytokines like TNF-α in astrocytes and macrophages (28) and TNF-α, in turn, is known to stimulate CXCL8 production (232). Extracellular HIV-1 Tat induces CCL2 production in human fetal astrocytes (35). Further, CCL2 recruits monocytes from across BBB to sites of inflammation in the brain. Consequently monocytes have been shown to release TNF-α, which impairs glutamate metabolism in astrocytes (63). Recent study by our group has shown that TNF-α

efficiently contributes to CXCL8 secretion by astrocytes. Thus HIV-1 Tat can significantly alter chemokine balance of brain contributing to neuroinflammation during HIV-1 infection (43).

In vivo studies using HIV-1 Tat transgenic model, which mimics HIV-1 as a chronic disease, have shown extent of neurotoxicity by this protein. The unique 'inducible' HIV-1 Tat transgenic mouse model, characterized by astrogliosis and activated microglia in the cortex and hippocampus, demonstrates that HIV-1 Tat protein alone can generate neuropathogenesis equivalent to HIV-1 infection (110). Synaptic dysfunction and memory deficit is an important hallmark of such mice (64). HIV-1 Tat not only led to growth of tumors comprising of mouse blood cells in HIV-1 Tat transgenics, but also exhibited upregulation of several chemokines where CXCL8 was amongst them (166). These studies suggest that viral proteins like HIV-1 Tat exert their neurotoxic effects *via* upregulation of chemokines like CXCL8.

d) HIV-1 Nef

The HIV-1 protein Nef is a 23-35 kDa peripheral membrane protein and it is expressed by most primate lentiviruses (98). Based on molecular epidemiological data, in humans Nef has regulatory function and plays a key role in viral pathogenesis. Animal model studies have shown that presence of Nef leads to the development of full-blown acquired immunodeficiency syndrome (AIDS) (184). Nef interaction and CXCL8 production is reported in dendritic cells (DC) (143), with some relation to maturation stage of DC. Several studies have focused on viral proteins individually and their interactions with

cells of immune/nervous system. Nonetheless it would be interesting to know how function of one viral protein affects another because in the system all viral proteins interact towards the progression of neuropathogenesis. In this context, recently it was shown that HIV-1 gp120 downregulates Nef induced IL-6 production in dendritic cells. Further, HIV-1 gp120 induced CXCL10 in immature dendritic cells which when bound to CXCL10 receptors activated signaling cascades leading to downregulation of IL-6 (184). Therefore, crosstalk between viral proteins eventually contributes to the cytokine and chemokine balance in the brain.

Nef promotes HIV-1 replication in primary T lymphocytes and macrophages, mechanism being not well known (45, 144). Moreover, HIV-1 infected long term nonprogressors exhibit defective *Nef* gene confirming the important role played by Nef in viral replication (112, 139). Also CXCL8 enhances viral replication in T-lymphocytes and macrophages (120). Since HIV-1 replication is a complex mechanism involving both host and viral factors, detailed investigation is required to understand whether Nef enhances HIV-1 replication *via* modulation of chemokines like CXCL8.

1.7 CXCL8 and NeuroAIDS

Cytokine imbalance is linked to neuropathogenesis and CXCL8 is an important contributor in context of HIV-1 infection. Increased cytokine production, mainly IL-6 and CXCL8, follows after the virus binds to human astrocytes, independent of virus entry and replication (124)). Levels of CXCL8 in CSF correlate with the

degree of neurocognitive impairment in HAND in the post-ART era suggesting that CXCL8 can serve as important biomarker for HAND (228).

Interestingly, HIV-1 matrix protein p17 induces monocyte activation *via* binding to CXCR1. The p17/CXCR1 interaction results in adhesion and chemotaxis of human monocytes, similar to that directed by CXCL8 (77). Elevated CXCL8 levels in brain microenvironment during HIV-1 infection have been linked to increased matrix metalloproteinase (MMP) production. The MMP may aid in disrupting the BBB leading to increased infiltration of inflammatory cells from the periphery (42).

Neuroprotective role of CXCL8

Chemokine CXCL8 has dual role; out of which one of the important functions is neuroprotection. CXCL8 protects mouse hippocampal neurons against Aβ-induced death, indicating its importance in neuronal survival and maintenance (214). Further, CXCL8 is shown to protect human neurons from Aβ induced toxicity (7). It has been shown that elevated levels of CXCL8 potentially protect in perinatal asphyxic brain injury. Despite few studies indicating CXCL8 as neuroprotective, the possible mechanisms behind neuroprotection by CXCL8 remain unclear. The neuroprotective role of CXCL8 is yet unclear because inhibition of its receptors CXCR1/2 improves neurologic deficits and reduces long-tem inflammation in permanent and transient rat cerebral ischemia (210). The role of CXCL8 during HIV-1 infection of CNS is summarized in Scheme 1.

1.8 Chemokine CXCL8 as Potential Therapeutic Agent in HIV-1 Infection

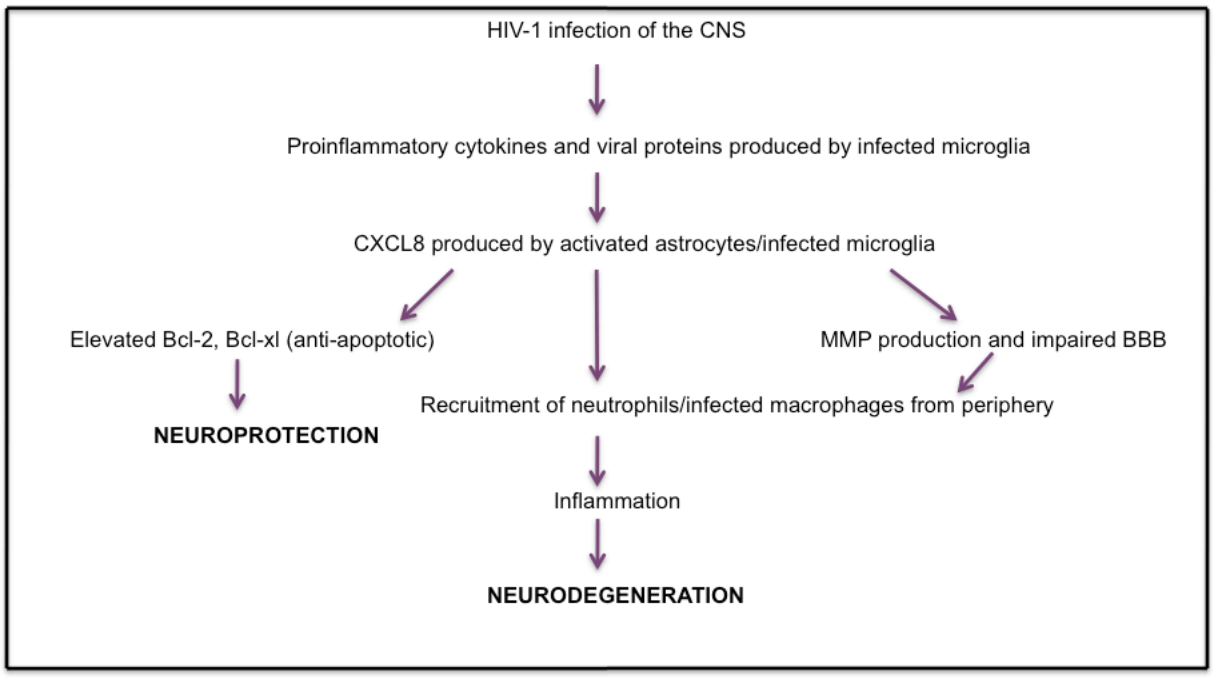
Chemokine CXCL8 is an important factor in HIV-1 infection and thus it is imperative that it is considered as target in HIV-associated neuroinflammation. However, the dual role of CXCL8 is complex. On one side, as a chemoattractant it recruits target cells resulting in progressive infection by enhancing HIV-1 replication, whereas on contrary it is also involved in reduced HIV-1 replication during early infection (120, 161). Also, it imparts survival properties by inducing anti-apoptotic proteins Bcl-2 and Bcl-xl (123).

Since CXCL8 levels are enormously high in brain microenvironment, direct targeting of the chemokine may not be a favored mechanism in HIV-1 therapy. Instead, targeting and blocking of receptors CXCR1/2 may be a preferred mechanism to block the effects of CXCL8. Blocking of CXCL8 receptor CXCR1 by reparixin reduces short-term neutrophil infiltration and infarct size. Considering the success of CXCR1 inhibitor, a group designed a dual inhibitor of both CXCR1 and CXCR2 receptors with longer half-life as compared to reparixin. Remarkably, the inhibitor successfully decreased polymorphonuclear lymphocyte infiltration and improved neurological function in transient rat cerebral ischemia (68). Since neutrophil infiltration and neurological deficits are hallmarks of HAD as well, therefore CXCL8 and its signaling pathways are important pharmacological targets in HAND treatment.

1.9 Conclusions

Cytokines and chemokines are important factors in disease progression during HIV-1 infection of CNS. Chemokine CXCL8 plays an important role in recruitment of neutrophils to the site of infection, thus contributing towards

neuroinflammation. Recently, CXCL8 has become a candidate to be targeted for treatment of HAD. CXCL8 contributes towards direct/indirect neuroprotection as well as neuroinflammation. Therefore, to develop CXCL8 based therapeutic strategies, it is important to study regulation mechanisms involved in its production in various cellular systems. It is equally important to understand the functions of CXCL8 receptors in various physiological responses and downstream signaling initiated by receptor activation. Also, detailed investigation of neuroinflammatory networks regulated by CXCL8 would have application in glial biology and clinical relevance.



Scheme 1.2: Dual role of CXCL8 in CNS. CXCL8 produced in the CNS following HIV-1 infection plays a dual role. It protects neurons *via* upregulation of

anti-apoptotic proteins. CXCL8 impairs BBB *via* MMP production. Recruitment of neutrophils and infected macrophages from peripheral blood, eventually contributes towards neuroinflammation.

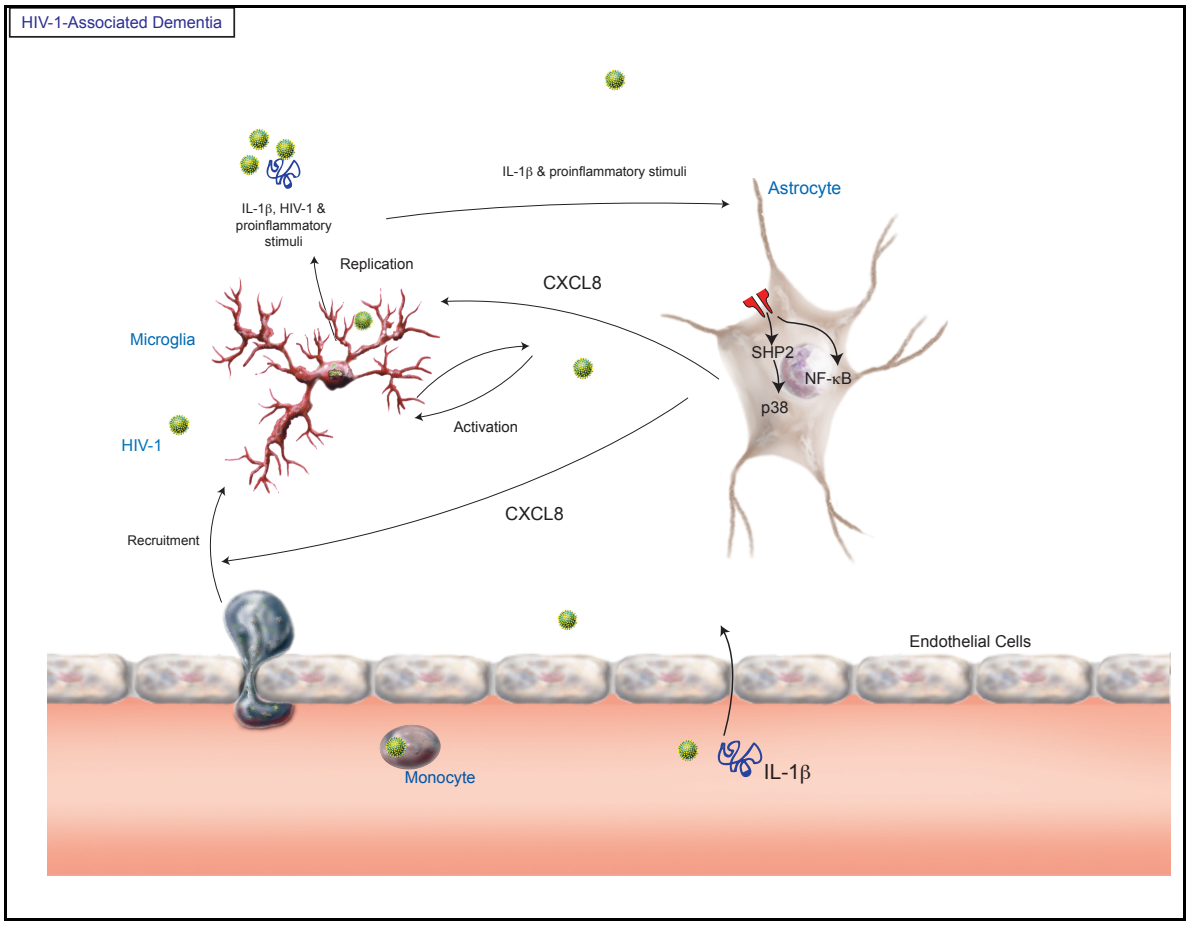
Objectives of the present study: Despite the advent of ART, HIV-1 persists in infected individuals as a latent reservoir and continues to replicate. Also, neurological complications develop in individuals infected with HIV-1, which alters the cytokine/chemokine balance. Chemokines and their receptors are involved in pathogenesis of various neurological disorders. One of the inflammatory pathways in HAD is linked to upregulation of the chemokine CXCL8. Microglial infection and activation in the CNS leads to upregulation of proinflammatory cytokines like IL-1β and TNF-α, which activate astrocytes and enhance CXCL8 production.

Our ***goal*** is to find the 'missing link' between HIV-1 infection of the brain and productive infection in the microglia. We ***hypothesize*** that chemokine CXCL8 plays a key regulatory role in HIV-1 CNS infection by mediating intercellular interactions between astrocytes and microglia. CXCL8 secreted by activated astrocytes is a link between initial viral infection in the brain and subsequent enhanced viral replication as well as microglial activation, which is a major step in neuroinflammation. Overall, we portray CXCL8 as an important contributor of HIV-1 infection neuroinflammatory processes.

The following ***specific aims*** will be addressed in this proposal:

Specific Aim 1: ***To investigate the regulation mechanisms of astroglial CXCL8 production.***

Specific Aim 2: ***To evaluate role of CXCL8 in HIV-1 replication in mononuclear phagocytes.***



Scheme 1.3 Overall scheme for proposed studies. HIV-1 infected monocytes cross the blood-brain barrier and activate astrocytes by secretion of proinflammatory stimuli like IL-1β. We propose that activated astrocytes produce CXCL8, which aids in recruitment of more microglia across the blood-brain barrier and enhances HIV-1 replication in macrophages/microglia.

CHAPTER 2

Src Homology-2 Domain-Containing Protein Tyrosine Phosphatase (SHP) 2 and p38 Regulate the Expression of Chemokine CXCL8 in human astrocytes.

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Abstract: CXCL8, one of the first chemokines found in the brain, is upregulated in the brains and cerebrospinal fluid of HIV-1 infected individuals suggesting its potential role in human immune deficiency virus (HIV)-associated neuroinflammation. Astrocytes are known to be the major contributors to the CXCL8 pool. Interleukin (IL)-1b activated astrocytes exhibit significant upregulation of CXCL8. In order to determine the signaling pathways involved in CXCL8 regulation in astrocytes, we employed pharmacological inhibitors for non-receptor Src homology-2 domain-containing protein tyrosine phosphatase (SHP) 2 and mitogen-activated protein kinases (MAPK) pathway and observed reduced expression of CXCL8 following IL-1β stimulation. Overexpression of SHP2 and p38 enzymes in astrocytes led to elevated CXCL8 expression; however, inactivating SHP2 and p38 with dominant negative mutants abrogated CXCL8 induction. Furthermore, SHP2 overexpression resulted in higher SHP2 and p38 enzyme activity whereas p38 overexpression resulted in higher p38 but not SHP2 enzyme activity. Phosphorylation of SHP2 was important for phosphorylation of p38, which in turn was critical for phosphorylation of extracellular signal regulated kinase (ERK). Thus, our findings suggest an important role for SHP2 in CXCL8 expression in astrocytes during inflammation, as SHP2, directly or indirectly, modulates p38 and ERK MAPK in the signaling cascade leading to CXCL8 production. This study provides detailed understanding of the mechanisms involved in CXCL8 production during neuroinflammation.

Introduction

Chemokines, or chemotactic cytokines, have the ability to recruit leukocyte subsets into sites of inflammation. CXCL8, formerly called interleukin (IL)-8, was identified as the first member of a still growing chemokine family (10). Besides attracting neutrophil subsets into sites of inflammation by chemotaxis, CXCL8 can also activate monocytes and T cells. In this study, we focused on chemokine CXCL8 based on recent research implicating this chemokine in neuropathogenesis during several neurodegenerative disorders such as Alzheimer's disease and human immune deficiency virus (HIV)-1 infection (67, 232). Chemokine CXCL8 levels are elevated in serum, lymphoid tissue, plasma and cerebrospinal fluid (CSF) of HIV-1 infected individuals (24, 120). CXCL8 is produced and released in the brain microenvironment by a variety of cells including microglia and astrocytes (3, 54). Together, immune cells and immune mediators, which are comprised of cytokines and chemokines, contribute to the disruption of neuronal homeostasis leading to neurodegeneration (158). The effect of CXCL8 on neurons is a major area of interest. Research in our laboratory has shown that CXCL8 protects human neurons from amyloid-b-induced neurotoxicity (7). We also reported that astrocytes transfected with an HIV-1_{YU-2}-expressing plasmid, demonstrated elevated CXCL8 (135). Sources of elevated CXCL8 include wide variety of cells including activated microglia and astrocytes in the brain (42, 224). Astrocytes are the major cell type of central nervous system (CNS), and are known to secrete CXCL8 in response to

inflammation. However, the regulatory mechanisms of CXCL8 production in astrocytes are not well defined. Since CXCL8 is a potential mediator of neutrophil-induced inflammation, in this study we investigated the underlying astrocytic signaling networks involved in chemokine CXCL8 production.

Understanding of the inflammatory responses of astrocytes is of particular importance to unravel the process of neuropathogenesis in HIV-associated dementia (HAD) and several neurodegenerative diseases. CXCL8 is expressed by astrocytes in HIV-1 encephalitic tissue and is upregulated in an astrocytic cell line infected with HIV-1 (40, 183). This chemokine also stimulates HIV-1 replication in macrophages and T-cells (120). Thus, CXCL8 upregulation by activated astrocytes contributes to the inflammatory disease process. However, the mechanisms of CXCL8 production are not completely understood in human astrocytes.

Src homology-2 domain-containing protein tyrosine phosphatase (SHP) 2 (also known as PTPN11, PTP1D, SHPTP-2) is a ubiquitously expressed cytoplasmic protein tyrosine phosphatase (PTP) which acts downstream of many tyrosine kinases and cytokine receptors (reviewed in (207)). SHP2 in its native form is autoinhibited by N-terminal SH2 domains blocking the active site of the enzyme. Its catalytic activation requires phosphorylation at specific residues, which opens the conformation and relieves the autoinhibition. It is reported that following epidermal growth factor or platelet-derived growth factor stimulation, the growth factor receptors bind to the SHP2 N-terminal SH2 domains, which in turn binds to Grb2-Sos and leads to Ras/ mitogen-activated protein kinases (MAPK)

activation. MAPK are a family of serine/threonine kinases comprising of extracellular signal regulated kinase (ERK), p38 and c-jun N-terminal kinases. SHP2 is known to activate ERK in human fibroblasts; however, SHP2 is implicated to act downstream or parallel to the Ras/MAPK pathway (186). Overexpression of catalytically inactive form of SHP2 (SHP2CS) is shown to exert dominant negative effect on Ras/MAPK stimulation in different cellular models (5). Phosphorylation and activation of ERK and p38 is reported when mixed glial cells are activated with IL-1β (226). While strong evidence supports involvement of SHP2 in the MAPK pathway in other cells, it has never been reported in astrocytes and the mechanisms involved are still unclear.

In the present study, we hypothesized that SHP2 and MAPK participate in the upregulation of astrocyte CXCL8 expression following stimulation with HIV-1-relevant stimuli such as IL-1β. We propose that IL-1β receptors directly or indirectly lead to phosphorylation or activation of SHP2, which in turn modulates MAPK to increase CXCL8 expression in astrocytes. In this report, we further delineated the order in which SHP2 and p38/ERK MAPK regulate CXCL8 expression in activated astrocytes. Our data demonstrates that CXCL8 is upregulated in HIV-1 infected individuals and that astrocytes produce elevated levels of CXCL8 following activation by proinflammatory stimuli. We show for the first time that SHP2 mediates p38 MAPK signaling to regulate the expression of the chemokine CXCL8 in human astrocytes. The data presented in this study provide insights into the regulation of CXCL8 in astrocytes during inflammation,

which may provide means to therapeutically modulate the levels of this chemokine.

Methods

Preparation of human brain tissue extracts: Human brain specimens from the frontal cortex were provided by the National NeuroAIDS Tissue Consortium, Center for Neurovirology and Neurodegenerative Disorders brain bank and Rapid Autopsy Program at the University of Nebraska Medical Center (75). The institutional review boards of both the Universities of Nebraska Medical Center and North Texas Health Science Center approved the collection of human tissues for research. All donors gave informed written consent, which permits research use of their tissues and informed them of possible conflicts of interest. Brain samples are from the cohort as described in (62). Briefly, the HIV-1 infected patients included seven males and four females between the ages of 25 and 55 years; the age-matched control donors included two males and three females between the ages of 37 and 52 years. Brain lysates and RNA were isolated as previously described (200). Protein concentration was determined by bicionconic acid method as suggested by the manufacturer (Pierce, Rockford, IL, USA).

Isolation and cultivation of primary human astrocytes: Human astrocytes were isolated from first- and early second-trimester aborted specimens, ranging from 82 to 127 days, obtained from the Birth Defects Laboratory, University of Washington, Seattle, WA in full compliance with the ethical guidelines of the NIH. The institutional review boards of both the Universities of Washington and North

Texas Health Science Center approved the collection of human tissues for research. The Birth Defects Laboratory obtained written consent from all tissue donors. Human astrocytes were isolated as previously described in (69). Briefly, brain tissues were dissected and mechanically dissociated. Cell suspensions were centrifuged, suspended in media, and plated at a density of 20×10^6 cells/150 cm^2. The adherent astrocytes were treated with trypsin and cultured under similar conditions to enhance the purity of replicating astroglial cells. The astrocyte preparations were routinely >99% pure as measured by immunocytochemistry staining for glial fibrillary acidic protein and microglial marker CD68 determine possible microglial contamination and contribution of microglia in inflammatory responses.

RNA extraction and gene expression analyses: RNA was isolated (as described in (26)) from astrocytes treated as described in subsequent sections and gene expression was assayed by real-time PCR using StepOnePlus (Life Technologies, Carlsbad, CA). Commercially available TaqMan® gene expression assays were used to measure CXCL8 (Life Technologies, Cat# Hs00174103_ml) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat# 4310884E) mRNA levels. GAPDH, a ubiquitously expressed housekeeping gene, was used as an internal normalizing control. The 30 ml reactions were carried out at 48ºC for 30 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min in 96-well optical, real-time PCR plates. Samples were analyzed in triplicates. Relative quantities were determined by comparison to an experimental

standard curve with known cDNA quantities. Gene expression is expressed as relative quantity of CXCL8 normalized to that of GAPDH.

Astrocyte treatment and activation: Primary astrocytes were treated with or without p38 inhibitors: SB203580 and SB202190 (20 μM, Santa Cruz Biotechnology, Santa Cruz, CA); ERK inhibitors: PD98059 and U0126 (20 μM, Sigma Aldrich Inc., St Louis, MO); SHP2 inhibitor: phenylhydrazonopyrazolone sulfonate (PHPS1) (20 μM, Sigma); or PTP inhibitor: sodium orthovanadate (Na_3VO_4) (1 mM, Sigma) for 2 h prior to activation with IL-1β (20 ng/ml, R&D Systems, Minneapolis, MN), as previously described (12, 116, 135). This dose of IL-1β is well within the range of 5-100 ng/ml currently used to activate astrocytes (127) and levels induced in animal models (47, 61).

Plasmid constructs and transfection into astrocytes: Overexpression vectors were obtained from the non-profit plasmid repository, Addgene, Cambridge, MA. The SHP2 overexpression constructs: wild-type SHPWT (Addgene plasmid 8381) and dominant negative mutant SHP2CS (Addgene plasmid 8382) were deposited by Ben Neel (16); and the p38 overexpression constructs: p38 (Addgene plasmid 20351) and dominant negative mutant p38agf (Addgene plasmid 20352) were deposited by Roger Davis (56). Astrocytes were transfected using the P3 primary cell kit, nucleofector device and shuttle attachment (Lonza Inc., Walkersville, MD, USA) as previously standardized (135). Briefly, astrocytes were suspended in nucleofector solution and plasmid constructs (1 mg / 1.5 million cells). Transfected cells were supplemented with

astrocyte media and incubated for 30 min at 37°C prior to plating. Twelve to 24 h post-plating, cells were washed and treated as described above.

Quantification of CXCL8 by ELISA. CXCL8 protein levels were determined from culture supernatants by a CXCL8 specific sandwich ELISA (R&D Systems, Cat# S800C) according to manufacturer's protocol. Absorbance was determined by Spectromax M5 microplate reader using SoftMax Pro V5 software (Molecular Devices, Sunnyvale, CA).

Determination of in vitro phosphatase activity: SHP2 phosphatase activity was measured in whole cell protein lysates using SHP2 assay kit (R&D Systems, Cat# DYC2809). Briefly, transfected astrocytes grown to confluence in tissue culture flasks (8 million cells/75 cm^2 flask) were scraped and suspended in M-PER mammalian protein extraction reagent with protease inhibitors (Thermo Fisher Scientific, Rockford, IL). Cell lysates containing 100 ng/ml of total protein were immunoprecipitated with SHP2-specific beads, incubated with a synthetic phosphopeptide as substrate and phosphate released was measured. Absorbance of the reaction mixture was measured at 620 nM.

Determination of in vitro kinase activity: p38 kinase activity was measured in whole cell protein lysates using p38 MAP kinase assay kit (Cell Signaling, Cat# 9820). Briefly, 50 μg/ml of total protein were incubated with immobilized phosphorylated (P)-p38 primary antibody overnight at 4ºC. After elution with kinase buffer, eluent was incubated with ATP and activating transcription factor (ATF)-2 as substrate, for 30 min at 30ºC. Phosphorylation of ATF-2 is

proportional to p38 kinase activity, which was visualized by western blot analysis as described below.

Western blot analysis: Equal amounts of protein samples (25 mg) were boiled with 4X NuPAGE loading sample buffer (Life Technologies) for 5-10 min, resolved by NuPage 4-12% Bis tris gel and subsequently transferred to a nitrocellulose membrane using i-Blot (Life Technologies, Carlsbad, CA, USA). The membrane was incubated with individual primary antibodies (Cell Signaling; SHP2 #3752, P-SHP2 #3751, p38 #9212 and P-p38 #9216) at a dilution of 1:1000 overnight at 4ºC, washed and then incubated with anti-mouse or anti-rabbit goat antibody IgG conjugated to horseradish peroxidase (1:10,000, Bio-Rad) for 2 h at room temperature. The membrane was then developed with SuperSignal west femto substrate (Thermo) in a Fluorochem HD2 Imager (ProteinSimple, Inc. Santa Clara, CA). β-actin (1:1,000, Cell Signaling) immunoblotting was used as a loading control.

Determination of astrocyte metabolic activity: Following experimental manipulations described above, five percent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent in astrocyte medium was added to astrocytes and incubated for 20-45 min at 37°C. MTT is metabolically reduced to purple formazan crystals by living cells. The MTT solution was removed and crystals were dissolved in DMSO for 15 min with gentle agitation. The absorbance of the DMSO/crystal solution was assayed at 490 nm, as previously described (138). MTT ratio to control was calculated to normalize ELISA levels.

Statistical analyses: Statistical analyses were carried out using Prism V5.0 (GraphPad Software, La Jolla, CA) with one-way analysis of variance (ANOVA) and Newman-Keuls post-test for multiple comparisons Student's t-test was performed for paired observations. Significance was set at $p<0.05$ and data represents means +/- standard error of the mean (SEM). Data presented is representative of a minimum of three independent experiments with two or more independent donors.

Results

HIV-1 infection elevates chemokine CXCL8 levels in human brain

Chemokine CXCL8 levels are upregulated in CSF of HAD patients when compared to HIV-1 infected individuals (232). We examined CXCL8 levels in brain lysates isolated from frontal cortex of eleven HIV-1 infected patient brains and five age-matched controls. CXCL8 levels, as measured in mRNA by RT-PCR and protein lysates by ELISA, were higher in samples obtained from HIV-1 infected patients at both mRNA and protein levels, as compared to control samples. The mRNA levels in HIV-1 infected patients (mean 2.66) exhibited a 9.5-fold increase when compared to control samples (mean 25.48), with the lowest value observed in HIV-1 infected group being greater than the highest value in control samples (Figure 2.1 A, $p=0.009$). In accordance with increased mRNA levels, a 2.9 fold increase in CXCL8 protein levels was measured in HIV-1 infected brain lysates (mean 0.06103) as compared to controls (mean 0.02102) expressed as ng/μg of total protein (Figure 2.1 B, $p=0.04$).

Proinflammatory cytokines increase astrocyte CXCL8 expression

Since astrocytes are the most abundant cell in the CNS, we next examined whether they are a significant source of CXCL8. During HIV-1 infection in the brain, proinflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α are released by infiltrating HIV-1 infected macrophages (209). To study the effect of these cytokines on CXCL8 production, astrocytes were treated with IL-1β and TNF-α (20 ng/ml). RNA was isolated at 8 h and supernatants were collected 24 h post-treatment. While control astrocytes showed low or undetectable levels of CXCL8, a robust increase in CXCL8 levels was observed following treatment with IL-1β (90,000-fold) or TNF-α (10,000-fold). CXCL8 levels increased significantly at both RNA and protein levels indicating release of CXCL8 by stimulated astrocytes **(Figure 2.2, $p<0.001$)**. Secreted CXCL8 protein levels also increased in a dose-dependent manner upon astrocyte activation with either IL-1β (0.1 to 100 ng/ml) or TNF-α (1 to 100 ng/ml), as assayed by ELISA (data not shown). Taken together, stimulation of astrocytes by proinflammatory cytokines leads to release of chemokine CXCL8.

IL-1β -mediated increase in CXCL8 is SHP2 and MAPK dependent

To investigate signaling pathways involved in astrocyte CXCL8 production following activation, we used a panel of pharmacological inhibitors against SHP2 and MAPK (p38 and ERK). MAPK are implicated in CXCL8 gene regulation during inflammation (93). Since we observed significant upregulation in CXCL8 expression in IL-1β-activated astrocytes, we evaluated IL-1β-mediated CXCL8 expression during independent inhibition of SHP2, p38 and ERK pathways. While

basal CXCL8 levels remained unchanged following pretreatment of astrocytes with SB202190 (p38 inhibitor), and U0126 (ERK inhibitor), each of the inhibitors significantly reduced the induction of CXCL8 mRNA and protein expression by IL-1β . Dimethyl sulphoxide (DMSO) was used as a solvent control. **(Figure 2.3 A, B; $p<0.001$).** Another ERK inhibitor PD98059 was also employed in the experiment and yielded significant decrease in CXCL8 mRNA and protein levels in astrocytes treated with IL-1β (data not shown). Similarly, pretreatment of astrocytes with Na_3VO_4 (PTP inhibitor) and PHPS1 (SHP2 inhibitor) led to significant inhibition of the IL-1β-mediated increase in CXCL8 mRNA and protein levels **(Figure 2.3 C, D; $p<0.001$).** Thus SHP2, p38 and ERK were each implicated in modulation of CXCL8 levels in IL-1β-activated astrocytes. These results suggested that both SHP2 and MAPKs regulate CXCL8 mRNA and protein expression in activated astrocytes, either as part of one signaling cascade or independently in different signaling cascades eventually converging to CXCL8 expression.

Next, we employed overexpression of wild-type and dominant negative SHP-2 and p38 in order to elucidate the interaction of these two enzymes in regulation of CXCL8. We overexpressed SHP2 and inhibited p38, and vice versa, to understand whether SHP2 and p38 were part of the same signal transduction cascade leading to CXCL8 expression in astrocytes.

Overexpression of SHP2 and p38 in astrocytes increases CXCL8 production

To further validate the role of SHP2 and p38 MAPK in astrocyte CXCL8 production, we transiently transfected wild-type SHP2 (SHP2WT) and p38 overexpression constructs into astrocytes. The dominant negative mutants SHP2CS and p38agf were transfected into astrocytes, in parallel, for comparison. CXCL8 protein levels were measured by ELISA in astrocyte culture supernatants 24 h post-transfection. Interestingly, we found significantly increased CXCL8 protein levels in SHP2WT- and p38-transfected astrocytes as compared to dominant negative controls and mock (**Figure 2.4A**, $p<0.001$ and $p<0.01$, respectively)**.** Increased CXCL8 protein in astrocyte supernatants resulted from SHP2 and p38 overexpression, even in the absence of IL-1β stimulation. Supernatants from IL-1β-activated astrocytes were used as a positive control, demonstrating a 2400-fold induction in CXCL8 protein levels similar to that shown during SHP2WT overexpression, as compared to 1500-fold during p38-overexpression **(Figure 2.4A)**. *In vitro* SHP2 phosphatase assay, a measure of SHP2 function in whole cell protein lysates, showed high SHP2 enzyme activity in SHP2WT-transfected astrocytes as compared to mock- and SHP2CS-transfected indicating efficient transfection, expression and activity of SHP2 protein. Concurrently, SHP2 phosphatase activity increased by two-fold following IL-1β-activation of mock-transfected astrocytes **(Figure 2.4B, $p<0.05$)**. Thus, IL-1β stimulation lead to elevated CXCL8 expression in astrocytes in a SHP2-dependent manner. Similarly, p38 kinase activity in astrocytes was measured by *in vitro* p38 kinase assay following transfection. As expected, p38 kinase activity in p38-transfected astrocytes increased by 40-fold, indicating efficient

transfection, expression and activity **(Figure 2.4C)**. Furthermore, p38 kinase activity was evident in IL-1β-activated astrocytes, demonstrating CXCL8 expression is regulated in p38-dependent manner in IL-1β-activated astrocytes. In comparison, p38agf-transfected astrocytes exhibited basal p38 enzyme activity, similar to that of mock-transfected astrocytes **(Figure 2.4C)**. The dominant negative mutants expressed the corresponding inactive enzymes efficiently. Surprisingly, we observed increased levels (~25-fold) of active p38 kinase in SHP2WT-transfected astrocytes, thus elucidating the order in which the two enzymes act in regulation of astrocyte CXCL8 expression.

p38 acts downstream of SHP2-mediated increase in astrocyte CXCL8 expression

p38 MAPK is known to act upstream of SHP2 in CXCL8 regulation in hepatocytes (11). To explore the order in which the two enzymes, a phosphatase and a kinase, act to regulate astrocyte CXCL8 expression, SHP2 was overexpressed in astrocytes followed by independent inhibition of SHP2 and p38 activity using pharmacological inhibitors. The SHP2-mediated increase in CXCL8 expression remained below detection in culture supernatants of SHP2WT-transfected astrocytes in the presence of PTP and p38 inhibitors, Na_3VO_4 and SB203580, respectively **(Figure 2.5A, B)**. However, treatment with a negative control inhibitor, SB202474, did not inhibit SHP2WT-induced CXCL8 expression **(Figure 2.5C)**. These data indicated that p38 is important for regulation of CXCL8 protein expression in astrocytes, and SHP2 overexpression alone could not bypass p38 activation. In parallel, p38 and p38agf-transfected astrocytes

were treated with Na_3VO_4 and SB203580. The p38-mediated CXCL8 increase was unchanged upon treatment with PTP inhibitor Na_3VO_4 **(Figure 2.6A)**; however, CXCL8 protein levels were undetectable, as expected, after treatment with p38 inhibitor SB203580 **(Figure 2.6B)**. The negative control inhibitor had no effect on the p38-mediated CXCL8 increase **(Figure 2.6C).** Therefore, p38 was implicated as acting downstream of SHP2 in the regulation of astrocyte CXCL8 expression.

Phosphorylation of SHP2 is important for downstream phosphorylation of p38 and ERK

Phosphorylation of both SHP2 and p38 is important for the activation of their enzymatic functions (206). Phosphorylation of p38 and ERK is stimulated by IL-1β in astrocytes (52, 205). Therefore, the phosphorylation of SHP2, p38 and ERK was compared in SHP2WT-, SHP2CS-, p38- and p38agf-transfected astrocytes with or without IL-1β stimulation for 5, 15 and 25 min. Phosphorylation of SHP2, p38 and ERK increased during SHP2WT overexpression, which further increased upon IL-1β stimulation **(Figure 2.7A)**. Interestingly, ERK phosphorylation was evident during p38 overexpression with little to no SHP2 phosphorylation **(Figure 2.7B)**, suggesting that SHP2 phosphorylation regulates p38 phosphorylation, which in turn, regulates ERK phosphorylation.

Discussion

In this study we investigated the intracellular signaling mechanisms of chemokine CXCL8 in astrocytes in the context of neuroinflammation during HIV-1

infection. CXCL8 is an important chemokine upregulated in CSF of HAD patients (232). Consistent with prior studies, our data also showed elevated CXCL8 levels in brains of HIV-1 infected patients. We found robust increases in astrocyte CXCL8 expression in response to proinflammatory stimuli as previously reported (3). Our study shows that SHP2, p38 and ERK are involved in regulating IL-1β-mediated increase in CXCL8 production by human astrocytes. In this study, we have identified SHP2 as an important signal transducer upstream of p38 and ERK directing upregulation of CXCL8 in activated astrocytes.

During the course of HIV-1 CNS infection, astrocytes respond to proinflammatory stimuli by release of several cytokines and chemokines, which are important mediators of HIV-1-induced neuronal damage (108). CXCL8 is involved during innate immune responses in the CNS, and likely contributes to intercellular interactions leading to neuroinflammation. Several studies relate elevated CXCL8 levels with neurodegeneration; however, the molecular mechanisms involved are incompletely understood. Increased CXCL8 levels are observed during congenital human cytomegalovirus infection of brain vascular pericytes, which often leads to CNS abnormalities (2). Interferon-β, an anti-inflammatory mediator, repressed CXCL8 gene expression in peripheral blood cells implicating a possible mechanism behind its anti-inflammatory properties through repression of CXCL8 (173). Astrocytes activation and overexpression of cytokines and chemokines like IL-1β, TNF-α and CXCL8 have been associated with Alzheimer's disease (89, 220). We report high levels of CXCL8 in HIV-1 infected individuals, which may be a likely candidate accounting for progressive

neurodegeneration associated with chronic HIV-1 infection. Elevated levels of CXCL8 have been reported after ischemic brain injury (115). A recent study showed high levels of CXCL8 expressed by human brain endothelial cells from patients with multiple sclerosis (199). Our results on HIV-1 infected patients' CXCL8 levels, combined with previous reports, strongly implicate CXCL8 to be involved in neuroinflammation during variety of neurodegenerative conditions, including HIV-1 infection.

Sources of CXCL8 include activated microglia, astrocytes and endothelial cells. Neutrophils are also shown to produce CXCL8 possibly to amplify leukocyte recruitment (131). Astrocytes are reportedly major producers of CXCL8 in the CNS during an inflammatory response (50). In accordance with several groups, we also found upregulation in CXCL8 an astrocytic response to proinflammatory cytokines like IL-1β and TNF-α (3, 232). Furthermore, the increase is found at both mRNA and protein levels, suggesting the induction of astrocyte CXCL8 gene expression in response to cytokines. This observation highlights involvement of signal transduction pathways communicating between extracellular environment of astrocytes and inside of nucleus. Since astrocytes are associated with the endothelial cells in blood-brain barrier, it is likely that proinflammatory cytokines activate astrocytes, which release chemokines to attract leukocytes from the periphery into the CNS, a process that eventually leads to neuroinflammation. Another study shows elevated cytokine levels, including CXCL8, when astrocyte cell lines were cocultured with peripheral blood mononuclear cells (97).

Regulation of CXCL8 has been studied in a variety of cell types (11, 132, 178, 232). MAPK is an important signaling pathway studied in context of CXCL8 regulation in different cell types. The observed repression of IL-1β-induced CXCL8 upregulation when p38 and ERK pathways were inhibited using specific inhibitors, clearly implicated the involvement of MAPK in astrocyte CXCL8 regulation. Since SHP2 has been studied in relation to cytokine receptors in different cellular systems, we extended this observation to astrocytes in the present study. SHP2 is a ubiquitously expressed non-receptor PTP, implicated in signaling events downstream of receptors for growth hormones and cytokines (169). SHP2 is also known to control cell growth and differentiation (60, 219). Mutations in the SHP2 gene are associated with genetic disorders, such as Noonan syndrome and Leopard syndrome, which are thought to result from abnormal MAPK activation (53).

We report that SHP2 acts to promote MAPK activity in activated astrocytes, which is consistent with reports in other cell systems (90, 192). In fibroblasts, treatment with fibroblast growth factor or platelet-derived growth factor leads to SHP2 phosphorylation and binding to Grb2, facilitating ERK activation (5). Another docking protein, Gab1, is known to associate with SHP2 in epidermal growth factor-induced ERK activation (22, 41, 174) Our study highlights the function of SHP2 in modulating p38 activation, an important event in astrocytes for ERK activation, leading to increased expression of CXCL8 during inflammation. Although MAPKs are known to be involved in expression of certain proinflammatory genes, the upstream and downstream regulators are not

well described. We show that expression of chemokine CXCL8 is significantly upregulated in astrocytes transfected with SHP2WT and p38 overexpression constructs; however, transfection with dominant negative mutants, incapable of becoming phosphorylated, did not show increased CXCL8 levels in human astrocytes. Elevated levels of CXCL8 resulted as direct effect of SHP2 and p38 enzyme function, which was evident from high phosphatase and kinase activity in the SHP2- and p38-overexpressing astrocytes.

In exploring the interaction of SHP2 with Ras/MAPK pathway in astrocytes, interestingly we found that inhibition of p38 could not block SHP2-overexpression-induced CXCL8 expression in astrocytes. In contrast, inhibition of SHP2 had no effect on p38-stimulated CXCL8 upregulation. This clearly suggested that SHP2 activity is important for p38 activity. Our interpretation of the results was strengthened by observing high p38 kinase activity in SHP2-overexpression. However, SHP2 phosphatase activity remained at basal levels during p38-overexpression. Furthermore, in SHP2-deficient cells, there was little or no phosphorylation of p38 and ERK following IL-1β stimulation; inactivation of SHP2 resulted in suppression of the P-p38 and P-ERK signals. SHP2 is a phosphatase and our results support its role in p38 and ERK phosphorylation, indicating that p38 and ERK are not direct substrates for SHP2 phosphatase activity. However, SHP2 is reported to be required for ERK activation in some, but not all growth factors and may act upstream, downstream or parallel to Ras/MAPK. A recent report shows CXCL8 production in hepatocytes in response to Hepatitis C virus and HIV-1 envelope proteins. However, p38 acts upstream to

SHP2 in regulation of chemokine CXCL8 production by hepatocytes (11). It is thus imperative to assess cellular system specific signaling mechanisms regulated by SHP2 and MAPK, and we report for the first time the involvement of SHP2 in the modulation of CXCL8 expression in human astrocytes.

Our data indicate that IL-1β-activated astrocytes exhibit increased SHP2 phosphorylation, which correlates with the phosphorylation of p38. Furthermore, the loss of p38 and ERK activation in SHP2-deficient astrocytes implicates SHP2 as an important mediator in the p38/ERK MAPK activation. It suggests that SHP2-mediated p38 and ERK MAPK signaling regulate expression of chemokine CXCL8 in human astrocytes **(Figure 2.8)**. The data presented in this study provide insights into the regulation of CXCL8 in astrocytes during inflammation.

Conclusions

Astrocytes produce CXCL8 in response to inflammatory stimuli, which is regulated by activation of SHP2 upstream to activation of p38 and ERK MAPK. The study provides insights into the signaling mechanisms of CXCL8 in human astrocytes that may be employed, in future, to therapeutically modulate CXCL8 levels *in vivo* in the context of neuroinflammation. Also, we have delineated a novel-signaling pathway in astrocytes, where phosphorylation of SHP2 is an important event in downstream signaling for production of CXCL8. Clearly, phosphorylation of SHP2 can be targeted for preventing downstream events. Future studies may be directed towards understanding SHP2 substrates in astrocytes. Also, the exact mechanisms of direct/indirect interaction of SHP2 with

various docking proteins like Gab1 and Grb2 require further investigation. It will help understand how signals are transmitted downstream of IL-1β receptors, *via* various docking proteins to SHP2 and further down to MAPKs. Detailed understanding of astrocyte signaling will be relevant to glial-neuronal interactions, which are central to neuroinflammation in HIV-1 and many other neurodegenerative conditions.

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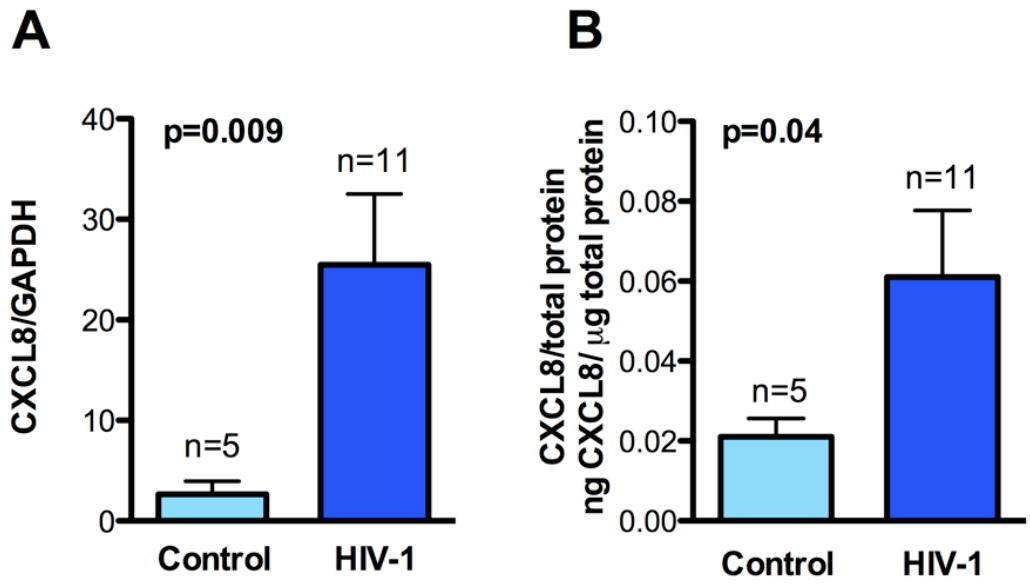

Figure 2.1: CXCL8 levels are elevated in brain lysates of HIV-1 infected patients. Human brain mRNA and protein lysates collected from frontal cortex of HIV-1 infected individuals and age matched controls were analyzed for CXCL8 levels. **A)** CXCL8 mRNA expression analyzed by real-time PCR normalized to GAPDH. **B)** CXCL8 protein levels assayed by ELISA shown as ng/mg total protein. Results are expressed as mean ± SEM of indicated number of donors. Statistical analyses were performed using unpaired student's t-test.

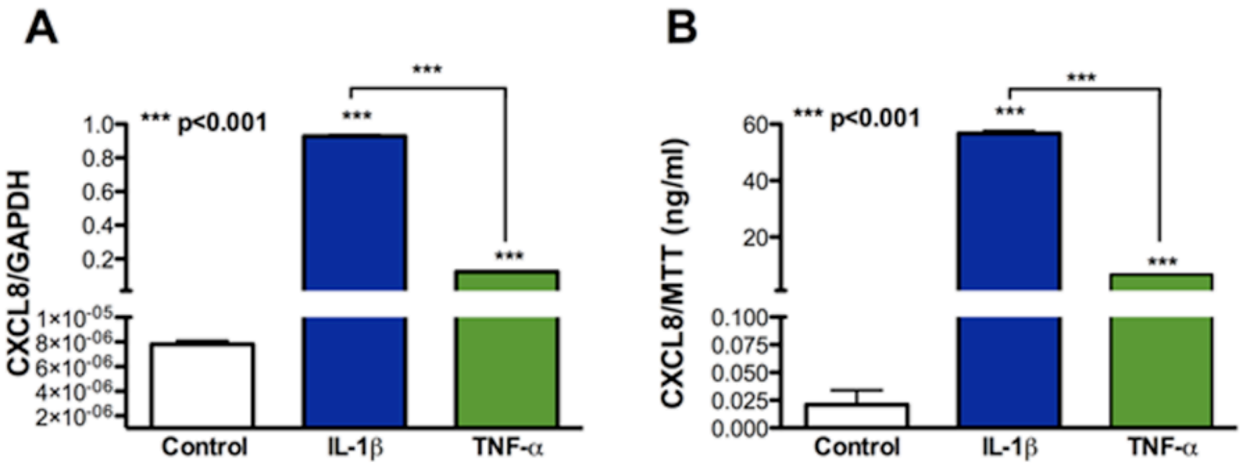

Figure 2.2: Increased CXCL8 expression in astrocytes activated by IL-1β and TNF-α. Cultured human astrocytes were treated with IL-1β (20 ng/ml) or TNF-α (20 ng/ml). **A)** CXCL8 mRNA expression 8 h post-activation normalized to GAPDH determined by real-time PCR. **B)** CXCL8 protein levels 24 h post-activation normalized to MTT determined by ELISA. Results are representative of three independent experiments performed in triplicates. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

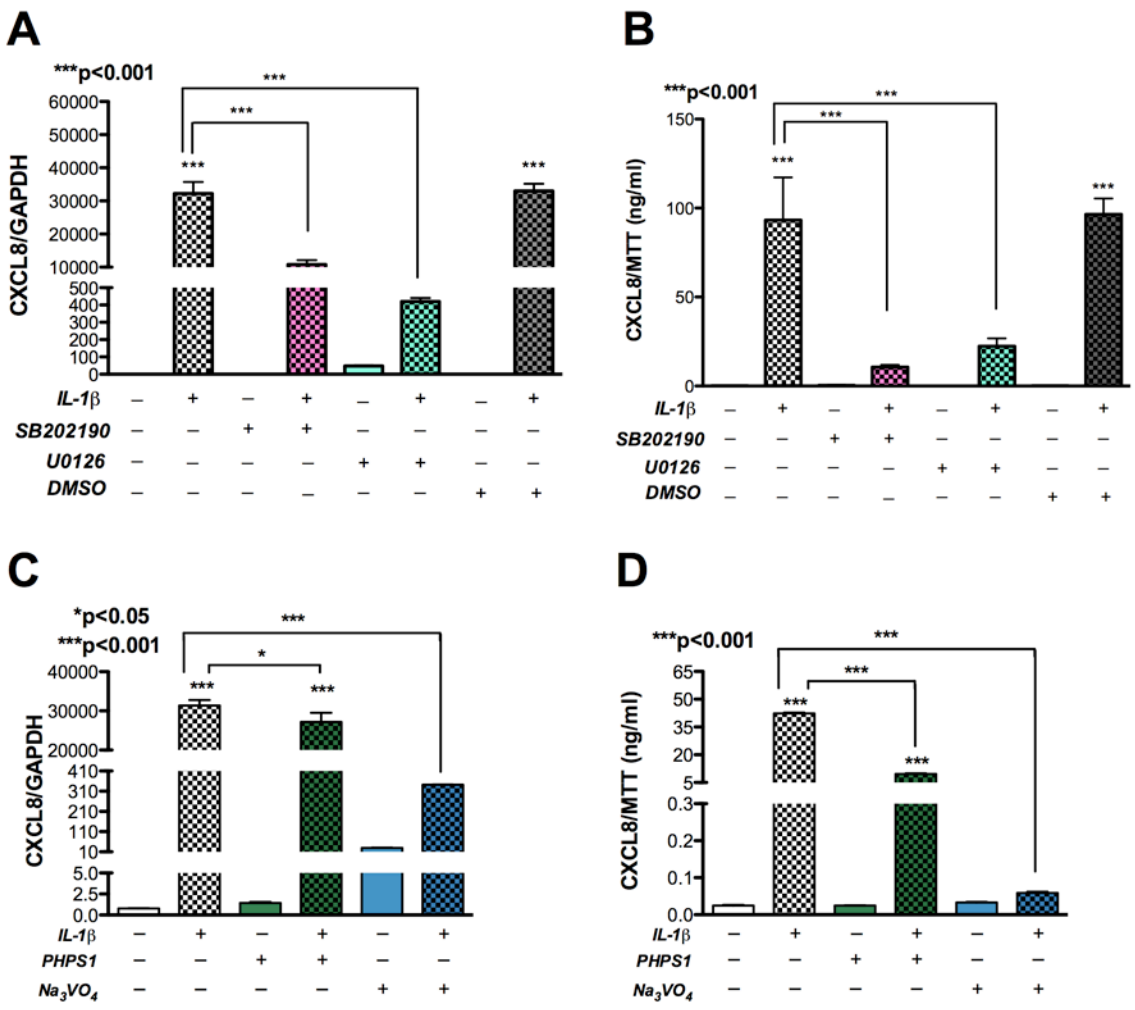

Figure 2.3: MAPK and SHP2 regulate IL-1β-mediated increased CXCL8 expression in astrocytes. Cultured human astrocytes were pretreated with MAPK and SHP2 inhibitors for 2 h, followed by activation with IL-1β (20 ng/ml). Untreated controls were maintained in parallel. RNA was isolated 8 h post-activation and supernatants were collected 24 h post-activation. CXCL8 mRNA was measured using real-time PCR, while protein expression was measured by ELISA. **A)** CXCL8 mRNA levels normalized to GAPDH after pretreatment with inhibitors specific to p38, SB202190 and ERK, U0126. **B)** CXCL8 protein levels normalized to MTT after pretreatment with inhibitors

specific to p38, SB202190 and ERK, U0126. DMSO was used as a solvent control **C)** CXCL8 mRNA levels normalized to GAPDH after pretreatment with inhibitors specific to SHP2, PHPS1 and PTP, Na_3VO_4. **D)** CXCL8 protein levels normalized to MTT after pretreatment with inhibitors specific to SHP2 (PHPS1) or PTP (Na_3VO_4). Results are representative of three independent experiments performed in triplicate and expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

Figure 2.4

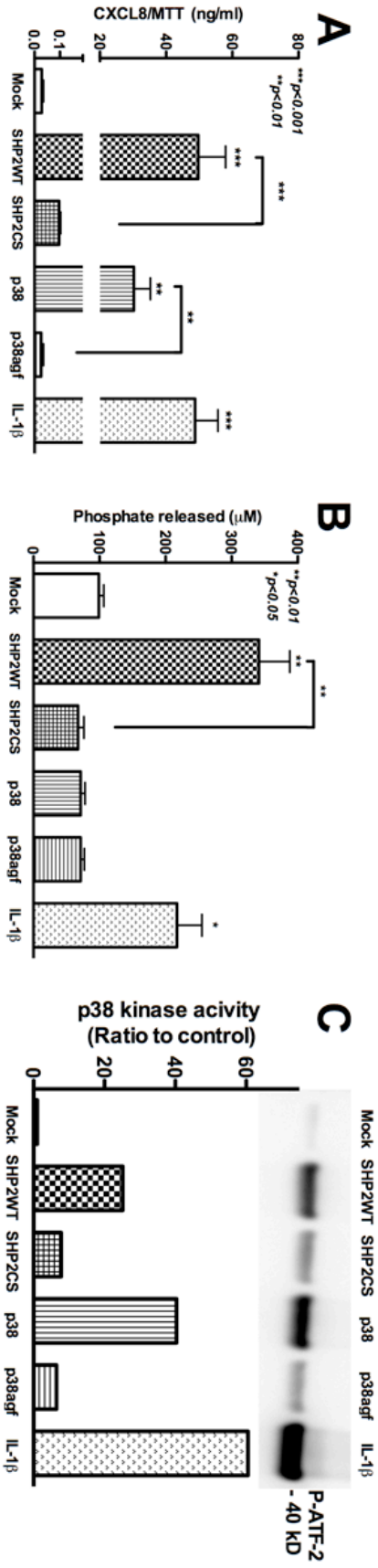
A
CXCL8/MTT (ng/ml)
0.0
0.1
20
40
60
80
***p<0.001
**p<0.01
Mock
SHP2WT
SHP2CS
p38
p38agf
IL-1β
B
Phosphate released (μM)
0
100
200
300
400
**p<0.01
*p<0.05
C
p38 kinase acivity
(Ratio to control)
P-ATF-2
- 40 kD

Figure 2.4: CXCL8 upregulation by SHP2 and p38 overexpression in **astrocytes.** Primary human astrocytes were transfected with SHP2WT and p38 overexpression plasmids with corresponding dominant negative mutants SHP2CS and p38agf. Twenty-four h post-transfection supernatants were analyzed for CXCL8 protein levels by ELISA. 24 h post-transfection astrocytes were treated with IL-1β (20 ng/ml) for 24 h, protein lysates and supernatants were collected and analyzed for SHP2 and p38 enzyme activity. **A)** CXCL8 protein levels assayed by ELISA normalized to MTT. **B)** SHP2 *in vitro* phosphatase assay showing phosphate released as a measure of SHP2 phosphatase activity. Results are representative of three independent experiments performed in triplicate and expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons. **C)** *In vitro* p38 kinase assay based on immunoprecipitation of p38, followed by incubation with ATF-2 as substrate. P-ATF-2 band at 40 kD, which is a function of higher p38 kinase activity, is shown with densitometry. Results are representative of three independent experiments.

Figure 2.5

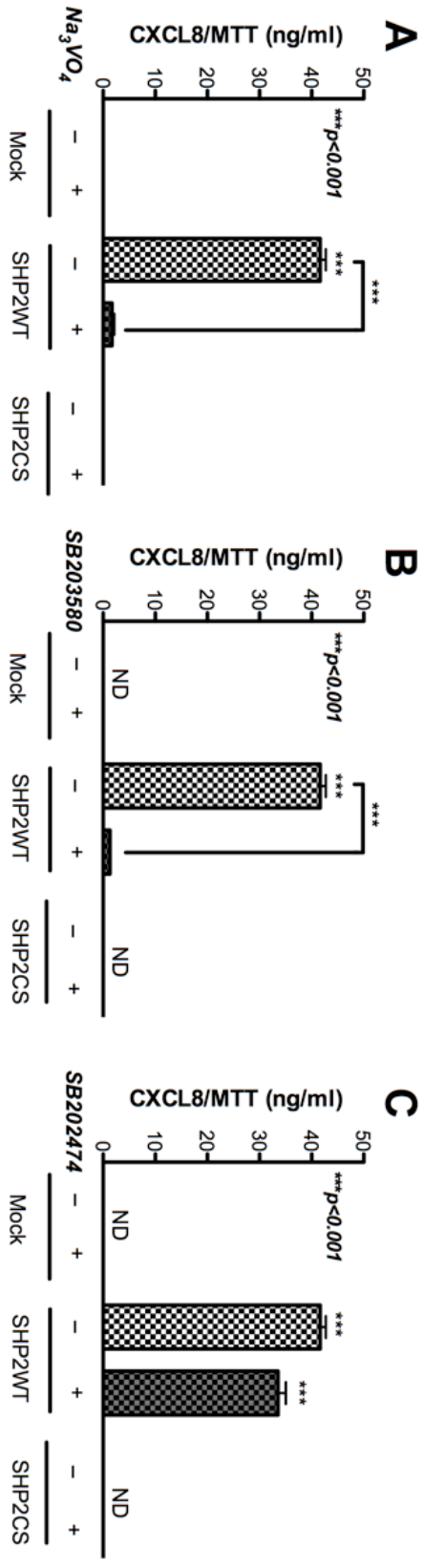
A
CXCL8/MTT (ng/ml)
Na_3VO_4
0
10
20
30
40
50
***p<0.001
***
***
Mock
SHP2WT
SHP2CS
– + – + – +
B
CXCL8/MTT (ng/ml)
SB203580
ND
***p<0.001
***
***
ND
Mock
SHP2WT
SHP2CS
– + – + – +
C
CXCL8/MTT (ng/ml)
SB202474
ND
***p<0.001
***
***
ND
Mock
SHP2WT
SHP2CS
– + – + – +

Figure 2.5: Effect of SHP2 overexpression and p38 inhibition on CXCL8 levels. SHP2WT- and SHP2CS-transfected astrocytes were treated with PTP inhibitor, Na_3VO_4; p38 inhibitor, SB203580, or negative control inhibitor, SB202474. CXCL8 levels measured by ELISA in cellular supernatants collected 24 h post-treatment with **A)** Na_3VO_4 **B)** SB203580 **C)** SB202474. ND denotes not detectable. Results are representative of three independent experiments performed in triplicate and expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons or student's t-test.

Figure 2.6

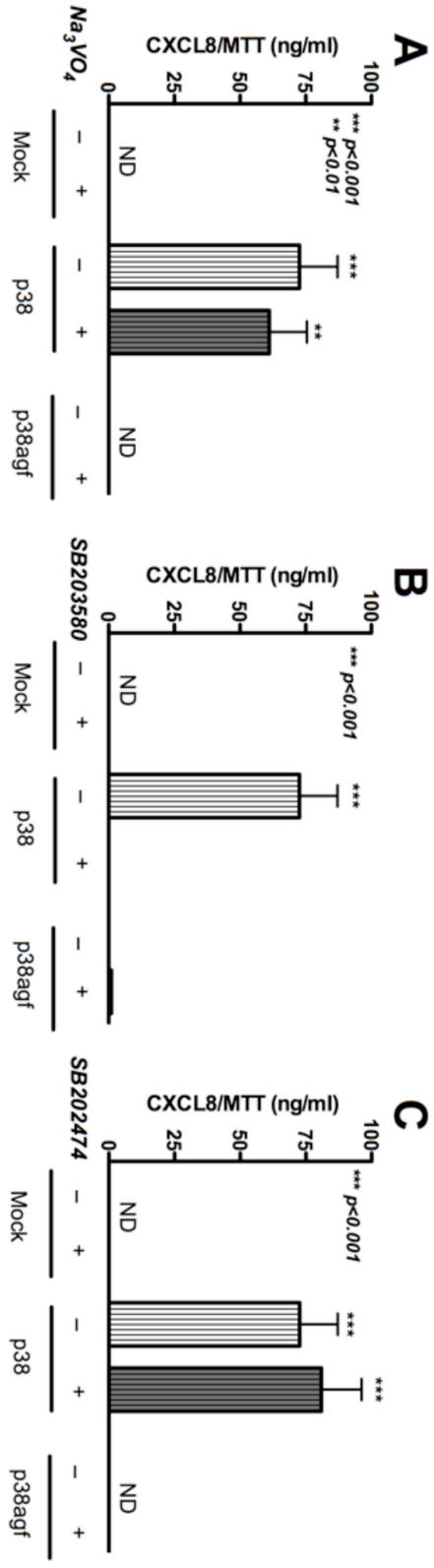
A
Na_3VO_4
CXCL8/MTT (ng/ml)
0
25
50
75
100
*** p<0.001
** p<0.01
ND
***
**
ND
Mock
p38
p38agf
B
SB203580
CXCL8/MTT (ng/ml)
*** p<0.001
ND
***
Mock
p38
p38agf
C
SB202474
CXCL8/MTT (ng/ml)
*** p<0.001
ND
***
***
ND
Mock
p38
p38agf

Figure 2.6: Effect of p38 overexpression and SHP2 inhibition on CXCL8 levels. p38- and p38agf-transfected astrocytes were treated with PTP inhibitor, Na_3VO_4; p38 inhibitor, SB203580, or negative control inhibitor, SB202474. CXCL8 levels measured by ELISA in cellular supernatants collected 24 h post-treatment with **A)** Na_3VO_4 **B)** SB203580 **C)** SB202474. ND denotes not detectable. Results are representative of three independent experiments performed in triplicate and expressed as mean ± SEM, analyzed by student's t-test.

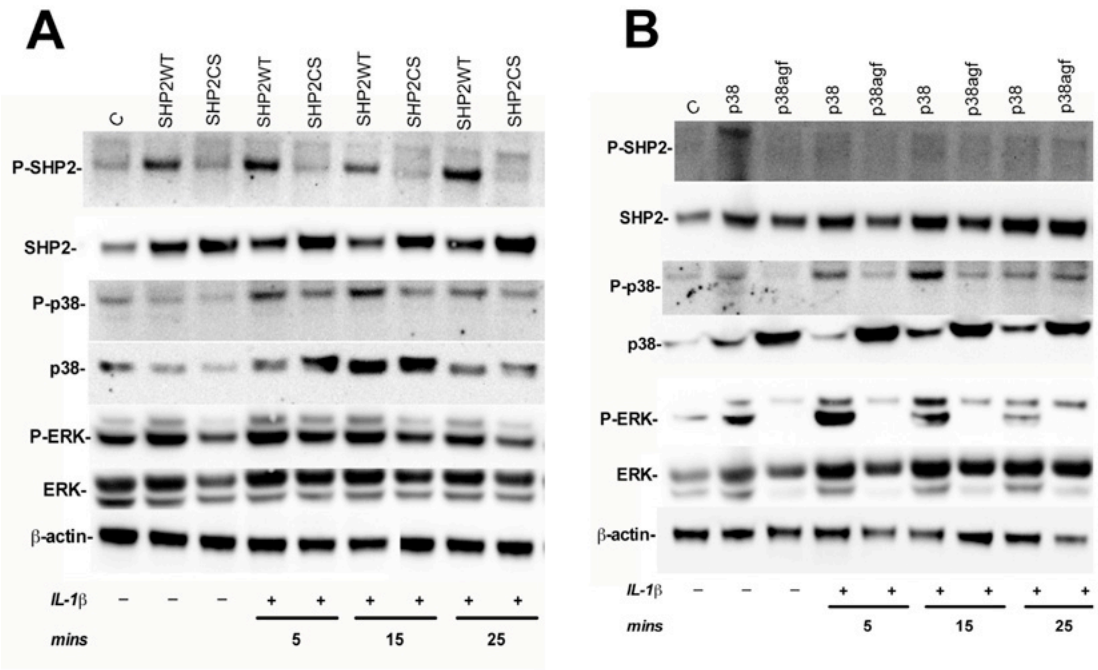

Figure 2.7: SHP2, p38 and ERK phosphorylation following IL-1β stimulation of SHP2WT-, SHP2CS-, p38- and p38agf-transfected astrocytes. Astrocytes were transfected with SHP2WT, SHP2CS, p38 or p38agf plasmids and stimulated with or without IL-1β for 5, 15 and 25 min. Whole cell protein lysates were collected and equivalent amounts were resolved by SDS-PAGE, transferred and immunoblotted for P-SHP2, SHP2, P-p38, p38, P-ERK and ERK. β-actin was used as loading control. **A)** Immunoblot of SHP2WT- and SHP2CS-transfected astrocytes following stimulation with IL-1β. **B)** Immunoblot of p38- and p38agf-transfected astrocytes following stimulation with IL-1β.

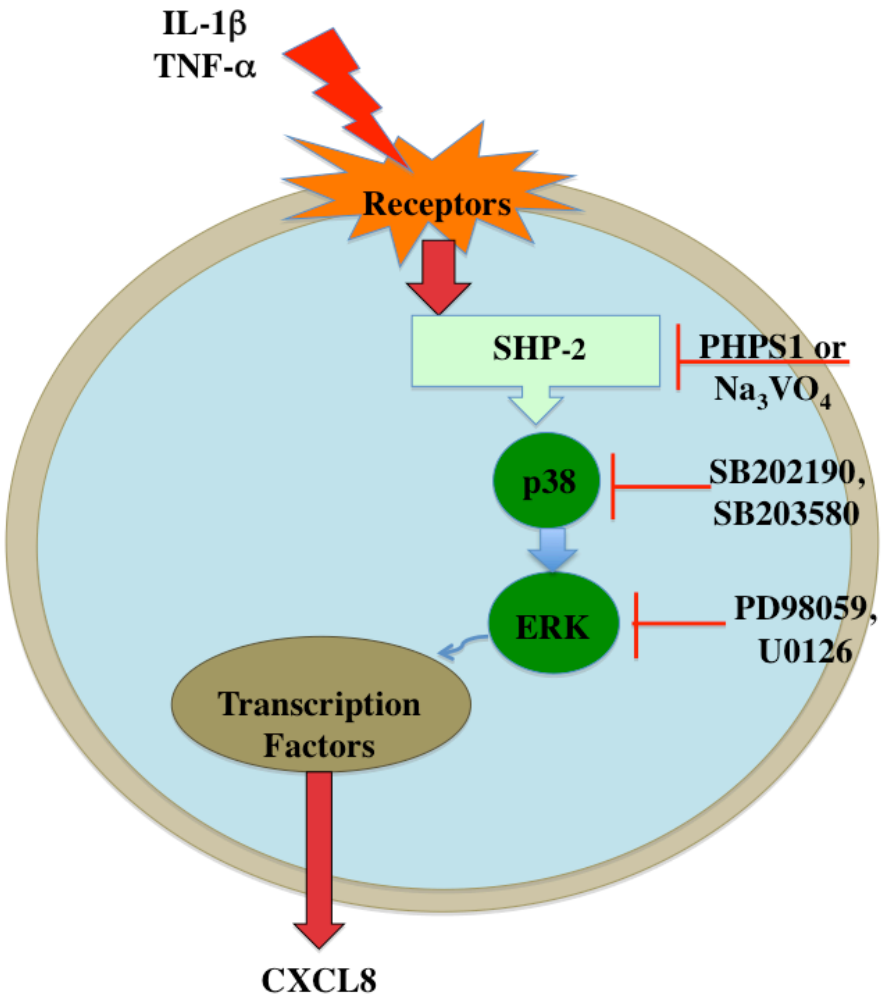

Figure 2.8: **Signaling pathways involved in increased CXCL8 production in activated human astrocytes.** Proinflammatory cytokines, such as interleukin (IL)-1b and tumor necrosis factor (TNF)-a, stimulate astrocytes in turn activating protein tyrosine phosphatase SHP2. Specific inhibitors are indicated adjacent to target. SHP2 is upstream of p38, which directly or indirectly modulates extracellular signal regulated kinase (ERK) mitogen-activated protein kinases activity, leading to increased expression of CXCL8 protein.

CHAPTER 3

Chemokine CXCL8 promotes HIV-1 replication in human monocyte-derived macrophages and primary microglia *via* NF-κB pathway

Manmeet K. Mamik[a] and Anuja Ghorpade[a]#

(*Submitted to Retrovirology*)

ABSTRACT

Background: Chemokine CXCL8 is an important neutrophil chemoattractant implicated in various neurodegenerative disorders. Cytokine/chemokine imbalance, with an increase in proinflammatory cytokines like interleukin (IL)-1β and tumor necrosis factor (TNF)-α within the CNS is a hallmark of human immunodeficiency virus (HIV)-1 infection. We previously reported that treatment with TNF-α and IL-1β upregulates CXCL8 in human astrocytes. Chemokines play crucial roles in trafficking of leucocytes and in the case of HIV-1 infection, the trafficking of infected monocytes across the blood-brain barrier (BBB) results in transmission of virus to perivascular macrophages and microglial cells. In the post-antiretroviral therapy era, a low level of productive HIV-1 replication in the brain is a critical component of neuropathogenesis. The present study investigated the effect of CXCL8 on productive infection of HIV-1 in human monocytes-derived macrophages (MDM) and primary human microglia.

Results: Human MDM and microglia were infected with blood-derived HIV-1_{ADA} and brain-derived HIV-1_{JRFL} viral isolates. Human promonocytic cell line U937 was employed for efficient transfection. Treatment with CXCL8 significantly upregulated HIV-1*p24* levels in supernatants of both HIV-1 infected MDM as well as microglia. In addition, the formation of 2-long terminal repeat (LTR) circles, a measure of viral genome integration, was significantly higher in CXCL8-treated HIV-1-infected MDM and microglia. Transient transfection of U937 cells with HIV-1 LTR luciferase reporter construct resulted in increased promoter activity when treated with CXCL8. Moreover, increased nuclear translocation of nuclear factor

(NF)-κB was seen in HIV-1-infected MDM following CXCL8 treatment. Blocking CXCL8 receptors CXCR1 and CXCR2 abrogated the CXCL8-mediated enhanced HIV-1 replication.

Conclusion: Our results show that CXCL8 mediates productive infection of HIV-1 in MDM and microglia *via* receptors CXCR1 and CXCR2. These results demonstrate that CXCL8 exerts its downstream effects by increasing translocation of NF-κB into the nucleus, thereby promoting HIV-1 LTR activity.

INTRODUCTION

Human immunodeficiency virus (HIV)-1 infects CD4+ T cells and monocytes in peripheral blood, which differentiate into tissue specific macrophages. Microglia, the resident macrophages of the brain, and perivascular macrophages that migrate into the brain are prime targets for HIV-1 productive infection in the brain (175, 233). The glycoprotein (gp) 120 in the viral envelope binds to CD4 receptor on host cells. Macrophage (M)-tropic viruses primarily use CCR5 as a co-receptor (29, 46, 51). HIV-1 replication is a complex mechanism involving both host and viral factors. In the central nervous system (CNS), astrocytes are not productively infected and the neurons are not targets for HIV-1 infection (82, 180). Therefore, majority of viral replication in CNS occurs in perivascular macrophages and/or microglia within brain parenchyma (9, 185, 201). Due to poor penetration of anti-retroviral drugs and other factors, macrophages and/or microglia continue to harbor and release infectious viral particles, viral proteins and other soluble factors, which are potentially neurotoxic and lead to inflammation in CNS (17, 119). Although low plasma levels of HIV-1 are maintained by anti-retroviral therapy, still intracellular reservoir of virus persists. Immune activation markers such as interleukin (IL)-6 and sCD14 determine the level of viral replication in HIV-1 infected population (25). Persistence of HIV-1 in the brain gradually leads to HIV-associated neurological disorders (HAND) in almost 50% of infected individuals (70). Thus, a complete understanding of factors contributing towards HIV-1 replication in CNS is important for better therapeutic strategies to combat HAND.

HIV-1 viral proteins affect inflammatory responses by altering cytokine and chemokine production (146, 227). Chemokine CXCL8 is one of the first chemokines found in the brain and is produced by almost all cells in CNS; astrocytes, microglia and neurons (78, 80, 168). Elevated levels of CXCL8 have been reported in plasma, serum and cerebrospinal fluid (CSF) of HIV-1-infected individuals suggesting its potential role in neuroinflammatory processes and neurodegeneration in HAND (24, 120). Increase in proinflammatory cytokines like IL-1β, IL-6 and tumor necrosis factor (TNF)-α follows soon after initial HIV-1 infection. A previous study from our group indicated that CXCL8 expression is robustly increased in astrocytes treated with IL-1β and TNF-α by src homology-2 domain-containing protein tyrosine phosphatase (SHP2) and mitogen activated protein kinases (MAPK) pathways (136). In the present study, we extended this observation to unravel effect of CXCL8 on HIV-1 replication in human monocyte-derived macrophages (MDM) and primary human microglia. Cytokines and chemokines have been shown to induce HIV-1 replication in variety of cell types (30, 155). TNF-α alters permeability of blood-brain barrier (BBB) that allows infiltration of HIV-1 infected cells into the brain (86). Stimulation of HIV-1 replication by CXCL8 has been reported in macrophages and T-lymphocytes (120). However, the mechanisms linking enhanced chemokine levels and HIV-1 replication are not well understood. While there has been considerable development in understanding of mechanisms that regulate CXCL8 production in CNS, more detailed investigation into the role of this chemokine on HIV-1 replication is required.

Various HIV-1 isolates have been widely used to study HIV-1 infection in macrophages and microglia (36, 39). Blood and brain derived isolates HIV-1_{ADA} and HIV-1_{JRFL} preferentially infect mononuclear phagocytes and both were selected for our present study for investigation of strain-dependent effects from a panel of macrophage-tropic viral isolates. The promonocytic cell line U937, characterized by ability to differentiate into macrophages, forms an *in vitro* model system for transfection studies, which have always been challenging to be carried out in human macrophages (229). Previous studies have shown that chemokines like CCL3 and CCL5 suppress HIV-1 replication in T cells and monocytes (33, 34). In contrast some reports have shown such chemokines to aid in viral replication (49, 81, 208). We show that CXCL8 increases translocation of nuclear factor (NF)-κB into the nucleus thereby increasing HIV-1 long terminal repeat (LTR) activity. HIV-1 LTR promoter is known to have NF-κB binding sites facilitated by chromatin accessibility at the LTR promoters (145). We also show that the CXCL8 receptors CXCR1 and CXCR2 are important for downstream events initiated by CXCL8 in macrophages. The data demonstrate that CXCL8 can potentiate HIV-1 replication in both human macrophages and microglia *via* NF-κB dependent mechanisms. The present study provides insight into the role of chemokines in HIV-1 infection in the brain and thus their contribution towards HIV-1 associated neurodegeneration.

MATERIALS AND METHODS

Isolation and cultivation of primary human microglia: Human microglia were isolated as previously described (72, 75) from first- and early second-trimester aborted specimens, ranging from 14 to 20 weeks, obtained from the Birth Defects Laboratory, University of Washington, Seattle, WA in full compliance with the ethical guidelines of the NIH. The institutional review boards of both the Universities of Washington and North Texas Health Science Center approved the collection of human tissues for research. The Birth Defects Laboratory obtained written consent from all tissue donors. Briefly, tissue was dissected mechanically followed by several Hank's balanced salt solution (HBSS) washes. Tissue pieces were treated with 0.25% trypsin. After 14 days in culture, the nonadherent microglia cells were collected and purified by preferential adhesion (76). Cells were used in experimental protocols when morphological differentiation was apparent (usually 2-5 days). Microglia obtained by this procedure were routinely > 98% pure as measured by immunocytochemistry staining for microglial marker CD68.

Isolation and cultivation of human MDM: Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque (Fisher Scientific, Waltham, MA) density centrifugation from whole blood donated by healthy volunteers (Carter Blood Care. Fort Worth, TX). Monocytes were enriched from freshly isolated PBMC using magnetic-activated cell sorting (MACS), CD14+ beads and MACS LS Columns (Miltenyi Biotec, Auburn, CA), yielding an average 98% purity. To differentiate PBMC into MDM, PBMC were cultured in Dulbecco's

modified Eagle's medium (Life Technologies, Carlsbad, CA) with 10% heat-inactivated pooled human serum (Atlas Biologicals, Fort Collins, CO), 50 μg/ml gentamicin (Life Technologies), 10 μg/ml ciprofloxacin (Sigma, St. Louis, MO) and 50 ng/ml macrophage-colony stimulating (MCSF) for two weeks. MDM were cultured as adherent cells in 48-well plates (4×10^5 / well) or 6-well plates (3×10^6) for virus infection or as non-adherent cultures in teflon flasks (2×10^6 cells/ml, 150×10^6 cells in flask) for transfection assays. Cultures were maintained by half-media exchange twice weekly.

Cell lines and culture conditions: The promonocytic cell line U937 was obtained from the ATCC and maintained in suspension culture in RPMI-1640 medium (Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Neomycin (PSN) at 37°C in a humidified atmosphere of 5% CO_2. U937 cells were induced to differentiate by treating the cells with 20 ng/ml of phorbol 12-myristate 13-acetate (PMA; Fisher Scientific) overnight followed by complete media exchange. Cultures were maintained by half-media exchange every 3-4 days.

HIV-1 infection of MDM: HIV-1_{JRFL} (159) was obtained from the AIDS Research and Reference Reagent Program, Division of National Institute of Allergy and Infectious Diseases (NIAID). It was previously isolated from brain tissue of an HIV-1-infected individual with encephalitis. HIV-1_{ADA} was isolated from PBMC of an individual with AIDS and was propagated as previously described (71). All isolates were prepared as viral stocks free of mycoplasma and endotoxin contamination. Concentration of HIV-1*p24* 10 ng/ml was used for infection.

Following 2 weeks in culture, MDM were infected with HIV-1 as described (73, 74, 164). Following day, virus was washed off the cells and supplemented with media with or without varying concentrations (10-100 ng/ml) of human recombinant CXCL8 (R&D Systems, Minneapolis, MN). Heat-inactivated CXCL8, generated by heating at 70°C for 10 minutes, was used as a control. Heating disrupts the protein structure and thus interferes with efficient binding to its receptors. Culture supernatant samples of infected MDM were collected by half-media exchange twice weekly. Lipopolysaccharide (LPS; 2 ng/ml) was used as a positive control for activation.

DNA isolation and quantification of 2-LTR circles: To quantify 2-LTR circles, 3×10^6 MDM were plated in 6-well plates and infected with HIV-1_{ADA} or HIV-1_{JRFL}. To isolate DNA, cells were washed with phosphate-buffered saline (PBS), and DNA was harvested using the DNeasy tissue kit (Qiagen). Two-LTR circles were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using 100 ng of the DNA template and primer sets as described in (19). The PCR products were detected with TaqMan® gene expression assays using Step One Plus (Life Technologies). Absolute copy numbers were determined by normalization to standard curves generated from a serially diluted pTA2LTR/CCR5 plasmid. The plasmid pTA2LTR/CCR5 harbors the sequence of 2-LTR junction and was a generous gift from Dr. Mario Stevenson, University of Miami (20, 191). In all cases the DNA standards were diluted into 100 ng of uninfected cellular DNA to match the cellular DNA samples.

Plasmids and DNA transfection into MDM and U937 cells: The plasmid construct pBlue3'LTR-luc was obtained from AIDS Research and Reference Reagent Program, NIAID, deposited by Dr. Reink Jeeninga and Dr. Ben Berkhout (96, 113). MDM were transfected with pBlue3'LTR-luc, *Renilla* luciferase (pRL-SV40, Promega), siRNA specific to NF-κB p65 (si-p65, Cell Signaling Technology, Danvers, MA) or scrambled non-targeting siRNA (si-Con, Thermo Scientific, Waltham, MA) using the Amaxa® Human Macrophage Nucleofector® kit (Lonza, Walkersville, MD). Briefly, 1 million MDM were suspended in 100 μl nucleofector solution per cuvette with 2 μg plasmid DNA or 100 nM siRNA and transfected using a Nucleofector® II (Lonza) device. U937 cells were transfected using Amaxa® Cell Line Nucleofector® Kit C with 2 million cells/cuvette. Transfected cells were supplemented with MDM or U937 media (with 20 nM PMA) and plated in 48 well plates at a density of 4 x 10^5 / well. Twenty-four hours post-transfection cells were treated with indicated concentrations of CXCL8, anti-CXCL8 IgG or mouse IgG (R&D Systems).

Measurement of HIV-1 LTR promoter activity by luciferase assay: Twenty-four hours post-treatment, 4 x 10^5 transfected cells/well were harvested and lysed using 65 μl lysis buffer. An aliquot (20 μl) of the cell lysate was used to measure luciferase activity using the dual-luciferase reporter assay kit (Promega, Madison, WI). The firefly luciferase activity was normalized to the *Renilla* luciferase activity. All experiments were carried out in triplicate, and the data are presented as the mean luciferase activities ± standard error of the mean (SEM).

Quantification of CXCL8 and HIV-1p24 by ELISA: CXCL8 protein levels were determined from culture supernatants by a CXCL8 specific sandwich ELISA (R&D Systems) and HIV-1*p24* ELISA (Advanced Biosciences Laboratories, Rockville, MD) according to manufacturer's protocol. Absorbance was determined on a Spectromax M5 microplate reader using SoftMax Pro V5 software (Molecular Devices, Sunnyvale, CA).

Western blot analysis: MDM were cultured as adherent monolayers in 6 well-plates at a density of 3 X 10^6 cells/well and infected with HIV-1_{ADA} overnight. Following day, cells were washed with PBS and supplemented with fresh medium with or without CXCL8. LPS (2 ng/ml) was added to uninfected MDM. Three hours later, cells were collected by scraping in PBS. Cytoplasmic and nuclear protein extracts were isolated using nuclear and cytoplasmic extraction kit (NE-PER, Thermo Fisher Scientific, Pittsburgh, PA). Equal amounts of protein samples (15 μg) were boiled with 4X NuPAGE loading sample buffer (Life Technologies) for 5-10 min, resolved by NuPage 4-12% Bis tris gel and subsequently transferred to a nitrocellulose membrane using i-Blot (Life Technologies). The membrane was incubated with primary antibody against NF-κB p65 (1:1000, Cell Signaling) overnight at 4ºC, washed and then incubated with anti-mouse or anti-rabbit goat antibody IgG conjugated to horseradish peroxidase (1:10,000, Bio-Rad) for 2 h at room temperature. The membrane was then developed with SuperSignal west femto substrate (Fisher Scientific) in a Fluorochem HD2 Imager (ProteinSimple, Inc. Santa Clara, CA). GAPDH (1:1000,

Santa Cruz Biotechonology) and Lamin A/C (1:1000, Cell Signaling Technology) were used as loading controls for cytoplasmic and nuclear extracts, respectively.

Determination of metabolic activity: Following experimental manipulations, five percent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Fisher Scientific) in fresh medium was added to MDM and/or microglia and incubated for 20-45 min at 37°C. MTT is metabolically reduced to purple formazan crystals by living cells. Representative images were taken using a phase-contrast microscope. The MTT solution was removed and crystals were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific) for 15 min with gentle agitation. The absorbance of the DMSO/crystal solution was assayed at 490 nm, as previously described (138). MTT ratio to control was calculated to normalize ELISA levels.

Immunocytochemistry: MDM and microglia were fixed with cold acetone:methanol 1:1 (V/V) solution for 20 min at -20°C and blocked with PBS with 2% bovine serum albumin (BSA) and 0.1% triton X-100 for 1 h at room temperature. Cells were then incubated with primary antibodies specific to CD68 (1:50, Abcam, Cambridge, MA) and HIV-1*p24* (1:50, Abcam) in blocking buffer overnight at 4°C and washed incubated with AlexaFluor® secondary antibodies, anti-rabbit (488 nm, green) and anti-mouse (594 nm, red) (1:100, Life Technologies). Nuclei were visualized with diamidino-2-phenylindole (DAPI; 1:800, Life Technologies). Micrographs were taken on a Nikon Eclipse Ti-4 (Nikon Inc., Melville, NY) using the NLS-Elements BR. 3.0 software.

Statistical analyses: Statistical analyses were carried out using Prism V5.0 (GraphPad Software, La Jolla, CA) with one-way analysis of variance (ANOVA) and Newman-Keuls post-test for multiple comparisons. Significance was set at $p<0.05$ and data represents means +/- standard error of the mean (SEM). Data presented is representative of a minimum of three independent experiments with two or more independent donors.

RESULTS

Chemokine CXCL8 increases HIV-1*p24* levels in MDM

Secretion of viral antigen HIV-1*p24* is an important indicator of viral protein production by infected cells. We examined HIV-1*p24* levels in MDM infected with macrophage tropic blood-derived isolate HIV-1_{ADA} and brain-derived isolate HIV-1_{JRFL}. To determine the role of CXCL8 in HIV-1 infection, MDM were infected with HIV-1_{ADA} or HIV-1_{JRFL} followed by treatment with or without varying concentrations of CXCL8. HIV-1*p24* levels were measured 1 week post-infection by ELISA. As shown in Figure 3.1A and B, MDM treated with CXCL8 showed significant dose-dependent increase in HIV-1*p24* levels when compared to infected cells without CXCL8 treatment. Significant increase in HIV-1*p24* was observed with CXCL8 concentration as low as 10 ng/ml, for both HIV-1_{ADA} and HIV-1_{JRFL} infection ($p<0.01$ and 0.001 respectively). HIV-1*p24* levels were increased by more than 2-fold when CXCL8 concentration was increased from 1 to 100 ng/ml for both HIV-1_{ADA}- and HIV-1_{JRFL}-infected MDM ($p<0.001$). Also, the basal levels of HIV-1*p24* were more in HIV-1_{ADA}-infected MDM as compared to HIV-1_{JRFL}-infected MDM, which is in agreement with previous findings demonstrating higher infectivity with HIV-1_{ADA} as

compared to HIV-1_{JRFL} (95). Heat-inactivated CXCL8, used as a control, did not significantly alter HIV-1*p24* levels in infected MDM. In parallel, we investigated the cellular expression of HIV-1*p24* in CXCL8-treated HIV-1-infected MDM. One week post-infection, MDM were immunoassayed for HIV-1*p24* (red) and macrophage marker CD68 (green). *HIV-1p24* antigen expression could be seen in HIV-1-infected MDM but as expected, not in control cells (Figure 3.1C, D). Furthermore, CXCL8-treated MDM showed intense staining with HIV-1*p24* and multinucleated giant cells (MGC) as compared to infected MDM without CXCL8 treatment (Figure 3.1E, F). Taken together, CXCL8 treatment led to dose-dependent increase in HIV-1*p24* release as well as enhanced cellular expression in HIV-1 infected MDM.

Endogenous CXCL8 stimulates HIV-1*p24* levels in HIV-1-infected MDM

Chemokine CXCL8 is produced by a variety of cells including astrocytes, macrophages and microglia (78, 80, 168). To determine whether HIV-1-infected MDM secreted CXCL8, we first measured levels in culture supernatants following HIV-1_{ADA} or HIV-1_{JRFL} infection at different time points. While the basal levels of CXCL8 in uninfected cells remained unchanged over a period of three weeks, CXCL8 significantly increased as viral infection progressed from one week to three weeks in both HIV-1_{ADA} - and HIV-1_{JRFL}-infected MDM (Figure 3.2 A, B; $p<0.001$). To determine the effect of endogenous CXCL8 on HIV-1*p24* production, HIV-1-infected MDM were treated with CXCL8 neutralizing antibody the day following infection with HIV-1. Consistent with our hypothesis, by two weeks, significant downregulation in HIV-1*p24* levels was noted upon CXCL8 neutralization in both HIV-1_{ADA}- and HIV-1_{JRFL}-infected MDM. Treatment with mouse IgG, used as a

control, did not alter HIV-1*p24* levels in both HIV-1_{ADA}- and HIV-1_{JRFL}-infected MDM (Figure 3.2 C, D; $p<0.001$). These results corroborated those obtained above and showed that both endogenous and exogenous CXCL8 increased HIV-1*p24* production in HIV-1-infected MDM.

CXCL8 increases HIV-1 LTR promoter activity in U937 cells and MDM

The HIV-1 LTR promoter activity is dependent upon host cell transcriptional machinery once the viral genome integrates into the host chromosome (14, 111). Since transcription is a requisite for production of viral proteins, we analyzed the effect of CXCL8 on HIV-1 LTR promoter activity. We utilized luciferase reporter assay with the pBlue3'LTR-luc plasmid (96, 113). We employed the promonocytic cell line U937 for the promoter-reporter studies because of better transfection efficiency and viability following transfection. Briefly, U937 cells were cotransfected with pBlue3'LTR-luc and *Renilla* plasmid followed by treatment with varying concentrations of CXCL8 24h post-transfection. CXCL8 induced a significant dose-dependent increase in HIV-1 LTR promoter activity, as indicated by increase in luciferase activity (Figure 3.3 A; $p<0.001$). No significant changes in HIV-1 LTR promoter activity were observed with heat-inactivated CXCL8. Since we observed robust increase in CXCL8 production by PMA treated differentiating U937 cells (Figure 3.3B), we next evaluated the HIV-1 LTR promoter activity following neutralization of endogenous CXCL8. The luciferase activity significantly dropped upon CXCL8 neutralization ($p<0.001$), whereas no change was observed when control IgG was added to the transfected U937 cells (Figure 3.3C). In conclusion,

these findings indicate an important role of chemokine CXCL8 in regulating HIV-1 LTR promoter activity.

CXCL8 promotes formation of 2-LTR circles in HIV-1 infected MDM

HIV-1 entry into the cells is followed by reverse transcription of viral RNA into a linear double strand cDNA copy, which is then transported into the nucleus (59, 107). Soon after nuclear import, viral cDNA integrates into the host genome. The HIV cDNA that does not integrate then circularizes to form 2-LTR circles and can be used as a marker for nuclear import of viral DNA (106). To investigate the effect of CXCL8 on formation of 2-LTR circles, MDM were infected with HIV-1_{ADA} or HIV-1_{JRFL} with or without CXCL8 treatment (106). Two weeks post-infection, 2-LTR circle copy numbers were quantified by real-time PCR assay using a standard curve generated by pTA-2LTR/CCR5 (Figure 3.4A). We observed that treatment with CXCL8 led to significant increase in number of 2-LTR circles compared to non-treated HIV-1_{ADA}- and HIV-1_{JRFL}-infected MDM (Figure 3.4B; $p<0.01$). These data demonstrate that CXCL8 treatment enhanced formation of 2-LTR circles in HIV-1-infected cells along with elevated infection as evident from high HIV-1*p24* levels.

CXCL8 treatment increased HIV-1*p24* and 2-LTR circle formation in human microglia

Microglia are resident macrophages of the brain and constitute important targets for HIV-1 infection in the CNS. To confirm the results obtained in MDM and to assess whether CXCL8 promotes HIV-1 infection in human microglia, we infected cultured human microglia with viral isolates HIV-1_{ADA} or HIV-1_{JRFL}. HIV-1*p24* levels,

as measured by ELISA, showed significant upregulation when infected microglia were treated with CXCL8. Interestingly, CXCL8 treatment led to 2-fold increase in HIV-1*p24* levels in HIV-1_{ADA}-infected microglia, whereas the increase was more than 4-fold in HIV-1_{JRFL}-infected microglia (Figure 3.5A; $p<0.001$). Given the limitation in culturing microglia, only selected conditions and time-points were analyzed. Two weeks post-infection DNA was isolated and assayed for 2-LTR circles. Consistent with trends observed in HIV-1*p24* levels, CXCL8 treatment increased 2-LTR circle copy numbers by only 2-fold in HIV-1_{ADA}-infected microglia as compared to a 10-fold increase in HIV-1_{JRFL}-infected human microglia (Figure 3.5B; $p<0.001$). Therefore, these data corroborate the results obtained with MDM and indicate that CXCL8 treatment leads to elevated HIV-1*p24* levels and 2-LTR circles in primary human microglia.

MTT is reduced to purple formazan by viable cells, thus formazan staining can be used to evaluate changes in cell morphology or proliferation (149). Primary human microglia were incubated with MTT and images were taken by light microscopy. Uninfected control cells exhibited amoeboid bipolar morphology with smaller individual cells. Formazan aggregates could be seen in HIV-1_{ADA}-infected microglia revealing MGC formation. Treatment with CXCL8 exacerbated formation of giant cells, as indicated by formazan aggregates (Figure 3.5C-E). In parallel, microglia were fixed and immunostained for CD68 and HIV-1*p24.* Intense HIV-1*p24* antigen expression could be seen in HIV-1_{ADA}-infected microglia but not in control cells (Figure 3.5F, G). Furthermore, CXCL8-treated HIV-1_{ADA}-infected microglia showed bright HIV-1*p24* staining and MGC as compared to infected untreated cells (Figure

3.5H). These data demonstrate that CXCL8 enhanced HIV-1 infection in primary human microglia with both increased nuclear import of viral DNA as well as viral protein HIV-1*p24* expression.

CXCL8 promotes HIV-1 LTR promoter activity *via* NF-κB dependent pathway in U937 cells and MDM

The HIV-1 LTR promoter controls transcription of viral genes and is reported to have NF-κB binding sites (152, 197). The transcription factor NF-κB can translocate to nucleus in response to various internal or external stimuli (218). The p65 subunit of NF-κB is essential in transcriptional regulation and forms a vital component of the transcription factor. We next examined the effect of silencing the p65 subunit in two cellular systems, U937 and MDM and then assayed the HIV-1 LTR promoter activity. For this, U937 cells and MDM were cotransfected with pBlue3'LTR-luc plasmid, *Renilla* and siRNA targeting p65. We found a significant reduction in CXCL8-induced HIV-1 LTR promoter activity after silencing p65 subunit of NF-κB. The trend was similar in both U937 cells and MDM (Figure 3.6A, B respectively; $p<0.001$). To further elucidate whether CXCL8 stimulated nuclear translocation of NF-κB, HIV-1_{ADA}-infected and uninfected MDM were stimulated with CXCL8 and cytoplasmic and nuclear protein lysates were collected. Immunoblot analysis showed that CXCL8 served as a stimulus for the nuclear translocation of NF-κB p65 into the nucleus, and that CXCL8 facilitated this translocation in infected as well as uninfected MDMs. Together, these data demonstrate that NF-κB p65 is important for CXCL8-induced activation of HIV-1 LTR.

CXCL8-induced enhanced HIV-1 infection is both CXCR1 and CXCR2 dependent

Human MDM express G-protein coupled receptors CXCR1 and CXCR2, through which CXCL8 exerts its effects (80, 187). To evaluate the role of these receptors in CXCL8-mediated enhanced HIV-1 replication, we infected human MDM with HIV-1_{ADA} followed by addition of antibodies to CXCR1 and CXCR2. Both CXCR1 and CXCR2 played important role in CXCL8-mediated HIV-1*p24* elevation, as evident by significantly reduced HIV-1*p24* levels following CXCR1 and CXCR2 neutralization in HIV_{ADA}-infected MDM (Figure 3.7A; $p<0.001$). qRT-PCR assay revealed similar trends in 2-LTR circle copy numbers. Neutralization of CXCR1 or CXCR2 abrogated 2-LTR circle responses, which were further reduced when both the receptors were neutralized concurrently (Figure 3.7B; $p<0.001$). These results suggest that either of the two receptors, CXCR1 and CXCR2, is required for CXCL8-mediated increase in HIV-1 infection in MDM.

DISCUSSION

Increased expression of chemokines has been correlated with neurodegeneration in the brain (121). More importantly, chemokine CXCL8 has been implicated in NeuroAIDS (42). The correlation of CXCL8 and HIV-1 infection was demonstrated very early when increased circulating levels of this chemokine were found in HIV-1-infected individuals (140, 204) and also in HIV-1 encephalitis (HIVE) (183). *In vitro* studies confirmed that CXCL8 could be independently produced by HIV-1-infected microglia and astrocytes (40, 195). HIV-1

predominantly resides and productively replicates in peripheral blood mononuclear cells and microglia, which are the resident macrophages in the brain. The infected cells release viral particles, viral proteins, cytokines and chemokines and immune response generated by them contributes towards HIV-1 associated neurodegeneration (108, 109, 218). In the current study we investigated the mechanism of HIV-1 replication in human macrophages and microglia. We provide several lines of evidence to support the observation that CXCL8 treatment increased HIV-1 replication in human macrophages and microglia. We found that CXCL8 treatment enhanced HIV-1 LTR promoter activity in monocytic cell line U937 and MDM. The expression and secretion of structural protein HIV-1*p24* was greater with CXCL8 treatment. CXCL8 also activated transcription factor NF-κB translocation to the nucleus, which is a regulator of HIV-1 LTR promoter. We also demonstrate that both CXCR1 and CXCR2 receptors mediate CXCL8 signaling in MDM. Overall, our study shows that CXCL8 enhances HIV-1 infection in macrophages and microglia through receptors CXCR1 and CXCR2 by downstream activation of NF-κB.

We found significant effect of CXCL8 treatment on HIV-1 protein expression in infected macrophages and microglia. High levels of intracellular and secreted HIV-1*p24* suggest that CXCL8 increases viral protein expression, which is an indicator of increased viral replication (106). The chosen CXCL8 concentrations were based on previous reports by our group and others showing robust increase in CXCL8 levels by activated astrocytes (136, 232). Astrocytes are the major cell type of CNS contributing to elevated CXCL8 levels in brain

microenvironment. Presence of CXCL8 in abundance possibly aids in migration of monocytes through BBB leading to increased viral entry. Previous reports suggest that it may activate monocytes/macrophages and thus indirectly promote higher level of viral replication (83). We also observed HIV-1*p24* positive MGC with CXCL8 treatment. Infected macrophages/microglia forming MGC through HIV envelope-dependent fusion are a hallmark of progressive viral infection. HIV-1*p24* positive MGC have been reported during *in vivo* expression analysis of proinflammatory cytokines in HIVE brains (223). We report increased MGC with CXCL8 treatment in HIV-1-infected macrophages and primary microglia, which suggest a plausible mechanism by which CXCL8 may contribute towards neuropathogenesis in tissue microenvironment.

The HIV-1 LTR promoter contains DNA elements, which control viral gene expression and promoter activation increases production of viral proteins and particles in the brain. We report that CXCL8 increases HIV-1 LTR promoter activity in two separate model systems, U937 cells and MDM. Monocytic cell line U937 differentiates into macrophage like cells after stimulation with agents like PMA and is an efficient model system for transfection studies. Luciferase gene under the control of HIV-1 LTR promoter showed increased activity after CXCL8 stimulation and blocking the chemokine reduced the activity. Consistent results were observed in macrophages. Viral proteins like Tat and gp120 play an important role in HIV-1 associated neurotoxicity (43, 104). Therefore, it is plausible that CXCL8 may potentiate neurotoxicity indirectly *via* activation of HIV-1 LTR promoter, thus increasing viral protein production. HIV-1-infected macrophages/microglia

themselves are a significant source of CXCL8; however, not as robust as activated astrocytes. Therefore, the observed increase in HIV-1*p24* levels may in part be contributed by endogenous CXCL8 secreted by macrophages during the course of HIV-1 infection. To address this issue, we neutralized CXCL8 in culture supernatants of HIV-1-infected macrophages and U937 cells and found decreased HIV-1*p24* levels and HIV-1 LTR promoter activity, respectively. This observation was consistent with previous report in macrophages and T-lymphocytes (120).

2-LTR circles are formed after joining of the 5′ and 3′ ends of unintegrated viral DNA. These circles are shown to be unstable in cells and serve as a useful marker for ongoing infection and viral replication in patients undergoing highly active antiretroviral therapy (HAART) (20, 191). Despite the controversy regarding stability of 2-LTR circles, the level of 2-LTR circles is proportional to the level of nuclear import of viral DNA (137). The observed rise in 2-LTR circles possibly suggests a role for CXCL8 in promoting nuclear translocation of viral DNA. Another possibility explaining the observed results may be a rise in number of infected cells following CXCL8 treatment. It may, thus, contribute to increase in 2-LTR circles as a whole instead of increased 2-LTR circle levels per cell. In contrast, studies have shown 2-LTR circles persisting in long-lived nondividing cells and in patients undergoing HAART (19, 165). In such cases, 2-LTR circles cannot be considered as a direct marker for ongoing viral replication. Since our present study focused on acute infection, analysis of 2-LTR circles is a reliable method to assess early steps of HIV-1 infection. Therefore, our results establish that CXCL8 treatment increases nuclear transport of viral cDNA and promotes viral infection.

Activation of NF-κB leads to expression of various chemokines including CXCL8 in astrocytes, macrophages and microglia (42, 129, 190). Interestingly, the secreted chemokines may exhibit feedback activation of NF-κB to maintain their constitutive expression (87, 177). Also, the NF-κB binding regions in HIV-1 LTR promoter are well documented and thus transcription factor NF-κB is implicated in regulating HIV-1 LTR activity (145). Our findings explore the role of CXCL8 in NF-κB activation. We demonstrated that CXCL8 was efficient in activating signaling cascades leading to nuclear translocation of NF-κB in macrophages, even in the absence of HIV-1 infection. The next logical question was to investigate the role of translocated NF-κB in HIV-1 LTR promoter activity, for which we utilized both the U937 system and macrophages. We found that p65 subunit of NF-κB was important for CXCL8-induced HIV-1 LTR activity. Interestingly, NF-κB activation is linked to generation of inflammatory responses and release of CXCL8 in U937 and macrophages (125, 212). Therefore, present data indicate that NF-κB induces CXCL8 expression that in turn further activates NF-κB, and this loop is beneficial for viral replication. More detailed investigation could be carried out to unravel molecular signaling involved upstream of NF-κB, following CXCL8 treatment in macrophages.

Since CXCL8 is a key mediator of inflammation during HIV-1 infection and our study highlights its importance in promoting HIV-1 replication, blocking the production or effects of CXCL8 can be a therapeutic strategy. Complex signaling pathways interact to produce CXCL8 in various cellular systems, therefore, blocking the production completely may not be feasible *in vivo*. Instead, indirect

approaches need to be employed. A recent study demonstrated use of dominant-negative CXCL8 decoy proteins *in vivo* to block CXCL8-induced inflammatory cascade (58). In another approach, the receptors CXCR1 and CXCR2 may be targeted to block the effects of CXCL8 in brain microenvironment. Blocking of CXCL8 receptor CXCR1 by inhibitor reparixin has been shown to reduce short-term neutrophil infiltration and infarct size. Inhibiting both CXCR1 and CXCR2 decreased polymorphonuclear lymphocyte infiltration and improved neurological function in permanent and transient rat cerebral ischemia (68, 210). Reparixin has been tested in human clinical trials as a combinatorial therapy in diabetes and cancer (32). Our findings compliment these studies and confirm the role of chemokine CXCL8 and receptors CXCR1/2 in regulating HIV-1 infection in MDM and microglia.

In summary, our data suggest a mechanism where elevated CXCL8 levels during HIV-1 infection of the brain promote HIV-1 infection and HIV-1*p24* release in human macrophages and microglia. CXCL8 binds to receptors CXCR1/2 and initiates NF-κB translocation into the nucleus, thereby promoting HIV-1 LTR promoter activity, which in turn increases viral gene expression. Considering its affect on HIV-1 infection in macrophages and microglia, CXCL8 can be a potential therapeutic target for controlling persistence of viral infection in the brain. CXCR1/2 inhibition in combination with HAART might prove to be a better therapy for control of neuroinflammation in HIV-1 infection.

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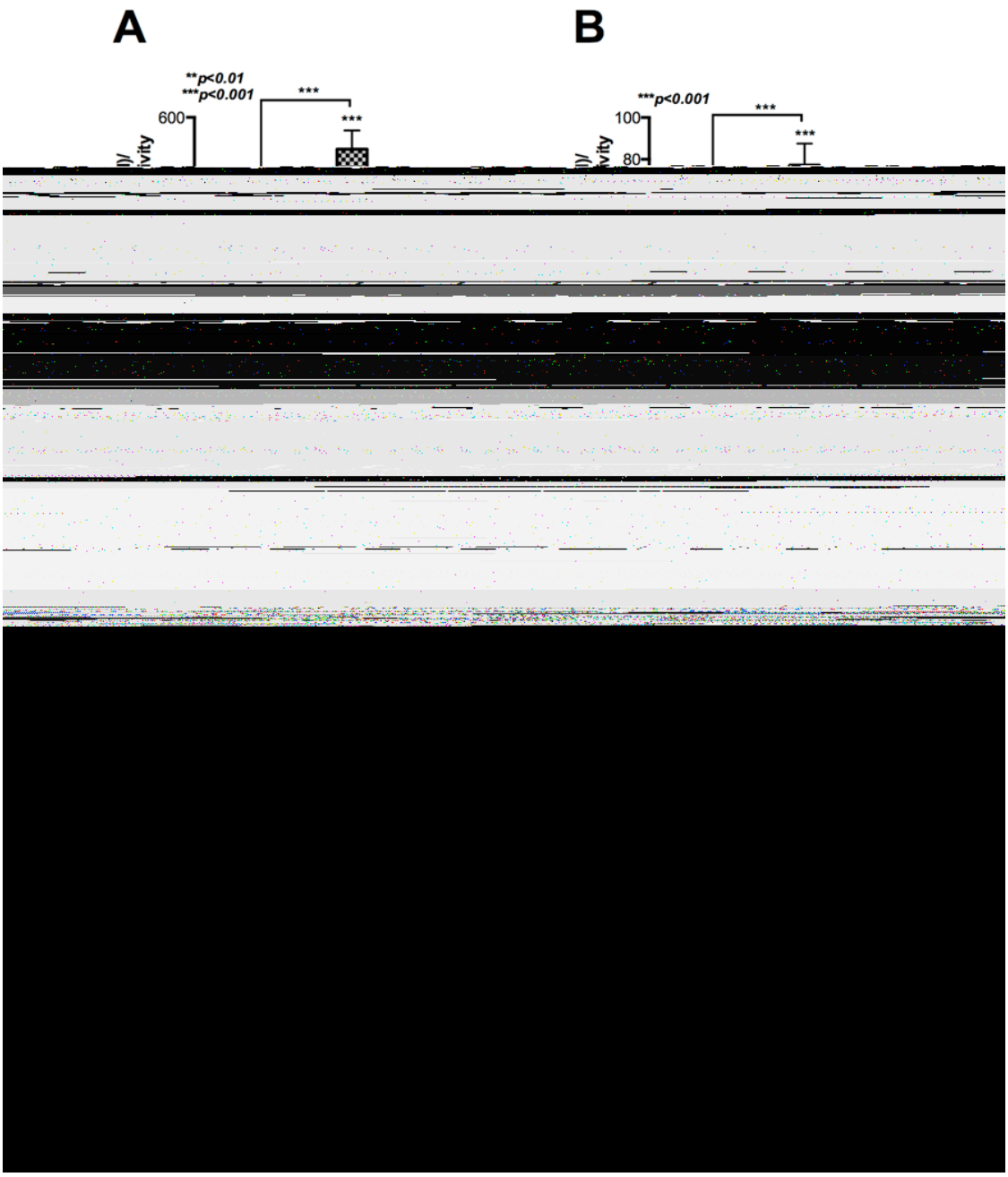


Figure 3.1: CXCL8 induces a dose-dependent upregulation of HIV-1*p24* in HIV-1-infected MDM. Human MDM were infected with HIV-1_{ADA} or HIV-1_{JRFL} (10 ng/ml *p24*) overnight, followed by treatment with indicated concentrations of CXCL8. Cell culture supernatants were collected 1 week post-infection and HIV-1*p24* levels were measured by ELISA in MDM infected with **(A)** HIV-1_{ADA} or **(B)** HIV-1_{JRFL}. Expression of macrophage marker CD68 and HIV-1*p24* was measured by immunocytochemistry in HIV-1_{ADA}-infected MDM 1 week post-infection. Nuclei were stained in blue by DAPI. **(C)** Control, CD68-positive cells

(green) **(D)** Co-localization (yellow) of CD68 and HIV-1*p24* (red) in HIV-1_{ADA}-infected MDM **(E, F)** HIV-1*p24* expression and multinucleated giant cells in CXCL8 treated HIV-1_{ADA}-infected MDM. Original magnification 200X. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

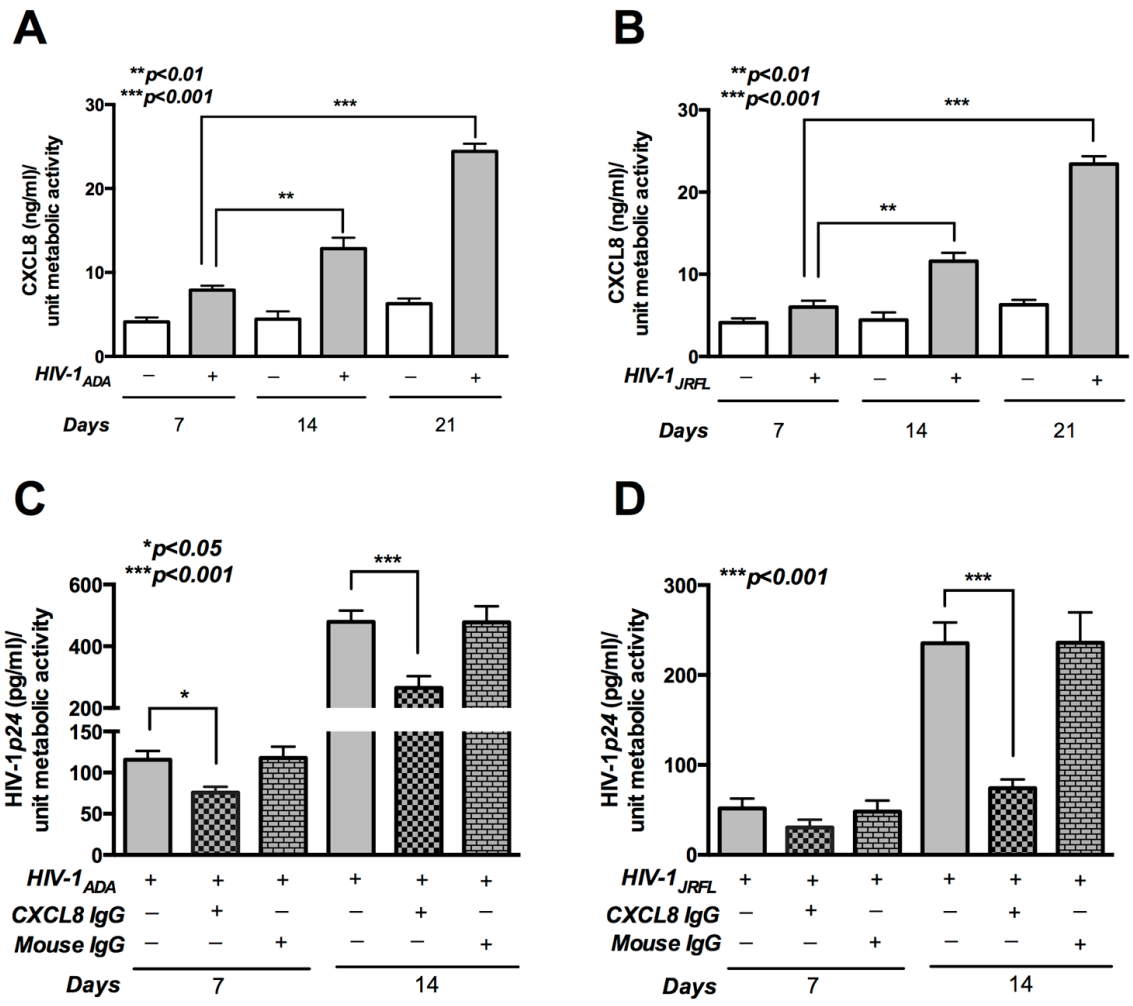


Figure 3.2: Endogenous CXCL8 stimulates HIV-1*p24* levels in HIV-1-infected MDM. Human MDM were infected with HIV-1_{ADA} or HIV-1_{JRFL} (10 ng/ml *p24*) overnight, followed by treatment with/without anti-CXCL8 IgG (1.2 µg/ml). Mouse IgG (1.2 µg/ml) was used as a control. Cell culture supernatants were collected at indicated time points. CXCL8 levels were determined by ELISA in MDM infected with **(A)** HIV-1_{ADA} or **(B)** HIV-1_{JRFL} **(C)** HIV-1*p24* levels as determined by ELISA in MDM infected with HIV-1_{ADA} or **(D)** HIV-1_{JRFL}. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

Figure 3.3

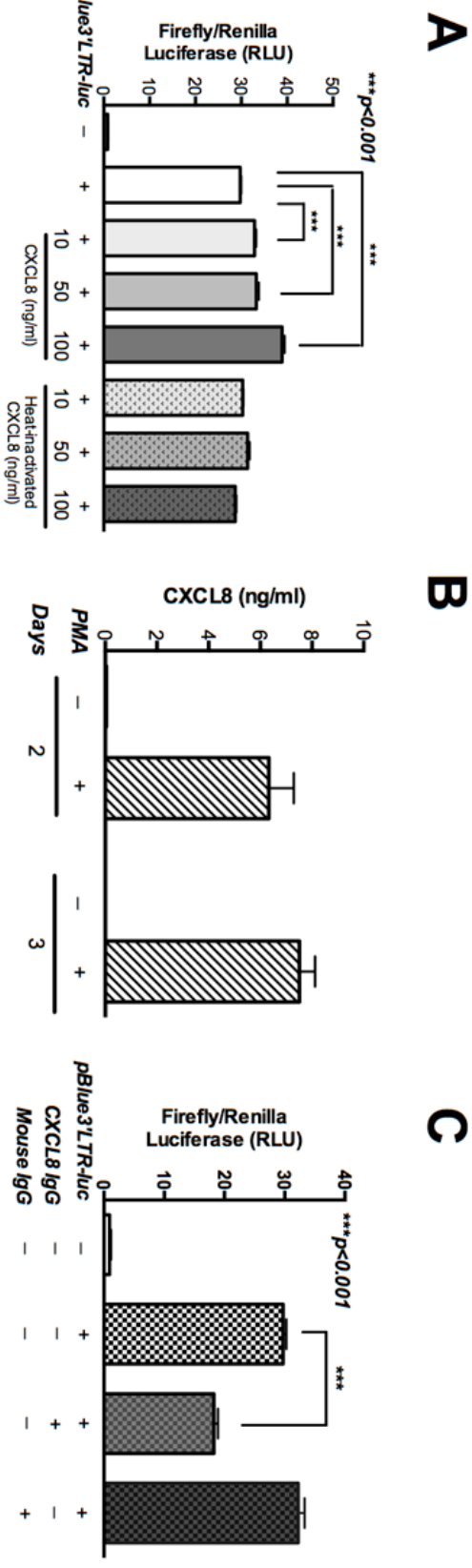

A
Firefly/Renilla Luciferase (RLU)
0
10
20
30
40
50
***p<0.001
***
***
***
pBlue3'LTR-luc
–
+
+
+
+
+
+
+
CXCL8 (ng/ml)
10
50
100
Heat-inactivated CXCL8 (ng/ml)
10
50
100
B
CXCL8 (ng/ml)
0
2
4
6
8
10
PMA
–
+
–
+
Days
2
3
C
Firefly/Renilla Luciferase (RLU)
0
10
20
30
40
***p<0.001
***
pBlue3'LTR-luc
CXCL8 IgG
Mouse IgG
–
–
+
–
–
+
+
–
–
+
+
+

Figure 3.3: CXCL8 stimulates HIV-LTR promoter activity in U937 cells. U937 cells were transfected with pBlue3'LTR luc along with a *Renilla* transfection control. Cells were plated in 48-well plates with 20 nM PMA overnight. Following day, cells were washed and treated with indicated concentrations of CXCL8 or anti-CXCL8 IgG (1.2 µg/ml). Mouse IgG (1.2 µg/ml) was used as a control. **(A, C)** Cell lysates were collected 24 h post-treatment and relative luciferase activity was determined. **(B)** Levels of CXCL8 were measured by ELISA in PMA treated U937 cells. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

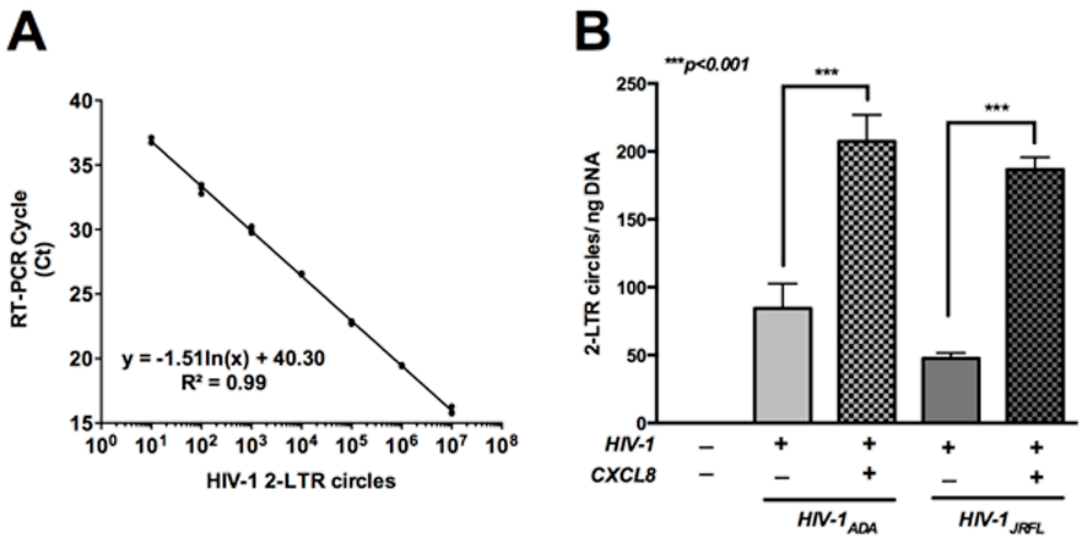


Figure 3.4: CXCL8 increases 2-LTR circle formation in HIV-1-infected MDM. Human MDM were plated in 6-well plates and infected with HIV-1_{ADA} or HIV-1_{JRFL} (10 ng/ml *p24*) overnight. Following day, MDM were treated with/without CXCL8 (50 ng/ml). DNA was extracted 1 week post-infection and 2-LTR circle junctions were amplified by real-time PCR. **(A)** Standard curve generated using pTA2LTR/CCR5 plasmid with 2-LTR copy numbers at X-axis and real-time PCR threshold counts at Y-axis. **(B)** Comparison of 2-LTR circle copy numbers in HIV-1_{ADA} or HIV-1_{JRFL} infected MDM. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

Figure 3.5

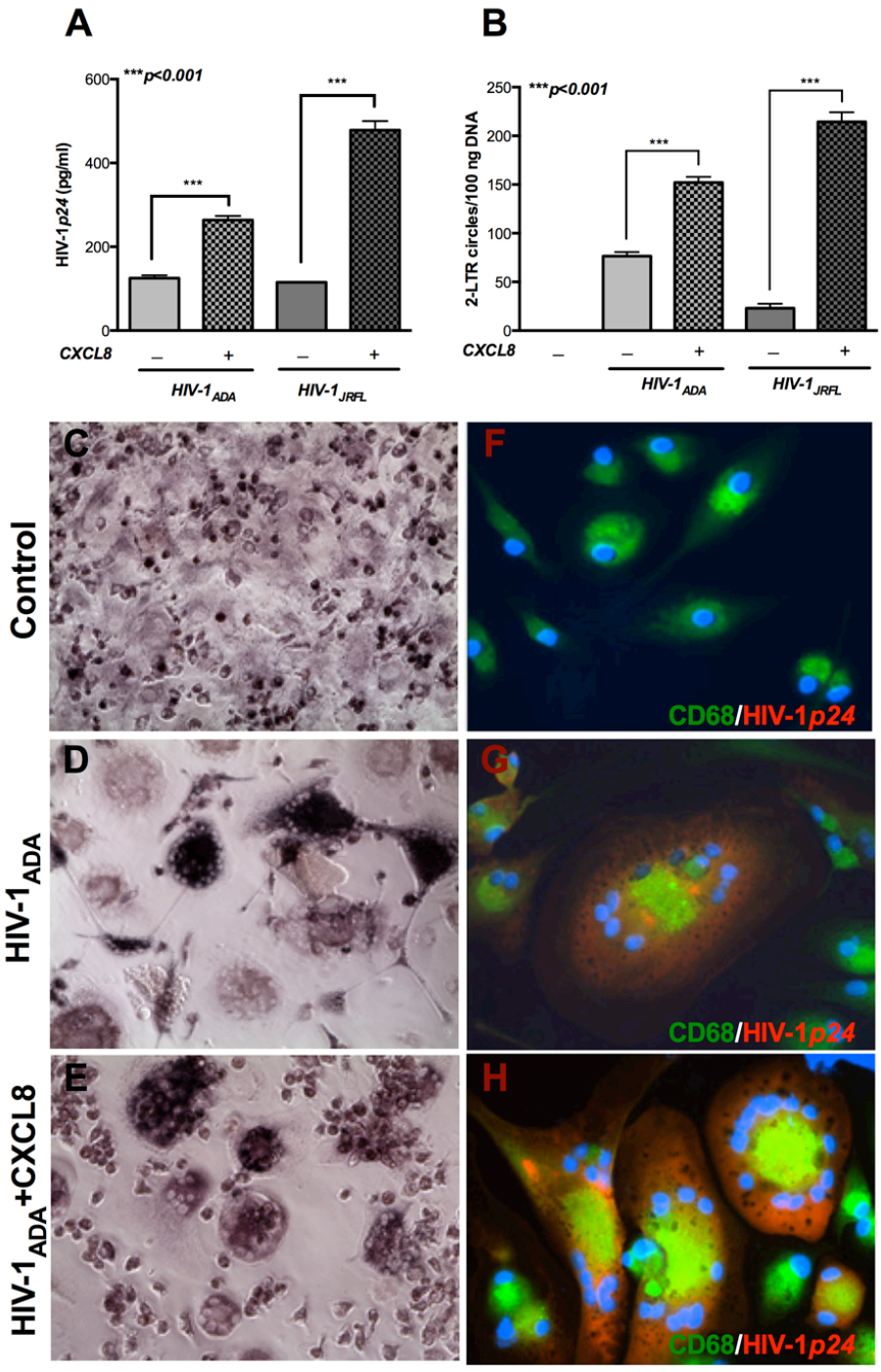

A
B
***p<0.001
***
HIV-1p24 (pg/ml)
600
400
200
0
CXCL8
–
+
HIV-1ADA
HIV-1JRFL
2-LTR circles/100 ng DNA
250
150
100
50
Control
HIV-1ADA
HIV-1ADA+CXCL8
C
D
E
F
G
H
CD68/HIV-1p24

Figure: 3.5 CXCL8 increases HIV-1*p24* and 2-LTR circles in HIV-1 infected primary human microglia. Primary human microglia were infected with HIV-1_{ADA} or HIV-1_{JRFL} (10 ng/ml *p24*) overnight, followed by treatment with 50 ng/ml CXCL8. **(A)** Supernatants were collected 2 weeks post-infection and HIV-1*p24* levels were measured by ELISA. **(B)** DNA was isolated and 2-LTR circle junctions were amplified by real-time PCR . **(C-E)** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to microglia and incubated for 15 minutes at 37°C. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons. Phase contrast pictures showing **(C)** cell morphology of uninfected primary human microglia. **(D)** Multinucleated giant cells formed with HIV-1_{ADA} infection **(E)** HIV-1_{ADA} infection and CXCL8 treatment**. (F-H)** Expression of CD68 (green) and HIV-1*p24* (red) was measured by immunocytochemistry 2 weeks post-infection with HIV-1_{ADA}. Nuclei were stained in blue by DAPI. **(F)** Uninfected primary human microglia **(G)** co-localization (yellow) of CD68 and HIV-1*p24* in HIV-1_{ADA}-infected microglia. **(H)** HIV-1*p24* expression and multinucleated giant cells in HIV-1_{ADA}-infected microglia. Original magnification 200X.

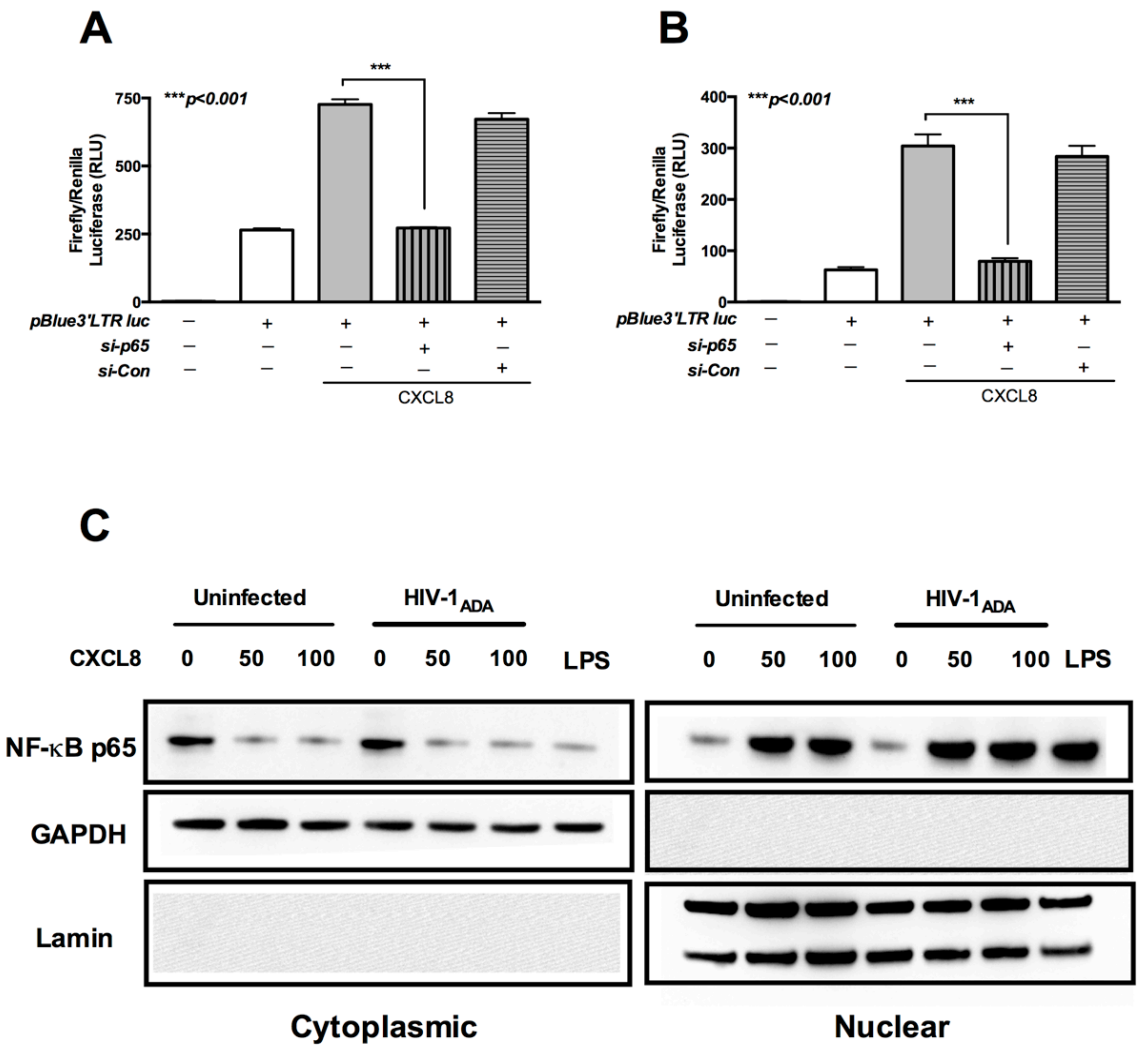


Figure 3.6: p65 subunit of NF-κB is essential for HIV-LTR promoter activity and CXCL8 translocates NF-κBp65 to the nucleus. Human MDM were transfected with pBlue3'LTR luc and siRNA for NF-κB p65. Cells were plated in 48-well plates overnight. Following day, cells were washed and treated with 50 ng/ml CXCL8. Cell lysates were collected 24 h post-treatment and relative luciferase activity was determined in **(A)** U937 cells and **(B)** Human MDM. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons. **(C)** HIV-1_{ADA}-infected and uninfected MDM were stimulated with

indicated concentrations of CXCL8 and protein lysates were collected for cytoplasmic and nuclear components 3 h post-simulation. NF-κB p65 was detected by western blotting. GAPDH and lamin were used as loading control for cytoplasmic and nuclear protein, respectively. Data are representative of two independent donors.

Figure 3.7

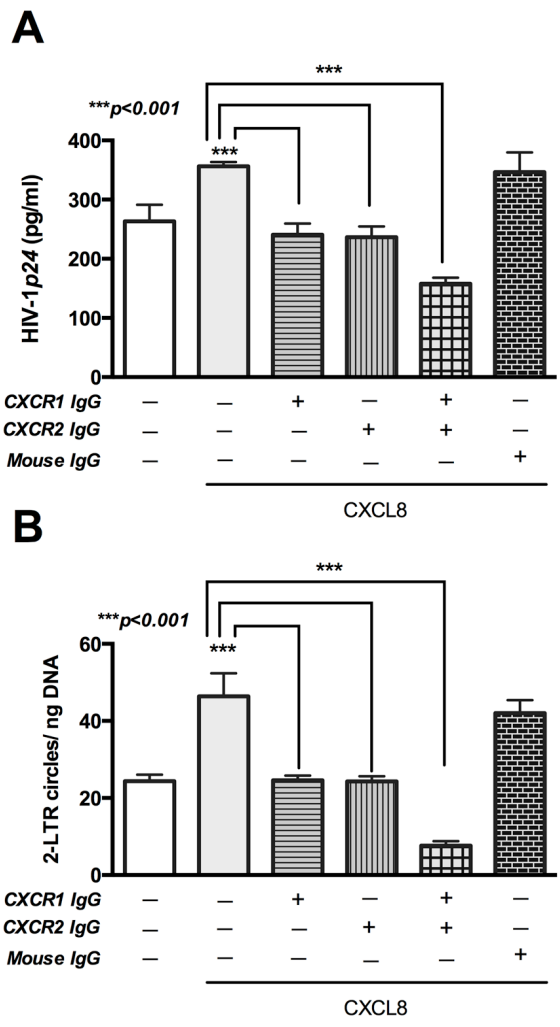

A
***
***p<0.001
***
HIV-1p24 (pg/ml)
400
300
200
100
0
CXCR1 IgG – – + – + –
CXCR2 IgG – – – + + –
Mouse IgG – – – – – +
CXCL8
B
***
***p<0.001
***
2-LTR circles/ ng DNA
60
40
20
0
CXCR1 IgG – – + – + –
CXCR2 IgG – – – + + –
Mouse IgG – – – – – +
CXCL8

Figure 3.7: Neutralization of CXCR1 and CXCR2 reduces CXCL8-induced HIV-1*p24* levels and 2-LTR circle formation in HIV-1-infected MDM. Human MDM were infected with HIV-1_{ADA} (10 ng/ml *p24*) overnight. Following day, cells were washed and supplemented with macrophage media with/without anti-CXCR1 (20 µg/ml), anti-CXCR2 (10 µg/ml) or both and 50 ng/ml CXCL8. Mouse IgG (25 µg/ml) was used as a control. **(A)** Cell culture supernatants were collected 1 week post-infection and HIV-1*p24* levels were determined by ELISA. **(B)** DNA was extracted 1 week post-infection and 2-LTR circle junctions were amplified by real-time PCR. Results are expressed as mean ± SEM, analyzed by one-way ANOVA and Newman-Keuls post-test for multiple comparisons.

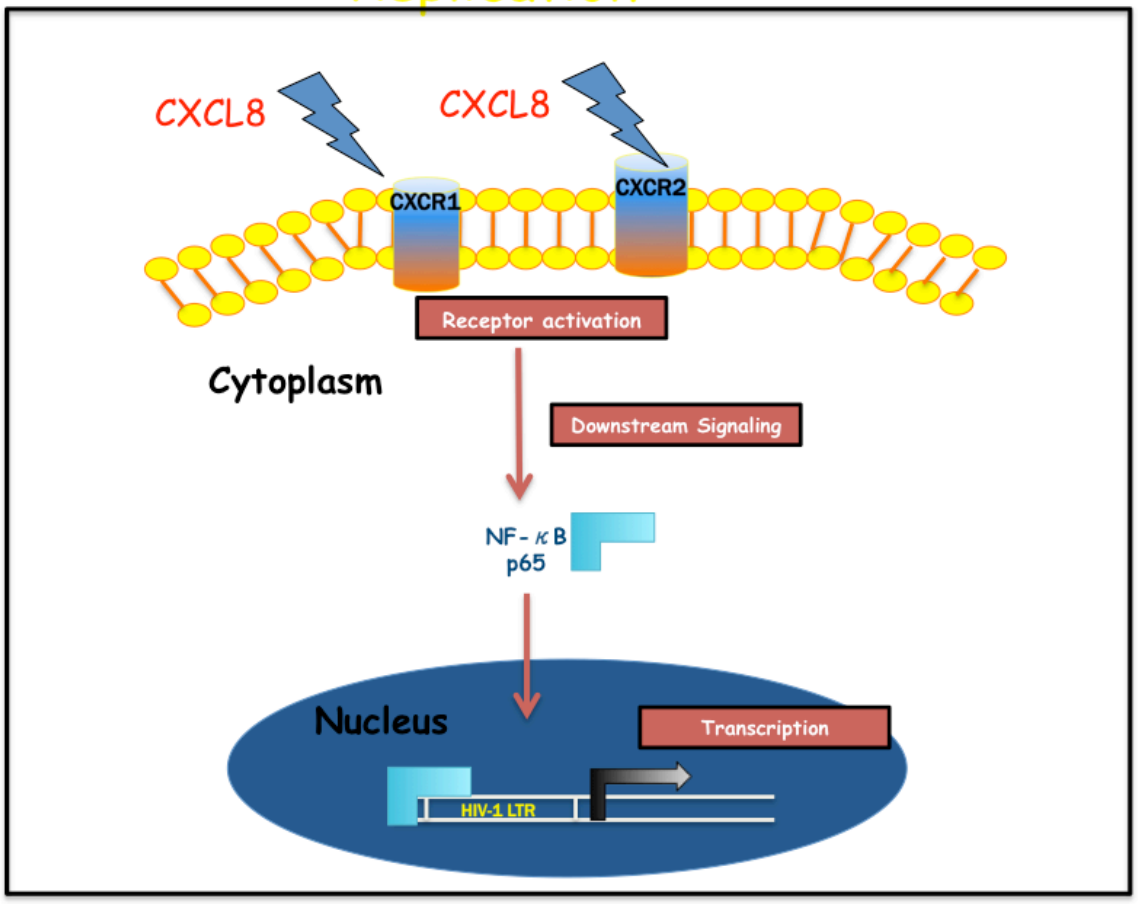


Figure 3.8: Proposed mechanism of CXCL8-induced HIV-1 replication. The schematic shows a mononuclear phagocyte. CXCL8 activates the receptors CXCR1 and CXCR2, which in turn activate downstream signaling events leading to the activation of transcription factor NF-κB. NF-κB translocates into the nucleus and binds to the HIV-1 LTR promoter. This leads to transcription of viral genome, eventually promoting HIV-1 replication in macrophages/microglia.

CHAPTER 4

GENERAL DISCUSSION

The pathogenesis of various neurodegenerative disorders is linked to an imbalance in the cytokine/chemokine profile (171). Chemokines and their receptors are particularly important in the trafficking of leukocytes, thus eliciting immune responses in the CNS. Chemokine CXCL8 has emerged as a potential candidate in the context of neuroinflammation during HIV-1 infection. It is important to understand the sources and signaling of chemokine production in the CNS during HIV-1 infection in order to control chemokine expression, eventually leading to the control of neuroinflammation. This study advances our knowledge about the regulation of CXCL8 production in human astrocytes after activation with proinflammatory stimuli and the effect of elevated CXCL8 on HIV-1 replication in microglial cells. Astrocytes are major cellular source of CXCL8 and we show a robust increase in CXCL8 in astrocytes activated with HIV-1-relevant stimuli like IL-1β and TNF-α.

Since the levels of chemokine CXCL8 are upregulated in serum, plasma and CSF of HIV-1 infected individuals (24, 120, 232); it strongly implicates CXCL8 involvement in neuroinflammation during HIV-1 infection. Consistent with prior studies, we reported upregulation of CXCL8 RNA and protein levels in brain lysates of HIV-1 infected patients, as shown in Chapter 2. Next, we were interested in understanding how CXCL8 secretion is regulated in astrocytes. We utilized astrocytes as a cellular system for regulation mechanisms of CXCL8 as

astrocytes are the predominant cellular source of CXCL8 in CNS (50). Our group reported robust increase in CXCL8 RNA and protein levels by activated astrocytes, as shown in Chapter 2. We chose CXCL8 as a candidate in neuroinflammatory networks during HIV-1 infection, based on our gene microarray analysis (12). Astrocytes were activated with IL-1β as a proinflammatory stimulus since it is one of the first responses generated by activated microglia and macrophages in the event of CNS HIV-1 infection. CXCL8 showed 14 to 22 fold increase in microarray analysis performed on activated astrocytes. Activation of astrocytes by IL-1β in the brain microenvironment contributes to neurodegeneration (100). However, the molecular mechanisms underlying neurodegeneration in HIV-1-infected brain are largely unknown. Thus, we analyzed CXCL8 production and regulation in IL-1β-activated astrocytes. We investigated potential signaling pathways underlying CXCL8 release in human astrocytes.

The results of these studies showed significant upregulation of CXCL8 following treatment with IL-1β as well as another proinflammatory stimulus TNF-α, as previously reported (3). Also, the increase was robust at both RNA and protein levels, as measured by RT-PCR and ELISA respectively. The CXCL8 promoter has binding sites for transcription factors like NF-κB. Cytokines like IL-1β and TNF-α activate signaling cascades converging to NF-κB (141). This explains the observed upregulation of CXCL8 in astrocytes activated by IL-1β and TNF-α. Similar results have been reported in other cellular systems like epithelial cells, cancer cells and macrophages (99, 232). Next, we evaluated

signal transduction pathways communicating between extracellular environment of astrocytes and inside of the cell. MAPK is an important signaling pathway involved in CXCL8 regulation in variety of cell types like hepatocytes and macrophages (11, 178). We observed downregulation of IL-1β-induced CXCL8 production when astrocytes were treated with inhibitors for p38 and ERK pathway. We also looked at SHP2 involvement in CXCL8 signaling mechanisms in astrocytes. SHP2 is a ubiquitously expressed non-receptor protein tyrosine phosphatase, implicated in events downstream of cytokine receptors (169). The rationale for looking at SHP2 and MAPK in astrocytes is based on reports by various groups indicating regulation of MAPK by SHP2 (27, 202). Our data supports crosstalk between MAPK and SHP2 signaling molecules as reported in Chapter 2 (136).

In addition to treatment with specific inhibitors, we employed overexpression constructs for SHP2 and p38 in order to delineate the order of signaling. SHP2 is known to activate ERK in human fibroblasts, and there are reports indicating it acts either downstream or parallel of MAPK pathway (186). Astrocytes can be efficiently transfected and therefore we utilized this property to study the order of SHP2 and MAPK signaling in astrocytes. Overexpression of SHP2WT and p38 showed significant upregulation of CXCL8 in human astrocytes, as shown in Figure 2.4. Also, we show that SHP2 acts upstream of MAPK, which is consistent with reports in other cell systems (91, 192). Inhibition of SHP2 had no effect on p38-stimulated CXCL8 upregulation; however, inhibition of p38 blocked SHP2-induced CXCL8 expression in astrocytes.

Therefore, we reported for the first time involvement of SHP2 in the modulation of CXCL8 expression in human astrocytes *via* MAPK pathway. It has been shown that p38 acts upstream to SHP2 in the regulation of CXCL8 in hepatocytes (11), therefore it is important to note that signaling mechanisms are a function of distinct cellular systems.

Overall, SHP2 and MAPK signaling pathways are critical for activation of astrocytes leading to elevated CXCL8 production. SHP2 is an important signal transducer upstream of p38 and ERK directing upregulation of CXCL8 in activated astrocytes. Thus, our findings of CXCL8 regulation in astrocytes may prove useful in therapeutic strategies to target controlled CXCL8 production *in vivo*. However, limitation of such strategies may be in the fact that MAPK is an important signaling cascade involved in many cellular functions for growth and survival.

Astrocytes are the predominant cells of the CNS, however, microglia; the residual macrophage in the CNS, is a prime target for HIV-1 infection and the virus predominantly resides in microglia (230). Given the fact that macrophages/microglia express receptors for CXCL8 (40, 117), we next investigated the effect of CXCL8 on HIV-1 replication in macrophages/microglia. The specific mechanisms linking astrocyte activation and microglial infection, eventually leading to neuron damage, remain unresolved. We proposed that CXCL8 is a link between astrocyte responses and microglial viral infectivity. Since, isolation and cultivation of primary human microglia is challenging and a limiting step, we employed human MDM as a model system for microglial

activation and confirmed critical observations using primary human microglia. However, macrophages are professional phagocytes and harder to transfect, therefore promonocytic U937 cells were employed for better transfection efficiency and cell viability post-transfection.

Various readouts can be evaluated to study viral replication ranging from viral protein secretion to HIV-1 LTR activation. Macrophage tropic isolates HIV-1_{ADA} and HIV-1_{JRFL} were used as blood and brain derived isolates, respectively. The two strains were selected based on previously reported significant infection heterogeneity among macrophage-tropic isolates (73, 196). We compared viral protein HIV-1*p24* production in HIV-1_{ADA} or HIV-1_{JRFL}-infected MDM. The results indicated that treatment with CXCL8 increased HIV-1 *p24* production by MDM and that the effect was dose dependent. Thus, as reported earlier in MDM and T-lymphocytes (120), CXCL8 enhanced viral replication in MDM. Interestingly, similar results were obtained when HIV-1_{ADA} and HIV-1_{JRFL} infection was extended to cultured primary human microglia. An important observation was that HIV-1_{JRFL} exhibited higher infectivity than HIV-1_{ADA} in microglia as compared to MDM, as indicated by higher HIV-1*p24* and more 2-LTR circle numbers. One possible explanation is that HIV-1_{JRFL} being a brain derived viral isolate may exhibit preferential infectivity towards microglia, since microglia are residents of brain. These data illustrate that although initial infectivity by HIV-1_{ADA} and HIV-1_{JRFL} may differ in macrophages and microglia, CXCL8 increases HIV-1 replication in both MDM and microglia.

In order to study HIV-1 LTR promoter activity, luciferase promoter reporter construct was employed and efficiently transfected into U937 cells. Additionally, we successfully transfected MDM using Amaxa nucleofection developed recently to bypass transfection restriction in MDM. The HIV-1 LTR promoter contains DNA elements, which control viral gene expression and production of viral proteins in the brain. CXCL8 increased HIV-1 LTR promoter activity in both U937 cells and MDM. Viral proteins like Tat and gp120 play an important role in HIV-1 associated neurotoxicity (43, 104). This would explain how CXCL8 may potentiate neurotoxicity indirectly *via* activation of HIV-1 LTR promoter, thus increasing viral protein production. HIV-1-infected macrophages/microglia themselves are a significant source of CXCL8; however, not as robust as activated astrocytes. Therefore, the observed increase in HIV-1*p24* levels may in part be contributed by endogenous CXCL8 secreted by macrophages during the course of HIV-1 infection. To address this issue, we neutralized CXCL8 in culture supernatants of HIV-1-infected macrophages and U937 cells and found decreased HIV-1*p24* levels and HIV-1 LTR promoter activity, respectively. This observation was consistent with previous report in macrophages and T-lymphocytes (120).

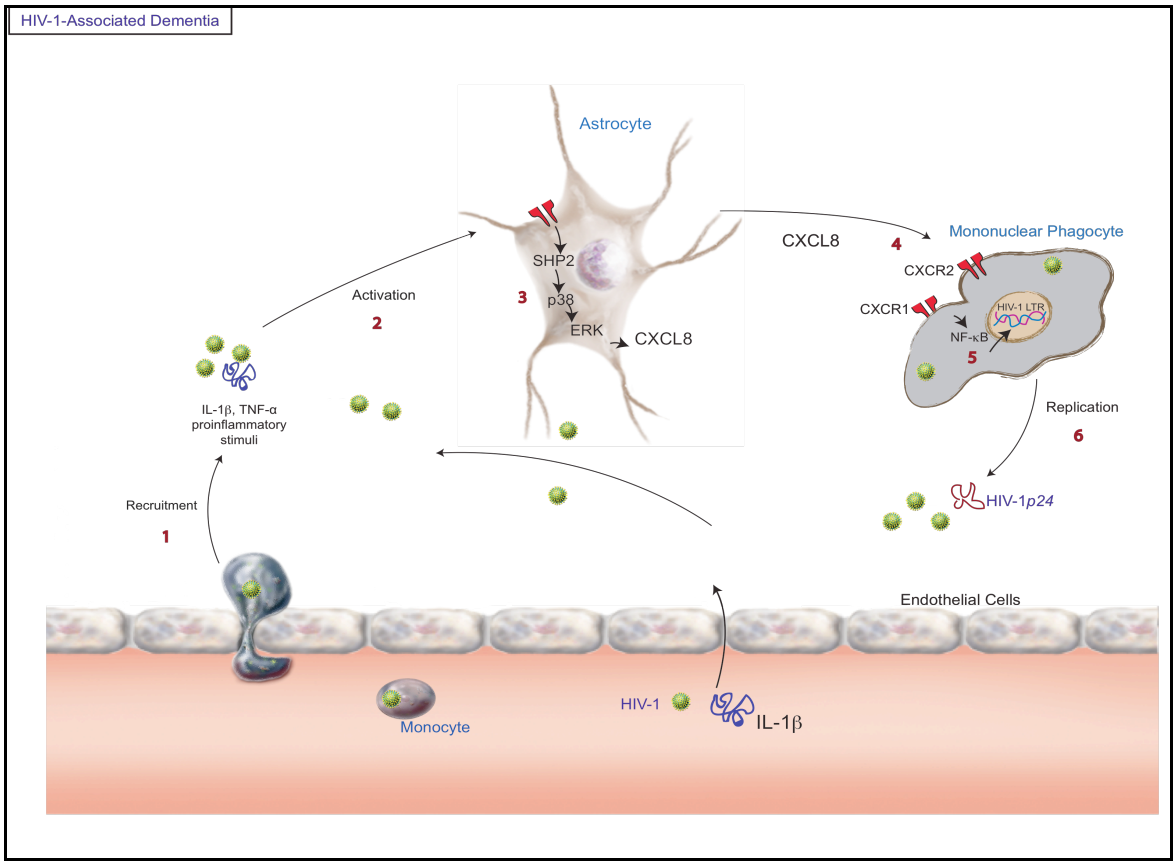
CXCL8 exerts its biological effects by binding to two GPCRs, CXCR1 and CXCR2. Although significant progress has been made towards understanding of CXCR1/2 signaling in cancer cells, very little is known about the signaling cascades in macrophages/microglia. CXCR1/2 activation leads to activation of downstream signaling molecules like Akt, protein kinase C (PKC) and/or MAPK pathways. These signaling pathways, in turn, regulate various transcription

factors like STAT3, AP-1 and NF-κB by promoting their nuclear translocation (215). Consistently in our report, NF-κB nuclear translocation was increased by CXCL8 treatment in macrophages. What effect could this have on HIV-1 replication? Following infection and integration into the host chromosome, the HIV-1 gene regulation under the HIV-1 LTR promoter is dependent on host cellular factors including NF-κB (14, 111, 122). NF-κB has been shown to regulate viral transcription *via* the two NF-κB sites in the HIV-1 LTR promoter (154). Our results also show that NF-κB is essential for CXCL8-induced HIV-1 LTR promoter activity, which further supports the general mechanism that CXCR1/2 signal through NF-κB. However, a detailed understanding of how NF-κB activation is regulated upstream is important to decipher CXCR1/2 signaling in macrophages. These data suggest that CXCR1/2 can be potential targets for CXCL8 inhibition *in vivo*. Recent report highlights successful use of decoy proteins, in human clinical trials, similar in structure to CXCL8 to block CXCR1/2 without activation (58). Furthermore, it will be important for these preclinical studies to determine the potential side effects and toxicities of blocking these chemokine receptors, since CXCR1 and CXCR2 signaling is critical in modulating neutrophil function.

In conclusion, consistent with previous reports, we report here that CXCL8 is present in abundance in brain lysates of HIV-1 infected individuals. The chemokine CXCL8 enhances HIV-1 replication in macrophages and microglia, accounting for itself as a candidate in progressive neurodegeneration associated with HIV-1 infection (summarized in Scheme 4.1). Several studies relate elevated

CXCL8 levels with neurodegeneration (67, 221); however, the molecular mechanisms are incompletely understood. To develop CXCL8 based therapeutic strategies it is important to delineate the regulatory mechanisms and neuroinflammatory networks mediated by CXCL8. This report has a high level of significance and advancement in glial biology and neuroinflammation. The intercellular neuroinflammatory networks resulting from CXCL8 production in the CNS are highly complex and intriguing.

Although significant progress has been made in application of CXCL8 based therapy in humans for several inflammatory disorders, this study yields important information with both basic and clinical relevance in the field of HIV-1 associated neuroinflammation.



Scheme 4.1: Overall Summary.

1. HIV-1 infected cells enter the CNS and release proinflammatory stimuli including IL-1β and TNF-α.

2. Astrocytes are activated in response to proinflammatory stimuli.

3. Activated astrocytes produce CXCL8 by SHP2 and MAPK dependent mechanisms.

4. CXCL8 binds to receptors CXCR1 and CXCR2 on HIV-1 infected mononuclear phagocytes.

5. Transcription factor NF-KB translocates into nucleus and increases HIV-1 LTR promoter activity.

6. Transcription of viral genome leads to increased HIV-1 replication.

FUTURE DIRECTIONS

The first and foremost application of the present study is advancement in the field of CXCL8 as a therapeutic target in HIV-1 infection. Since we report CXCL8 as an important trigger for enhanced viral replication, it is logical to investigate the effects of downregulation of CXCL8 in the periphery. Although we present mechanism for CXCL8 regulation in astrocytes, it may not be feasible to block those signaling cascades, controlling important cellular functions, to attain CXCL8 downregulation. Therefore, it will be interesting to develop modes of indirect modulation of CXCL8 activity. Also, it is noteworthy that CXCL8 is produced by macrophages and microglia when infected with HIV-1. Therefore it will be worthwhile to determine the signaling mechanisms of CXCL8 production in macrophages/microglia.

Our study identified NF-κB as an important transcription factor involved in downstream signaling mediated by CXCL8 *via* receptors CXCR1 and CXCR2. However, the effector molecules upstream of NF-κB need to be determined to elucidate complete cellular mechanism of CXCL8 signaling in macrophages. Intracellular signaling pathways involving PKC, PI3K, ERK1/2 and p38 MAPK are shown to be activated upon CXCR1/2 binding by CXCL8 (108, 203). This understanding will aid in developing better therapeutic strategies to combat the inflammatory effects of CXCL8.

The receptors CXCR1 and CXCR2 have been defined on astrocytes, microglia as well as neurons. Reports from other groups and our lab show

CXCL8 to be neuroprotective (7, 214), though the direct effect of CXCL8 on neurons is unclear and the phenomenon needs to be unraveled. Considering this, it will be of equal importance to study the neuronal cell survival/apoptosis by CXCL8. Since we show that CXCL8 is a key regulator for intercellular interactions in activated astrocytes and microglia, it will be logical to investigate mechanisms of CXCL8-induced microglial recruitment and activation. These studies will further envisage the intercellular interactions mediated by CXCL8 in HIV-1 infection of CNS.

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