THE ROLE OF ADAPTIVE STRESS RESPONSES IN HIV REPLICATION AND MACROPHAGE-MEDIATED NEUROTOXICITY

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Dedication

For my mom, Charlie.

Acknowledgments

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ABSTRACT

THE ROLE OF ADAPTIVE STRESS RESPONSES IN HIV REPLICATION AND MACROPHAGE-MEDIATED NEUROTOXICITY

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Despite antiretroviral therapy (ART), HIV infection promotes cognitive dysfunction and neurodegeneration through persistent inflammation and neurotoxin release from infected and/or activated macrophages. Inflammation and immune activation within both the central nervous system (CNS) and periphery correlate with disease progression and morbidity in ART-treated individuals. Accordingly, drugs targeting these pathological processes are needed for effective, adjunctive therapy. Using our *in vitro* model of HIV-mediated neurotoxicity, in which HIV infected monocyte-derived macrophages (HIV/MDM) release excitatory neurotoxins, we demonstrate that HIV infection dysregulates adaptive stress responses, including the antioxidant response and the unfolded protein response (UPR). HIV infected macrophages have dramatic reductions in heme oxygenase-1 (HO-1) levels. Activation of the antioxidant response attenuates HIV replication and restoration of HO-1 expression, specifically, reduces neurotoxin release from HIV/MDM, even with robust HIV replication. We propose that dysregulation of the antioxidant response during HIV infection drives macrophage-mediated neurotoxicity and that pharmacological inducers of the antioxidant response could serve as adjunctive neuroprotectants and HIV disease modifiers in ART-treated individuals. Additionally, we found that HIV infection activates the UPR in macrophages, increasing phosphorylated eIF2α in our *in vitro* system and macrophagic BiP in HAND frontal cortex. Pharmacological induction of the UPR, which attenuates viral replication, enhances macrophage-mediated neurotoxicity. Therefore, processes that induce the UPR in macrophages may enhance neurotoxin production and contribute to pathological processes

underlying HAND. Understanding how HIV infection affects adaptive stress responses and neurotoxin production pathways in the macrophage will improve our ability to develop effective adjunctive therapies for the neurological consequences of HIV infection.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ARE	antioxidant response element
ART	antiretroviral therapy
BBB	blood-brain barrier
CCL2	chemokine (C-C motif) ligand 2
CLT	clotrimazole
CoPP	cobalt (III) protoporphyrin IX chloride
DMF	dimethyl fumarate
DPI	day post infection
eIF	eukaryotic initiation factor
EFZ	efavirenz
ER	endoplasmic reticulum
GPX1	glutathione peroxidase 1
HAND	HIV-associated neurocognitive disorders
HO-1	Heme oxygenase-1
HPI	hour post inefection
IL	interleukin
IFN	interferon
MAP2	microtubule-associated protein 2
MDM	monocyte-derived macrophages
MMF	monomethyl fumarate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PBMC	peripheral blood mononuclear cell
ROS	reactive oxygen species
RT	reverse transcriptase
SnMP	tin (IV) mesoporphyrin IX dichloride
tBHQ	*tert*-butylhyrdoquinone
TNFα	tumor necrosis factor-alpha
UPR	unfolded protein response

CHAPTER 1

INTRODUCTION

At the end of 2009, an estimated 30.8 million adults and 2.5 million children were living with HIV globally (WHO, 2010). Current antiretroviral therapy (ART) regimens for HIV infection have greatly improved virological control and clinical outcomes in infected individuals. However, despite the efficacy of ART, the prevalence of HIV-associated neurocognitive disorders (HAND) has persisted, with prevalence and associated morbidity estimated at up to 50% (McArthur et al., 2003; Robertson et al., 2007; Sacktor et al., 2002). While ART will remain the mainstay of HIV therapy, there is a need for adjunctive neuroprotective therapies that address the pathological processes persisting in ART-treated individuals.

HIV-1 infection of the central nervous system (CNS) can result in cognitive, motor, and behavioral abnormalities, collectively known as HIV-associated neurocognitive disorders (HAND) (Antinori et al., 2007; McArthur et al., 2010). Pathological processes in both the brain and periphery, involving multiple cell types, contribute to the neurological complications of AIDS. Early in the course of infection, HIV traffics into the brain via infected monocytes and lymphocytes (Dunfee et al., 2006). Despite ART, HIV persists in the CNS in parenchymal microglia and perivascular macrophage reservoirs (Ho et al., 1985; Koenig et al., 1986; Petito et al., 1986). And because HIV cannot infect neurons, HIV-induced neuronal damage is mediated indirectly by the release of neurotoxins from infected and/or activated macrophages, microglia and astrocytes. *In vitro* and *in vivo* studies have identified viral proteins, pro-inflammatory cytokines, interferons, excitatory amino acids, phospholipids, and reactive oxygen species as neurotoxic factors produced by macrophages/microglia following HIV infection (Boven et al., 1999; Brenneman et al., 1988; Brew et al., 1995; Gelbard et al., 1993; Gelbard et al., 1994; Gendelman et al., 1998; Genis et al., 1992; Jiang et al., 2001; Maragos et al., 2003; Scorziello et al., 1998; Song et al., 2003; Wesselingh et al., 1993). Furthermore, the severity of pre-mortem HAND correlates with increased numbers of microglia and macrophages in the CNS, supporting the hypothesis that these cell types are principal mediators of HIV-induced neurological impairment (Anthony et al., 2005; Glass et al., 1995; Petito et al., 1986).

The persistence of HAND in individuals effectively controlled for systemic viral replication is incompletely explained, although recent evidence suggests that prolonged inflammation in both the CNS and periphery may be responsible (Ancuta et al., 2008; Brenchley et al., 2006b; Eden et al., 2007). Multiple pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 are elevated in the CNS and/or CSF of patients with HAND (Achim et al., 1993; Foli et al., 1997; Oster et al., 1987; Perrella et al., 1992). The pro-inflammatory environment within the CNS is a result of cytokine release from immune activated astrocytes (Kramer-Hammerle et al., 2005; Sabri et al., 2003) and monocytes/macrophages stimulated by direct viral infection, shed viral proteins or proinflammatory mediators (Rappaport et al., 1999; Sundar et al., 1991). Inflammation can alter the permeability of the blood-brain barrier, enhance entry of infected monocytes into the CNS, and drive peripheral processes that contribute to the neurological complications of HIV. Accordingly, drugs targeting inflammatory-mediated processes in the CNS and systemic compartments are needed for effective, adjunctive therapy in HAND.

Numerous physiological and pathological stimuli can initiate and/or propagate the inflammatory response. Of the many relevant pathways, this work focuses on the interactions between oxidative stress, endoplasmic reticulum (ER) stress and inflammation. The ER, as a protein folding compartment and dynamic calcium store, is primed to sense cellular stress and initiate adaptive stress responses, including the unfolded protein response (UPR). Intracellular calcium signals and free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO), act as messengers in coordinating several of the adaptive stress responses. Calcium release from the ER, during states of stress, can induce the generation and release of ROS from the mitochondria. This in turn activates the antioxidant response, inflammatory pathways and with sustained activation, cellular apoptosis. Furthermore, ROS can feedback onto the ER and target calcium channels and resident-ER chaperones to exacerbate ER calcium release and ER stress. As a consequence, misfolded proteins accumulate in the ER, induce the UPR, and activate signaling cascades to promote the inflammatory response, antioxidant response, apoptosis and other

cellular adaptive stress responses (Ron and Walter, 2007; Zhang and Kaufman, 2008). The effect of HIV infection on the antioxidant response, the UPR and the interactions between these adaptive stress responses and inflammation has not yet been explored.

The antioxidant response maintains redox balance and counteracts oxidative damage by inducing the transcriptional upregulation of proteins that are involved in detoxification of reactive oxygen species (ROS). These genes have a common promoter element, the antioxidant response element (ARE), and are regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) (Figure 1.1). Following exposure to ROS or electrophiles, Nrf2 translocates to the nucleus to drive expression of numerous genes, including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione peroxidase 1 (GPX1), and genes responsible for the synthesis of glutathione, the principal antioxidant produced by the cell. Recent studies have demonstrated that viral infection can affect the cellular oxidation state and induce the activation of the antioxidant response (Chen et al., 2011; Schaedler et al., 2010). HIV infected patients have evidence of increased ROS production and depressed levels of glutathione (Dworkin et al., 1986), suggesting that the antioxidant response is altered following HIV infection.

We hypothesized that HIV infection of macrophages would result in suppression of the antioxidant response and that induction of the antioxidant response would attenuate HIV replication. Furthermore, restoration of the cellular redox state in HIV-infected macrophages could dampen inflammatory processes if they are driven by virus-induced generation of excessive ROS. The studies presented in this body of work begin to characterize the consequences of HIV infection on the antioxidant response and the link between the dysregulation of the antioxidant response and macrophage-mediated neurotoxicity. Most importantly, we demonstrate that HIV infection dramatically reduces levels of HO-1 in macrophages and that restoration of HO-1 decreases macrophage mediated neurotoxicity. We also show that induction of the antioxidant response attenuates NF-κB and TNFα signaling, a major mediator of inflammation and immune

activation. Our results provide evidence that therapeutics that ameliorate the dysregulation of the antioxidant response following HIV-infection should be considered as adjunctives to ART for the treatment and prevention of HAND.

We also examined the role of HIV-infection of macrophages on activation of the UPR and the interaction between activation of the UPR and the antioxidant response. Virus replication and assembly can strain the capacity of the endoplasmic reticulum (ER) and result in the accumulation of misfolded proteins and induction of the UPR. The UPR is designed to eliminate misfolded proteins and promote cellular recovery by attenuating translation and upregulating the expression of chaperones, degradation factors, and regulators of metabolic and redox states. Activation of the signaling pathways comprising the UPR can also induce the transcription of inflammatory genes via activation of NF-κB pathways, among others.

The UPR is a quality control mechanism that can be activated during physiological stress (e.g., glucose deprivation, oxidative stress) and viral infection (Figure 1.1). The UPR attempts to eliminate excessive misfolded proteins in the ER through two mechanisms; 1) attenuation of protein translation, in order to reduce the flux of proteins entering into the ER and 2) induction of chaperone proteins and degradation factors to refold and/or eliminate misfolded proteins. Three proteins have been identified as sensors of ER stress: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). Under normal conditions, the ER chaperone immunoglobulin heavy-chain-binding protein (BiP) is bound to the sensor domain of PERK, ATF6 and IRE1 in the ER lumen. When misfolded proteins accumulate in the ER, BiP is sequestered away from these sensors to bind to misfolded proteins, resulting in activation of PERK, ATF6 and IRE1 (Bertolotti et al., 2000). Activation of PERK initiates a signaling cascade that results in the inhibition of new protein translation, mediated by phosphorylated eIF2α, and the induction of genes important for cellular recovery from ER stress, as mediated by ATF4 transcriptional activity. ATF6 activates genes carrying ER stress response

elements (ERSEs) in their transcriptional promoters (Mori, 2000). ERSEs are found in genes encoding for cellular chaperones including BiP, protein-disulfide isomerase (PDI) and calreticulin (Harding et al., 2003). Phosphorylation of IRE1 results in the alternative splicing of X box binding protein 1 (XBP-1) mRNA, which encodes an active transcription factor capable of inducing genes regulated by the ERSE (Lee et al., 2003). In addition, sXBP-1 can also activate the transcription of genes containing UPR elements (UPRE), thereby enhancing the capacity of ER-stressed cells to degrade irrevocably misfolded proteins via ER-associated protein degradation (ERAD) (Hosokawa et al., 2001; Yoshida et al., 2003). Chronic activation of the UPR, which occurs when the cell cannot manage or recover from ER stress, results in the activation of signaling pathways that will commit the cell to apoptosis (Ferri and Kroemer, 2001; Oyadomari et al., 2002a).

In some cases, viral infection induces ER stress and activates components of the three branches of the UPR. Enveloped viruses utilize the ER as the primary site of envelope glycoprotein biogenesis and several viruses undergo genomic replication and particle assembly in the ER compartment. Increasing the burden on the ER, especially at times of high viral production, could increase the accumulation of misfolded proteins in the ER lumen and activate the UPR. However, while activation of the UPR is essential for host cell survival during viral infection, some of the consequences of UPR activation, such as inhibition of protein translation and enhanced ERAD, would be detrimental to viral replication. Consequently, many viruses that induce the UPR have evolved mechanisms to regulate pathways of the UPR in order to promote efficient viral replication. While UPR activation has been reported following cellular infection by several different viruses, the effect of HIV infection on activation of the UPR and the role of the UPR in modulating HIV replication has not yet been investigated.mon

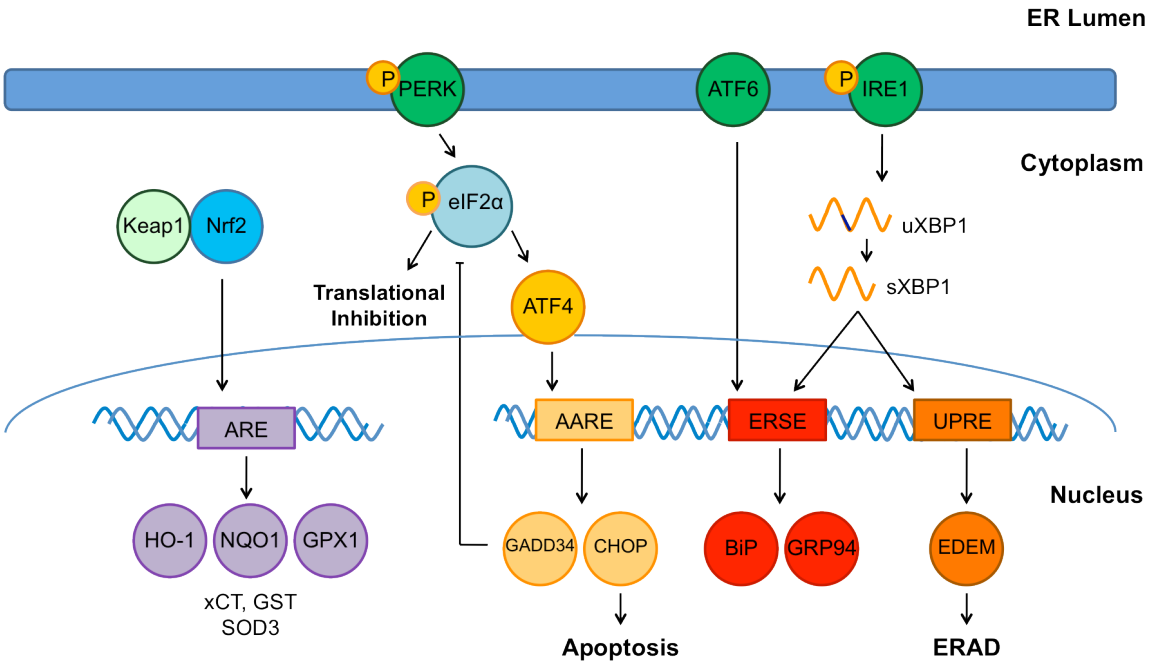

Figure 1.1 The signaling pathways comprising the antioxidant response and the Unfolded Protein Response (UPR). The antioxidant response maintains the redox state of the cell and counteracts oxidative damage through induction of proteins that are involved in detoxification of reactive oxygen species (ROS). The transcription of these genes is regulated by Nrf2-dependent transcription of the antioxidant response element (ARE). During states of low oxidative stress, Nrf2 is kept transcriptionally inactive by Keap1, which sequesters Nrf2 in the cytoplasmic compartment. Following exposure to ROS or electrophiles, Keap1 is degraded by the proteasome and Nrf2 translocates to the nucleus to drive expression of HO-1, NQO1, and glutathione peroxidase 1 (GPX1), among others. Accumulation of misfolded protein in the ER causes ER stress and results in the activation of the UPR, an adaptive stress pathway. PERK, IRE1 and ATF6 have sensor domains that monitor levels of misfolded protein in the ER lumen. Following activation, each of these pathways initiates a signaling cascade that results in the transcriptional upregulation of genes that will help the cell reduce, manage or recover from ER stress. Phosphorylation of eIF2α by activated PERK results in the attenuation of all cap-dependent protein translation, in order to reduce the incoming burden of new proteins into the ER. Increased levels of phosphorylated eIF2α also result in the translational upregulation of ATF4, a transcription factor that increases amino acid response element (AARE)-regulated genes in order to modulate cellular metabolism and redox state during cellular recovery from ER stress. ATF4 also increases levels of GADD34, which restores protein translation by mediating eIF2α dephosphorylation as part of a negative feedback loop. In addition to PERK, three other kinases (PKR, GCN2 and HRI) can phosphorylate eIF2α and initiate the UPR, although instead of misfolded protein levels, they respond to double stranded RNA, amino acid limitation and heme levels, respectively. ATF6, upon activation, translocates to the Golgi where is it cleaved into an active form, which also functions as a transcriptional regulator of ER stress-element (ERSE)-regulated genes. ERSEs regulate genes encoding for a variety of cellular chaperones, which promote the proper folding of misfolded proteins in the ER, including BiP and GRP94. Activation of IRE1 results in its auto-phosphorylation and the subsequent activation, by a splicing event, of XBP1 mRNA. Spliced XBP1 (sXBP1) encodes a transcription factor capable of upregulating genes with ERSEs or UPR elements (UPREs). Transcriptional upregulation of UPRE-regulated genes results in increased levels of EDEM, which enhances the degradation of misfolded proteins in the ER by ER-associated degradation (ERAD). If the UPR is unable to manage the stress to the ER, the cell will undergo CHOP-mediated apoptosis.

We hypothesized that HIV infection would induce the UPR during times of high viral replication, when viral protein translation, glycoprotein biogenesis and viral particle assembly would be highest. We found that HIV infection increases levels of phosphorylated eIF2α in our *in vitro* model system and that macrophagic BiP was increased in the macrophages of HAND frontal cortex. Interestingly, pharmacological induction of the UPR, which attenuates viral replication, is associated with increased macrophage-mediated neurotoxicity. This finding has important implications for the development of adjunctive therapies for HAND. Therapeutics or processes that induce the UPR in macrophages, regardless of the effect on HIV replication, could enhance neurotoxin production and contribute to the pathological processes underlying HAND.

HIV infection results in the alteration of several adaptive stress pathways in the macrophage. Suppression of the antioxidant response and induction of the UPR both associate with high levels of viral replication and neurotoxin production. And while pharmacological induction of either stress pathway attenuates HIV replication, only induction of the antioxidant response results in decreased macrophage-mediated neurotoxicity. Therefore, activation of the UPR by inflammation, oxidative stress or ART is predicted to enhance neurotoxin production in macrophages, regardless of the level of HIV replication. Understanding how HIV infection affects adaptive stress responses and neurotoxin production pathways in the macrophage will continue to improve our ability to develop effective adjunctive therapies for HAND.

CHAPTER 2

NEUROAIDS AS AN INFLAMMATORY DISORDER[1]

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Abstract

The pathological basis for the neurological complications of AIDS involves complex interactions between multiple cell types within both the brain and the periphery. This review focuses on the role for inflammation in the development of HIV-associated neurocognitive disorders (HAND). Specifically, evidence for inflammation in HAND pathogenesis in the pre-antiretroviral therapy (ART) and post-ART eras is discussed. The biology of HIV infection and subsequent CNS invasion is emphasized, with particular focus on chemokines and chemokine receptors. Chronic neuroinflammation has also been implicated in other infectious and non-infectious CNS disorders, of which multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) are reviewed. The status of current adjunctive therapies for HAND and their relationship to such neuroinflammatory disorders is also included. Understanding common pathways of neuroinflammation will lead to the identification of biomarkers for classification, diagnosis, and clinical prognosis, as well as to the development of novel treatment modalities for HAND and other neuroinflammatory disorders of the CNS.

Introduction

Advances in antiretroviral therapies for HIV infection have improved virological control and greatly increased the life expectancy of infected individuals. However, despite the availability of these potent antiviral drugs, the prevalence of HIV-associated neurocognitive disorders (HAND) has persisted and even increased (McArthur et al., 2003; Robertson et al., 2007; Sacktor et al., 2002), emphasizing the need for effective adjunctive neuroprotective therapies. Inflammation in the brain and in the periphery is a driving force in the neuropathogenesis of HAND, MS, HAM/TSP, and probably also AD and PD. As these conditions share common features in their pathogenic processes, identifying common mechanisms and developing targeted treatment modalities will not only benefit HAND patients, but likely also be effective in other neuroinflammatory disorders.

2.1 - Pathological Characteristics of NeuroAIDS

Clinical Characteristics of HIV-Associated Neurocognitive Disorders (HAND)

According to recent estimates, over 33 million people are currently living with HIV-1 worldwide (WHO, 2009). HIV-1 infection has devastating consequences for the immune system, resulting in immunodeficiency marked by profound CD4+ T-cell depletion. In addition, neurologic disorders involving the CNS and the peripheral nervous system (PNS) affect between 40–70% of HIV-positive individuals at some point during the course of infection (McArthur et al., 2005). Although opportunistic infections of the CNS and PNS associated with HIV-induced immunodeficiency have become far less common because of the availability of antiretroviral therapy (ART) (Habata et al., 1999; Mamidi et al., 2002; Roullet, 1999), the prevalence of primary HIV-induced neurological disorders has increased (Antinori et al., 2007). Conditions directly induced by HIV-1 include peripheral neuropathies, vacuolar myelopathies, and HIV-associated neurocognitive disorders (HAND) (Antinori et al., 2007; Childs et al., 1999; McArthur et al., 2005; McArthur et al., 2003).

HAND are comprised of three conditions of increasingly severe cognitive impairment and interference with activities of daily living: (1) HIV-associated asymptomatic neurocognitive impairment (ANI), (2) HIV-associated mild neurocognitive disorder (MND), and (3) HIV-associated dementia (HAD) (Antinori et al., 2007; Grant, 2008). The diagnosis of ANI, the least severe of the HAND conditions, describes individuals who have mild cognitive impairment, revealed by formal neuropsychological testing, that does not affect day-to-day functioning and does not meet criteria for delirium or dementia. In the more severe MND, increased cognitive impairment interferes with daily functioning as determined by self-reporting or by observation of others. Again, individuals with MND do not meet the criteria for delirium or dementia. HAD, the most severe form of HAND, is typically diagnosed during end-stage HIV infection, primarily in patients with low CD4+ T-cell counts. In HAD, cognitive impairment is associated with marked interference with day-to-day functioning and HAD is considered a significant independent risk factor for death due to AIDS (Liner et al., 2008). Since the inception of ART, the incidence of HAD, defined as the percentage of new HAD cases diagnosed in a given year, has decreased (McArthur et al., 2004; Nath et al., 2008; Sacktor et al., 2002). However, the prevalence of HAD, defined as the overall number of HAD cases, is rising owing to the increased life span of HIV patients (McArthur et al., 2003; Robertson et al., 2007; Sacktor et al., 2002). There is also evidence of ongoing neurodegeneration that may manifest as ANI or MND in patients without evidence of active HIV disease, contributing to the continued prevalence of HAND in the ART era. Moreover, the onset of HAND during ART-controlled clinical latency underscores the need for adjunctive neuroprotective therapies, because no current therapies would be expected to improve neurologic outcomes in the absence of viral replication (Antinori et al., 2007; Brew, 2004; Sacktor et al., 2002).

Pathology of HAND: Evidence for Neuroinflammation

HIV-1 enters the brain early in the course of infection via infected macrophages and lymphocytes (Ho et al., 1985; Koenig et al., 1986; Petito et al., 1986). Subsequently, the virus persists in the

CNS primarily in perivascular macrophages and microglia and increased numbers of macrophages and microglia have been found to correlate with the severity of HAND (Glass et al., 1995). In general, intrathecal replication of HIV-1 is controlled by CD8+ T cells (McCrossan et al., 2006; Sadagopal et al., 2008), and cerebral spinal fluid (CSF) viral load has been found to correlate with both viral load in the brain and the degree of cognitive dysfunction in HAND (Brew et al., 1997; Ellis et al., 2000; Ellis et al., 1997; Ellis et al., 2002; McArthur et al., 1997). Importantly, in addition to the initial neuroinvasion and infection of macrophages and microglia, it is believed that HIV infection and immune activation in the periphery and ongoing neuroinvasion of activated monocytes play a role in the development of HAND (Banks et al., 2006; Gartner, 2000).

The pathological hallmarks of HIV infection in the brain in the pre-ART era, collectively termed HIV encephalitis (HIVE), include monocyte infiltration and accumulation of perivascular monocyte-derived macrophages (MDM), formation of microglial nodules and multinucleated giant cells (syncytia) due to HIV-driven fusion of MDM/microglia, widespread reactive microgliosis and astrogliosis, and myelin pallor indicative of oligodendrocyte damage (Adle-Biassette et al., 1999; Gendelman et al., 1994; Lawrence and Major, 2002; Masliah et al., 1997; Petito et al., 1986; Wiley and Achim, 1994). Since the initiation of widespread ART (1996/7), however, at least one study suggests the presence of “burnt out” HIVE in some individuals dying with AIDS (see below) (Gray et al., 2003). MDM and microglia are the primary CD4+ cells in the CNS and the major sources of productive HIV infection in the brain (Gonzalez-Scarano and Martin-Garcia, 2005; Kaul et al., 2001; Kolson and Gonzalez-Scarano, 2000; McArthur et al., 2003) and clinical disease severity correlates more strongly with the amount of monocyte infiltration and MDM/microglia activation than with the quantity of infected cells or viral load (Adle-Biassette et al., 1999; Glass et al., 1995), suggesting that MDM/microglia play a predominant role in the neuroinflammation and neurotoxicity seen in HAND. Immune activation of MDM/microglia is demonstrated by expression of CD14 (lipopolysaccharide receptor), CD16, CD68, and MHC class II in vivo (Anderson et al.,

2002; Bell, 2004; Fischer-Smith et al., 2004; Swindells et al., 1999). Furthermore, CSF markers of immune activation and inflammation are commonly detected in individuals with HAND. These markers include CCL2 (monocyte chemoattractant protein-1, MCP-1) (Chang et al., 2004; Conant et al., 1998), β2 microglobulin (Brew et al., 1992; Brew et al., 1996; Enting et al., 2000; McArthur et al., 1992), quinolinic acid (Achim et al., 1993; Brew et al., 1995; Heyes et al., 1991; Heyes et al., 2001), arachidonic acid metabolites (Genis et al., 1992; Griffin et al., 1994), oxidative stress markers (Haughey et al., 2004; Schifitto et al., 2009a), and platelet activating factor (PAF) (Gelbard et al., 1994).

Although most studies demonstrate that neurons are not infected by HIV, neuronal loss is common in HAND and post-mortem studies of HAND patients have revealed morphological changes in neurons including loss of synaptic density, dendritic simplification, and vacuolization (Masliah et al., 1997; Petito et al., 1986). This neuronal damage induced by HIV infection affects multiple regions of the brain and several neuronal subtypes. HIV antigen is commonly detected in the thalamus, basal ganglia, and central white matter and neuronal damage and loss has been reported in the frontal cortex, cerebellum, putamen, and substantia nigra, although the distribution of HIV antigen might be altered in individuals receiving ART (Everall et al., 1991; Ketzler et al., 1990).

Changes in Neuropathology of HAND in the Era of ART

The most effective current therapy for HAND is treatment of the underlying HIV infection with ART. Neuropsychological performance is improved in AIDS patients treated with ART and the CNS penetration of antiretroviral drugs directly correlates with decreased CSF viral loads and improved neurocognitive performance (Letendre et al., 2008). However, some recent studies suggest that ART drugs might demonstrate CNS neurotoxicity in treated patients, with associated poorer neurocognitive performance (Marra et al., 2009; Schweinsburg et al., 2005). Unfortunately, although ART can improve cognition, it does not fully eradicate impairments. In addition, patients

who have had ART exposure before developing HAND do not appear to respond as well to a change in their ART regimen, indicating the presence of drug-resistant viruses in the CNS or the emergence of a burn-out phase of the disease (Brew et al., 2007).

The era of ART has changed the neuropathology of HIV-1 infection (Table 2.1) (Anthony et al., 2005; Boisse et al., 2008; Brew, 2004). Before the introduction of ART, neuroinflammation was frequently observed in HIV-infected patients and usually increased throughout the progression of disease from the asymptomatic stage to AIDS and HAD. Inflammation is less severe during ART, but it appears to be persistent within the macrophage/microglial populations (Gray et al., 2003). Furthermore, ART seems to have limited the severity of pathological changes characteristic of HIVE. As described by Gray and colleagues (Gray et al., 2003), the persistent pathological findings in ART-experienced individuals include neuronal loss with apoptosis, astrocytosis, myelin pallor, and at least a few activated microglia and perivascular macrophages. Distinctly absent are multinucleated giant cells and microglial nodules.

Although for the most part ART has limited the persistent infiltration of HIV-infected lymphocytes into the CNS (Anthony and Bell, 2008), it should be noted that an exception to this occurs during neurologic immune reconstitution inflammatory syndrome (neuroIRIS). NeuroIRIS is a relatively rare (less than 1% of those who initiate ART) consequence of the introduction of ART in highly immunosuppressed patients and is marked by a severe deterioration in neurologic status that is characterized by massive lymphocytosis, extensive demyelination, and white matter damage (Anthony et al., 2005; Boisse et al., 2008). In neuroIRIS there is a paradoxical clinical deterioration in spite of improved CD4+ T-cell counts and decreased viral loads (McCombe et al., 2009). Despite the overall effectiveness of ART in limiting the infiltration of infected cells into the CNS, neuroinflammation still persists. However, the primary sites of neuroinflammation are different (Table 2.1); a strong involvement of the basal ganglia was observed pre-ART, whereas post-ART specimens display prominent signs of inflammation in the hippocampus and adjacent

parts of the entorhinal and temporal cortex (Anthony et al., 2005; Ho et al., 1985). Notably, these autopsy studies have demonstrated microglial activation in brains of individuals treated with ART comparable to those of patients with fully developed, pre-ART AIDS, although HIVE (when defined as the presence of HIV-infected multinucleated giant cells) is much less common now. Overall, these studies confirm the notion that neuroinflammation continues to be associated with HIV CNS infection in ART-experienced individuals, albeit without HIVE.

Table 2.1 - Changes in the neuropathology of HAND in the era of HAART

	Pre HAART	Post HAART
CSF viral load	viral load correlates with cognitive dysfunction (Brew et al., 1997; Ellis et al., 2000; Ellis et al., 1997; Ellis et al., 2002; McArthur et al., 1997)	CSF viral load is decreased with treatment (Letendre et al., 2008)
cognitive dysfunction	severe in HAD and improved when patients begin HAART (Letendre et al., 2008)	variable, from mild to severe (Antinori et al., 2007; McArthur et al., 2004)
predictive biomarkers of dementia	CSF viral load, decreased $CD4^+$ T-cells, increased levels of CCL2 (Bandaru et al., 2007; von Giesen et al., 2005)	increased levels of CCL2, β2 microglobulin, quinolinic acid, arachidonic acid metabolites, oxidative stress markers, platelet activating factor (Bandaru et al., 2007)
major pathological findings	multinucleated giant cells, microglial nodules, neuronal loss, astrocytosis, myelin pallor, activated microglia and perivascular macrophages	neuronal loss, astrocytosis, myelin pallor, activated microglia and perivascular macrophages.
multinucleated giant cells and microglial nodules	present	absent
neuroinflammation	severe and progressive	less severe, but chronic
site of neuroinflammation	basal ganglia (Ho et al., 1985)	hippocampus and entorhinal and temporal cortex (Anthony et al., 2005; Ho et al., 1985)
microglial activation	present	present
CNS complications from Tx	N/A	NeuroIRIS (rare <1%) (Boisse et al., 2008)

2.2 - Biology of HIV Infection and Invasion of the Brain

As mentioned above, HIV-1 traffics into the brain early in the course of infection via infected monocytes and lymphocytes (Dunfee et al., 2006). Despite retroviral therapy, HIV-1 persists in the CNS throughout the duration of infection in parenchymal microglia and perivascular macrophages (Ho et al., 1985; Koenig et al., 1986; Petito et al., 1986). Because increased numbers of microglia and macrophages correlate with the severity of pre-mortem HAND, these cell types probably mediate neurological impairment (Anthony et al., 2005; Glass et al., 1995; Petito et al., 1986). Multiple pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 are elevated in the CNS and/or CSF of patients with HAD (Achim et al., 1993; Foli et al., 1997; Oster et al., 1987; Perrella et al., 1992). The pro-inflammatory environment within the CNS is a result of cytokine release from monocytes/macrophages stimulated by either direct viral infection or by shed viral proteins (Rappaport et al., 1999; Sundar et al., 1991) and astrocyte activation without productive infection but with immune activation (Kramer-Hammerle et al., 2005; Sabri et al., 2003). Inflammatory mediators modulate the permeability of the blood-brain barrier, the entry of infected monocytes into the CNS, and peripheral processes that contribute to the neurological complications of HIV. Thus, understanding the mechanisms responsible for HIV entry into the CNS and the modulation of replication within the monocyte/macrophage reservoir are important for developing targeted therapeutics for HAND.

Integrity of the Blood-Brain Barrier (BBB): Role for Neuroinflammation

The blood-brain barrier (BBB) separates the CNS from the periphery and modulates the traffic of low-molecular-weight nutrients, peptides, proteins, and cells in and out of the brain. The integrity and traffic across the BBB can be impacted by many factors, including HIV-dependent cytotoxicity towards cellular BBB components, chemotactic gradients, and the regulation of adhesion molecules and tight junction proteins (Figure 2.1). Progressive HIV infection and immune compromise result in the breakdown of the BBB (Dallasta et al., 1999; Kanmogne et al., 2002;

Persidsky et al., 2000), which permits the entry of free virus, lymphocytes, and infected and/or activated monocytes into the CNS.

The BBB is composed of brain microvascular endothelial cells (BMECs) that are connected by intercellular junctions to form a semipermeable monolayer. HIV infection increases the permeability of the BBB by compromising the integrity of the tight junctions. Brain regions with HIVE demonstrate an accumulation of activated/infected perivascular macrophages and a decrease in the tight junction membrane proteins zonula occludens (ZO-1) and occludin (Dallasta et al., 1999; Persidsky et al., 2006). TNF-α production by activated/infected macrophages can directly increase the permeability of the BBB to free virus (Fiala et al., 1997). However, these profound structural changes of the BBB are late events associated with encephalitis and HIV neuroinvasion is an early and continuing process.

HIV enters the CNS compartment through infected monocytes that cross the BBB via trans-endothelial migration, and this process is particularly enhanced by monocyte immune activation (Figure 2.1). Infected and immune-activated monocytes induce adhesion molecules on BMECs, thereby increasing transmigration during HIV infection. In HIVE brain tissue, viral load and pro-inflammatory cytokines correlate with levels of the adhesion molecules E-selectin and VCAM-1 (Hurwitz et al., 1994; Nottet et al., 1996; Persidsky et al., 1997; Sasseville et al., 1994). Exposure to TNF-α stimulates astrocytes to produce ICAM-1, VCAM-1, IG9, and E-selectin, all of which promote monocyte attachment and transmigration (Hurwitz et al., 1994). Following HIV infection, inflammatory cytokines promote the expression of adhesion molecules on BMECs and thereby promote the transmigration of activated/infected monocytes into the brain. Whether compromise to the BBB is as common now in the ART era as in the pre-ART (HIVE) era remains to be determined.

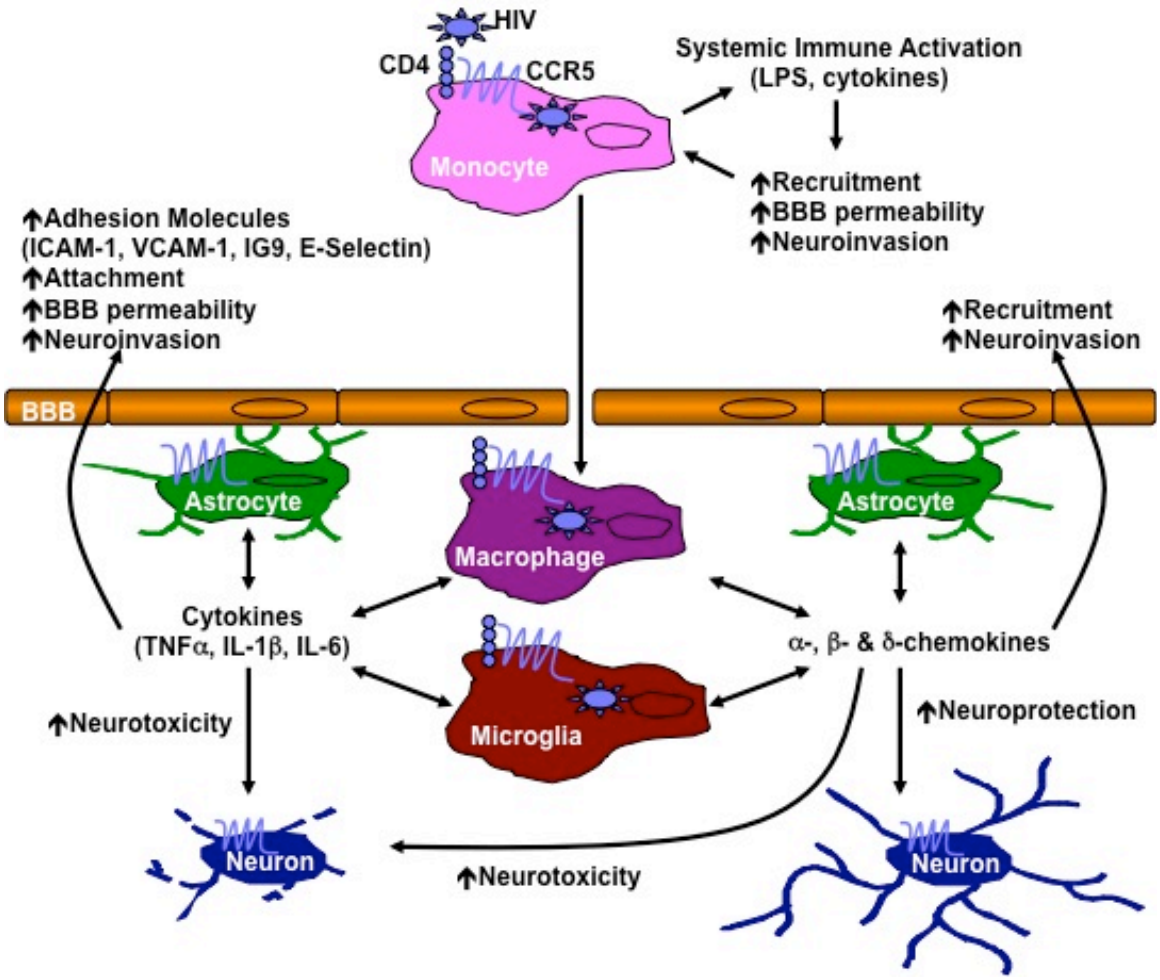

Figure 2.1 Role for inflammation via cytokines and chemokines in HIV neuroinvasion and neurodegeneration. HIV-infected monocytes cross the BBB, differentiate into macrophages, and productively infect microglia and other macrophages. Infected and/or activated macrophages/microglia and immune activated astrocytes release pro-inflammatory cytokines which promote monocyte neuroinvasion by increasing expression of adhesion molecules required for monocyte attachment and increasing BBB permeability. This pro-inflammatory environment is enhanced by LPS- and cytokine-induced systemic immune activation, which promotes monocyte recruitment and further BBB permeability. Together, the resulting neuroinflammation can directly and indirectly cause neuronal loss. Infected and/or activated macrophages/microglia and astrocytes also release chemokines, which can serve as both neurotoxic pro-inflammatory factors and neuroprotectants against HIV-induced neurotoxicity. Cytokines and chemokines can undergo reciprocal modulation by additional neurotoxic factors released from infected and/or activated macrophages/microglia, including excitatory amino acids (EAAs), phospholipids and reactive oxygen species (ROS).

Inflammation in the Periphery

Pathology in the periphery may also be involved in the development of HAND (Figure 2.1) (Gartner, 2000). Neurological complications following HIV infection have been associated with an expanded population of circulating blood monocytes expressing markers of activation (Pulliam et al., 1997). These activated CD14+/CD16+ monocytes produce TNF-α and IL-1β, which contribute to inflammation in the periphery and further activation of other immune cells (Thieblemont et al., 1995). CD16+ monocytes are particularly susceptible to HIV infection, are capable of tissue invasion, and they compose the majority of the accumulated perivascular macrophages in patients with HIVE (Ellery et al., 2007; Jaworowski et al., 2007; Shiramizu et al., 2005). Peripheral activation of monocytes leads to an enhancement of infected and invasive monocytes that can be recruited into the CNS by chemokines (chemotactic cytokines), as discussed in detail below.

In the gastrointestinal tract, HIV infection also causes microbial translocation, in which HIV-driven depletion of gut-associated lymphoid tissue (GALT) results in leakage of bacteria into the bloodstream and subsequently increases systemic bacterial lipopolysaccharide (LPS) (Brenchley et al., 2006b; Douek, 2007). LPS increases monocyte transmigration into the CNS by contributing to the systemic immune activation of chronic HIV infection. In addition to priming peripheral monocytes for neuroinvasion, bacterial LPS can also compromise the integrity of the BBB. In vitro studies demonstrate that LPS-stimulated macrophages create gaps between endothelial cells of an artificial BBB, resulting in enhanced monocyte transmigration (Persidsky et al., 1997; Wang et al., 2008; Zhou et al., 2006). Notably, studies in SIV-infected rhesus macaques and HIV-infected humans correlate higher levels of plasma LPS, LPS-binding protein, and soluble CD14 with increased HAND severity (Ancuta et al., 2008) and more rapid progression towards AIDS (Brenchley et al., 2006b). Thus, the neurological complications of HIV are not exclusively mediated by pathological processes within the CNS, and components of systemic immune activation also play a significant role in the development of HAND.

In summary, inflammatory mediators contribute to HIV infection of the brain by affecting components of both the CNS and the peripheral compartments. Pro-inflammatory cytokines activate a neuroinvasive subset of monocytes in the periphery, compromise the integrity of the BBB, and promote the transmigration of infected monocytes into the CNS. In addition to mediating HIV neuroinvasion, inflammatory mediators also promote the accumulation of macrophages in the brain. These cells serve as a primary reservoir for HIV in the CNS and contribute to the pathogenesis of HAND via the release of neurotoxic and inflammatory cellular products, which can activate noninfected cells (macrophages/microglia, astrocytes). Understanding the role of inflammation in mediating HIV infection of the CNS could provide new therapeutic targets for HAND and other neuroinflammatory diseases.

2.3 - Mechanisms of HIV-Induced Neurodegeneration: Roles for Chemokines, Chemokine Receptors, and Inflammation

Monocyte infiltration and macrophage/microglia activation are thought to initiate HAND pathogenesis through both systemic and CNS inflammatory signaling (Kaul et al., 2001; Kraft-Terry et al., 2009; Yadav and Collman, 2009). Following systemic inflammation and monocyte infiltration, activated and/or infected macrophages/microglia within the CNS can release a variety of neurotoxic factors, including viral proteins (gp120, Tat), pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), interferons (IFN-α, IFN-β, IFN-γ), excitatory amino acids (glutamate, quinolinic acid), phospholipids (platelet activating factor, arachidonic acid), and reactive oxygen species (Boven et al., 1999; Brenneman et al., 1988; Brew et al., 1995; Gelbard et al., 1993; Gelbard et al., 1994; Gendelman et al., 1998; Genis et al., 1992; Jiang et al., 2001; Maragos et al., 2003; Scorziello et al., 1998; Song et al., 2003; Wesselingh et al., 1993). Many of these factors can undergo reciprocal modulation by chemokines. Indeed, chemokines and chemokine receptors expressed within the CNS have central roles in HIV neuropathogenesis, from the function of chemokine receptors in mediating infection of the macrophage/microglia reservoir, to the seemingly dichotomous roles of chemokines as neurotoxic pro-inflammatory factors and neuroprotectants

against HIV-induced neurotoxicity (Collman and Yi, 1999; Doms, 2000; Gonzalez-Scarano and Martin-Garcia, 2005; Martin-Garcia et al., 2002). Here, we focus on the α-, β-, and δ-chemokine subfamilies in relation to HAND pathogenesis.

α-Chemokines

The α-chemokines, which bind CXCR chemokine receptors, affect HAND neuropathogenesis by enhancing neuroinvasion, promoting astrocyte activation, and directly acting as neurotoxic or neuroprotective factors (Figure 2.1). Increased expression of several α-chemokines has been documented in the CSF and brain tissue of HAD patients, including CXCL10 (γ-interferon-inducible protein 10, IP-10) and CXCL12 (stromal cell-derived factor-1, SDF-1) (Cinque et al., 2005; Kolb et al., 1999; Rostasy et al., 2003; Zhang et al., 1998). In vitro studies demonstrate that CXCL10 is expressed in microglia and astrocytes, and that gp120, Tat, TNF-α and IFN-γ can act independently or synergistically to increase CXCL10 release (Asensio et al., 2001; D'Aversa et al., 2004; Dhillon et al., 2008a; Kutsch et al., 2000; Williams et al., 2009a; Williams et al., 2009b). In turn, soluble CXCL10 can directly induce apoptosis in neurons (Sui et al., 2004; Sui et al., 2006a; van Marle et al., 2004) and increase neuroinflammation through leukocyte recruitment into the CNS (Asensio et al., 2001; Dhillon et al., 2008a; Kolb et al., 1999).

Like CXCL10, CXCL12 is a potent chemoattractant that can increase recruitment, adhesion, and transendothelial migration of monocytes into the CNS (Malik et al., 2008; Peled et al., 1999; Peng et al., 2006; Rostasy et al., 2003; Wu et al., 2000). CXCL12 is expressed in astrocytes, microglia, and neurons, and exposure to LPS or IL-1β from HIV-infected and/or activated macrophages can increase CXCL12 release (Bajetto et al., 1999; Ohtani et al., 1998; Peng et al., 2006). Conversely, CXCL12 can trigger astrocytic release of TNF-α and glutamate, causing neuronal damage and apoptosis (Bezzi et al., 2001). Like CXCL10, CXCL12 can also act as a direct neurotoxin. It can undergo proteolytic cleavage by matrix metallic proteinase-2, which changes its co-receptor specificity from CXCR4 to CXCR3, the receptor for CXCL10, and enhances its

neurotoxicity (Vergote et al., 2006; Zhang et al., 2003). The neurotoxic properties of native, non-cleaved CXCL12 are controversial as CXCL12 exposure produces either neuroprotective or neurodegenerative responses depending on the experimental conditions (Hesselgesser et al., 1998; Kaul and Lipton, 1999; Khan et al., 2004; Lazarini et al., 2000; Zheng et al., 1999a; Zheng et al., 1999b).

β-Chemokines

Like α-chemokines, β-chemokines, which bind CCR receptors, can mediate neurotoxic and neuroprotective effects against HIV-induced neurotoxicity (Figure 2.1). Several ligands in the β-chemokine subfamily are expressed at increased levels during HIV infection, including CCL2, CCL3 (macrophage inflammatory protein-1α, MIP-1α), CCL4 (MIP-1β), and CCL5 (regulated on activation, normal T cell expressed and secreted (RANTES; (Kelder et al., 1998)). Interestingly, CCL2 CSF levels increase in SIV-infected macaques and HIV-infected individuals prior to neurocognitive impairments and correlate with severity of dementia, suggesting that CCL2 can be a predictive marker for clinical HAND (Cinque et al., 1998; Dhillon et al., 2008b; Kelder et al., 1998; Ragin et al., 2006; Sevigny et al., 2004; Sevigny et al., 2007; Zink et al., 2001; Zink et al., 1998). In vitro studies demonstrate that CCL2 is expressed and released from endothelial cells, macrophages, microglia, and astrocytes during HIV infection and in response to gp120, Tat, TNF-α, IL-1β, IFN-β, and IFN-γ (Choe et al., 2001; Conant et al., 1998; D'Aversa et al., 2004; Gu et al., 1997; Guillemin et al., 2003; Lehmann et al., 2006; McManus et al., 2000; Mengozzi et al., 1999). HIV infection of macrophages increases their expression of the CCL2 receptor, CCR2, and concomitantly increases CCL2-mediated recruitment and transmigration of HIV-infected monocytes into the CNS (Eugenin et al., 2006; Park et al., 2001). Thus, inflammation following HIV infection establishes a cycle of monocyte/macrophage activation and neuroinvasion mediated by CCL2. However, CCL2 also provides neuroprotection against Tat-induced neurotoxicity (Eugenin et al., 2003; Yao et al., 2009), suggesting that increased expression of CCL2 during HIV infection may play a destructive and/or protective role. Additionally, the potential

CNS destructive role for CCL2 in HIV infection thus appears to be driven through its enhancement of monocyte transendothelial migration and not direct effects on neurons.

While elevated CCL2 CSF levels are associated with an increased risk of HAND, the association of CCL3, CCL4, and CCL5 with HAND is unclear (Letendre et al., 1999). CCL3, CCL4, and CCL5 can all serve as ligands for the CCR5 receptor and can suppress CCR5-mediated HIV infection (Cocchi et al., 1995). Studies also demonstrate that CCL3, CCL4, and CCL5 are expressed and released from microglia and astrocytes during HIV infection and in response to Tat (Cota et al., 2000; D'Aversa et al., 2004; El-Hage et al., 2005; Si et al., 2002), and can ameliorate excitatory amino acid- and gp120-induced neurotoxicity in vitro (Bruno et al., 2000; D'Aversa et al., 2004; Kaul and Lipton, 1999; Meucci et al., 1998; Meucci et al., 2000). The neuroprotective effects of CCL5 could be mediated by CCL2 induction (Eugenin et al., 2003). Together, these studies suggest that β-chemokines play important roles in modulating neuroinflammation and neurotoxicity during HIV infection and that they could express either protective or destructive effects within the CNS inflammatory microenvironment.

δ-Chemokines

CX3CR1 and its unique ligand, CX3CL1 (fractalkine), are the only known receptor-ligand pair in the δ-chemokine subfamily. Furthermore, CX3CL1 is the only chemokine expressed in higher amounts in the CNS than in peripheral tissues, suggesting its critical role in modulation of HAND pathogenesis (Figure 2.1) (Bajetto et al., 2001; Cotter et al., 2002; Re and Przedborski, 2006). It is expressed as a membrane-anchored form on the cell surface or as a soluble form, which can be proteolytically released from the cell. CX3CL1 expression is elevated in serum, CSF, and brain tissue of HAD patients (Erichsen et al., 2003; Pereira et al., 2001; Sporer et al., 2003) and in vitro studies suggest that exposure to purified HIV virus, gp120, TNF-α, IL-1β, IFN-γ, or glutamate increases expression of soluble and membrane-bound forms of CX3CL1 in neurons and astrocytes (Chapman et al., 2000a; Erichsen et al., 2003; Maciejewski-Lenoir et al., 1999; Pereira

et al., 2001; Sporer et al., 2003). Both forms of CX3CL1 can serve as potent CNS leukocyte chemoattractants and can mediate monocyte adhesion and transendothelial migration across the BBB (Ancuta et al., 2003; Chapman et al., 2000b; Harrison et al., 1998; Imai et al., 1997; Tong et al., 2000). In addition to recruiting activated peripheral blood monocytes, fractalkine can trigger the production of IL-6 and CCL2 by CD16+ monocytes (Ancuta et al., 2003). Elevated CCL2 levels can lead to further recruitment of monocytes to the brain while IL-6 can activate and promote the differentiation of monocytes into macrophages, which could promote neurodegeneration. CX3CL1 differentially modulates other neuroinflammatory factors as well, increasing secretion of TNF-α and IL-8, from monocytes and macrophages (Ancuta et al., 2003; Ancuta et al., 2006; Cotter et al., 2002), while inhibiting TNF-α, IL-1β, and IL-6 release from LPS-activated microglia and attenuating cytokine-mediated neuronal loss in vivo and in vitro (Cardona et al., 2006; Mizuno et al., 2003).

Like β-chemokines, CX3CL1 can also promote neuronal survival, as it can protect against gp120- and Tat-induced neurotoxicity in vitro (Deiva et al., 2004; Limatola et al., 2005; Meucci et al., 1998; Meucci et al., 2000). While it is unclear what factors maintain the in vivo balance between these neurotoxic and neuroprotective functions of CX3CL1 and other chemokines, the dysregulation of chemokine signaling likely significantly contributes to HAND pathogenesis. These processes are central to neurodegenerative diseases such as HIV infection and multiple sclerosis, and have implications for other neuroinflammatory diseases (Kaul and Lipton, 2006; Li and Ransohoff, 2008; Ransohoff, 1999; Ransohoff and Zamvil, 2007; Savarin-Vuaillat and Ransohoff, 2007).

2.4 - Inflammatory Markers in NeuroAIDS and Other Neuroinflammatory Diseases

Chronic neuroinflammation and elevated levels of pro-inflammatory cytokines and chemokines are associated with several neurodegenerative disorders of the CNS including HAND, MS, AD, PD, and HAM/TSP (Block and Hong, 2005; Grant et al., 2002; Mrak and Griffin, 2005; Sawada et

al., 2006). In the CNS, macrophages/microglia are the principal mediators of inflammation and, when activated, secrete pro-inflammatory cytokines (including TNF-α, IL-1β, and IL-6), chemokines (including CCL2 and CCL3), and adhesion molecules (ICAM-1, VCAM-1) that promote inflammation. Regardless of the factor initiating microglia activation, a chronic inflammatory response in the brain can contribute to the death of vulnerable neuronal populations and understanding common mechanisms in these neuroinflammatory disorders could identify common targets for broadly protective drugs. Additionally, more reliable biomarkers for neuroinflammatory diseases are needed in order to make early and accurate diagnoses, monitor the course of disease progression, and predict a patient's response to therapy.

Inflammation in Neurodegenerative Diseases Associated with Aging

HAND shares clinical features with normal aging including the deterioration of cognitive abilities and working memory (Alirezaei et al., 2008; Kaul, 2009). Of interest, diffusion tensor imaging studies, where diffusion is an indicator of neuroinflammation, have shown altered water diffusion within specific brain regions in HIV-infected patients in comparison to normally aging control patients (Chang et al., 2008). These data suggest that even a well-controlled HIV infection may accelerate aging and promote neurodegeneration. In addition, there are several pathological features shared between HAND and neurodegenerative diseases associated with aging, including AD and PD (Brew et al., 2009; Chang et al., 2008; Esiri et al., 1998; Khanlou et al., 2009). These include neuroinflammation, oxidative stress, and cellular degradation pathways (Brew et al., 2009; Lovell and Markesbery, 2007; Nath et al., 2008).

The neuropathology of HAND is clearly distinguishable from AD at autopsy, although there are some shared features. HAND demonstrates less atrophy and fewer neurofibrillary tangles in plaques, which are the hallmarks of AD. Amyloid beta (Aβ) deposition, which precedes symptoms in AD, has also been described in the brains of HIV-infected individuals, although its potential role in HAND symptoms is controversial (Brew et al., 2009; Esiri et al., 1998). Aβ deposition has pro-

inflammatory effects that accelerate neurodegeneration in vitro (Craft et al., 2006). In AD brains, microglia localize to amyloid plaques (McGeer et al., 1987) and upregulate human leukocyte antigen-DR (HLA-DR) and complement in addition to several pro-inflammatory cytokines that are also implicated in HAND pathogenesis (IL-1β, IL-6, and TNFα) (Eikelenboom et al., 2002; McGeer and McGeer, 2010; McGeer et al., 1987; Sheng et al., 1998). Notably, a transgenic mouse model for a familial AD mutation of amyloid precursor protein recapitulates this proinflammatory effect of Aβ deposition and astrocytes and microglia expressing IL-1β, IL-6, TGF-β, and TNF-α surround plaques (Qiao et al., 2001). In addition to common expression of cytokines that are believed to be involved in the pathogenesis of AD and HAND, LPS concentrations are elevated in both disorders (Ancuta et al., 2008; Herber et al., 2006).

As with AD, HAND also shares some similar neuropathological features with PD. The presence of α-synuclein-positive inclusions in the cell bodies (Lewy bodies) and processes (Lewy neuritis) in the substantia nigra is the neuropathological hallmark of PD. Lewy bodies have also been observed in autopsied brains of HIV-infected individuals (Brew et al., 2009; Esiri et al., 1998; Kaul, 2009; Khanlou et al., 2009) and dopamine deficiency is found in both disorders. However, whether neuroinflammation in PD is a consequence or a cause of the selective loss of dopaminergic neurons in the substantia nigra is unknown. Evidence for neuroinflammation in PD includes increased CSF levels of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, TGF-β, and IFN-γ (Hunot et al., 1999; Vawter et al., 1996). Moreover, single nucleotide polymorphisms associated with increased production of cytokines and chemokines are overrepresented in PD cohorts and may confer increased susceptibility to PD (Hakansson et al., 2005a, b; Kruger et al., 2000).

Although the role for neuroinflammation in AD and PD is not completely understood, inflammatory mediators may be used as both biomarkers and targets for drug development for these disorders. For example, platelet inflammatory biomarkers, like cyclooxygenase 2 (COX-2) and

phospholipase A2 could be exploited as peripheral inflammatory biomarkers (Casoli et al., 2010). COX-2 and its homolog COX-1 are pro-inflammatory proteins that are used by activated microglia to synthesize a variety of inflammatory mediators and are elevated in AD brains. Both COX-1 and COX-2 are targets of non-steroidal anti-inflammatory drugs (NSAIDS), and there is a link between chronic use of NSAIDS and a reduced risk for AD and PD (Breitner et al., 1995; Chen et al., 2003). Moreover, biomarkers identified in AD, PD, and other diseases related to aging may also be relevant in HAND. In a recent study, β-Amyloid(1–42)(Aβ42) measurements in the CSF of HAND patients were found to be similar to those found in patients with AD and significantly decreased compared to those in normal controls and HIV-infected individuals with normal cognitive function (Clifford et al., 2009). However, HAND patients had normal or slightly depressed levels of CSF tau and tau phosphorylated at threonine 181 (p-Tau181), which distinguished them from patients with AD (Clifford et al., 2009). The detection of pro-inflammatory proteins in the periphery may, in the future, be exploited as useful biomarkers in a wide spectrum of neurodegenerative disorders.

Other Chronic Progressive, Inflammatory Disorders of the CNS with Known or Suspected Viral Etiology

Neuroinflammation is a major component of other disorders of the CNS with known or suspected viral etiology including HAM/TSP and MS. The human retrovirus HTLV-1 (human T-lymphotropic virus type I) causes HAM/TSP in a small percentage (<5%) of infected individuals. The incubation period between infection with HTLV-I and the development of HAM/TSP is typically long (20–30 years) and the clinical hallmark of this chronic progressive neurologic disorder is a gradual onset of lower extremity weakness (McFarlin and Blattner, 1991; Osame et al., 1990). HTLV-I is predominantly CD4+T-cell-tropic. However, CD8+ T cells, astrocytes, monocytes/macrophages, and microglia may also become infected and serve as viral reservoirs (Hoffman et al., 1992; Nagai et al., 2001a; Nagai et al., 2001b; Watabe et al., 1989). As in HAND, monocytes/macrophages are believed to contribute to the pathogenesis of HAM/TSP. However,

in contrast to the pathogenesis of HAND/HIV-1 infection of the CNS, much of the neurodegeneration resulting from HTLV-I infection is caused directly by HTLV-I Tax-specific cytotoxic T cells (CTLs) restricted to immunodominant epitopes of HTLV-I gene products (predominantly Tax) (Elovaara et al., 1993; Jacobson et al., 1990b). These HTLV-I-specific CTLs are readily detected in the peripheral blood lymphocytes (PBLs), CSF, and active inflammatory lesions of HAM/TSP patients (Greten et al., 1998; Levin et al., 1997). HTLV-I Tax-specific CTLs secrete many proinflammatory cytokines and chemokines including IFN-γ, TNF-α, CCL3, CCL4, and IL-16 (Biddison et al., 1997). In addition, HTLV-I Tax trans-activates many host genes and HTLV-I-infected astrocytes secrete high levels of IL-1α, IL-6, TNF-α, and matrix metalloproteinases (MMPs) (Szymocha et al., 2000a; Szymocha et al., 2000b). Increased levels of HTLV-I tax mRNA, an increased frequency of HTLV-I Tax-specific CD8+ T cells, and high proviral loads correlate with disease severity in HAM/TSP and provide good biomarkers of disease progression (Yamano et al., 2002). Other candidate HAM/TSP biomarkers, CD244 (a signaling lymphocyte activation molecule [SLAM] family receptor) and SLAM-associated protein (SAP), were found to be significantly higher in HAM/TSP compared to asymptomatic carriers and uninfected individuals and both may be used as biomarkers for neurodegeneration in HTLV-I-infected individuals (Enose-Akahata et al., 2009).

Another prototypic neuroinflammatory disease with some neuropathological similarities to HAND is MS. MS is the most common inflammatory disease of the CNS, with a prevalence that ranges between 2 and 150 per 100,000 (Rosati, 2001). The etiology of MS is unknown. In part, this is attributable to the variability of this disease, suggesting that many factors may be involved in the development of MS (reviewed in (Soldan et al., 2008)). However, it is generally believed that genetic, immunological, and environmental factors contribute to MS pathogenesis. Infectious agents have been implicated in the pathogenesis of MS for over 100 years but no single causative agent has been identified. Again, unlike HAND, MS is chiefly a T-cell-mediated neuroinflammatory disorder. However, in both MS and HAND an inflammatory cascade

contributes to disease pathogenesis. Although the neuroinflammatory nature of MS has been confirmed at all stages, inflammation tends to decrease after decades of disease, as irreversible neuronal degeneration accumulates. Such neuroinflammation is detectable through brain magnetic resonance imaging (MRI) as the presence of gadolinium-enhancing lesions, which represent areas of inflammation associated with leakiness of capillary endothelial cells and infiltration of serum components into the brain parenchyma. Analysis of CSF often reveals markers of neuroinflammation, as does examination of autopsied brain specimens (see below).

The overexpression of several proinflammatory cytokines, including TNF-α, IFN-g, IL-12, IL-6 and CXCL10 have been demonstrated in MS brain specimens (Bartosik-Psujek and Stelmasiak, 2005; Drulovic et al., 1998; Drulovic et al., 1997; Frohman et al., 2006; Miljkovic et al., 2002; Ubogu et al., 2006). Importantly, an increase in TNF-α expression in peripheral blood mononuclear cells has been found to precede MS relapses and inflammatory activity (Rieckmann et al., 1995). In contrast, anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) and other chemokines (CCL2 and CCL5) are downregulated during MS disease exacerbations (Mahad et al., 2002a, b; Malmestrom et al., 2006; Rieckmann et al., 1995). The altered expression of these cytokines promotes disease pathogenesis by upregulating MHC and adhesion molecule expression on endothelial and glial cells, activating macrophages, recruiting TH-1 cells, and/or by directly damaging oligodendrocytes and myelin sheaths. In addition, soluble adhesion molecules such as ICAM-1 and E-selectin are elevated in the sera of MS patients while soluble vascular cell adhesion molecules VCAM-1 and E-selectin are increased in the CSF of MS patients, thereby promoting the trafficking of activated T cells into the CNS (Dore-Duffy et al., 1995).

Another long-standing diagnostic biomarker for MS is the presence of oligoclonal bands, which represent intrathecally expressed immunoglobin. While the presence of oligoclonal bands clearly suggests neuroinflammation in the CSF, the MS-relevant reactive antigens have not yet been identified. CSF oligoclonal bands are also present in HAM/TSP and in these individuals they have

been found to be chiefly directed against HTLV-I (Jacobson et al., 1990a). More reliable biomarkers that can be assayed in the peripheral blood and CSF of MS patients are still being sought due to the high cost of frequent MRI and its poor reliability to detect neuronal degeneration, axonal loss, and to some extent, spinal cord lesions.

There are many molecules with the potential for more specific clinical diagnostic and prognostic application in MS including auto-antibodies, virus antibodies, transcription factors, molecules in the nitric oxide pathway, neuronal breakdown products, apolipoprotein E (a marker for cognitive dysfunction in AD), molecules in the amyloid precursor protein pathway, cytokines, and chemokines among others (reviewed in (Harris and Sadiq, 2009)). Several biomarkers of MS inflammatory disease activity are also of interest in HAND, including IL-6 and osteopontin, which are both upregulated in HAND and in MS lesions (Burdo et al., 2008; Cannella and Raine, 1995; Frei et al., 1991) and CCL2, which is downregulated during MS exacerbations and increased in HAND (Kelder et al., 1998). Further assessment of these biomarkers and the use of proteomics and microarray technologies to discover new and specific biomarkers for neuroinflammatory diseases will ultimately improve diagnosis and treatment of these disabling neuroinflammatory disorders.

2.5 - Therapeutic Considerations

Given the complexity of the pathogenesis of HIV-associated neurodegeneration, multiple cell types and cellular processes are under investigation as therapeutic targets. Ongoing clinical trials are examining drugs that target overall neuronal survival in addition to reducing neuroinflammation. It is likely that a combination of classes of drugs will be necessary to ameliorate the neurocognitive decline in HIV patients receiving ART.

ART is increasingly effective in reducing morbidities and mortality in HIV-1-infected patients. While ART has significantly decreased the incidence of HAD, presumably by lowering systemic

viral loads, current efforts are focused on improving the penetration of ART past the BBB. Improved penetration or retention of bioavailable ART drugs in the CNS may slow HAND progression by decreasing CNS viral replication and reducing concomitant release of neurotoxins from infected and activated macrophages/microglia (Ellis et al., 2007; Letendre et al., 2008; Spitzenberger et al., 2007). However, a recent study demonstrated that ART regimens with strong CNS penetration and reduced CSF viral loads were associated with poorer neurocognitive outcomes (Marra et al., 2009). While further clinical studies are needed, this study highlights the importance of considering drug toxicity when promoting ART in the CNS. Furthermore, effective therapies for HAND will likely require combination therapy that not only targets viral load, but also addresses the indirect pathways known to contribute to HIV-associated neurodegeneration.

Clinical similarities between HIV-associated neurodegeneration and other neurodegenerative diseases have prompted investigation into currently approved neuroprotective therapies in HAND (Table 2.2). Memantine (Namenda), approved in the treatment for Alzheimer's disease, is a non-competitive NMDA receptor antagonist that also increases levels of brain-derived neurotrophic factor (BDNF) and conserves dopamine function in SIV-infected macaques (Meisner et al., 2008). Both in vitro and in vivo animal studies have shown that memantine inhibits gp120 and Tat-induced neurotoxicity (Anderson et al., 2004; Nath et al., 2000; Toggas et al., 1996). A short-term clinical trial in HAND patients demonstrated that memantine improved neuronal metabolism (as judged by magnetic resonance spectroscopy/MRS), indicative of neuroprotection, but did not cause significant neurocognitive improvement (Schifitto et al., 2007a). Nevertheless, this study suggests a potential beneficial effect of memantine, even following short-term administration, although a longer-term follow-up study in this patient cohort failed to reveal a clinically demonstrable neurological benefit (Zhao et al., 2010).

Selegiline (Deprenyl), a monoamine oxidase B (MAO-B) inhibitor used in the treatment of early-stage Parkinson's disease, has shown some promise in clinical trials for HAND. Selegiline is

proposed to act as a neuroprotectant by reducing the antioxidant burden of the cell (Magyar and Szende, 2004). Early trials with orally or transdermally administered selegiline demonstrated some improvement in psychomotor speed (Sacktor et al., 2000; The Dana Consortium, 1998). However, recent studies have shown neither a reduction in biomarkers of oxidative stress nor evidence of cognitive improvement with short-term (24 weeks) transdermal selegiline (Schifitto et al., 2009a; Schifitto et al., 2007b). While memantine and selegiline have shown some effectiveness, they are clearly not potential monotherapies for HAND. In addition to using such drugs prior to neurocognitive decline, effective neuroprotective therapies will likely have to be used in combination and over the duration of viral infection in order to have maximal clinical benefit.

Table 2.2 Therapeutics under consideration for HAND

Generic (Brand) Name	Molecular Target	Effects/Role	References
Memantine (Namenda)	NMDA receptor antagonist	Increases BDNF levels, conserves dopamine function, inhibits gp120 and Tat-induced neurotoxicity, improves neuronal metabolism, short-term treatment provided no neurocognitive impairment	(Anderson et al., 2004; Meisner et al., 2008; Nath et al., 2000; Schifitto et al., 2007a; Toggas et al., 1996; Zhao et al.)
Selegiline (Deprenyl)	MAO-B Inhibitor	Proposed to reduce antioxidant burden of the cell, may improve psychomotor speed, no clinical evidence of cognitive improvement over 24 week treatment	(Magyar and Szende, 2004; Sacktor et al., 2000; Schifitto et al., 2009a; Schifitto et al., 2007b; The Dana Consortium, 1998)
Sodium valproate and lithium	GSK-3β inhibitor	Reduces neurotoxicity, improved neuropsychological performance	(Ances et al., 2008; Dou et al., 2003; Everall et al., 2002; Letendre et al., 2006; Schifitto et al., 2006; Schifitto et al., 2009b; Tong et al., 2001)
SSRIs – citalopram, paroxetine	Serotonin transporter	May decrease HIV viral levels in CSF, improved adherence to ART	(Ances et al., 2008; Letendre et al., 2007)
Minocycline	5-lipoxygenase and others	Decreases CCL2 levels in CSF, improves encephalitis, suppresses HIV replication and inhibits secretion of TNFα, IFNγ and IL-2 by lymphocytes	(Colovic and Caccia, 2003; Copeland and Brooks, 2010; Si et al., 2004; Szeto et al., 2010; Zink et al., 2005)
PMS-601	platelet-activating factor (PAF) receptor antagonist	Reduces neurotoxicity, microgliosis and TNFα, CCL3, CCL4 and CCL5 secretion by macrophages	(Eggert et al., 2009b; Martin et al., 2000)
Copolymer-1 (Copaxone)		Decreases microgliosis, astrogliosis, neurotoxicity and TNFα and IL-12 levels	(Gorantla et al., 2007; Gorantla et al., 2008)
CEP-1347		Anti-apoptotic, decreases monocyte secretion of TNFα, reduces microgliosis and neurotoxicity	(Bodner et al., 2002; Eggert et al., 2009a; Sui et al., 2006b)

In addition to therapies for neurodegenerative diseases, compounds in clinical use for neuropsychiatric disorders are also under investigation in HAND (Ances et al., 2008). Sodium valproate (VPA) and lithium are approved for treatment of bipolar disorder and related mood disorders, and both inhibit glycogen synthase kinase-3β and provide neuroprotection against HIV-induced toxicity in vitro and in mouse models of HIVE (Dou et al., 2003; Everall et al., 2002; Tong et al., 2001). Several small pilot studies have demonstrated improved neuropsychological performance in HAND patients following short-term VPA or lithium therapy (Letendre et al., 2006; Schifitto et al., 2006; Schifitto et al., 2009a). Additional studies using selective serotonin reuptake inhibitors (SSRIs), including citalopram and paroxetine, are also under consideration as adjunctive therapies for HAND (Ances et al., 2008; Letendre et al., 2007).

Other adjunctive therapies for HAND are focused on targeting inflammation cascades that contribute to neurotoxicity. Minocycline is a broad-spectrum tetracycline antimicrobial that is currently in phase I clinical trials for HAND (Copeland and Brooks, 2010). Minocycline is capable of efficiently crossing the BBB and can suppress HIV replication in microglia, macrophages, and lymphocytes (Colovic and Caccia, 2003; Si et al., 2004; Szeto et al., 2010; Zink et al., 2005). In addition, minocycline has anti-inflammatory properties and can inhibit the secretion of the inflammatory cytokines TNF-α, IFN-γ, and IL-2 by lymphocytes (Szeto et al., 2010). Experimental studies using SIV-infected macaques demonstrated that minocycline decreased CSF levels of CCL2, a marker of CNS inflammation, and decreased the severity of encephalitis (Zink et al., 2005).

In addition to minocycline, several other therapies that have anti-HIV and anti-inflammatory effects are also being considered as adjunctive therapy for HAND. PMS-601, a platelet-activating factor (PAF) receptor antagonist, reduces neurotoxicity, microgliosis, and macrophage secretion of the inflammatory mediators TNF-α, CCL3, CCL4, and CCL5 in vitro and in a mouse model of HIVE (Eggert et al., 2009b; Martin et al., 2000). Copolymer-1 (COP-1 or Copaxone) is a clinically

approved immune modulator used in the treatment of MS. In addition to decreasing levels of TNF-α and IL-12, COP-1 decreases microgliosis, astrogliosis, and neurotoxicity in a mouse model of HIVE (Gorantla et al., 2007; Gorantla et al., 2008). CEP-1347 is an anti-apoptotic immune modulator that decreases monocyte secretion of TNF-α and macrophage secretion of chemokines, including CCL4 and CXCL10 (Eggert et al., 2009a; Sui et al., 2006b). CEP-1347 reduces microgliosis and HIV-mediated neurotoxicity in vitro and reduces signs of HIVE in a mouse model (Bodner et al., 2002; Eggert et al., 2009a; Sui et al., 2006b). As our understanding of the complex nature of HAND pathogenesis evolves, it is becoming clear that adjunctive therapies that address not only viral burden in the CSF but also the contribution of processes such as inflammation are critical for successful clinical management of HAND. In addition, the development of adjunctive therapies for HAND will contribute to and likely improve the clinical management of other neuroinflammatory disorders, including AD, PD, and MS.

CHAPTER 3

MATERIALS AND METHODS

Reagents

Stock solutions of dimethyl fumarate and monomethyl fumarate (Sigma, St. Louis, MO), *tert*-butylhyrdoquinone (tBHQ; Acros Organics/Thermo Fischer Scientific, Geel, Belgium), thapsigargin (Tocris Bioscience, Ellisville, MO), Sal003 (Calbiochem/EMD Biosciences, La Jolla, CA), clotrimazole (Sigma) were prepared in DMSO and stored at -20°C until use. Tin (IV) mesoporphyrin IX dichloride (SnMP), cobalt (III) protoporphyrin IX chloride (CoPP), and hemin (Frontier Scientific, Logan, UT) were prepared in 1N NaOH and stored at -20°C until use. Stock solutions of Ara-C (Sigma), phytohemagglutinin (PHA; Sigma), TNFα (R&D Systems, Minneapolis, MN) and CCL2 (Peprotech, Rocky Hill, NJ) were prepared in filter-sterilized distilled water and stored at -20°C. Stock solutions of efavirenz (NIH AIDS Research and Reference Reagent Program, Germantown, MD) were prepared in DMSO and frozen at -80°C until use.

Isolation and culture of human monocyte-derived macrophages (MDM)

All human studies were reviewed and approved by the Institutional Review Board at the University of Pennsylvania. Human monocytes were prepared from PBMCs of healthy donors and isolated by Ficoll density gradient centrifugation as previously described (Chen et al., 2002; O'Donnell et al., 2006). Monocytes were plated at 1×10^6 cells per well to Cell-Bind 6-well plates (Corning, Lowell, MA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10% horse serum, 1% non-essential amino acids with 50 U/mL penicillin/streptomycin at 37°C, 6% CO_2. Cells were cultured for 7-8 days and visually inspected for MDM differentiation before use in HIV-infection experiments. MDM were cultured for 7-10 days before use in non-infectious experiments.

HIV infection of MDM

MDM were pretreated with efavirenz, MMF, DMF or tBHQ, at the indicated concentrations, for 1 hour. All wells were normalized for the vehicles appropriate for drug treatments (DMSO and/or NaOH). Differentiated MDM were exposed to 50ng (p24 ELISA, equivalent to 1.82 ± 0.22

kcpm/μL by reverse transcriptase (RT) activity assay) of HIV-1 Jago (R5 strain) or 89.6 (R5/X4 strain) for 24 hours. HIV-Jago is a macrophage tropic, CSF isolate from a patient with confirmed HIV-associated dementia (Chen et al., 2002). Virus stocks were prepared by the University of Pennsylvania Center for AIDS Research Virology Core. For some experiments, HIV/MDM were exposed to drug treatments, or appropriate vehicle controls, for 6 (0.5μM thapsigargin) or 24 hours (10μM clotrimazole or 50μM Sal003) beginning at day 6 post infection. Following drug exposure, wells were washed x1 with DMEM before cells were put into fresh, drug-free media for the remainder of the infection time course. Supernatants from HIV-infected or non-infected (Mock) MDM were collected every 2-4 days and stored at -80°C. Supernatants were monitored for HIV replication by quantifying viral RT activity, as analyzed by the amount of radiolabeled deoxythymidine incorporation.

Subcellular fractionations and Western blot analysis

For whole cell lysate collection, cells were rinsed twice with ice-cold PBS and lysed in 75mM Tris-HCl (pH 6.8), 15% glycerol, 3.75mM EDTA, 3% SDS and supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science).

To assess for nuclear translocation of NF-κB proteins, differentiated MDM were treated with DMF for 24 hours, exposed to TNFα (1 ng/mL) for 10 minutes and fractionated. To prepare nuclear extracts, cells were rinsed twice in ice-cold PBS and lysed on ice for 10 minutes in 10mM HEPES (pH 7.9), 10mM KCl, 10mM EDTA, 1mM DTT, 0.4% Nonidet P-40, supplemented with protease and phosphatase inhibitors. Nuclei were pelleted for 3 minutes at 16,000 × *g* and the supernatant (cytoplasmic fraction) was collected and stored at -20°C. The nuclear pellet was resuspended in 20mM HEPES (pH 7.9), 400mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT, protease and phosphatase inhibitors and incubated at 4°C on a rocking platform at 200rpm for 2 hours. After centrifugation at 16,000 × *g* for 5 minutes, supernatants (nuclear fractions) were collected and

stored at -20°C. All protein concentrations were determined by the Detergent Compatible (*DC*) protein assay (Bio-Rad Laboratories, Hercules, CA).

Cell lysates were subjected to SDS-PAGE as previously described (O'Donnell et al., 2006) using the following antibodies: rabbit anti-HO-1 (Stressgen/Enzo Life Sciences, Farmingdale, NY), mouse anti-NQO1 (Abcam, Cambridge, MA), mouse anti-Nrf2 (R&D Systems), rabbit anti-RelB (Cell Signaling Technologies, Danvers, MA), rabbit anti-NF-κB p65 (Cell Signaling), rabbit anti-NF-κB p50 (Cell Signaling), rabbit anti-poly (ADP-ribose) polymerase (PARP) (Cell Signaling), rat anti-GRP94 (Stressgen), mouse anti-BiP (BD Transduction Laboratories/BD Biosciences, Franklin Lakes, NJ), rabbit anti-phosphorylated eIF2α (Invitrogen, Carlsbad, CA), mouse anti-eIF2α (Cell Signaling), rabbit anti-β-tubulin (Cell Signaling), mouse anti-GAPDH (Advanced Immunochemical, Long Beach, CA), and species-specific HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA or Cell Signaling). For densitometry analysis, films were scanned and a fixed cursor area centered over each band was assessed for pixel density using ImageJ (NIH, Bethesda, MD).

MDM-mediated neurotoxicity

Rat cerebrocortical neuronal cultures were prepared from embryos of Sprague-Dawley rats at day 17 of gestation, as previously described (O'Donnell et al., 2006). All procedures were within the ARRIVE guidelines for animal research, and in accordance with protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Cells were plated in tissue culture dishes pre-coated with poly-L-lysine (Peptides International, Louisville, KY) and maintained in neurobasal media plus B27 supplement (Invitrogen) at 37°C and 5% CO_2. Forty-eight hours after plating, cells were treated with 10μM Ara-C. After 7 days *in vitro* (DIV), approximately one-half volume of fresh media was added to the cells in order to counteract effects of evaporation. All cultures were used between 14 and 16 DIV.

Cell-based microtubule-associated protein 2 (MAP2) ELISAs were performed on primary rat cerebrocortical cells plated at a density of 6×10^4 cells per well in 96-well plates. Following a 24 hour exposure to HIV/MDM supernatant, cultures were fixed and fluorescently labeled as described (Wang et al., 2007; White et al., 2011) using the following reagents: mouse anti-MAP2 (Covance, Princeton, NJ), goat anti-mouse β-lactamase TEM-1 conjugate (Invitrogen), and Fluorocillin Green substrate (Invitrogen). Fluorescence intensity was measured using a fluorometric plate reader with the 480/520 nm filter set. Macrophage supernatant was applied at a 1:10-1:50 dilution; the dilution that gave values within the linear range of the assay is presented.

Immunofluorescence

Primary rat cerebrocortical cells were plated at a density of 2×10^5 cells per 35mm dish with glass coverslips. Following exposure to HIV/MDM supernatant for 24 hours, cultures were fixed and fluorescently labeled as described (O'Donnell et al., 2006) using the following reagents: mouse anti-MAP2 (Sigma) and species-specific Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories), and Hoescht 33342 (Invitrogen).

LDH assay

Soluble lactate dehydrogenase (LDH) in HIV/MDM culture supernatant was measured using the Cytotoxicity Detection KitPLUS (Roche Applied Science) according to manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA)

Following 24 hours of pretreatment with DMF, human MDM were exposed to 1 ng/mL TNFα for 10 minutes and nuclear protein extracts were isolated as described. 8μg of nuclear protein was assessed for NF-κB-DNA binding with an EMSA kit (Panomics, Santa Clara, CA), used according to manufacturer's directions. The labeled oligonucleotide for NF-κB p50 binding, 5'-AGTTGAGGGGACTTTCCCAGGC-3', was used.

Cytokine detection in culture supernatants

The concentration of TNFα in culture supernatants was detected using an ELISA kit (Invitrogen) and used according to manufacturer's instructions. Uninfected MDM were treated with 0.067% DMSO (vehicle), DMF or tBHQ for 24 hours prior to exposure to 10 µg/mL PHA for 6 hours. Supernatants were collected and frozen at -80°C until assayed.

Chemotaxis assay

Monocyte chemotaxis was assayed using the Chemicon QCM 96-well (5µM pore size) Migration kit (Millipore, Temecula, CA) according to manufacturer's directions. Freshly isolated human monocytes were plated at a density of $2x10^5$ cells/well in serum- and growth factor-free culture media to the upper chamber in the presence of DMF, MMF or vehicle (0.02% DMSO). CCL2 (300 ng/mL) was added to the lower chamber and cells were incubated at 37°C and 6% CO_2 for 6 hours (Chiou et al., 2011; Eugenin et al., 2006; Janic et al., 2008). Exposure of monocytes to 300 ng/mL CCL2 for 6 hours most consistently induced chemotaxis, with an average of 35.3 ± 20.2% above baseline. All cells that had migrated through the insert, including those adhered to bottom of the membrane, were collected. For quantification, cells were lysed and labeled with CyQuant GR dye. Fluorescence was read with the 480/520 nm filter set on a fluorometric plate reader.

Flow cytometry

Human PBMCs were cultured in RPMI supplemented with 10% FBS and 50 U/mL penicillin/streptomycin at 37°C and 5% CO_2. Following 6 or 36 hours of treatment with the indicated concentrations of DMF or DMSO vehicle, cells were washed with ice-cold FACS buffer (PBS, 1% BSA, 0.1% NaN_3) and stained with CD11b-PE (clone ICRF44, eBioscience, San Diego, CA), CD14-PE/Cy7 (M5E2, BioLegend, San Diego, CA), CCR2-PerCP/Cy5.5 (TG5, BioLegend), CD4-FITC (OKT4, BioLegend), CD195/CCR5-Alexa Fluor 700 (HEK/1/85a, BioLegend) and CD184/CXCR4-PerCP/Cy5.5 (12G5, BioLegend) antibodies. Mouse IgG2a-PerCP/Cy5.5 (MOPC-173, BioLegend) was used as the isotype control for CCR2 staining. Antibody-stained cell

suspensions were pretreated with 4'6'-diamidino-2-phenylindole (DAPI) to identify dead cells. Flow cytometry was performed on a LSR-II (BD Biosciences). Doublets were excluded using forward side scatter-height versus forward side scatter-width and side scatter-height versus side scatter-width parameters. Data were analyzed using FlowJo (Tree Star, Ashland, OR). Monocytes were identified as $CD11b^{+}CD14^{+}$ cells.

Indirect fluorescence assay

For immunofluorescent staining, paraffin-embedded tissue sections from the frontal cortices of control (*n* = 2) and HIV(+) (*n* = 12) human autopsy cases were obtained from the National Neuro-AIDS Tissue Consortium. Glass slides containing paraffin-embedded sections (5 μm) were deparaffinized and rehydrated as described previously (Chalovich et al., 2005). Endogenous peroxidase activity was inactivated using 3% H2O2 in methanol. Antigen unmasking was performed by target retrieval solution (Dako Corporation, Carpinteria, CA) at 95°C for 1 h. Tissue sections were then blocked with 10% normal goat serum in PBS. Mouse monoclonal antibodies to BiP (1:150, BD BioSciences) and the macrophage/microglia marker, HLA-DR (MHC class II protein, clone CR3/43,1:1000, Sigma) were used in IFA for macrophagic BiP. Rabbit polyclonal antibodies to phosphorylated eIF2α (1:1000, Invitrogen) and the macrophage/microglia marker, HAM56 (human alveolar macrophage, clone 56, 1:100, Dako Corporation) were used in IFA for macrophagic phosphorylated eIF2α. The tyramide amplification system (New England Biolabs, Beverly, MA) was used according to manufacturer's directions for the detection of HLA-DR and phosphorylated eIF2α. DNA was visualized by DAPI staining (5 mM, Molecular Probes, Carlsbad, CA). Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and analyzed by laser confocal microscopy on a Bio-Rad Radiance 2100 equipped with Argon, Green He/Ne, Red Diode and Blue Diode lasers (Bio-Rad), as described previously (Strachan et al., 2005). All images shown were captured with uniform threshold and intensity settings.

Post-acquisition analysis for immunofluorescent staining was performed using MetaMorph 6.0 image analysis software (Universal Imaging, Inc, Downingtown, PA, USA). Eight 212 μm × 212 μm images were captured randomly from the areas of positive staining in the midfrontal cortical grey matter for each case. HLA-DR, HAM56 and phosphorylated eIF2α area was determined by the measurement of the number of the pixels positive for the desired signal. Total macrophage specific BiP was determined by the measurement of integrated pixel intensity of BiP over pixels positive for HLA-DR, where integrated pixel intensity is defined as total pixel intensity per image times the area of pixels positive for the signal. Total HAM56 and phosphorylated eIF2α intensity was determined by the measurement of integrated pixel intensity for HAM56 or phosphorylated eIF2α per image, where the integrated pixel intensity is defined as total pixel intensity per image times the area of pixels positive for the signal. Total macrophage specific phosphorylated eIF2α was determined by the measurement of integrated pixel intensity of phosphorylated eIF2α over pixels positive for HAM56. Data is also presented for phosphorylated eIF2α colocalization normalized to the area of pixels positive for HAM56 (macrophagic phosphorylated eIF2α intensity per HAM56 area), to account for differences in HAM56 expression due to effects of disease.

Statistics

All quantifications are expressed as mean ± standard error of mean. Statistical comparisons were made by Student's *t*-test, one-way ANOVA plus Newman-Keuls *post hoc* test or *post hoc* test for linear trend, as indicated. All graphs were generated and statistical analyses were performed using GraphPad Prism software (San Diego, CA), and values of $p<0.05$ were considered significant.

Table 3.1 Summary of case data for autopsy tissue obtained from the National Neuro-AIDS Tissue Consortium brain bank for BiP and HLA-DR staining.

Case	HIV	Gender	Age (yrs)	Post mortem interval (hrs)	Neurocognitive diagnosis
HIV1	+	M	44	8.5	HAND
HIV2	+	M	33	20	HAND
HIV3	+	M	51	5	HAND
HIV4	+	M	33	6	HAND
HIV5	+	F	31	9	HAND
HIV6	+	M	49	12	HAND
HIV7	+	N/A	46	2.75	Normal
HIV8	+	N/A	45	13	Normal
HIV9	+	N/A	44	16.4	Normal
HIV10	+	N/A	34	5	HAND
HIV11	+	N/A	32	14	HAND
HIV12	+	N/A	57	5.5	HAND
Control1	-	M	44	21.5	Normal
Control2	-	M	52	17.5	Normal

Table 3.2 Summary of case data for autopsy tissue obtained from the National Neuro-AIDS Tissue Consortium brain bank for phosphorylated eIF2α and HAM56 staining.

Case	HIV	Gender	Age (yrs)	Post mortem interval (hrs)	Neurocognitive diagnosis
HIV1	+	M	35	9.25	HAND
HIV2	+	F	34	5	HAND
HIV3	+	M	57	5.5	HAND
HIV4	+	M	32	14.5	HAND
HIV5	+	M	38	5.5	HAND
HIV6	+	M	32	14	HAND
HIV7	+	M	36	2.5	HAND
HIV8	+	M	37	11.5	HAND
HIV9	+	M	31	8.83	HAND
HIV10	+	M	49	67.33	HAND
HIV11	+	M	42	27.33	HAND
HIV12	+	M	50	21	HAND
HIV13	+	M	43	unknown	HAND
HIV14	+	M	50	18	Normal
HIV15	+	M	46	2.75	Normal
Control1	-	M	46	27.65	Normal
Control2	-	F	47	19.18	Normal
Control3	-	M	40	14.66	Normal
Control4	-	F	51	21.75	Normal

CHAPTER 4

THE ANTIOXIDANT RESPONSE ATTENUATES HIV REPLICATION AND MACROPHAGE-MEDIATED NEUROTOXICITY[2]

[2] This work was originally published in *The Journal of Immunology*. Cross SA, Cook DR, Chi AWS, Vance PJ, Kolson LL, Wong BJ, Jordan-Sciutto KL, Kolson DL. 2011. Dimethyl fumarate, an immune modulator and inducer of the antioxidant response, suppresses HIV replication and macrophage-mediated neurotoxicity; a novel candidate for HIV-neuroprotection. J. Immunol. Vol. 187 (10): pp-pp.

Abstract

Despite antiretroviral therapy (ART), HIV infection promotes cognitive dysfunction and neurodegeneration through persistent inflammation and neurotoxin release from infected and/or activated macrophages/microglia. Furthermore, inflammation and immune activation within both the central nervous system (CNS) and periphery correlate with disease progression and morbidity in ART-treated individuals. Accordingly, drugs targeting these pathological processes in the CNS and systemic compartments are needed for effective, adjunctive therapy. Using our *in vitro* model of HIV-mediated neurotoxicity, in which HIV infected monocyte-derived macrophages (MDM) release excitatory neurotoxins, we show that HIV infection dysregulates the macrophage antioxidant response and reduces levels of heme oxygenase-1 (HO-1). Furthermore, restoration of HO-1 expression in HIV-infected MDM reduces neurotoxin release without altering HIV replication. Given these novel observations, we have identified dimethyl fumarate (DMF), used to treat psoriasis and showing promising results in clinical trials for multiple sclerosis, as a potential neuroprotectant and HIV disease-modifying agent. DMF, an immune modulator and inducer of the antioxidant response, suppresses HIV replication and neurotoxin release. Two distinct mechanisms are proposed; inhibition of NF-κB nuclear translocation and signaling, which could contribute to the suppression of HIV replication, and induction of HO-1, which is associated with decreased neurotoxin release. Finally, we found that DMF attenuates CCL2-induced monocyte chemotaxis, suggesting that DMF could decrease recruitment of activated monocytes to the CNS in response to inflammatory mediators. We propose that dysregulation of the antioxidant response during HIV infection drives macrophage-mediated neurotoxicity and that DMF could serve as an adjunctive neuroprotectant and HIV disease modifier in ART-treated individuals.

Introduction

HIV-1 infection of the central nervous system (CNS) can result in cognitive, motor, and behavioral abnormalities, collectively known as HIV-associated neurocognitive disorders (HAND) (Antinori et al., 2007; McArthur et al., 2010). Early in the course of infection, HIV traffics into the brain via infected monocytes and lymphocytes (Dunfee et al., 2006) and despite antiretroviral therapy (ART) persists in parenchymal microglia and perivascular macrophages (Ho et al., 1985; Koenig et al., 1986; Petito et al., 1986). HIV infection of the CNS results in the immune activation of resident glia, and because HIV cannot infect neurons, neuronal damage is mediated by neurotoxins released by these infected and/or activated macrophages, microglia and astrocytes. Although the severity of HAND has been significantly reduced through the widespread use of ART, the prevalence and associated morbidity remain high (~50%) (Robertson et al., 2007; Sacktor et al., 2002). The persistence of HAND in individuals effectively controlled for systemic viral load is incompletely explained, although recent evidence suggests that prolonged inflammation in both the CNS and periphery may be responsible (Ancuta et al., 2008; Brenchley et al., 2006b; Eden et al., 2007).

Chronic systemic inflammation is tightly linked to morbidity and mortality in ART-treated patients, which suggests that adjunctive anti-inflammatory drugs or immune modulators may improve clinical outcomes. Despite undetectable plasma viral loads, measures of systemic inflammation correlate to cerebral spinal fluid (CSF) immune activation, CNS inflammation and HAND (Ancuta et al., 2008; Brenchley et al., 2006b; Eden et al., 2007). It has been proposed that elevated peripheral inflammation mediates neurocognitive decline by increasing the transendothelial migration of infected and/or activated monocytes into the brain (Ancuta et al., 2008; Persidsky et al., 1997). An increased number of microglia and macrophages in the CNS correlates with the severity of pre-mortem HAND, demonstrating the importance of these cell types in mediating neurological impairment (Anthony et al., 2005; Glass et al., 1995; Petito et al., 1986). Some of the most striking evidence linking peripheral inflammation to HAND derives from the strong

association between early and persistent damage caused to gut-associated lymphoid tissue (GALT) by HIV infection (or SIV infection in macaques), increased microbial translocation, systemic immune/monocyte activation and HAND progression (Ancuta et al., 2008; Brenchley and Douek, 2008; Brenchley et al., 2006a; Brenchley et al., 2006b). Therefore, reducing inflammation in the periphery as well as within the CNS is expected to improve neurocognitive impairment in HIV-infected patients.

Fumaric acid esters (FAEs), including dimethyl fumarate (DMF) and its primary *in vivo* metabolite monomethyl fumarate (MMF), are a class of compounds that have anti-inflammatory and immune modulating effects *in vitro* and *in vivo*. Fumaderm, a formulation of DMF and other FAEs, has been used in Europe since 1995 as an effective treatment for psoriasis; its mechanism of action is attributed to modulation of T cell activation and infiltration into plaques (Hoxtermann et al., 1998). DMF is currently under investigation for use in multiple sclerosis (MS) and a recently completed Phase III study demonstrated a significant benefit in suppressing relapses, disease progression and brain lesion inflammation (2011). Using the rodent model of MS, experimental allergic encephalomyelitis (EAE), it was shown that DMF reduces the recruitment of monocytes into areas of active demyelination in the brain (Schilling et al., 2006). In *in vitro* model systems, DMF has been shown to inhibit pro-inflammatory cytokine production and NF-κB signaling via inhibition of nuclear translocation (Loewe et al., 2002; Schilling et al., 2006; Seidel et al., 2009; Stoof et al., 2001). Furthermore, DMF induces the expression of Nrf2-driven antioxidant response genes, including heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) (Lehmann et al., 2007; Linker et al., 2011). Notably, induction of HO-1 expression in human monocytes by hemin has been associated with suppression of HIV-1 replication (Devadas and Dhawan, 2006).

Because HIV replication can be strongly driven by NF-κB activation and nuclear translocation, we hypothesized that DMF treatment of HIV-infected monocyte-derived macrophages (HIV/MDM)

would result in attenuation of HIV replication, immune activation and neurotoxin production. Our *in vitro* system models macrophage-mediated neurotoxicity during HIV infection by utilizing human MDM and rat cerebrocortical neuronal cultures. In this system, HIV infection of MDM results in the release of low molecular weight excitotoxins that injure neurons through excessive activation of N-methyl-D-aspartate (NMDA) receptors (Kaul et al., 2005; Lipton, 2004; O'Donnell et al., 2006). In this study we demonstrate that DMF attenuates HIV replication, nuclear translocation of NF-κB subunits and TNFα production in human MDM. Furthermore, supernatants from DMF and MMF-treated HIV/MDM cultures are markedly less neurotoxic to primary neurons than those from non-treated HIV/MDM cultures. Suppression of neurotoxin production is mediated by induction of HO-1 in HIV/MDM, and this suppression of neurotoxin production can occur even without suppression of HIV replication. Finally, DMF and MMF also reduce CCL2-induced chemotaxis in human monocytes. This study demonstrates that DMF inhibits key steps in HAND pathogenesis through distinct effects on HIV replication and macrophage-mediated neurotoxin production and DMF should be considered as an adjunctive therapeutic for ameliorating the neurological complications of HIV infection.

Results

DMF and MMF inhibit HIV replication in human MDM

Dimethyl fumarate (DMF), and its *in vivo* primary metabolite monomethyl fumarate (MMF), inhibit NF-κB signaling, suppress the production of inflammatory mediators and induce an antioxidant response in a variety of cell types (Lehmann et al., 2007; Loewe et al., 2002; Schilling et al., 2006; Seidel et al., 2009; Stoof et al., 2001; Vandermeeren et al., 1997; Vandermeeren et al., 2001). NF-κB signaling has been established as a major pathway of HIV transcriptional regulation, and recent studies have implicated the antioxidant response enzyme, HO-1, as a negative regulator of HIV replication in monocytes (Devadas and Dhawan, 2006; Devadas et al., 2010). Therefore, we hypothesized that DMF could modulate HIV replication in human macrophages through one or both of these mechanisms. Human MDM were treated with DMF or MMF and then examined for virus replication. As shown in Figure 4.1, exposure of MDM to DMF (A) or MMF (B) attenuated HIV replication in a dose-dependent manner, as determined by culture supernatant reverse transcriptase levels. Suppression of replication in MDM was seen with the R5 CSF HIV strain, Jago (Figure 4.1) and the prototypic R5/X4 strain, 89.6 (Figure 4.2). As shown in Table 4.1, HIV replication was inhibited by an average of approximately 30% at MMF concentrations achieved *in vivo* after single dose administration (4.4μM in CSF and 6.5μM in plasma) (Linker et al., 2011; Litjens et al., 2004). No drug toxicity was detected at concentrations up to 100μM in HIV-infected MDM (HIV/MDM) (Figure 4.1C, 1D) and non-infected MDM (data not shown). DMF demonstrated additive effects in attenuating HIV replication when used in combination with efavirenz, a non-nucleoside reverse transcriptase inhibitor (Figure 4.3A). There was no observed cellular toxicity when DMF was used in combination with efavirenz (Figure 4.3B).

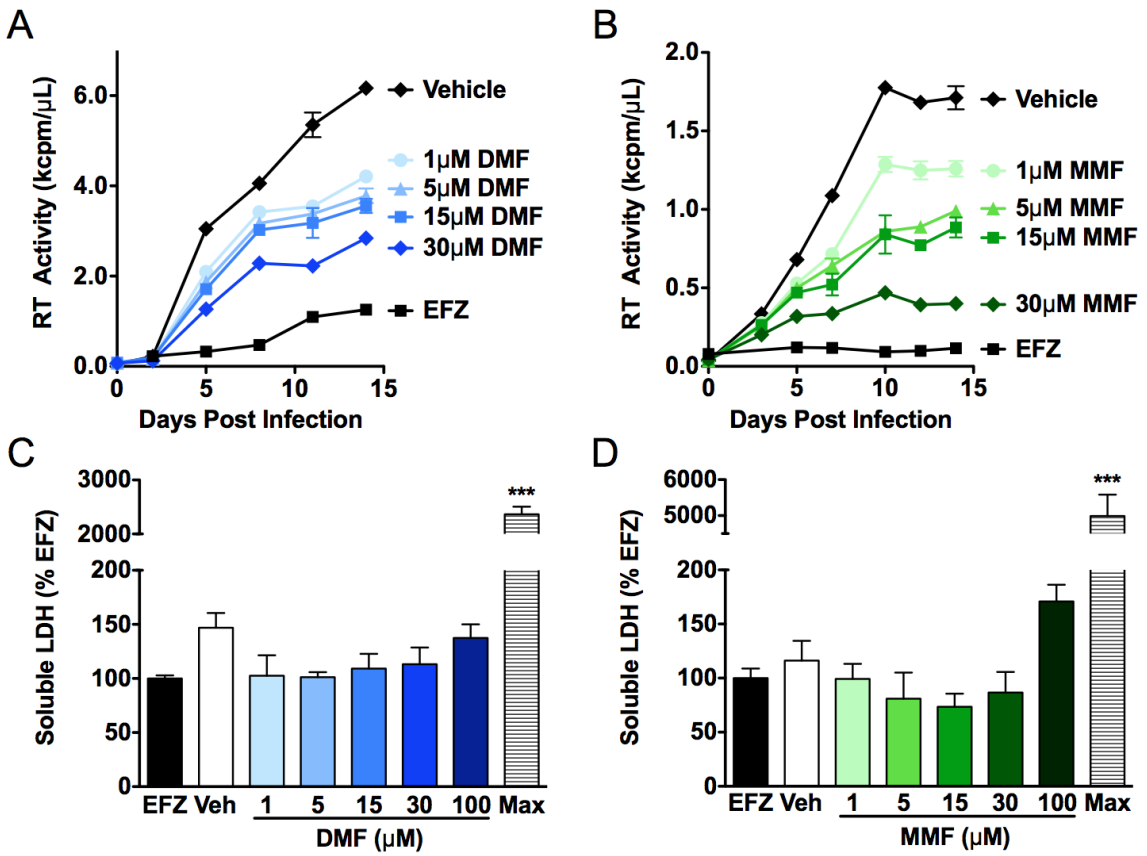

Figure 4.1 Dimethyl fumarate and monomethyl fumarate attenuate HIV replication in human MDM. Human MDM infected with 50ng HIV (p24 ELISA, equivalent to 1.82 ± 0.22 kcpm/µL by reverse transcriptase (RT) activity assay) were treated with DMF (**A**) or MMF (**B**) over the course of infection at the indicated concentrations (1-30µM) or with 20nM of the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ). Culture supernatants were collected every 2-3 days, as indicated, and HIV replication was quantified by RT activity. **C)** DMF and **D)** MMF cause no cytotoxicity in HIV/MDM as assessed by LDH assay of supernatants harvested at day 14 post infection. Maximum (Max) LDH release represents the soluble LDH release following cell lysis. RT curves are representative of 3-4 independent experiments, with each replicate performed on cell preparations from different donors. LDH assays represent data averaged from 3-5 individual donors. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, ***p<0.001 vs. EFZ.

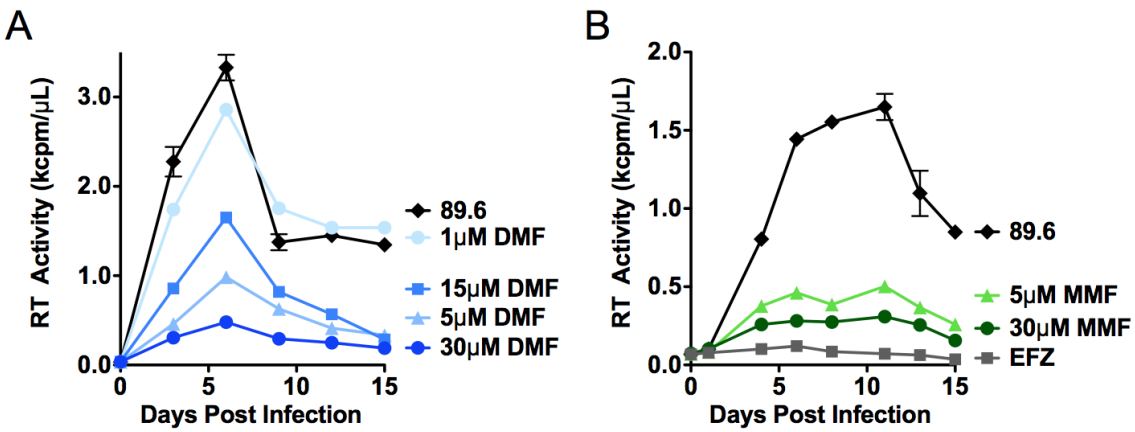

Figure 4.2 Dimethyl fumarate attenuates 89.6 HIV replication in human MDM. Human MDM infected with HIV-89.6 were treated with DMF (**A**) or MMF (**B**) over the course of infection at the indicated concentrations (1- 30µM) or with the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ). Culture supernatants were collected every 2-4 days, as indicated, and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 2-3 independent experiments, with each replicate performed on cell preparations from different donors.

Table 4.1. Reduction in RT activity, relative to vehicle control, with increasing concentrations of DMF and MMF among individual donors.

Donor #	5μM DMF	15μM DMF	30μM DMF
5	16.7 ± 11.0% n =4	27.8 ± 9.8% n =3	64.0 ± 13.4% n =5
36	10.0 ± 13.1% n =3	68.0 ± 3.6% n =1	52.7 ± 15.4% n =3
40	17.1 ± 6.7% n =2	66.0 ± 5.0% n =1	73.4 ± 20.9% n =2
7	59.6 ± 5.0% n =2	65.2 ± 9.2% n =1	60.6 ± 5.7% n =2
26	81.5 ± 12.4% n =1	91.3 ± 6.7% n =1	51.2 ± 15.1% n =3
38	38.7 ± 4.3% n =1	42.3 ± 4.2% n =1	53.9 ± 6.0% n =2
AVG	**26.5 ± 36.0% n =20 (12)**	**45.5 ± 26.4% n =13 (11)**	**55.0 ± 32.3% n =25 (13)**

Donor #	5μM MMF	15μM MMF	30μM MMF
33	29.5 ± 19.2% n =3	36.7 ± 14.6% n =3	66.1 ± 13.9% n =3
40	14.7 ± 21.5% n =2	57.0 ± 8.3% n =2	61.6 ± 8.3% n =2
38	29.5 ± 9.9% n =2	36.7 ± 2.9% n =1	66.1 ± 3.7% n =2
29	33.6 ± 93.9% n =2	76.8 ± 4.3% n =1	86.9 ± 1.7% n =1
AVG	**28.6 ± 32.4% n =12 (7)**	**47.8 ± 27.1% n =10 (7)**	**67.5 ± 20.5% n =12 (7)**

Values were calculated from RT data from the peak of infection and are presented as mean ± SD. For combined average (AVG) data, n = number of replicates averaged (number of unique donors). Only individual donors assayed more than once are included in the table.

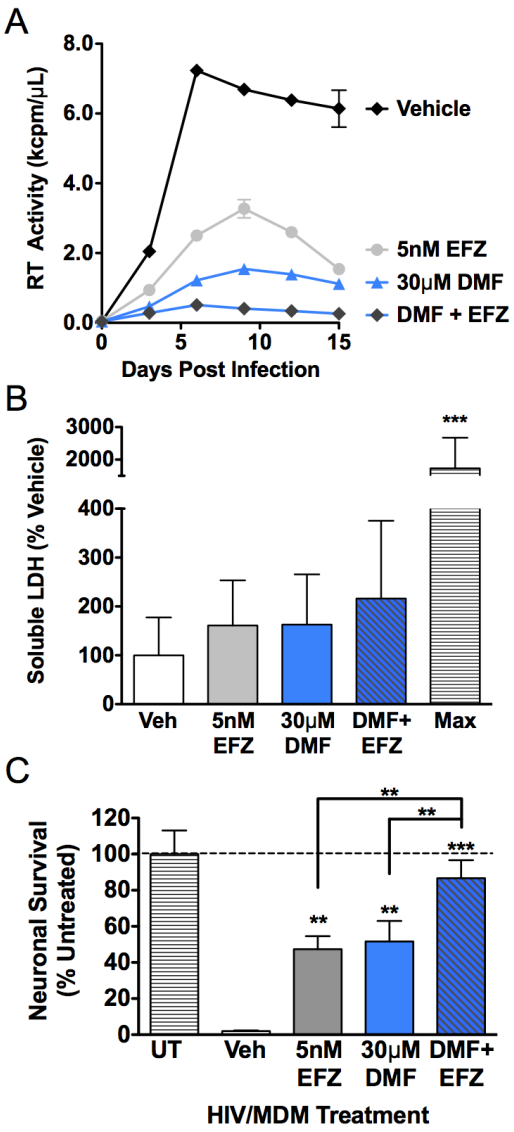

Figure 4.3 Dimethyl fumarate and efavirenz have additive effects on attenuation of HIV replication and neurotoxin production in human MDM. A) DMF and efavirenz (EFZ), a non-nucleoside reverse transcriptase inhibitor, have additive effects on the attenuation of HIV replication in human MDM. MDM were infected with HIV-Jago and treated with DMF and/or EFZ over the course of infection. Culture supernatants were collected every 3 days and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 2 independent experiments, with each replicate performed on cell preparations from a different donor. **B)** DMF and EFZ cause no cytotoxicity in HIV/MDM as assessed by LDH assay of supernatants harvested at day 15 post infection. Maximum (Max) LDH release represents the soluble LDH release following cell lysis. LDH assays represent data averaged from 2 individual donors. **C)** DMF and EFZ used in combination reduce macrophage-mediated neurotoxicity to a greater extent than either drug alone. Neuronal survival was assessed by MAP2 ELISA following exposure to supernatant of HIV-infected macrophages, which were treated with DMF and/or EFZ over the course of infection. MAP2 data is expressed as a percentage of untreated (UT) cultures (n = 6). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, **p<0.01 and ***p<0.001 vs. Vehicle.

DMF and MMF reduce HIV/MDM-mediated neurotoxicity

We and others have shown that HIV-infected MDM release potent neurotoxins that injure neurons through over-activation of N-methyl-D-aspartate receptors (NMDAR) and that this excitotoxicity is mediated by glutamate and other low molecular weight NMDAR agonists (Chen et al., 2002; Jiang et al., 2001; O'Donnell et al., 2006). Although the mechanisms underlying neurotoxin production in HIV/MDM are not fully understood, suppression of HIV replication in MDM generally suppresses such neurotoxicity, as demonstrated by treatment with efavirenz (Figure 4.4). Similarly, in addition to suppressing HIV replication (Figure 4.1), DMF (Figure 4.4A) and MMF (Figure 4.4B) also reduce HIV/MDM neurotoxin production in a dose-dependent manner, as assessed by neuronal survival in our *in vitro* HIV neurotoxicity model. Representative images of HIV/MDM-mediated neurotoxicity and the protective effects of DMF and MMF are shown (Figure 4.4C), where surviving neurons are labeled for MAP2 (microtubule-associated protein 2). DMF and EFZ used in combination resulted in additive effects on the suppression of macrophage-mediated neurotoxicity (Figure 4.3C), demonstrating that DMF may successfully reduce HIV replication and macrophage-mediated neurotoxicity that is not fully suppressed by ART. This neuroprotection is due to drug effects on the macrophages, as DMF and MMF do not prevent HIV/MDM mediated neurotoxicity when applied directly to the neurons prior to addition of HIV/MDM supernatants (data not shown).

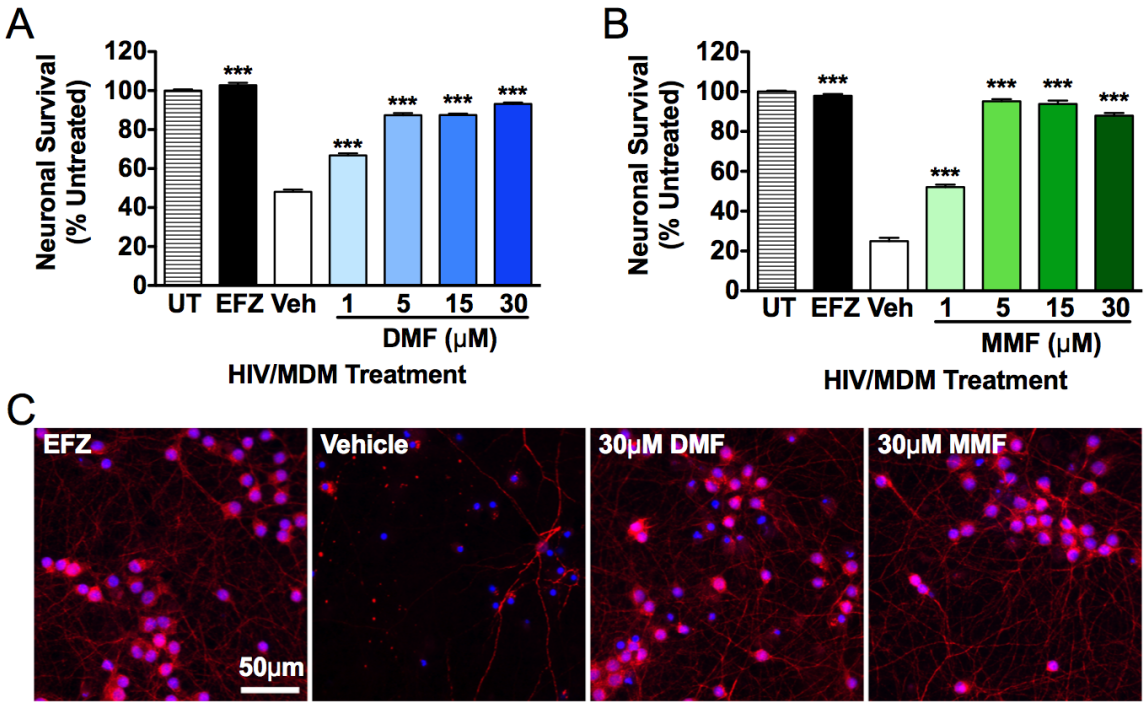

Figure 4.4 DMF and MMF reduce HIV/MDM mediated neurotoxicity. Rat cerebrocortical cultures were exposed to supernatant from HIV-infected macrophages that were treated with DMF (**A**) or MMF (**B**) at the indicated concentrations (1-30μM) during the course of infection. Neuronal survival was assessed by MAP2 ELISA and expressed as a percentage of untreated (UT) cultures (n = 6; ***p<0.001 vs. Vehicle). C) Representative images of rat cerebrocortical cultures immunofluorescently stained for MAP2 (red) and Hoescht 33324 (blue) following 24 hours treatment with the indicated HIV/MDM supernatant. Scale bar represents 50μm. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing.

DMF inhibits NF-κB nuclear entry, DNA binding and TNFα production in human MDM

NF-κB and TNFα are part of a positive feedback loop that regulates the transcriptional activity of the HIV long terminal repeat (LTR). In unstimulated cells, NF-κB is unable to bind DNA due to its association with inhibitory κB (IκB) proteins, which sequester NF-κB in the cytoplasmic compartment (Baldwin, 1996; Ganchi et al., 1992; Henkel et al., 1992). Following exposure to an activating stimulus such as TNFα, NF-κB is rapidly freed from the inhibitory complex and translocates into the nucleus to induce transcriptional activation of viral and host genes. NF-κB proteins are major modulators of the HIV LTR and are among the most potent activators of

proinflammatory and inflammatory genes. Five members of the mammalian NF-κB/Rel family have been described, including c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), and RelB. Functional NF-κB complexes are composed of heterodimer complexes containing p65, c-Rel, or RelB bound to p50 or p52 (Baldwin, 1996; Kilareski et al., 2009; Neumann et al., 2000). Exposure to activating stimuli, such as TNFα, induces the nuclear accumulation of NF-κB proteins, DNA binding by NF-κB p50 and transcription from the HIV LTR (Duh et al., 1989).

To determine if DMF and MMF inhibit the nuclear translocation of NF-κB proteins in MDM, DMF and MMF-treated MDM were stimulated with TNFα and subjected to subcellular fractionations before detection of NF-κB subunits by Western blotting. DMF and MMF each inhibited TNFα-induced nuclear accumulation of RelB, p65 and p50 in a dose-dependent manner (Figure 4.5A, 3B). We also demonstrate that DMF inhibited the formation of the NF-κB p50-DNA complex, as assessed by EMSA (Figure 4.5C). Because NF-κB signaling also induces expression of inflammatory mediators, we assessed the effects of DMF treatment on TNFα release from MDM. In agreement with previous reports of DMF decreasing the release of inflammatory mediators from multiple cell types, including TNFα, IL-1β and IL-6, (Lehmann et al., 2007; Wilms et al., 2010) we found that DMF suppresses release of TNFα from PHA-activated MDM (Figure 4.5D). Furthermore, DMF also markedly suppressed HIV-induced TNFα release from MDM (Figure 4.5E). Thus, DMF and its primary metabolite, MMF, inhibit NF-κB translocation and signaling events that contribute to the positive feedback loop that modulates HIV transcription in infected and activated MDM.

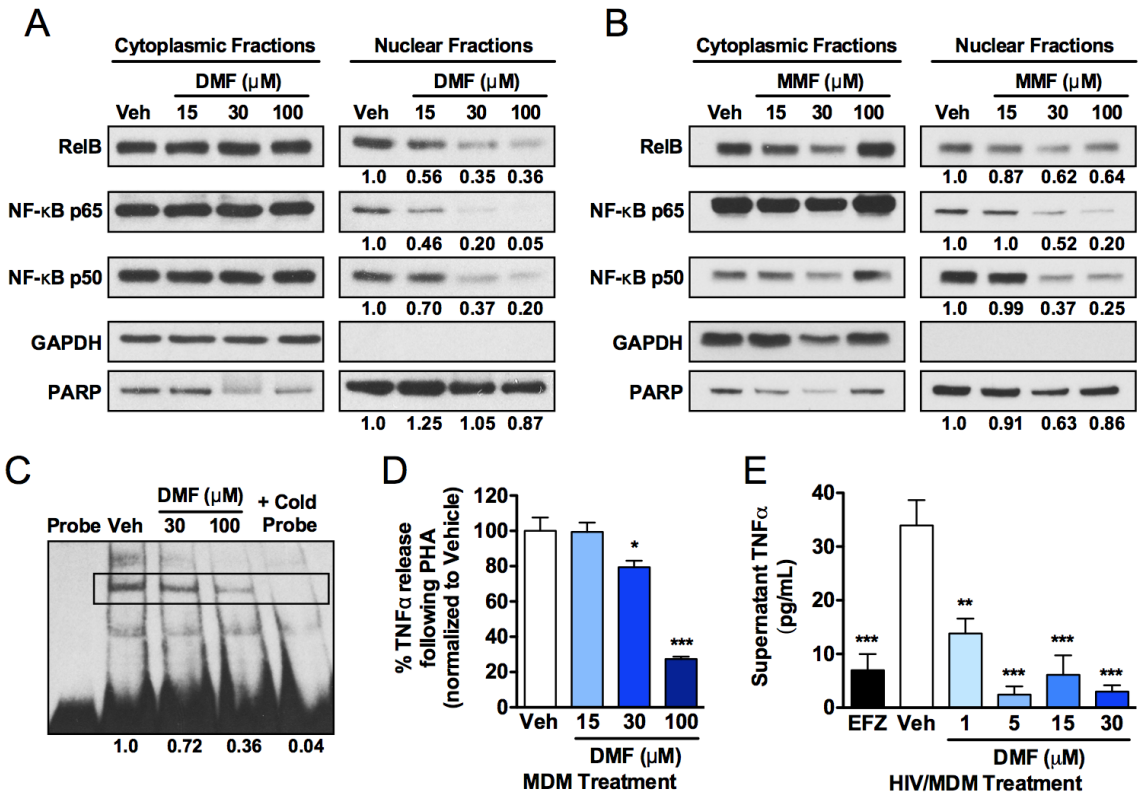

Figure 4.5 DMF inhibits NF-κB nuclear translocation, DNA binding and TNFα production in human MDM. A) DMF and **B)** MMF inhibit the nuclear translocation of the NF-κB proteins RelB, p65 and p50 in human MDM in a dose-dependent manner. Cells were treated with DMF or MMF for 24 hours, exposed to TNFα (10 min), separated into cytoplasmic and nuclear fractions and analyzed by Western blotting. Results of densitometry analysis are presented numerically under each panel as the ratio of NF-κB protein to PARP, a nuclear marker and loading control. Blots are representative of 4-6 independent experiments, with each replicate performed on cell preparations from different donors. **C)** DMF inhibits nuclear NF-κB p50 binding to DNA in TNFα stimulated MDM, as assessed by EMSA. Results of densitometry analysis were normalized to vehicle. **D)** DMF inhibits the production of TNFα in MDM stimulated with PHA (10μg/mL). Values are expressed as percent TNFα production relative to Vehicle treated cells (227 ± 11.9 pg/mL TNFα in Vehicle). Data are expressed as mean ± SEM and represent data averaged from 4 different donors. **E)** TNFα production in HIV/MDM is inhibited by DMF treatment. HIV/MDM were treated with DMF (1-30μM) or 20nM efavirenz (EFZ) over the course of infection and culture supernatants from day 14-15 post infection were assayed for TNFα by ELISA. Values represent the mean ± SEM of data averaged from 5 different donors. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing (*p<0.05, **p<0.01, ***p<0.01 vs. Vehicle).

DMF restores the antioxidant response suppressed by HIV infection in MDM

The antioxidant response is one of the cellular adaptive stress responses that can modulate virus replication and host cell survival, as shown in Hepatitis B and Dengue 2 infection models (Chen et al., 2011; Schaedler et al., 2010). The antioxidant response maintains redox balance and counteracts oxidative damage through induction of proteins that are involved in detoxification of reactive oxygen species (ROS). These proteins are produced by genes with a common promoter element, the antioxidant response element (ARE), and ARE transcription is mediated by Nrf2. Under conditions of low oxidative stress, Nrf2 is kept transcriptionally inactive by Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf2 in the cytoplasmic compartment (Itoh et al., 1999). Following exposure to ROS or electrophiles, Keap1 is degraded by the proteasome and Nrf2 translocates to the nucleus to drive expression of numerous genes including, HO-1, NQO1, glutathione peroxidase 1 (GPX1), and genes responsible for glutathione synthesis (glutamate cysteine ligase modifier, glutamate cysteine ligase catalytic subunit and glutathione synthetase). HIV infection is associated with increased ROS production and depressed levels of glutathione, the major intracellular antioxidant (Dworkin et al., 1986). We observed a marked reduction in the level of HO-1 expression in HIV/MDM across multiple human donors, with a more modest but nonetheless consistent reduction in GPX1 levels (Figure 4.6A, 4B). The effects of DMF on Nrf2 and NQO1 levels were more variable among HIV/MDM cultures from different donors, but trended towards increased expression relative to uninfected Mock/MDM (Figure 4.6A, 4B).

Upon exposure of HIV/MDM to DMF, expression of Nrf2, HO-1, GPX1 and NQO1 increased with increasing doses of DMF (Figure 4.6C), suggesting a restoration of antioxidant responses in HIV-infected MDM. Both HIV infection and DMF increase total levels of Nrf2, suggesting that while HIV infection stabilizes or induces total cellular Nrf2 levels, this is not sufficient for the coordinated transcriptional activation of ARE-regulated genes, such as HO-1 and GPX1. DMF and MMF treatment activates transcription of these ARE-regulated genes in HIV-infected macrophages, possibly by disrupting inhibitory Nrf2-Keap1 interactions (Linker et al., 2011). DMF restores levels

of HO-1 and GPX1 to those observed in uninfected MDM, while NQO1, which is not suppressed during HIV infection, is induced to levels exceeding those in uninfected MDM (Figure 4.6D). We have also confirmed that MMF can induce the antioxidant response in HIV/MDM (Figure 4.6E, 4F) and that both DMF and MMF induce the antioxidant response in uninfected MDM (data not shown). DMF induction of antioxidant responses in MDM occurs independently of HIV infection, which is consistent with previous findings describing induction of the antioxidant response by DMF in multiple cell types, including glia and neurons (Lehmann et al., 2007; Linker et al., 2011).

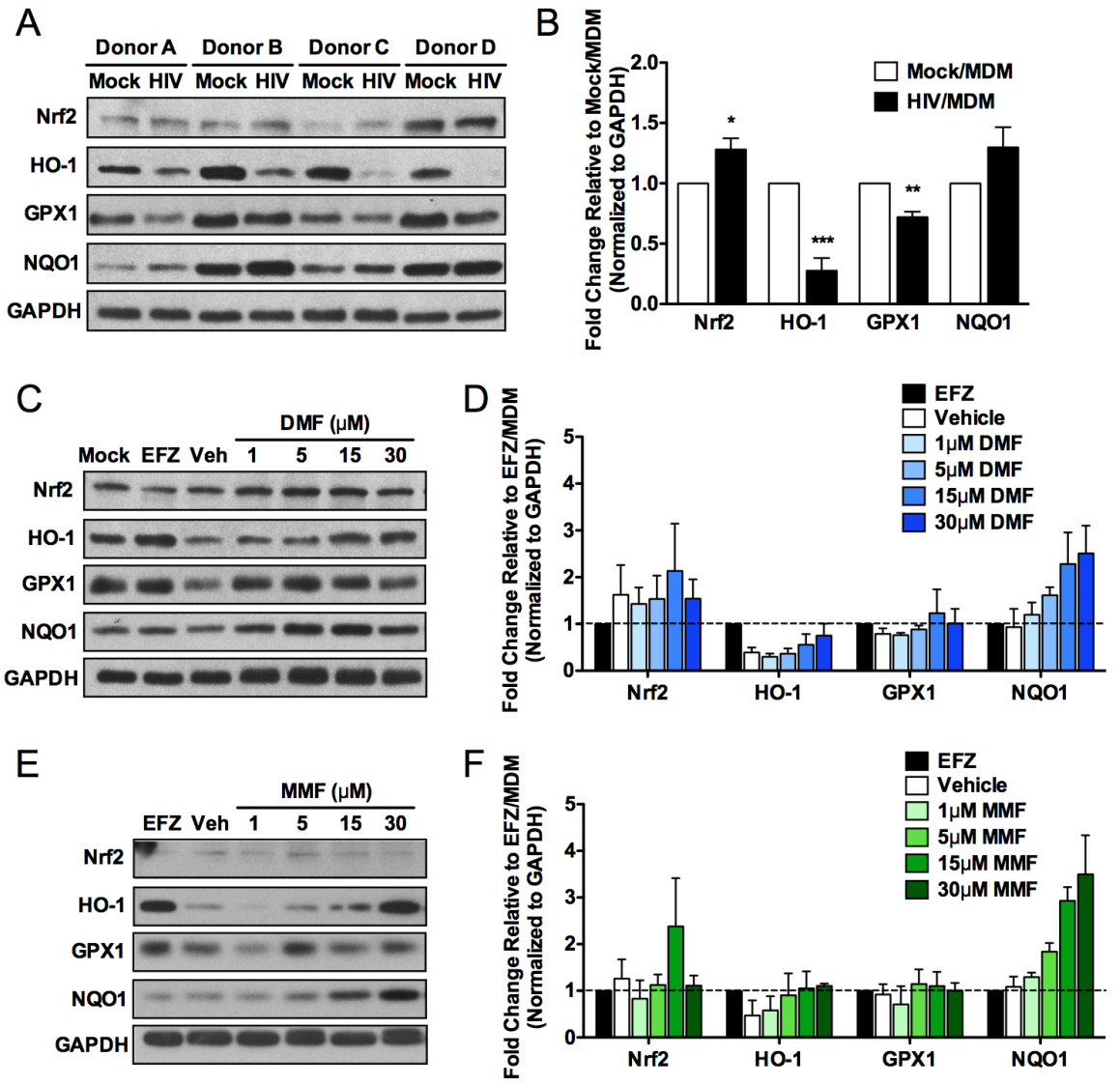

Figure 4.6 DMF restores the imbalance in the antioxidant response caused by HIV infection. HIV infection of human MDM reduces HO-1 and GPX1 expression, as assessed by Western blotting (**A**) and quantified by densitometry analysis (**B**). Values indicate mean ± SEM of 6 different donors. Statistical comparisons were made by two-tailed paired t-test (*p<0.05, **p<0.01 and ***p<0.001 vs. Mock). **C)** DMF activates the Nrf2-dependent antioxidant response in HIV/MDM and restores HO-1 and GPX1 levels to that found in uninfected Mock cells, as quantified by densitometry analysis (**D**). **E)** MMF activates the Nrf2-dependent antioxidant response in HIV/MDM and restores HO-1 and GPX1 levels to that found in uninfected Mock cells, as quantified by densitometry analysis (**F**). Blots are representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. Densitometry data are expressed as mean ± SEM and represent data averaged from 3 different donors.

DMF inhibition of HIV replication and NF-κB signaling is not mediated by HO-1

HIV infection of human MDM results in alterations to the antioxidant response with a striking reduction in HO-1 levels (Figure 4.6A, 4.6B). Induction of HO-1 by hemin has been reported to decrease HIV replication in human monocytes, suggesting that DMF's induction of HO-1 may underlie its antiviral effects (Devadas and Dhawan, 2006). We used a pharmacologic inhibitor of HO-1 enzymatic activity, tin mesoporphyrin (SnMP), to determine the potential role for HO-1 in DMF-mediated suppression of HIV replication and NF-κB translocation. As shown in Figure 4.7A, SnMP had no effect on DMF-mediated HIV suppression, which suggests that DMF does not suppress HIV replication through enhanced HO-1 expression and activity. We found no effect of SnMP on DMF-mediated suppression of HIV replication regardless of donor, level of infection, DMF dose or timing of SnMP addition (data not shown). We also confirmed that SnMP does not inhibit DMF's suppression of TNFα-induced nuclear accumulation of NF-κB (Figure 4.7B). In addition, we show that an inducer of HO-1 expression, cobalt protoporphyrin (CoPP) had no effect on TNFα-induced nuclear accumulation of NF-κB (Figure 4.7B). These results suggest that DMF's induction of HO-1 does not directly suppress HIV replication or NF-κB signaling.

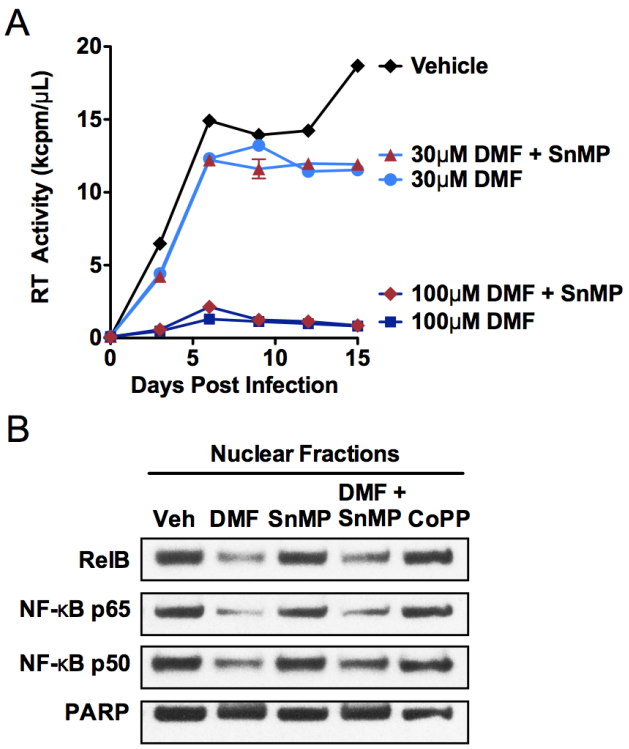

Figure 4.7 HO-1 does not mediate the attenuation of HIV replication or NF-κB signaling induced by DMF. A) SnMP, an inhibitor of HO-1 enzymatic activity, does not inhibit DMF-mediated attenuation of HIV replication. DMF and vehicle treated HIV/MDM were exposed to 10µM SnMP from day 6 through day 15 post infection. Culture supernatants were collected every 3 days and assessed for RT activity. RT curves are representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. **B)** SnMP and CoPP, a specific inducer of HO-1, do not directly affect or alter DMF-mediated inhibition of the nuclear translocation of NF-κB proteins, as assessed by western blotting. Human MDM were treated with 10µM SnMP, 10µM CoPP and/or 100µM DMF for 24 hours before treatment with 1 ng/mL TNFα (10min) and subcellular fractionation. Western blot is representative of 3 independent experiments, with each replicate performed on cell preparations from different donors.

Induction of HO-1 reduces neurotoxin production from HIV/MDM

We sought to determine whether the suppression of HIV/MDM neurotoxin production by DMF (Figure 4.4) was associated with DMF's suppression of HIV replication and/or induction of HO-1 expression. Inhibiting HIV replication in HIV/MDM can suppress neurotoxin release *in vitro*, as demonstrated by efavirenz treatment (Figure 4.4), and similar effects of ART drugs *in vivo* are thought to account for their ability to limit the severity of HAND in ART-experienced cohorts. While previous studies found that increased HO-1 activity is associated with decreased HIV replication in MDM (Devadas and Dhawan, 2006; Devadas et al., 2010), we found that neither inhibition of HO-1 activity by SnMP treatment of MDM (Figure 4.8A) nor induction of HO-1 expression by CoPP (Figure 4.8D, 6F) altered HIV replication. Remarkably, however, SnMP treatment significantly increased the neurotoxicity of MDM supernatant (Figure 4.8B), even when HIV replication was low or absent (Figure 4.8C). The increase in MDM-mediated neurotoxicity was a consequence of inhibiting HO-1 activity in the macrophage since SnMP was not toxic when added directly onto neurons (data not shown). And while CoPP does not attenuate HIV replication or inhibit NF-κB signaling, supernatant from CoPP-treated HIV/MDM is significantly less neurotoxic than untreated controls with a similar level of HIV replication (Figure 4.8E). These studies demonstrate that HO-1 is a critical modulator of neurotoxin production in HIV/MDM and that HO-1 levels can modulate HIV/MDM neurotoxicity without affecting HIV replication.

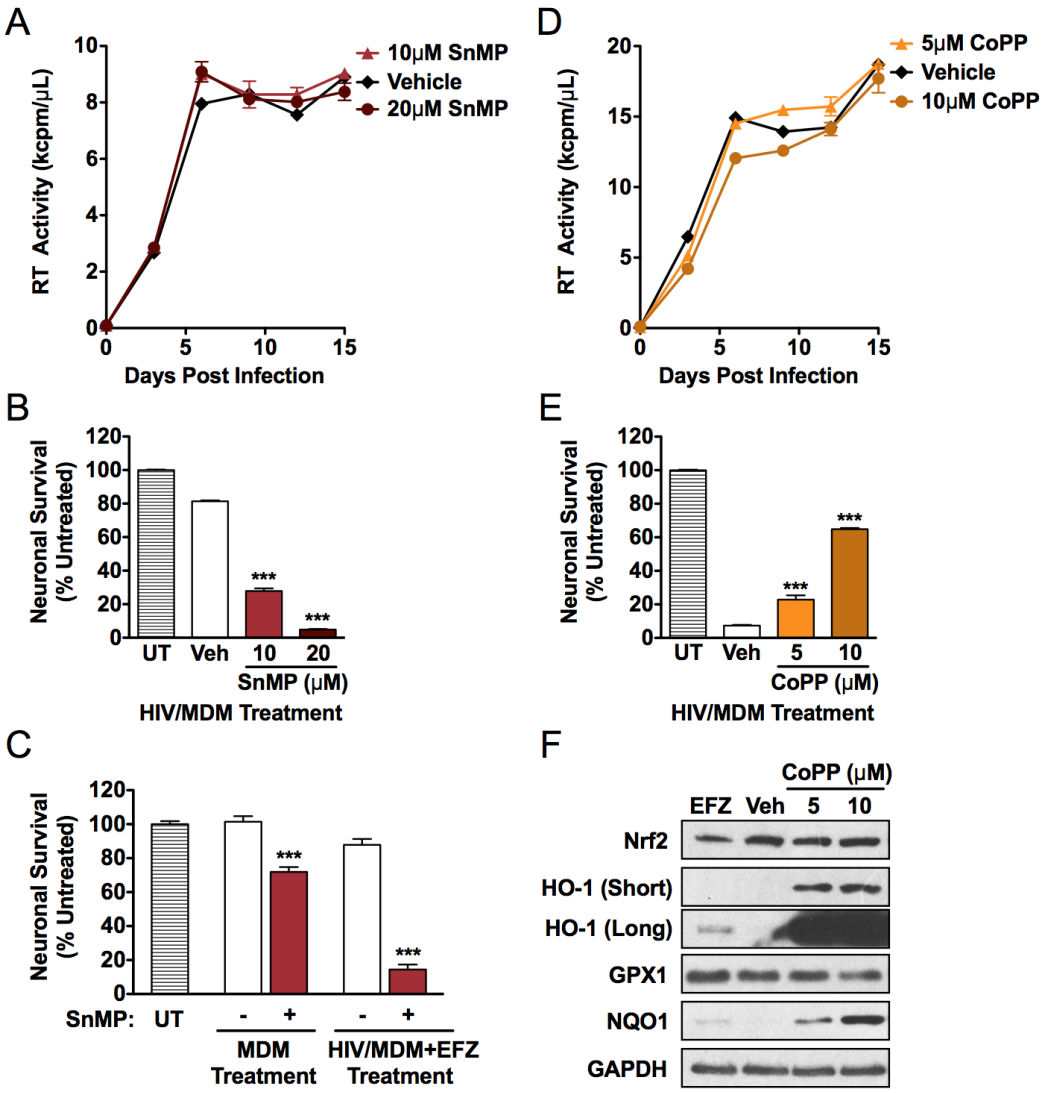

Figure 4.8 HO-1 induction reduces neurotoxin production in HIV/MDM without affecting HIV replication. A) SnMP, an inhibitor of HO-1 enzymatic activity, does not directly affect HIV replication and supernatant from these SnMP treated HIV/MDM are significantly more neurotoxic (**B**), despite equal levels of HIV replication. **C)** Uninfected Mock/MDM and 20nM efaverinz (EFZ) treated HIV/MDM, which normally produce minimal neurotoxins, are significantly more neurotoxic when treated with 10µM SnMP. **D)** CoPP, a specific inducer of HO-1 expression, does not directly affect HIV replication and supernatant from these CoPP treated HIV/MDM are significantly less neurotoxic (**E**), despite high levels of virus replication**. F)** CoPP treatment exponentially increases HO-1 levels without greatly altering the other components of the antioxidant response, as assessed by Western blotting. For A and C, SnMP or CoPP was added at day 6 post infection onwards and culture supernatants were collected every 3 days and assessed for RT activity. RT curves are representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. For neuronal survival assays, survival was assessed by MAP2 ELISA and expressed as a percentage of untreated (UT) cultures (n = 6; ***p<0.001 vs. vehicle treated paired-condition). Statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing. Western blot is representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. Two film exposures (short and long) of HO-1 are presented to demonstrate the extent of HO-1 induction over basal levels.

DMF and MMF inhibit CCL2 induced chemotaxis in human monocytes

The recruitment of activated and infected monocytes to the CNS in response to CCL2 is a key step in the pathogenesis of HAND (Cinque et al., 1998; Zink et al., 2001). In a previous DMF study using the mouse EAE model, DMF reduced macrophage infiltration into the spinal cord in areas of active demyelination (Schilling et al., 2006). We hypothesized that DMF could inhibit chemotaxis of human monocytes in response to chemotactic cytokines, such as CCL2. We found that DMF and MMF inhibited chemotaxis in freshly isolated human monocytes in response to CCL2 in a dose-dependent manner (Figure 4.9A, 7B). Furthermore, we found that DMF reduced the expression of the CCL2 receptor, CCR2, in freshly isolated human $CD11b^{+}CD14^{+}$ monocytes within 6 hours of treatment (Figure 4.9C, 7D), without causing death (Figure 4.9E). These results indicate that DMF and MMF can decrease monocyte chemotaxis in response to CCL2 and that this effect is associated with downregulation of CCR2 expression.

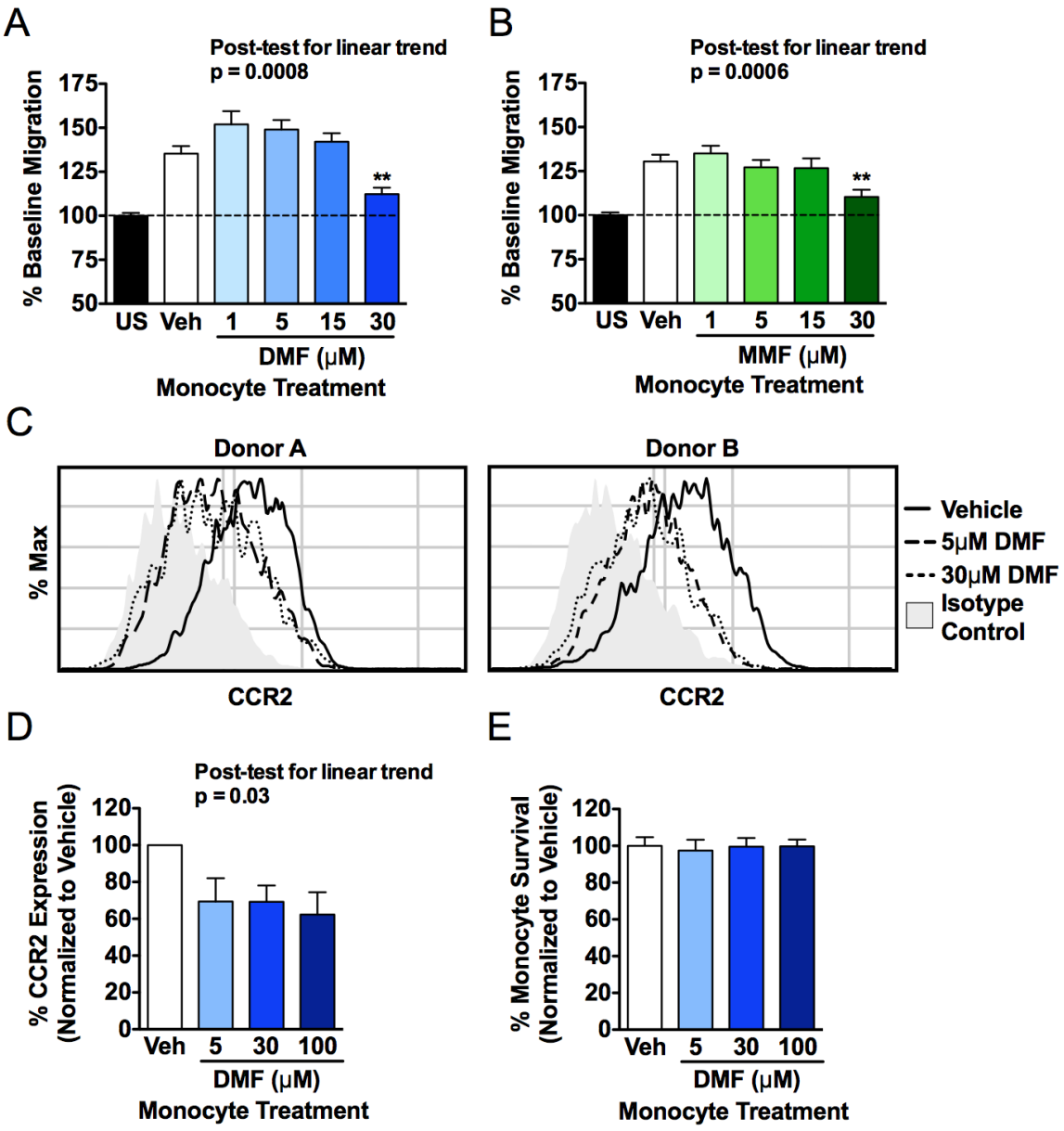

Figure 4.9 DMF and MMF reduce CCL2 induced chemotaxis in human monocytes. A) DMF and **B)** MMF inhibit CCL2-induced chemotaxis in freshly isolated human monocytes in a dose-dependent manner. Values are expressed as percent migration of unstimulated cells (US; 0 ng/mL CCL2) (n = 10-22; **p<0.01 vs. Vehicle). **C)** DMF decreases CCR2 expression on $CD11b^{+}CD14^{+}$ PBMCs following 6 hours of treatment, as quantified (**D**). **E)** DMF does not cause significant cell death over 6 hours of treatment in freshly isolated human monocytes, as measured by DAPI positivity in $CD11b^{+}CD14^{+}$ gated PBMCs. For all experiments, values represent data averaged from 3 different donors. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing. Results of post test for linear trend are also presented.

Discussion

Monocytes and macrophages are major reservoirs for HIV in both the periphery and CNS, and they facilitate the spread of virus to target cells, allow for viral persistence and serve as major contributors to inflammation-mediated pathology. Despite current ART, latently infected monocytes and $CD4^+$ T-lymphocytes persist, resulting in inflammation in the periphery and in the CNS in up to 50% of patients on ART (Robertson et al., 2007; Sacktor et al., 2002). While ART will remain the mainstay of HIV therapy, effective adjunctive therapies that suppress inflammation, improve morbidity and improve long-term cognitive outcomes are greatly needed. The immunomodulator DMF, which is effective for the treatment of psoriasis and which shows promising results for multiple sclerosis treatment in recent clinical trials, is an attractive candidate as a safe adjunctive neuroprotectant against HIV. We have demonstrated that physiologically relevant doses of DMF and its primary metabolite, MMF, (Linker et al., 2011; Litjens et al., 2004) affect key steps in the pathogenesis of HAND in our *in vitro* model system by inhibiting HIV replication, neurotoxin production, NF-κB signaling and TNFα production in human MDM and reducing monocyte chemotaxis in response to CCL2. These results suggest that DMF could serve as an effective neuroprotectant in HAND and have beneficial effects on systemic HIV-disease progression as well.

We have shown that DMF and MMF attenuate macrophage-mediated neurotoxicity following HIV infection by simultaneously attenuating viral replication and inducing HO-1 expression. Furthermore, induction of HO-1 can significantly decrease macrophage-mediated neurotoxicity even without decreasing HIV replication. Consequently, DMF may be an especially relevant therapeutic in patients who have relatively good virologic control but still suffer from neurological complications of HIV. We have shown that HIV infection of MDM results in a dysregulation of the antioxidant response with an especially prominent reduction in HO-1 levels, associated with supernatant neurotoxicity, and that DMF treatment restores HO-1 levels and reduces neurotoxin production in macrophages. In activated microglia, an oxidative burst is required for the release of

excitotoxic glutamate (Barger et al., 2007), demonstrating that alterations to cellular oxidative state can mediate the production and/or release of MDM neurotoxins. DMF's ability to decrease HIV replication and neurotoxin production by distinct mechanisms makes it an especially attractive therapeutic candidate for HAND. Furthermore, macrophage- and microglia-mediated neurotoxicity contribute to many other neurological disorders including multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease and stroke/reperfusion injury, for which therapeutics for restoring oxidative balance resultant from the disease state have been investigated/prescribed (Uttara et al., 2009).

Numerous proinflammatory factors contribute to HIV disease pathogenesis in both the peripheral and CNS compartments. TNFα, IL-6, IL-1β, IFN-γ and other proinflammatory cytokines are elevated in the blood and CSF of HIV-infected patients (Breen et al., 1990; Ciardi et al., 1994; Kobayashi S, 1990; Ownby et al., 2009). Among these, TNFα is the most potent mediator of inflammation and is induced early after HIV monocytic infection and its expression continues to increase over the course of infection (Esser et al., 1996; Folks et al., 1988; Koyanagi et al., 1988). It is well established that TNFα exposure upregulates HIV replication by initiating a signaling cascade that activates the nuclear translocation of NF-κB (Butera et al., 1993; Nabel and Baltimore, 1987; Poli et al., 1990; West et al., 2001). We have shown that DMF and MMF attenuate TNFα-mediated NF-κB signaling in human macrophages and reduce nuclear NF-κB levels, which are expected to decrease transcription from the HIV-LTR. However, the NF-κB and TNFα signaling loop may not entirely mediate DMF's antiviral activity. Attenuation of HIV replication occurs at low concentrations, as does induction of the ARE, while inhibition of NF-κB signaling may be more relevant at concentrations of 15μM and greater. Future studies are necessary to assess the role of the antioxidant response, including NQO1 and the cellular redox state, in mediating HIV infection and replication. DMF may alter the expression of the HIV co-receptors, CXCR4 and CCR5, similarly to the observed downregulation of cell surface CCR2. It has been reported that antioxidants decrease the stability of mRNA transcripts for CXCR4 and

CCR5 in human monocytes, suggesting that DMF treatment may directly reduce HIV entry into human monocytes (Saccani et al., 2000).

However, DMF's ability to inhibit NF-κB and TNFα signaling following both PHA stimulation and HIV infection has clear implications for the physiologic reduction of neuroinflammation and cytokine induced neuronal injury. Elevated TNFα levels increase monocyte entry into the brain, promote HIV replication, and drive inflammatory cascades, thereby enhancing the production of neurotoxins in the CNS from MDM, microglia and astrocytes (Fiala et al., 1997). Therefore, dampening TNFα-driven processes might also afford neuroprotection against HIV. Indeed, TNFα is linked to glutamine synthetase and glutamate import in macrophages (Porcheray et al., 2006), and DMF's inhibition of TNFα-driven processes may further decrease the release of excitatory neurotoxins, such as glutamate, in HIV/MDM. In human macrophages, we have shown that DMF is a potent suppressor of NF-κB nuclear translocation, subsequent binding to DNA and expression of NF-κB dependent genes. Therefore, DMF is a particularly good therapeutic candidate for pathological states characterized by macrophage driven inflammation and NF-κB signaling.

Although not directly dependent upon HO-1, DMF's antioxidant properties are likely mediating the inhibition of NF-κB activity. We hypothesize that such effects are due to DMF's modulation of the macrophage intracellular redox state as activation of the antioxidant response has been shown to block NF-κB activity and HIV transcription (Roederer et al., 1990; Schreck R, 1991; Staal FJ, 1993). Furthermore, classical (α and β), novel (δ) and atypical (ζ) PKC isotypes can modulate the nuclear translocation and transcriptional activity of NF-κB and PKC is activated by oxidative stress and inhibited by antioxidants (Asehnoune et al., 2005; Boscoboinik et al., 1991; Gopalakrishna et al., 1995; Gopalakrishna et al., 1997; Sun et al., 2000). In addition to potential effects on PKC, DMF may also affect the phosphorylation of IκB kinases (IKK) and subsequent phosphorylation and degradation of IκB proteins (Seidel et al., 2009; Vandermeeren et al., 2001).

Finally, DMF may affect NF-κB dependent transcription by modulating the preferred composition of NF-κB homo- and heterodimers that form after nuclear translocation has occurred. The intracellular oxidative state can affect levels of NF-κB p50 homodimers, which do not possess transactivation domains and are thought to act as transcriptional repressors of NF-κB heterodimer responsive genes (Cristofanon et al., 2009; Hoberg et al., 2006; Zhou et al., 2001). We are currently examining the role of DMF and MMF in modulating the activation state of the macrophage, which would affect the cell's relative sensitivity to pro-inflammatory signals and thereby contribute to decreased NF-κB signaling.

While other antioxidants have been considered as potential therapeutics for HAND, through direct effects on macrophages or neurons, DMF is unique in its ability to inhibit CCL2-induced monocyte chemotaxis. Monocyte transmigration across the blood-brain barrier is dependent upon production of chemokines, such as CCL2, in the CNS and the activation of monocytes in the periphery. Levels of CCL2 in the CSF correlate with CSF viral load and with the clinical severity of HAND (Cinque et al., 1998; Conant et al., 1998; Kelder et al., 1998; Letendre et al., 1999; Sozzani et al., 1997; Zink et al., 2001), and CCL2 is produced by brain macrophages, astrocytes and endothelial cells in response to inflammatory mediators and HIV proteins (Gu et al., 1997; Guillemin et al., 2003; Lehmann et al., 2006). Not only does DMF decrease TNFα production and NF-κB signaling in MDM, both of which have been implicated in CCL2 production, but DMF and MMF inhibit CCL2-driven monocyte chemotaxis, possibly by modulation of CCR2 expression. DMF and MMF may modulate the cell surface expression of CCR2 by inducing the antioxidant response and consequently altering the redox state of the cell. It has been demonstrated that direct antioxidants are capable of reducing the transcript stability of CCR2, which has been linked to decreased cell surface expression and CCL2-induced chemotaxis in human monocytes (Saccani et al., 2000). These findings in our *in vitro* model system predict suppression of transendothelial migration of monocytes into the CNS during HIV infection. Furthermore, it has been reported that DMF modulates adhesion molecule expression in human endothelial cells by

inhibiting TNFα-induced expression of ICAM-1, VCAM-1 and E-selectin (Vandermeeren et al., 1997). Expression of each of these adhesion molecules has been linked to monocyte entry into the CNS after HIV infection and down-regulation by DMF is expected to further inhibit monocyte entry into the CNS. Given these findings, DMF should be considered as a potential therapeutic for other neuroinflammatory diseases associated with CCL2-induced recruitment of leukocytes to the CNS.

With this study, we identify dimethyl fumarate as a candidate adjunctive therapy and potential neuroprotectant against HIV. To our knowledge, we are the first to demonstrate that HIV infection dysregulates components of the antioxidant response in human macrophages and that restoration of HO-1 levels, specifically, can reduce macrophage-mediated neurotoxicity. DMF is the first proposed neuroprotectant that reduces CCL2-mediated monocyte chemotaxis as a component of its mechanism of action. Furthermore, we have shown that DMF attenuates HIV replication associated with decreased TNFα and NF-κB signaling. Given these findings, we propose that DMF should be considered a relevant therapeutic candidate for neurological disorders and other complications of HIV-infection mediated by monocyte and macrophage inflammation.

Work in Progress

Induction of the antioxidant response with tBHQ attenuates HIV replication, macrophage-mediated neurotoxicity and NF-κB signaling

We have demonstrated that DMF and MMF treatment attenuate macrophage-mediated neurotoxicity in a dose-dependent manner and that these effects are due to induction of the antioxidant response, specifically restoration of HO-1 levels (Cross et al., 2011). We also hypothesized that induction of the antioxidant response and alteration of the cellular redox state is responsible for DMF and MMF's inhibition of NF-κB nuclear translocation following TNFα exposure and PHA-induced TNFα production in uninfected macrophages. tBHQ (*tert*-butylhydroquinone) is a well characterized and potent inducer of the antioxidant response. tBHQ possesses an oxidizable 1,4 diphenolic structure that dissociates the Keap1-Nrf2 complex, inducing Nrf2 translocation to the nucleus and transcription of ARE-regulated genes (De Long et al., 1987; Talalay, 1989; van Ommen et al., 1992). As shown in Figure 4.10, tBHQ treatment results in a robust increase in HO-1 protein levels, with a more moderate but significant increase in NQO1. And similar to DMF/MMF treatment, tBHQ treatment also results in a dose-dependent decrease in HIV replication in human macrophages with concomitant decreases macrophage-mediated neurotoxicity. Furthermore, tBHQ inhibits the nuclear translocation of NF-κB proteins following TNFα exposure and TNFα release following PHA stimulation (Figure 4.10D and E). tBHQ, a classic activator of the antioxidant response, has the same antiviral and anti-inflammatory properties as DMF and MMF treatment, supporting the hypothesis that alterations to the cellular redox state via activation of the antioxidant response are mediating the observed effects. These results also suggest that other pharmacologics that restore the dysregulation of the antioxidant response in HIV-infected macrophages should be considered as adjunctive therapies for HAND. Ongoing experiments are investigating the effect of tBHQ treatment on CCR2 expression and CCL2-induced monocyte chemotaxis.

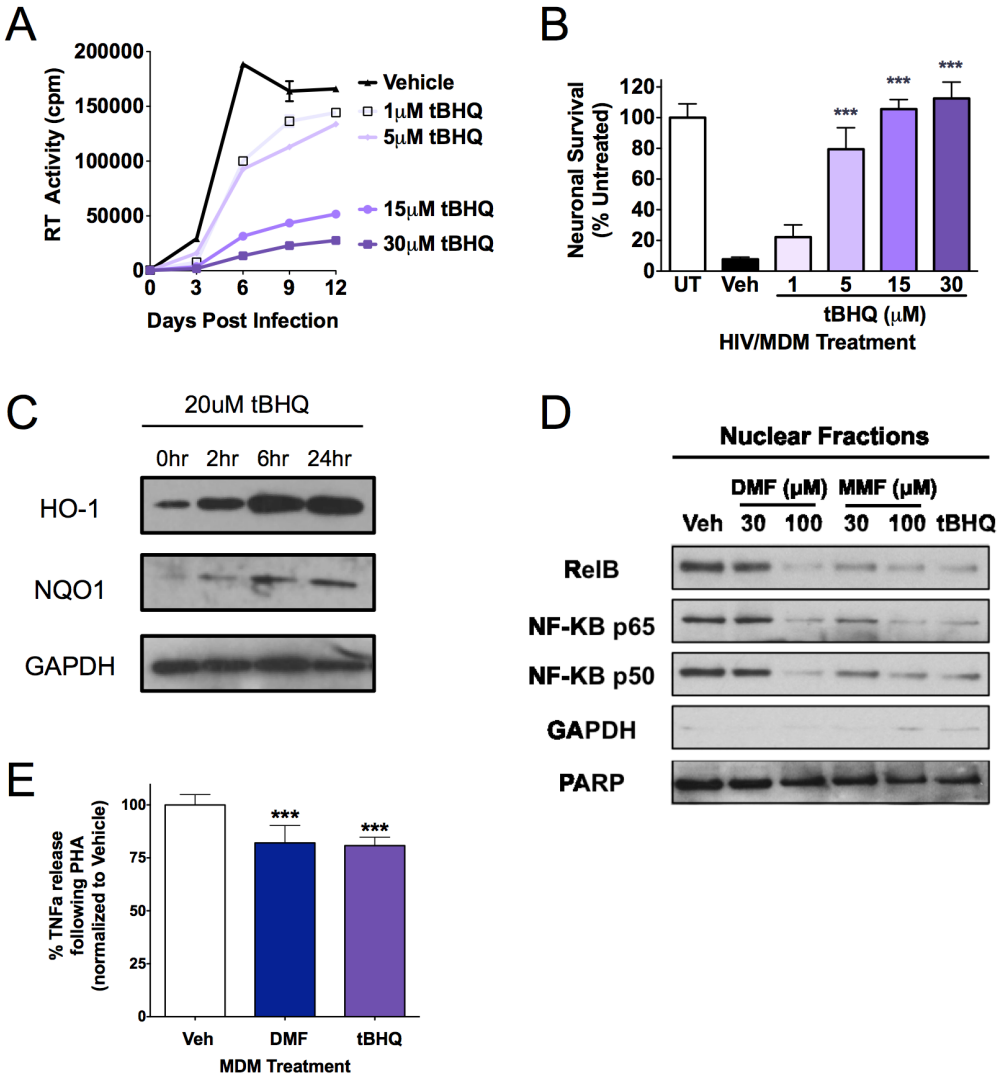

Figure 4.10 tBHQ, an inducer of the antioxidant response, attenuates HIV replication and macrophage-mediated neurotoxicity. A) tBHQ attenuates HIV replication in a dose-dependent manner. Human MDM infected with HIV-Jago were treated with tBHQ over the course of infection. Culture supernatants were collected every 3 days and assessed for RT activity. RT curves are representative of 2 independent experiments, with each replicate performed on cell preparations from a different donor. **B)** tBHQ reduces macrophage-mediated neurotoxicity following HIV infection. Neuronal survival was assessed by MAP2 ELISA following exposure of rat cerebrocortical neuronal cultures to supernatant of HIV-infected macrophages treated with tBHQ over the course of infection. MAP2 data is expressed as a percentage of untreated (UT) cultures (n = 6). **C)** tBHQ induces the antioxidant response in uninfected human MDM as assessed by Western blotting. **D)** Similarly to DMF and MMF, tBHQ inhibits the nuclear translocation of NF-κB proteins RelB, p65 and p50 in human MDM. Cells were treated with 100μM tBHQ for 24 hours, exposed to TNFα (10 min), separated into nuclear fractions and analyzed by Western blotting. Blots are representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. **E)** tBHQ inhibits the production of TNFα in MDM stimulated with PHA (10μg/mL) for 24 hours. Values represent data averaged from 3 different donors. Data are expressed as mean ± SEM. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, ***p<0.001 vs. Vehicle.

Induction of HO-1 expression with hemin attenuates macrophage-mediated neurotoxicity without altering HIV replication

Previous reports have associated hemin treatment, an inducer of HO-1, with attenuation of HIV replication (Devadas and Dhawan, 2006). However, we were unable to replicate these previously published results. As shown in Figure 4.11, hemin treatment of HIV-infected macrophages had no effect on viral replication, although it did induce levels of HO-1 above those seen in EFZ-treated controls. However, the extent of HO-1 induction following 20μM hemin treatment was several-fold less than that achieved with 100μM DMF treatment (Figure 4.11B). Hemin treatment also resulted in a small increase in NQO1 levels in HIV/MDM and previously published studies suggest that hemin is capable of activating several ARE-regulated genes, in addition to HO-1, through activation of Nrf2 (Iwasaki et al., 2006). However in our *in vitro* system, the effect of hemin in macrophages is similar to that seen with CoPP treatment, with preferential activation of HO-1 and no effect on HIV replication. And consistent with previous findings using CoPP (Figure 4.8), induction of HO-1 with hemin reduced neurotoxin production in HIV-infected macrophages, even in the absence of alterations to viral replication (Figure 4.11C). As previously demonstrated in Figure 4.8, treatment of HIV-infected macrophages with SnMP, which inhibits of HO-1 enzymatic activity despite increasing HO-1 expression, had no effect on HIV replication but enhanced neurotoxin production. While robust induction of the antioxidant response attenuates HIV replication and NF-κB driven inflammation, these effects are mediated by induction of ARE-regulated genes other than HO-1. However, the enzymatic activity of HO-1 is sufficient to alter neurotoxin production pathways, possibly by increasing iron, biliverdin or carbon monoxide (CO) levels, the catalytic byproducts following heme catabolism by HO-1. Future experiments will explore the role of HO-1 enzymatic activity in modulating known neurotoxin production pathways in macrophages.

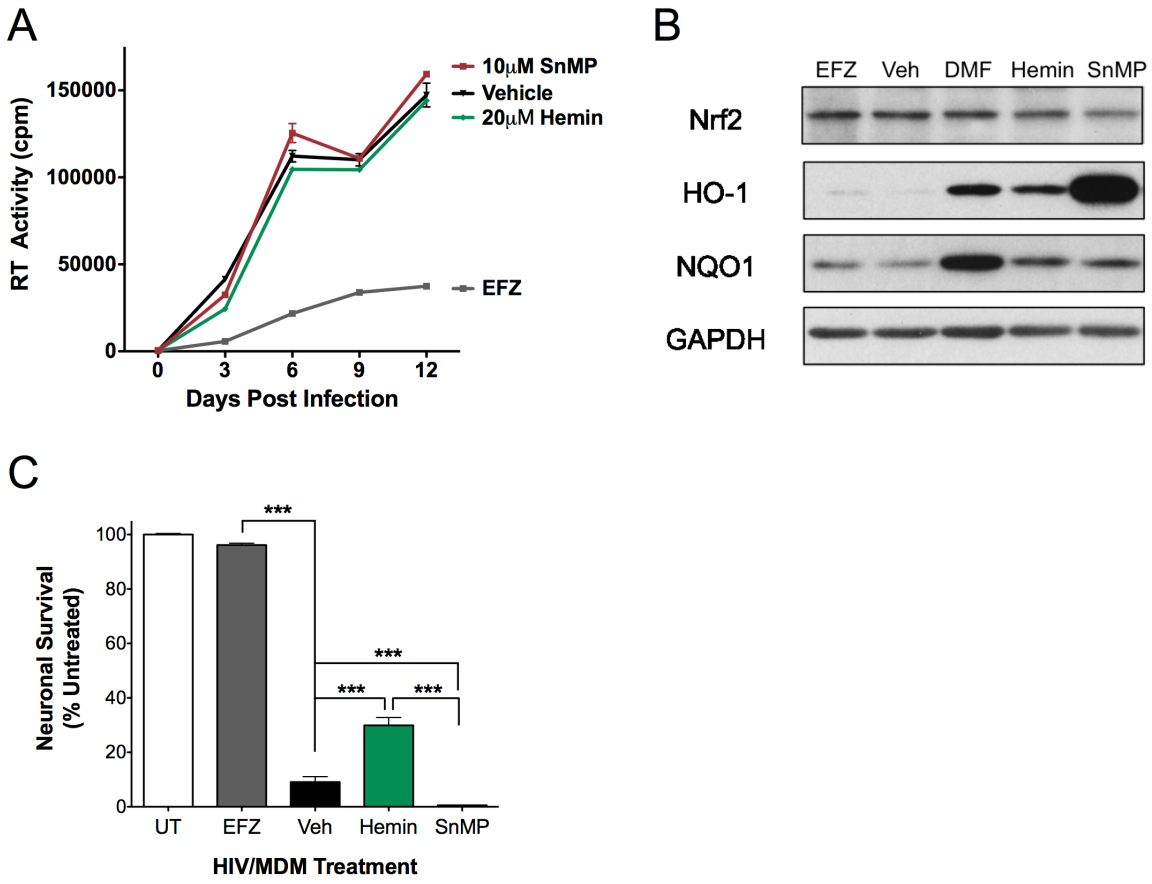

Figure 4.11 Hemin, an inducer of HO-1 and the antioxidant response, attenuates macrophage-mediated neurotoxicity without altering HIV replication. A) Human MDM were infected with HIV-Jago and treated with 20μM hemin, 10μM SnMP or the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ), over the course of infection. Culture supernatants were collected every 3 days and assessed for RT activity. Values indicate the mean ± SEM. **B)** 50μM hemin increases total levels of HO-1 and modestly increases NQO1 levels, as assessed by Western blotting in HIV-infected human macrophages. **C)** Hemin reduces macrophage-mediated neurotoxicity following HIV infection. Neuronal survival was assessed by MAP2 ELISA following exposure of rat cerebrocortical neuronal cultures to supernatant of HIV-infected macrophages treated with hemin over the course of infection. MAP2 data is expressed as a percentage of untreated (UT) cultures (n = 6). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, ***$p<0.001$.

DMF treatment reduces levels of the HIV co-receptors, CCR5 and CXCR4, on human monocytes

We have demonstrated that treatment of macrophages with DMF or MMF over the course of infection inhibits HIV replication (Figure 4.1). We hypothesized that DMF and MMF could inhibit HIV infection by decreasing levels of the HIV co-receptors, CCR5 and CXCR4, via alterations of the cellular redox state (Cross et al., 2011). As shown in Figure 4.12, $CD11b^{+}CD14^{+}$ PBMCs had significantly less cell surface expression of CXCR4 after 6 hours and CCR5 after 36 hours of DMF treatment. DMF treatment of $CD11b^{+}CD14^{+}$ PBMCs transiently increased cell surface levels of CD4 after 6 hours of treatment. However, CD4 cell surface expression was equal to or less than the vehicle control after 36 hours of DMF treatment. DMF's effect on receptor expression was not global, as levels of CD14 did not significantly change with treatment. These results suggest that DMF and MMF treatment may attenuate HIV replication in macrophages by inhibiting receptor-mediated viral entry. Furthermore, they suggest that DMF treatment can inhibit infection of human monocytes by both CCR5- and CXCR4-tropic viruses

We are currently investigating the effects of DMF treatment on early stages of viral replication through the use of virus pseudotyping. A pseudotyped virus is an enveloped virus particle, in this case HIV, which has been assembled with a foreign viral glycoprotein, such as vesicular stomatitis virus glycoprotein (VSV-G). VSV-G pseudotyped viruses can infect macrophages independently of the traditional HIV co-receptors. Luciferase assays, detecting transcription from the *nef* gene of the HIV genome, will be performed on infected macrophages, in the presence and absence of DMF, to assess if DMF treatment alters early events in viral infection (reverse transcription, nuclear translocation, incorporation into the genome and transcription/translation) in addition to viral entry.

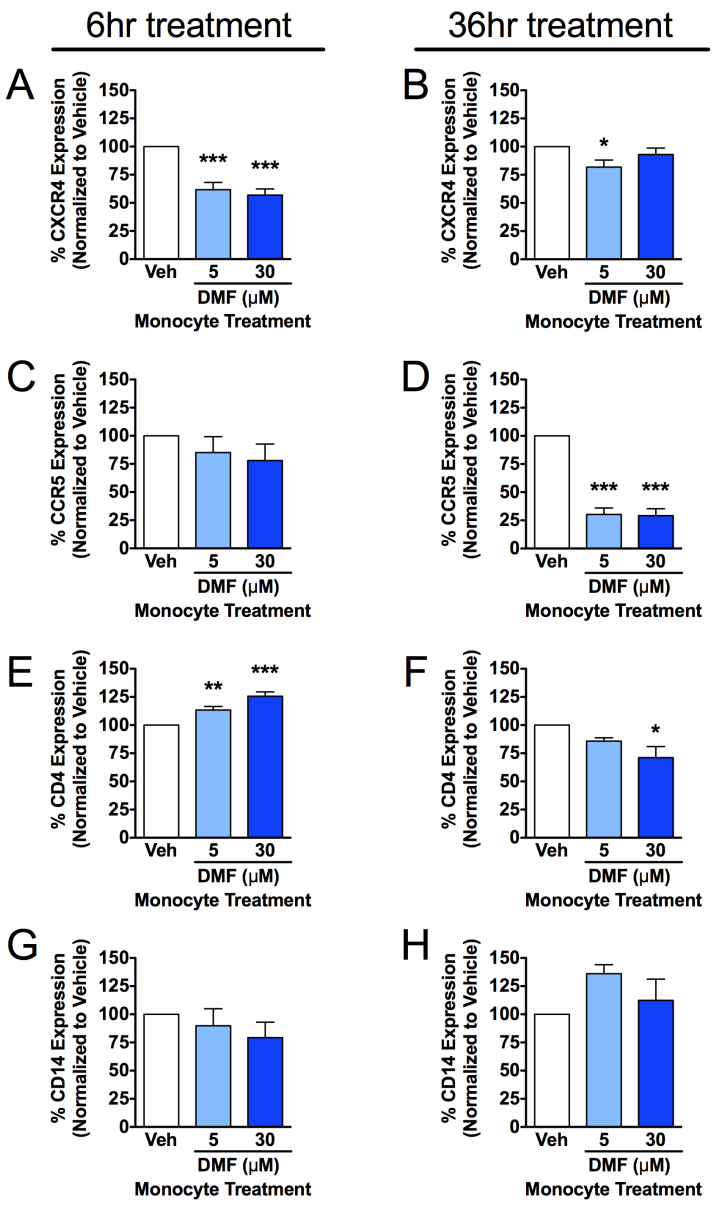

Figure 4.12 DMF decreases the cell surface expression of CXCR4 and CCR5 on human monocytes[3]. A) DMF significantly decreases CXCR4 cell surface expression on $CD11b^{+}CD14^{+}$ human PBMCs following 6 hours of treatment, **B)** but has little effect following 36 hours. **C**) DMF has no significant effect on CCR5 cell surface expression on $CD11b^{+}CD14^{+}$ human PBMCs after 6 hours of treatment, but **D)** significantly decreases CCR5 expression following 36 hours. **E)** DMF significantly increases CD4 cell surface expression on $CD11b^{+}CD14^{+}$ human PBMCs following 6 hours of treatment, **F)** but decreases CD4 expression following 36 hours of treatment. DMF has no significant effect on CD14 cell surface expression on $CD11b^{+}CD14^{+}$ human PBMCs after **G)** 6 hours or **H)** 36 hours of treatment. For all experiments, values represent data averaged from 6-8 different donors. All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ vs. Vehicle.

[3] All of the data contained in this figure was collected and analyzed by Anthony W.S. Chi, PhD

DMF treatment of HIV-infected macrophages has no effect on viral replication after infection is established; although DMF, but not efavirenz, decreases macrophage-mediated neurotoxicity

If DMF treatment decreases HIV infection by reducing the cell surface expression of the HIV co-receptors, then addition of DMF to an established infection would have little effect on HIV replication. Figure 4.13 demonstrates that as expected, the NNRTI, efavirenz, does not alter HIV replication when added to macrophages at day 9 post infection. Since efavirenz attenuates HIV replication by inhibiting the reverse transcription of the HIV RNA genome immediately following HIV entry, efavirenz application to an established infection would have no antiviral activity. Efavirenz treatment of HIV-infected macrophages supporting high levels of viral replication does not attenuate neurotoxin production (Figure 4.13B). When added to an established infection, DMF treatment has no effect on HIV replication (Figure 4.13C). This supports the hypothesis that DMF attenuates viral replication by inhibiting cell surface CCR5 and CXCR4 levels and thereby viral entry. However, when DMF was added to macrophages supporting high levels of HIV replication, there was significantly less neurotoxin production, as compared to vehicle control (Figure 4.13D). This finding is in agreement with previous studies demonstrating that induction of HO-1 reduces neurotoxin production in HIV-infected macrophages, even without attenuation of HIV replication.

These experimental results also suggest that DMF treatment has little effect on HIV replication kinetics after HIV has entered the cell and incorporated into the host genome. We had proposed that DMF's antiviral effects were a consequence of decreased transcription from the HIV LTR due to attenuation of NF-κB and TNFα signaling (Cross et al., 2011). However, if this hypothesis were true, addition of DMF to HIV-infected macrophages should have decreased viral transcription. DMF-mediated inhibition of NF-κB and TNFα signaling has important implications for inflammation mediated pathology of HAND, but our data suggests that this is not a major mechanism underlying DMF's antiviral effects. Rather, DMF treatment prevents the infection of macrophages and would limit viral spread of both CXCR4 and CCR5 tropic viruses. Furthermore,

DMF is an attractive adjunctive therapy for HAND, since treatment of infected macrophages attenuates neurotoxin production, whereas traditional antiretrovirals would have little effect.

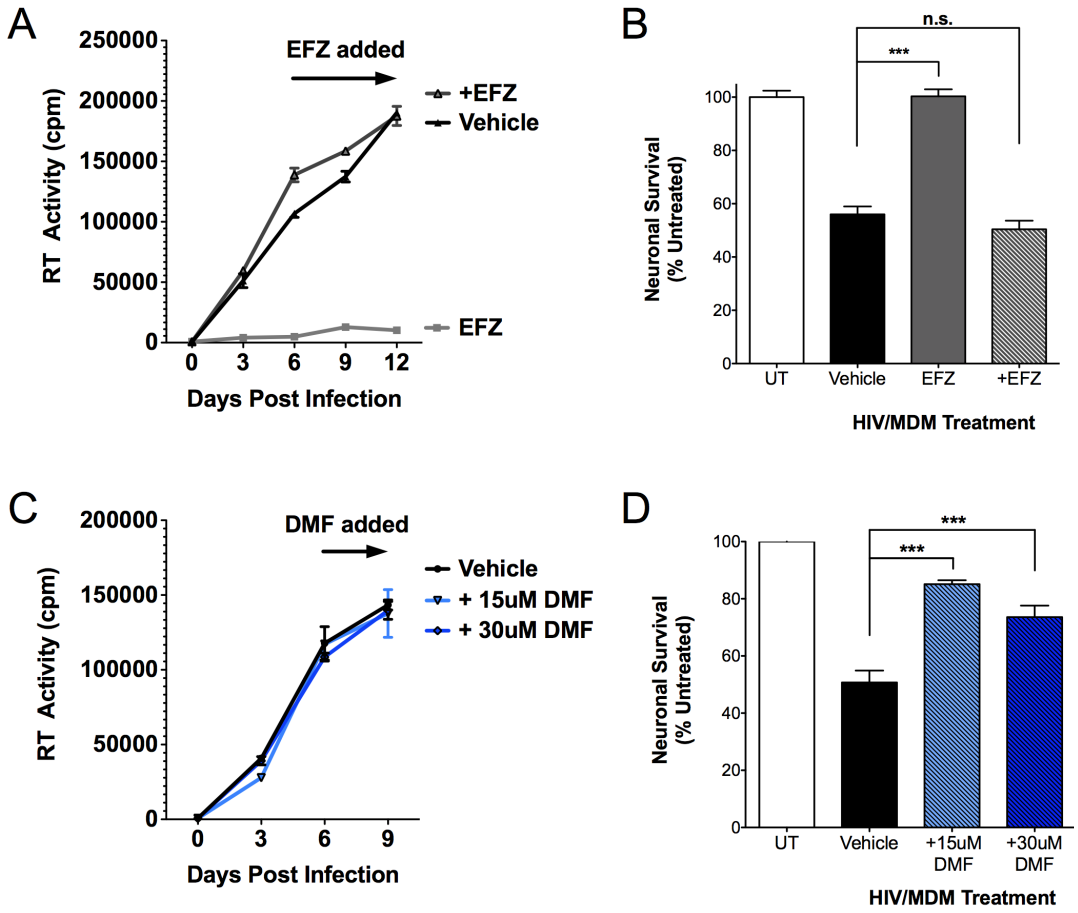

Figure 4.13 DMF, but not efavirenz, added to an established HIV infection decreases macrophage-mediated neurotoxicity without altering HIV replication. A) MDM infected with HIV-Jago were treated with 20nM of the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ), over the course of infection or were left untreated until day 6 post infection, and then treated with EFZ for the remainder of the time course (+EFZ). **B)** HIV/MDM treated with EFZ over the course of infection do not produce neurotoxins, whereas HIV/MDM treated with EFZ from day 6 through day 12 (+EFZ) cause as much neurotoxicity as vehicle controls. **C)** MDM infected with HIV-Jago were left untreated until day 6 post infection, and then treated with 15μM or 30μM DMF until day 9 post infection. **D)** HIV/MDM treated with 15μM and 30μM DMF from day 6 through day 9 post infection cause significantly less neurotoxicity than vehicle control, despite having equivalent levels of RT activity. For panels A and C, culture supernatants were collected every 3 days and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 2 independent experiments, with each replicate performed on cell preparations from a different donor. For panels B and D, neuronal survival was assessed by MAP2 ELISA following exposure of rat cerebrocortical neuronal cultures to supernatant of HIV-infected macrophages from the end of the time course (day 12 in panel B and day 9 in panel D). MAP2 data is expressed as a percentage of untreated (UT) cultures (n = 6). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, ***p<0.001 vs. Vehicle.

CHAPTER 5

THE UNFOLDED PROTEIN RESPONSE ATTENUATES HIV REPLICATION AND MACROPHAGE-MEDIATED NEUROTOXICITY

Abstract

Viral infection can result in the activation of host cell adaptive stress responses, including the antioxidant response and the unfolded protein response (UPR). Virus replication and assembly can strain the capacity of the endoplasmic reticulum (ER) and result in the accumulation of misfolded proteins and activation of the UPR. The UPR is designed to eliminate misfolded proteins and promote cellular recovery by attenuating translation and upregulating the expression of chaperones, degradation factors, and regulators of metabolic and redox states. Several viruses, including hepatitis C virus and herpes simplex virus, regulate antiviral components of the UPR in order to promote efficient viral replication. However, the effect of HIV infection on the UPR has not yet been investigated. We hypothesized that HIV infection would activate and modulate the UPR in infected macrophages. Using our *in vitro* model system, we found that HIV infection increases levels of phosphorylated eIF2α, which inhibits protein translational during states of cellular stress. We also found that the chaperone BiP, considered the master regulator of the UPR, was increased in the macrophages of HAND frontal cortex. While HIV infection activates components of the UPR in macrophages, pharmacological induction of the UPR attenuates HIV replication. This suggests that induction of phosphorylated eIF2α during HIV replication has some antiviral effect, although it is not sufficient to considerably attenuate HIV replication. Interestingly, pharmacological induction of the UPR, which attenuates viral replication, is associated with increased macrophage-mediated neurotoxicity. This finding has important implications for the development of adjunctive therapies for HAND. Therapeutics or processes that induce the UPR in macrophages, regardless of the effect on HIV replication, could enhance neurotoxin production and contribute to the pathological processes underlying HAND.

As we have previously described the effect of HIV replication on the antioxidant response, we assessed how restoration of the antioxidant response altered the UPR in HIV-infected macrophages. Activation of the antioxidant response with DMF and MMF attenuated HIV replication and decreased levels of phosphorylated eIF2α and BiP in HIV/MDM. Reductions in

phosphorylated eIF2α trend with increased levels of HO-1, but do not depend upon HO-1 enzymatic activity. In contrast, BiP levels decrease with induction of the antioxidant response, but these alterations are independent of HO-1. These findings suggest that while dysregulation of the antioxidant response reflects levels of HIV replication and HO-1 levels modulate neurotoxin production, levels of phosphorylated eIF2α and BiP do not predict HIV replication or extent of macrophage-mediated neurotoxicity. Ongoing studies are assessing the link between UPR activation, inflammation pathways and the antioxidant response as they relate to HIV infection and macrophage-mediated neurotoxicity.

HIV infection alters several adaptive stress pathways in the macrophage. The suppression of the antioxidant response and induction of the UPR both associate with high levels of viral replication and neurotoxin production. Pharmacological induction of either stress pathway attenuates HIV replication, however, induction of the antioxidant response, but not the UPR, decreases neurotoxin production. Therefore, activation of the UPR by inflammation, oxidative stress or ART is predicted to enhance neurotoxin production in macrophages, regardless of the level of HIV replication. Understanding how HIV infection affects adaptive stress responses and neurotoxin production pathways in the macrophage will continue to improve our ability to develop effective adjunctive therapies for HAND.

Introduction

Accumulation of misfolded proteins in the endoplasmic reticulum (ER) lumen results in the activation of an adaptive stress response known as the unfolded protein response (UPR). The UPR is a quality control mechanism that can be activated during physiological stress (e.g., glucose deprivation, oxidative stress) and viral infection. The UPR reduces the load of misfolded proteins in the ER by attenuating new protein translation via phosphorylated-eIF2α and upregulating chaperones that mediate protein folding, such as BiP. During viral infection, the cell can experience an excess flux of viral proteins passing through the ER. Several viruses, including human cytomegalovirus (CMV) and herpes simplex virus (HSV), are capable of regulating cellular responses to infection, including the UPR, in order to promote host cell survival while promoting efficient viral replication. The effect of HIV infection on the UPR has not yet been investigated.

The Unfolded Protein Response

The endoplasmic reticulum facilitates protein folding, is part of the protein-sorting pathway and is the site of post-translational modifications such as N-linked glycosylation and disulfide bond arrangement. These ER processes are tightly monitored in order to prevent improperly folded or modified proteins from being released into the cell. Under normal conditions, approximately 30% of newly synthesized proteins fail to achieve native structure due to errors in transcription, translation, post-translational modifications or protein folding (Schubert et al., 2000). If not managed, misfolded proteins accumulate in the ER and can result in toxic aggregates that interfere with the function of normal proteins (Romisch, 2004). In response to increased levels of misfolded proteins in the ER lumen, a signaling cascade known as the Unfolded Protein Response (UPR) or Integrated Stress Response (ISR) is activated. This signaling cascade limits the translation of new proteins, which decreases the incoming burden on the ER machinery, enhances the degradation of improperly folded proteins and upregulates levels of chaperones, which assist in protein folding/refolding. ER stress and induction of the UPR can occur under physiological conditions known to increase levels of misfolded protein, such as glucose

deprivation (Kaufman et al., 2002), heat shock (Kasuya et al., 1999; Matsumoto et al., 2005), oxidative stress (Holtz et al., 2006; Yan et al., 2008), hypoxia (Feldman et al., 2005; Koumenis and Wouters, 2006), increase demand of secretory function (B-cell antibody production) (Gass et al., 2002), and viral infection (Ambrose and Mackenzie, 2011; He et al., 1997; Isler et al., 2005; Yoshida et al., 2001).

Activation of the UPR attempts to eliminate misfolded proteins in the ER lumen through two mechanisms; 1) through attenuation of protein translation, in order to reduce the flux of proteins entering into the ER and 2) through the induction of chaperone proteins and degradation factors to refold and/or eliminate misfolded proteins. Three proteins have been identified as sensors of ER stress: protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). These proteins are transmembrane proteins with a sensor domain in the ER lumen and a kinase effector domain in the cytosolic compartment. Under normal conditions, the ER chaperone immunoglobulin heavy-chain-binding protein (BiP), also known as glucose regulated protein-78 (GRP78), is bound to the sensor domain of PERK, ATF6 and IRE1 in the ER lumen. When misfolded proteins accumulate in the ER, BiP is sequestered away from these sensors to bind to misfolded proteins (Bertolotti et al., 2000). BiP release from PERK and IRE1 results in the homodimerization of their luminal domains, causing autophosphorylation and activation of downstream signaling cascades (Figure 5.1). In contrast, BiP release from ATF6 unmasks a Golgi localization signal, resulting in the relocation of ATF6 to the Golgi, where it is cleaved into an activated form (Shen et al., 2002).

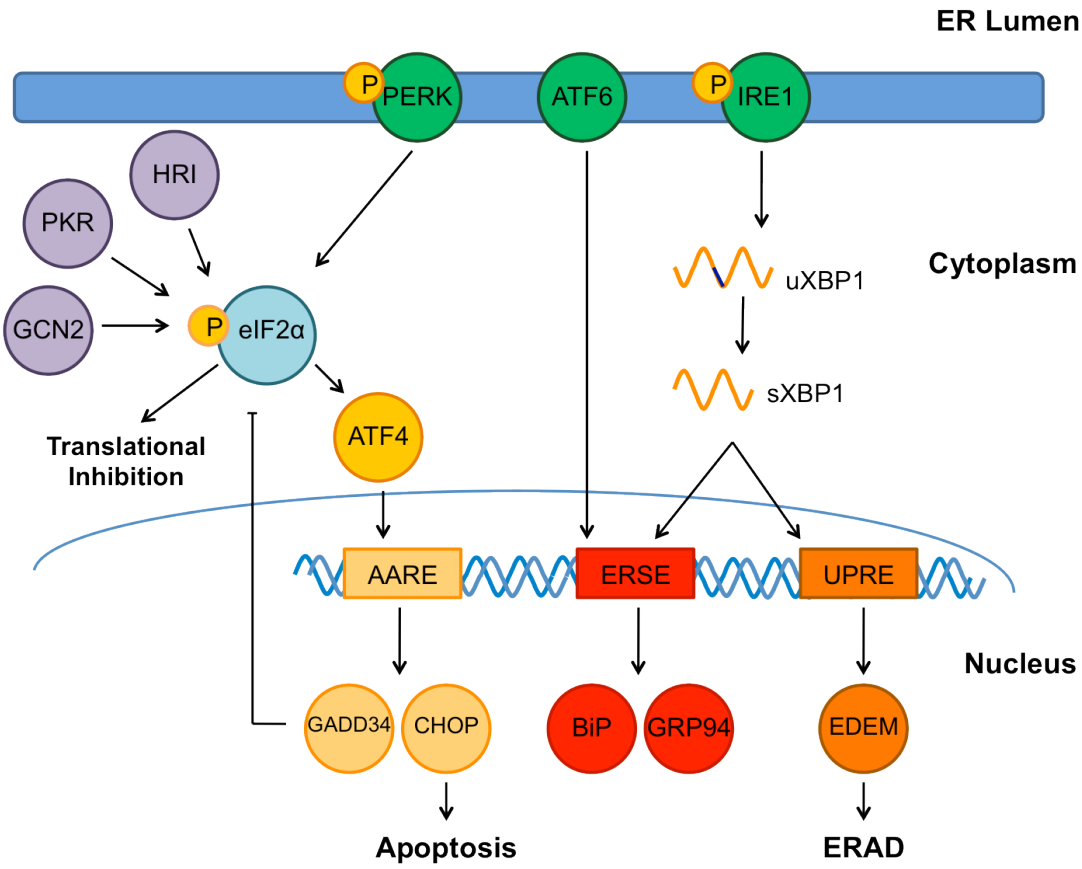

Figure 5.1 The signaling pathways comprising the Unfolded Protein Response (UPR). Accumulation of misfolded protein in the ER causes ER stress and results in the activation of the UPR, an adaptive stress pathway. PERK, IRE1 and ATF6 have sensor domains that monitor levels of misfolded protein in the ER lumen. Following activation, each of these pathways initiates a signaling cascade that results in the transcriptional upregulation of genes that will help the cell reduce, manage or recover from ER stress. Phosphorylation of eIF2α by activated PERK results in the attenuation of all cap-dependent protein translation, in order to reduce the incoming burden of new proteins into the ER. Increased levels of phosphorylated eIF2α also result in the translational upregulation of ATF4, a transcription factor that increases amino acid response element (AARE)-regulated genes in order to modulate cellular metabolism and redox state during cellular recovery from ER stress. ATF4 also increases levels of GADD34, which restores protein translation by mediating eIF2α dephosphorylation as part of a negative feedback loop. In addition to PERK, three other kinases (PKR, GCN2 and HRI) can phosphorylate eIF2α and initiate the UPR, although instead of misfolded protein levels, they respond to double stranded RNA, amino acid limitation and heme levels, respectively. ATF6, upon activation, translocates to the Golgi where is it cleaved into an active form, which also functions as a transcriptional regulator of ER stress-element (ERSE)-regulated genes. ERSEs regulate genes encoding for a variety of cellular chaperones, which promote the proper folding of misfolded proteins in the ER, including BiP and GRP94. Activation of IRE1 results in its auto-phosphorylation and the subsequent activation, by a splicing event, of XBP1 mRNA. Spliced XBP1 (sXBP1) encodes a transcription factor capable of upregulating genes with ERSEs or UPR elements (UPREs). Transcriptional upregulation of UPRE-regulated genes results in increased levels of EDEM, which enhances the degradation of misfolded proteins in the ER by ER-associated degradation (ERAD). If the UPR is unable to manage the stress to the ER, the cell will undergo CHOP-mediated apoptosis.

After activation, PERK initiates a signaling cascade that results in the inhibition of new protein translation and the induction of genes important for cellular recovery from ER stress. PERK directly phosphorylates the eukaryotic initiation factor eIF2α at serine 51 (Harding et al., 2000b; Harding et al., 1999). When phosphorylated, eIF2α inhibits cap-dependent protein translation by tightly binding eIF2B. This prevents eIF2B from catalyzing the GDP-GTP exchange and thereby stops the binding of the initiator Met-tRNA to the ribosome and initiation of translation (Krishnamoorthy et al., 2001). While inhibiting the majority of cellular protein translation, phosphorylated-eIF2α specifically increases the translation of select stress-responsive proteins, including activating transcription factor 4 (ATF4) (Harding et al., 2000b). ATF4 mRNA is efficiently translated due to small upstream open-reading frames (uORF) within the 5' untranslated region, resulting in a “uORF bypass scanning system” (Lu et al., 2004). ATF4 binds to the promoter regions of an array of different genes, including metabolism and redox regulatory factors involved in mediating recovery from ER stress. ATF4 deficiency results in increased sensitivity to cell death with a variety of stresses, including oxidative stress and amino acid deprivation (Harding et al., 2003). ATF4 also activates an inhibitory feedback loop to restore cellular protein translation by inducing levels of growth arrest- and DNA damage-inducible gene 34 (GADD34), which interacts with protein phosphatase 1 (PP1) and increases the dephosphorylation of eIF2α (Brush et al., 2003; Ma and Hendershot, 2003).

When BiP is sequestered away from ATF6 during ER stress, the inactive 90-kDa ATF6 precursor translocates to the Golgi where it is cleaved by site-1 and site-2 proteases into the transcriptionally active 50-kDa protein (Ye et al., 2000). ATF6, along with the constitutively expressed transcriptional regulator, nuclear factor Y (NF-Y), activate genes carrying ER stress response elements (ERSEs) in their transcriptional promoters [Mori, Cell, 2000]. ERSEs are found in genes encoding for cellular chaperones including BiP, GRP94, protein-disulfide isomerase (PDI) and calreticulin (Harding et al., 2003). Consequently, ATF6 activation results in the upregulation of ER chaperones and other proteins that are needed to promote proper protein

folding in an effort to relieve ER stress. Increased levels of BiP dampen PERK, IRE1 and ATF6 activation when the misfolded protein burden in the ER is managed. Furthermore, ATF6 can increase the amount of X box binding protein 1 (XBP-1) mRNA, providing a link between the ATF6 and IRE1 pathways (Lee et al., 2002; Yoshida et al., 2001).

Phosphorylation of IRE1, which has intrinsic endonuclease activity, results in the removal of a 26-nucelotide intron from the XBP-1 transcript (Lee et al., 2002). The spliced form of XBP-1 (sXBP-1) mRNA encodes an active transcription factor, capable of inducing genes regulated by the ERSE (Lee et al., 2003). In addition, sXBP-1 can also activate the transcription of genes containing UPR elements (UPRE), which are responsive to ER stress and are distinct from the ERSE (Lee et al., 2002; Yamamoto et al., 2004; Yoshida et al., 2001). One consequence of sXBP1 activation of the UPRE is the enhanced transcription of ER degradation enhancing α-mannosidase-like protein (EDEM). EDEM increases the capacity of ER-stressed cells to degrade irrevocably misfolded proteins via ER-associated protein degradation (ERAD) (Hosokawa et al., 2001; Yoshida et al., 2003). EDEM promotes the release of terminally misfolded proteins from the ER-resident chaperone calnexin, thereby making them available for transport to the cytoplasm for degradation by the ubiquitin proteasome system (UPS) (Kopito, 1997; Oda et al., 2003). Therefore, while the ATF6 pathway is important for protein refolding, the IRE1-XBP1 pathway mediates protein degradation via ERAD (Yoshida et al., 2003).

Chronic activation of the UPR, which occurs when the cell cannot manage or recover from ER stress, results in the activation of signaling pathways that will commit the cell to apoptosis (Ferri and Kroemer, 2001; Oyadomari et al., 2002a). At least three apoptosis pathways have been implicated in this apoptotic event. The first is transcriptional activation of C/EBP homologous protein (CHOP, also known as GADD153), primarily by ATF4 (Fawcett et al., 1999; Harding et al., 2000a; Ma et al., 2002), although the *CHOP* promoter can also be regulated by IRE1 and ATF6 (Wang et al., 1998; Yoshida et al., 2000). Induction of CHOP compromises cell viability

(Friedman, 1996; McCullough et al., 2001; Zhan et al., 1994) and targeted disruption of *CHOP* significantly protects cells from apoptosis following sustained ER stress (Oyadomari et al., 2002b; Zinszner et al., 1998). The second is activation of the cJUN NH2-terminal kinase (JNK) pathway, mediated by a complex between IRE1, TNF receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase1 (ASK1) (Nishitoh et al., 2002; Urano et al., 2000). The third pathway involved in initiating apoptosis in ER-stressed cells is mediated by caspase-12. Caspase-12, which is localized to the ER, is activated by specifically ER stress and not by apoptotic signals originating from the membrane or mitochondria (Nakagawa et al., 2000). All three of these apoptosis pathways ultimately lead to the activation of caspase-3 with BAX (Bcl-2–associated X protein) and BAK (Bcl-2 homologous antagonist/kiler) mediating the final steps of ER stress-mediated apoptosis (Wei et al., 2001).

Viral infection activates the UPR

In some cases, viral infection induces ER stress and activates components of the three branches of the UPR. BiP, considered the master regulator of the UPR, is induced in cells infected with respiratory syncytial virus (RSV) (Bitko and Barik, 2001), hanta viruses (Li et al., 2005), hepatitis C virus (HCV) (Liberman et al., 1999) and several members of the flavivirus family (Jordan et al., 2002; Su et al., 2002; Tardif et al., 2002). Activation of PERK has been reported in cells infected with herpes simplex virus (HSV) (Cheng et al., 2005; Mulvey et al., 2007) and cytomegalovirus (CMV) (Isler et al., 2005). Furthermore, the IRE1 and XBP-1 signaling pathway is activated with dengue infection (Yu et al., 2006) and the ATF6 pathway is activated in HCV infected cells (Tardif et al., 2004). Enveloped viruses utilize the ER as the primary site of envelope glycoprotein biogenesis and several viruses undergo genomic replication and particle assembly in the ER compartment. Increasing the burden on the ER, especially at times of high viral production, would easily increase the accumulation of misfolded proteins in the ER lumen and activate the UPR. However, while activation of the UPR is essential for host cell survival during viral infection, some of the consequences of UPR activation would be detrimental to viral replication. Consequently,

many viruses have evolved mechanisms to regulate the UPR and promote efficient viral replication.

Some of the consequences of UPR activation would be detrimental to efficient viral replication, while others would promote viral protein folding and assembly. Activation of the PERK pathway and phosphorylation of eIF2α results in the attenuation of protein translation for most transcripts in the host cell and viral genome dependent upon cap-dependent protein translation. Thus, a virus that causes phosphorylation of eIF2α must escape translational inhibition in order to express viral and cellular gene products necessary for viral replication. However, activation of ATF4 results in the transcriptional upregulation of genes involved in reestablishing cellular metabolism (as part of the UPR recovery effort), translation and buffering oxidative stress (Harding et al., 2003), and may therefore be beneficial to viral replication. ATF6 activation typically results in the activation of molecular chaperones, whose expression would benefit virus replication by promoting the proper folding of viral proteins. Activation of XBP-1, however, increases the transcription of EDEM and thereby enhances the degradation of misfolded protein by ERAD. Activation of IRE1 and XBP-1 could hamper viral replication by degrading viral proteins that are not efficiently folded within the ER lumen.

Viruses can modulate components of the UPR

Several viruses that are known to induce ER stress and activate components of the UPR have adapted strategies to avoid or shutdown signaling pathways that would attenuate viral replication. Infection by herpes simplex virus (HSV) results in the activation of PERK and phosphorylation of eIF2α (Wylie et al., 2009), however, the virus is able to escape from the resultant translational inhibition (He et al., 1997). The $\gamma_1$34.5 gene of HSV has homology to GADD34, whose expression level modulates the rate of eIF2α dephosphorylation by protein phosphatase 1 (PP1). HSV infection results in the transcription and translation of $\gamma_1$34.5. As HSV induces ER stress and phosphorylation of eIF2α, $\gamma_1$34.5 associates with PP1 and mediates the dephosphorylation of

eIF2α. HSV, which has a large genome, encodes a viral protein to modulate the UPR. For many other viruses, the mechanisms by which they modulate and avoid the consequences of the UPR are not as well understood.

Cytomegalovirus (CMV) infection results in the activation of PERK, phosphorylation of eIF2α and increased translation of ATF4. Despite this strong activation of the PERK-eIF2α pathway, CMV infection does not attenuate protein translation. The mechanism by which CMV overcomes the translational inhibition normally mediated by phosphorylated eif2α is unknown (Isler et al., 2005).

Infection with dengue virus also results in increased levels of phosphorylated eIF2α, although this induction does not attenuate protein translation of either host or viral transcripts (Edgil et al., 2006). During infection, dengue virus may sustain host and viral cell protein translation by simultaneously inducing the phosphorylation of eIF2α and increasing total levels of eIF2α (Umareddy et al., 2007), thereby reducing the activated fraction of total eIF2. Interestingly, pharmacological induction of ER stress in dengue-infected cells inhibits approximately 50% of cellular protein synthesis, while translation of dengue viral proteins is only inhibited 0-20% (Edgil et al., 2006). This finding suggests that translation of dengue viral proteins, as compared to most host cell proteins, can occur efficiently under conditions of impaired cap-dependent protein translation. While the exact mechanism is still unknown, dengue virus can replicate by a novel noncanonical, cap-independent mechanism that is independent of eIF4E (Edgil et al., 2006). eIF4E, the rate limiting component of cap-dependent translation, directs ribosomes to the cap structure of mRNA transcripts.

Many viruses actively antagonize the host translational machinery in order to shut off host cell protein translation, thereby preventing the activation of host-mediated antiviral responses. Picornavirus and poliovirus infection results in the cleavage of eIF4G isoforms by viral proteases and results in host translational shutdown (Gradi et al., 1998). In order for viral protein translation

to continue unimpaired, viruses such as poliovirus and hepatitis C virus (HCV) utilize internal ribosome entry sites (IRES) (Pelletier and Sonenberg, 1988). These are RNA elements that directly recruit ribosomes in a cap-independent manner, thereby requiring only a subset of the canonical eIFs. While the cellular mechanisms underlying IRES-dependent translation are still under investigation, the use of IRES by viruses has been more intensively studied. The HCV IRES directly bind the 40S ribosomal subunit in such a way that the HCV initiator codons enter the ribosomal P-site without mRNA scanning. Consequently, HCV IRES-dependent translation requires eIF2 and eIF3, but not eIF1 or eIF4 isoforms (Fraser and Doudna, 2007). In contrast, the IRES of picornaviruses does not attract the 40S ribosomal subunit directly, rather, a high-affinity binding site for eIF4G brings viral mRNA into contact with the ribosome. Translation from the IRES is dependent upon all of the standard eukaryotic translation initiation factors, except for the cap-binding protein eIF4E. However, picornavirus transcripts recruit several RNA-binding proteins that are not usually involved in translation, such as La protein and the polypyrimidine tract-binding protein (PTB) (Hunt and Jackson, 1999), to stimulate their IRES-mediated translation. How these recruited proteins and other IRES *trans*-acting factors (ITAFs) aid in IRES-dependent protein translation is still under active investigation.

In addition to modulation of the PERK-eIF2α pathway and translation initiation, viruses can also modulate the rate of ERAD in order to prevent viral protein degradation. HCV infection results in increased splicing of the uXBP1 transcript and elevation of sXBP1 protein levels (Tardif et al., 2004). However, the transcriptional upregulation of UPRE genes and ERAD activity is repressed in HCV-infected cells, suggesting that HCV dampens the transcriptional activity of sXBP1 (Tardif et al., 2004). This defect in IRE1-XBP1 pathway signaling may ultimately result in the translation of more viral protein, since functional knockdown of IRE1 results in increased IRES-dependent translation (Tardif et al., 2004). Based upon these studies, it is predicted that a host cell experiencing ER stress following HCV infection may promote viral translation over host cell protein translation due to HCV-mediated modulation of the UPR.

While activation of the UPR is primarily an adaptive stress response, it can also be responsible for virus-induced cellular pathology. For example, respiratory syncytial virus (RSV), a lytic virus, primarily spreads by inducing cellular apoptosis via caspase-12, which is specifically activated with sustained ER-stress. Functional knockdown of caspase-12 protects RSV-infected cells from apoptosis, demonstrating that activation of the UPR is part of the viral life cycle (Bitko and Barik, 2001). Moloney murine leukemia virus (MoMLV), a retrovirus that causes a non-inflammatory spongiform neurodegenerative disease, induces the UPR as a consequence of viral envelope misfolding in the ER (Dimcheff et al., 2003). Interestingly, the severity of neurological disease correlates to the folding instability of the viral envelope, suggesting that the observed spongiform neurodegeneration is the consequence of a virus-induced protein folding disorder (Portis et al., 2009). Viruses that induce ER-stress and the UPR have evolved many mechanisms to promote efficient replication and avoid the antiviral consequences of host-cell adaptive stress responses. The effect of HIV on the UPR and the role of the UPR in HIV replication and neurotoxicity have not yet been explored.

HIV proteins are processed in the ER and Golgi

The genome of HIV consists of 9.8 kilobases of single-stranded RNA that encode nine viral genes. The HIV genome encodes for three structural genes (*env*, *gag* and *pol*) and six regulatory genes, which control viral replication (*vif*, *rev*, *nef*, *tat*, *vpu* and *vpr*). The extensive glycosylation of the HIV-1 envelope glycoproteins (gp), gp120 and gp41, plays an important role in evasion of the host immune system and is required for infectivity (Wei et al., 2003). First synthesized in the endoplasmic reticulum, the160kD envelope precursor protein (cleaved into gp120 and gp41 in later processing stages) undergoes extensive processing with the addition of 25-30 N-linked glycosylations (Leonard et al., 1990). In fact, approximately 50% of the molecular weight of envelope glycoprotein is due to oligosaccharide modifications. In addition to requiring extensive folding and glycosylation in the ER, HIV proteins also require the host cell's Golgi and endosome

network in order to complete the viral lifecycle. Newly synthesized envelope proteins are sent to the plasma membrane, where they must be endocytosed and routed back to the plasma membrane via the *trans*-Golgi-network (TGN) (Rowell et al., 1995). The TGN is a critical site for HIV biogenesis, as it where envelope glycoproteins incorporate into infectious viral particles. In HIV-infected macrophages, virus release then proceeds through the subsequent release of mature viruses by exocytosis (Nguyen et al., 2003; Pelchen-Matthews et al., 2003). Given the extent of the involvement of the ER and Golgi in HIV replication, HIV infection and may induce the accumulation of misfolded proteins in the ER and activate the UPR, especially during times of high viral replication. Interestingly, it has been reported that HIV's envelope protein directly interacts with BiP (Earl et al., 1991).

HIV infection may induce and modulate the UPR

Given the extent of involvement of the ER and Golgi in HIV biogenesis, we hypothesize that HIV infection induces the UPR in macrophages, especially at times of high viral replication. However, since HIV replicates efficiently without causing cellular apoptosis in macrophages, HIV may also modulate the UPR in order to prevent the inhibition of protein translation and upregulation of CHOP. Furthermore, macrophages experiencing ER stress may be more prone to producing neurotoxins and/or inflammatory mediators that drive HAND pathology. In order to address these questions, we will use our *in vitro* model system and pharmacologic manipulations of the UPR in infected and non-infected human macrophages. We will assess the impact of HIV infection on the UPR, the role of the UPR in modulating HIV infection and the consequences of UPR induction on neurotoxin production.

Results

HIV infection induces phosphorylation of eIF2α in human MDM

It has become increasingly clear that viruses that activate adaptive stress responses in the host cell have adapted to modulate or take advantage of these responses in order to promote efficient viral replication. We hypothesized that HIV infection of human macrophages would result in the activation of the UPR, especially during periods of high viral replication. As shown in Figure 5.2B, HIV-infected monocyte derived macrophages (HIV/MDM) demonstrate a biphasic induction of phosphorylated eIF2α, while total eIF2, BiP and GRP94 (probe not shown) levels remain steady. Phosphorylated eIF2α is highest following the 24hours of infection and at times of high viral load. This increase in phosphorylated eIF2α is a consequence of active HIV replication, since HIV-infected macrophages treated with the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ), do not show elevations in phosphorylated eIF2α at either 24hours post infection (HPI) or at times of high viral reverse transcriptase (RT) activity (Figure 5.2D). EFZ treated HIV/MDM are exposed to viral inoculum and have normal levels of viral entry but the HIV-genome is not reverse transcribed, incorporated into the host genome or actively replicated. This suggests that HIV infection of the host cell increases levels of phosphorylated eIF2α via viral post-entry and post reverse transcription mechanisms. Furthermore, the biphasic induction of phosphorylated eIF2α may represent two separate signaling processes with two different time courses, both of which result in enhanced phosphorylation of eIF2α. Currently, we do not know the kinase responsible for phosphorylating eIF2α in HIV-infected macrophages.

In addition to PERK, the classic kinase mediating eIF2α phosphorylation in the UPR, there are three other kinases capable of phosphorylating eIF2α. These are protein kinase RNA regulated kinase (PKR), heme-regulated inhibitor kinase (HRI) and general control nonderepressible-2 kinase (GCN2). All of these kinases inhibit protein synthesis by phosphorylation of eIF2α, although their tissue distributions and activator domains make each kinase uniquely suited to detect different stress conditions. PKR is induced by interferon and double-stranded RNA

(dsRNA) and is a principal mediator of host-cell antiviral responses since dsRNA rarely occurs under normal conditions within the cell. PKR activation has been reported during human T-lymphotropic virus type I (HTLV-1) (Mordechai et al., 1995), Epstein-Barr virus (EBV) (Elia et al., 1996), Hepatitis D (Circle et al., 1997; Robertson et al., 1996) and HIV infection (Maitra et al., 1994; Roy et al., 1991), among others. GCN2, which is highly expressed in the liver and brain, is activated under conditions of nutritional deprivation and inhibits protein translation via phosphorylated eIF2α in order to conserve amino acids for essential metabolic processes (Sood et al., 2000). However, stressors unrelated to nutritional deprivation, such UV irradiation, proteasomal inhibition and viral infection can also activate GCN2 (Jiang and Wek, 2005). Sindbis virus (SV) genomic RNA directly binds to GCN2 and functional GCN2 decreases the permissiveness of cells to both SV and vesicular stomatitis virus (VSV) infection. These findings suggest that GCN2 contributes to the cellular antiviral response to RNA viruses (Berlanga et al., 2006). HRI, which is primarily expressed in cells of the erythroid lineage, attenuates protein synthesis in heme-deficient states, coordinating hemoglobin synthesis with iron availability (Chen and London, 1995). HRI protein is also present in macrophages and HRI regulates iron homeostasis and macrophage maturation (Liu et al., 2007). Less is known about the role of HRI in viral infection, although HRI can be transcriptionally and translationally upregulated in response to heat shock, treatment with a dsRNA mimic (Polyinosinic:polycytidylic acid; poly I:C) and infection with the aquareovirus, grass carp hemorrhagic virus (GCHV) (Zhu et al., 2006). While each of the four eIF2α kinases have a stimulus that they are particularly well suited to detect, it is clear that they can be broadly activated by several stresses and that any or all of these kinases could be activated during viral infection.

HIV infection in human macrophages induces the biphasic induction of phosphorylated eIF2α without altering total eIF2α or BiP levels (Figure 5.2). The increase in phosphorylated eIF2α observed at either 24HPI or at times of high viral replication may be mediated by any of the four known eIF2α kinases. At periods of high viral replication, the enhanced burden on the processing

capacity of the ER and Golgi by HIV glycoproteins could result in the accumulation of misfolded proteins and activation of PERK. The transactivation-responsive (TAR) element of HIV forms an elaborate RNA tertiary structure, including a 23-basepair hairpin with three bulges capable of dimerization, which is capable of activating PKR (Heinicke et al., 2009). GCN2 could be activated during periods of high viral replication if the rate of viral protein synthesis exceeded the availability of amino acids needed for basal host cell translation. Cellular iron homeostasis has been linked to rates of HIV transcription, suggesting that HRI activation and activity may impact HIV replication in macrophages (Debebe et al., 2007). In order to determine the kinase(s) responsible for eIF2α phosphorylation in HIV-infected macrophages, genetic manipulation of the human macrophage is necessary. With typical transfection protocols, only 2-5% of primary human macrophages will express the inserted gene of interest. However, recent work with Minivector DNA (a non-viral, supercoiled vector incorporating short hairpin RNA) has been reported to work well in primary cells such as macrophages and dendritic cells (Zhao et al., 2011). This highly biostable vector produces high cell transfection efficiency and gene silencing capacity, which would be required in order to detect effects on HIV replication levels (Zhao et al., 2011). Using the Minivector DNA system, we could knockdown each of the eIF2α kinases to determine the role of each in modulating phosphorylated eIF2α levels in basal conditions and during HIV infection.

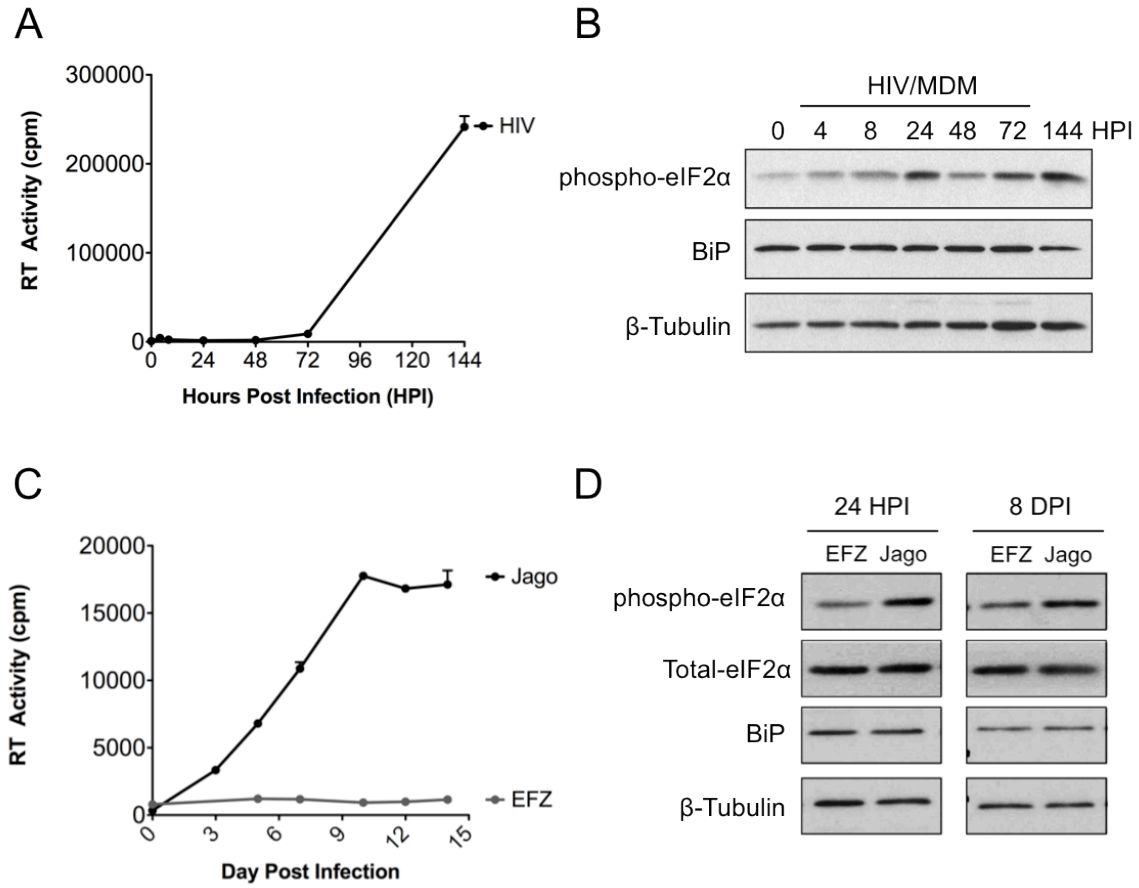

Figure 5.2 HIV infection induces phosphorylation of eIF2α in human MDM. A) MDM were infected with HIV-Jago (0-24 HPI) and culture supernatants were collected, as indicated, and assessed for reverse transcriptase (RT) activity. Values indicate the mean ± SEM. **B)** HIV infection results in a biphasic induction of phosphorylated eIF2α without an increase of BiP, as assessed by Western blotting. Maximum levels of phosphorylated eIF2α occur at the end of the 24-hour infection and at times of high viral production/release. Blot is representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. **C)** MDM were infected with HIV-Jago (day -1 to day 0) and culture supernatants were collected every 2-3 days, as indicated, and assessed for RT activity. Viral replication is effectively inhibited when MDM are treated with the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ, 20nM), over the course of infection. Values indicate the mean ± SEM. **D)** HIV infection results in increased levels of phosphorylated eIF2α at 24-hours post infection (24 HPI) and at times of high viral production/release (day 8 post infection), relative to EFZ-treated MDM, as assessed by Western blotting. Blot is representative of 3 independent experiments, with each replicate performed on cell preparations from different donors.

HIV infection increases levels of macrophage specific BiP in the human frontal cortex of HAND

Our *in vitro* model system of HIV-induced macrophage-mediated neurotoxicity utilizes primary human macrophages and rat cerebrocortical cultures. Following HIV-infection of human macrophages, cell culture supernatants are collected over the course of infection. This supernatant is applied to cerebrocortical cultures (in a 1:10 to 1:50 dilution) and neuronal survival is then assessed. While this model system utilizes primary cells and recapitulates the fundamental processes mediating HAND neuropathogenesis, many aspects of the human disease cannot be modeled. *In vitro*, the majority of our macrophages are infected with HIV over the course of the infection. In contrast, it is estimated that less than 0.1% of peripheral circulating monocytes are infected with HIV (Crowe et al., 2003). The proportion of HIV-infected CNS-resident macrophages is likely less than 5% during ART, although this is expected to be highly variable and dependent upon the CNS-penetrance of ART regimen, relative immune-activation state of the individual and extent of CNS neuroinvasion and inflammation, for which the susceptibility factors are not well understood. Since the overwhelming majority of cells in the CNS are astrocytes and neurons, techniques that selectively parse out effects in CNS-resident macrophages are required to assess the role of the macrophage in the neuropathology of HAND. We analyzed human frontal cortex for brain-resident macrophages by indirect immunofluorescence (IFA) in order to characterize the state of UPR activation in HIV-associated neurocognitive disorder (HAND).

IFA staining of BiP (Figure 5.3) and phosphorylated-eIF2α (Figure 5.4) in human frontal cortex from HIV-, HIV+ and HAND cases was performed. IFA results demonstrate that HIV infection associated with HAND significantly increases total macrophage specific BiP, relative to uninfected controls (HIV-) (Figure 5.3D). Cognitively normal, HIV+ cases had macrophage specific BiP levels in between the uninfected controls and the HAND cases. This increase in macrophage specific BiP in HAND cases occurred in the absence of increased macrophage area (Figure 5.3A and 5.3B), suggesting that BiP expression is increasing on the individual cell level. Phosphorylated-

eIF2α staining has proven more difficult. The signal for phosphorylated-eIF2α in macrophages has thus far been weak and highly variable, resulting in quantifications with large error bars and no statistical significance between conditions (Figure 5.4). At this point, we have no evidence that levels of macrophage-specific phosphorylated eIF2α are atlered with either HIV infection or neurocognitive status.

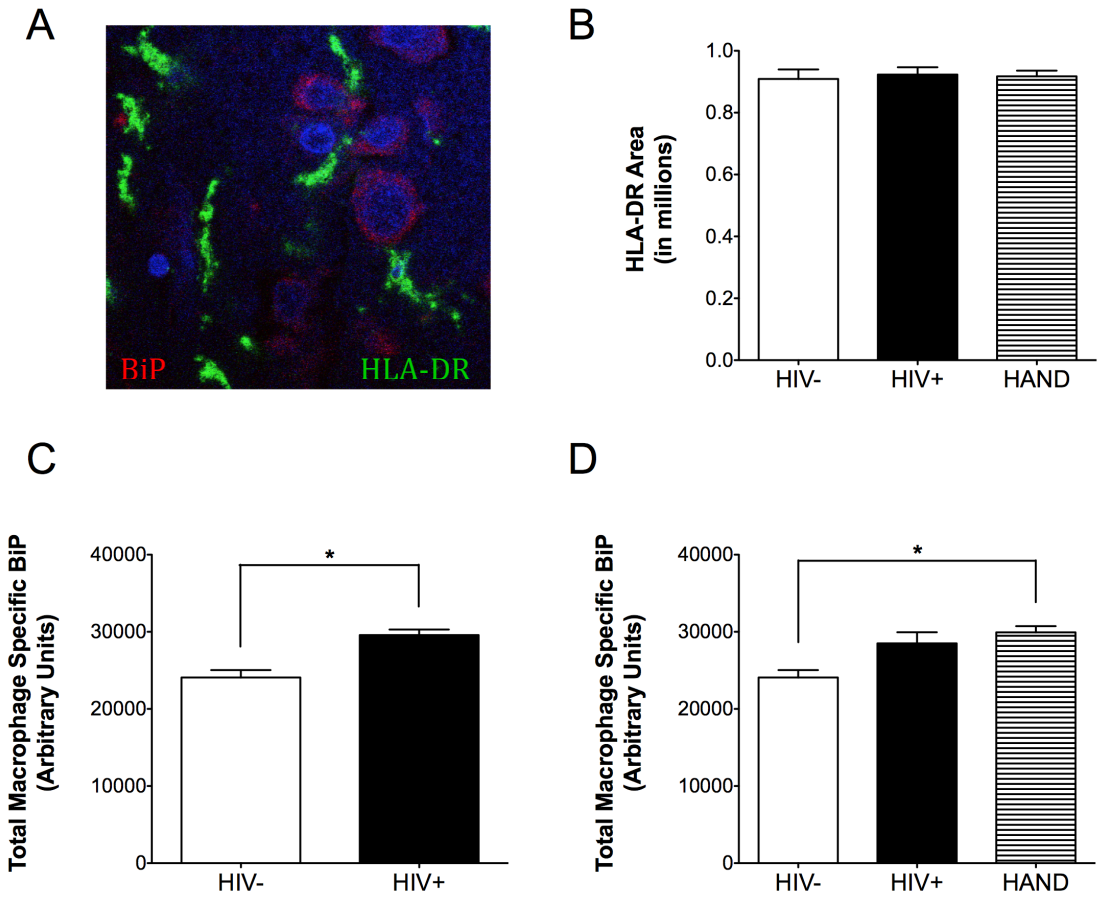

Figure 5.3 HIV infection increases levels of macrophage-specific BiP in the human frontal cortex of HAND cases. A) Frontal cortex from cognitively normal HIV- and HIV+ cases and neurocognitively impaired HIV+ (HAND) cases were labeled with BiP, HLA-DR (macrophage marker, CCR3/43 antibody) and DAPI (DNA marker, blue). Quantification of Macrophage/HLA-DR pixel area (**B**) shows no increase of HLA-DR-positive cells in HIV+ or HAND cases. Quantification of integrated pixel intensity of BiP colocalizing with HLA-DR (**C**, **D**) shows increased cell type-specific BiP expression with HIV infection and neurocognitive impairment (HAND). Statistical comparisons were made by two-tailed *t*-test in C and one-way ANOVA plus Newman-Keuls post hoc testing in D, *p<0.05. Data is expressed as mean ± SEM for n = 2 HIV-, n = 3 HIV+, cognitively normal and n = 9 HAND. For panels A and C, HIV+ represents all HIV+ cases (n = 12). Table 3.1, in the Methods section, provides further information on case history and tissue collection of individual samples.

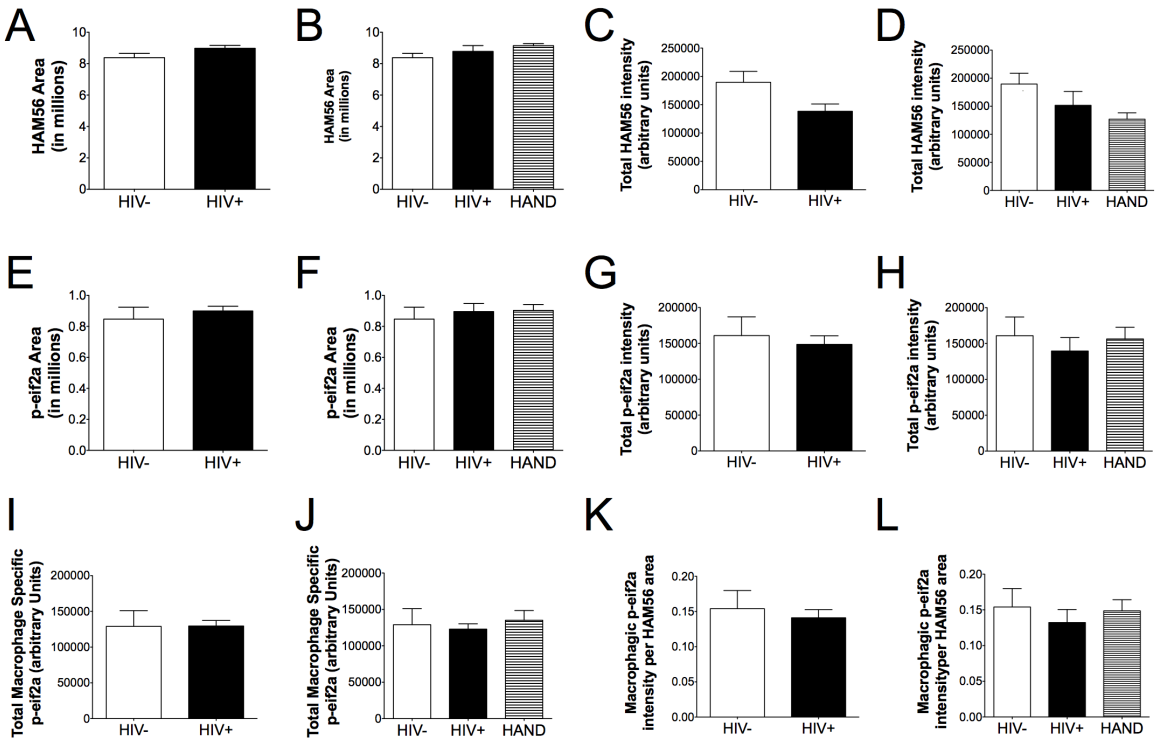

Figure 5.4 HIV infection does not alter levels of macrophage specific phosphorylated-eIF2a in the human frontal cortex of HAND cases. A) Frontal cortex from cognitively normal HIV- and HIV+ cases and neurocognitively impaired HIV+ (HAND) cases was labeled for phosphorylated eIF2α and HAM56, a macrophage/microglia marker. Quantification of HAM56 pixel area (**A**, **B**) and integrated pixel intensity of HAM56 (**C**, **D**) shows no change in the staining of HAM56-positive cells in HIV+ or HAND cases, as compared to HIV- cases. Quantification of phosphorylated eIF2α pixel area (**E**, **F**) and integrated pixel intensity of phosphorylated eIF2α (**G**, **H**) shows no change in the staining of phosphorylated eIF2α-positive cells in HIV+ or HAND cases, as compared to HIV- cases. Quantification of integrated pixel intensity of phosphorylated eIF2α colocalizing with HAM56 (**I**, **J**) and integrated pixel intensity of phosphorylated eIF2α colocalizing with HAM56, normalized to GFAP area (**K**, **L**), shows no increased macrophage-specific phosphorylated eIF2α expression with HIV infection or neurocognitive impairment. Statistical comparisons were made by two-tailed *t*-test or one-way ANOVA plus Newman-Keuls post hoc testing and no significant differences were found (significance was defined as $p<0.05$). Data is expressed as mean ± SEM for n = 4 HIV-, n = 2 HIV+, cognitively normal and n = 13 HAND. For panels comparing only HIV- and HIV+, HIV+ represents all HIV+ cases (n = 15). Table 3.2, in the Methods section, provides further information on case history and tissue collection of individual samples.

Pharmacologic induction of the UPR by thapsigargin attenuates HIV replication and enhances neurotoxin production in human MDM

Using our *in vitro* system, we found that HIV infection resulted in the activation of phosphorylated eIF2α at times of high viral replication (Figure 5.2). We have also demonstrated that the UPR is activated, with increased BiP expression, in CNS-resident macrophages of the frontal cortex in HAND (Figure 5.3). While the extent of UPR activation following HIV infection has not yet been fully characterized, initial studies suggest that HIV infection activates components of the UPR in macrophages and that the extent of this activation may correlate with the clinical appearance of HAND. To investigate the role of UPR activation in modulating HIV replication and neurotoxin production in infected macrophages, we used pharmacological inducers of the UPR in HIV/MDM in our *in vitro* model system.

Pharmacological agents that disrupt calcium homeostasis in the ER, such as thapsigargin, induce the UPR. Thapsigargin is a non-competitive inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and is considered a classic activator of the UPR. Thapsigargin treatment decreases ER calcium levels, decreases the activity of calcium-dependent chaperones and induces the accumulation of misfolded proteins in the ER lumen. However, inhibition of SERCA can result in the secondary activation of plasma membrane calcium channels, resulting in increased cytosolic calcium levels and alterations in the regulatory functions of Ca^{2+}-dependent kinases. While thapsigargin is considered a strong activator of the UPR, the signaling pathways induced by thapsigargin treatment vary according to cell type, concentration and duration of treatment and need to be empirically defined for each model system.

HIV-infected macrophages were pulsed with thapsigargin for 6 hours after infection was established and viral replication was robust. As shown in Figure 5.5, thapsigargin treatment drastically reduces HIV replication in the 48 hours following drug exposure. This reduction in viral replication is not a consequence of cell death, since HIV replication began to recover in the 2-4

days following thapsigargin exposure (Figure 5.5A). Thapsigargin treatment induced a robust increase in phosphorylated eIF2α and a moderate increase in levels of BiP following the 6 hour treatment (Figure 5.5B). And, these markers of the UPR were not altered in vehicle treated HIV/MDM. This pharmacological induction of the UPR was transient and could no longer be observed 6 days after thapsigargin treatment. These findings demonstrate that HIV-infection does not prevent the induction of the UPR and that infected macrophages are capable of responding to robust ER stress by increasing levels of ERSE regulated genes, such as BiP. Furthermore, robust activation of the UPR is capable of inhibiting viral replication, suggesting that HIV cannot efficiently escape the antiviral effects of the UPR, including translational inhibition and/or induction of ERAD. Since inhibition of virus replication has traditionally resulted in less neurotoxin production in HIV-infected macrophages (Figures 4.1 and 4.2), we assessed the consequence of the inhibition of viral replication due to thapsigargin treatment on macrophage-mediated neurotoxicity.

As previously discussed, attenuation of HIV replication with the nonnucleoside reverse transcriptase inhibitor (NNRTI), efavirenz (EFZ), significantly abrogates neurotoxin production in HIV/MDM (Figure 4.2 and Figure 5.5C). Interestingly, activation of the UPR and attenuation of HIV replication with thapsigargin treatment enhances neurotoxin production in HIV/MDM (Figure 5.5C). This demonstrates that the inhibition of virus replication does not always produce a concomitant decrease in neurotoxin production in HIV/MDM; an important implication for the development of adjunctive therapies for HAND. And as shown in Figure 5.5D, uninfected macrophages, which normally do not produce neurotoxins, cause significant neurotoxicity when treated with thapsigargin. These findings suggest that ER stress can modulate neurotoxin production in macrophages and that the elevations of phosphorylated eIF2α with HIV infection (Figure 5.2) and increased BiP levels in HAND (Figure 5.3) may contribute to HIV-induced macrophage-mediated neurotoxicity.

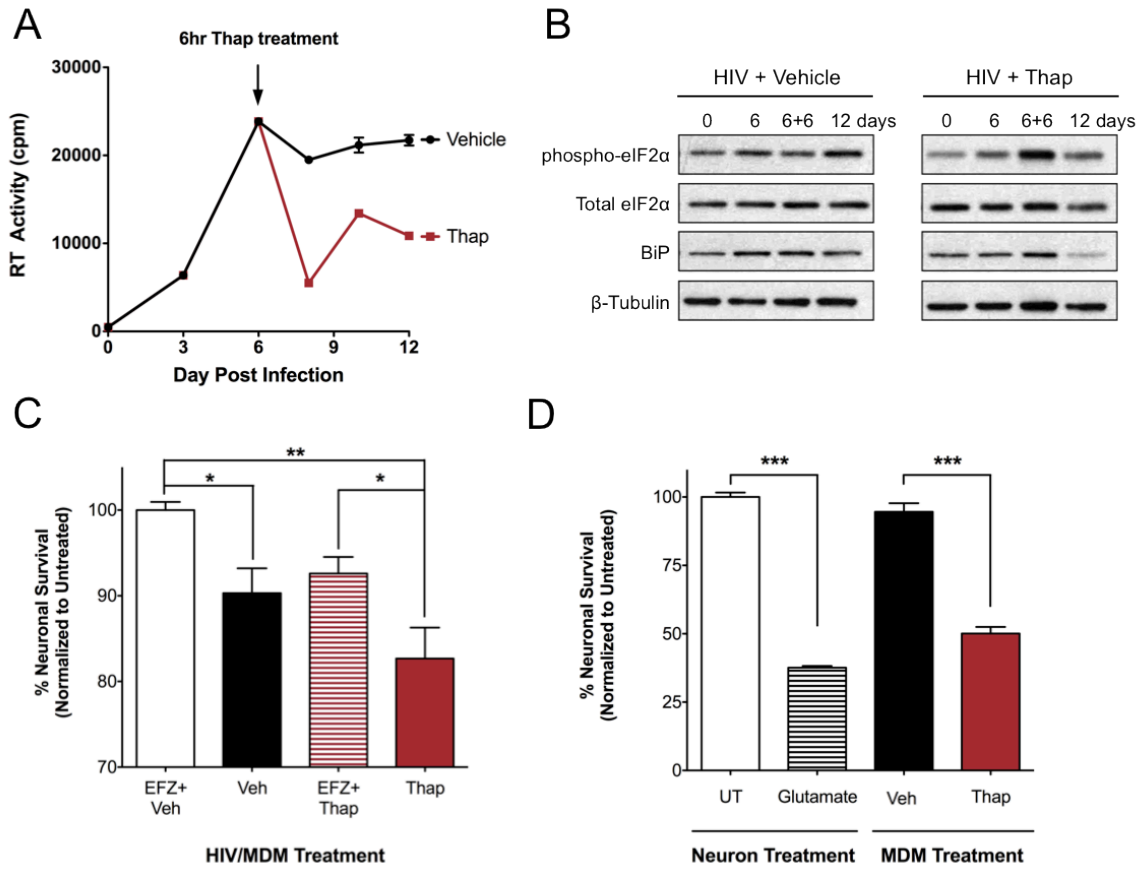

Figure 5.5 Thapsigargin attenuates HIV replication and enhances neurotoxin production in human MDM. A) Thapsigargin (Thap) attenuates HIV replication in human MDM. MDM were infected with HIV-Jago and then treated for 6 hours on day 6 post infection with 0.5μM Thapsigargin. Following drug exposure, cells were washed and put into fresh drug-free media. Culture supernatants were collected every 2-3 days, as indicated, and assessed for RT activity. Values indicate the mean ± SEM. RT curves are representative of 3 independent experiments, with each replicate performed on cell preparations from a different donor. **B)** Thapsigargin treatment results in increased levels of phosphorylated eIF2α and BiP, as assessed by Western blotting of lysates collected after the 6 hour thapsigargin treatment on day 6 post infection (6+6). Thapsigargin treatment enhances neurotoxin production in **C)** HIV-infected and **D)** uninfected human macrophages. For C, culture supernatants from day 8 post infection were assayed. For D, uninfected macrophages were treated with 0.5μM of thapsigargin for 24hours followed by drug washout and media replacement. Culture supernatant from 72 hours after thapsigargin exposure was then assayed. Neuronal survival was assessed by MAP2 ELISA and data is expressed as a percentage of untreated (UT) cultures (n = 6). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, *p<0.05, **p<0.01 and ***p<0.001.

Phosphorylated eIF2α attenuates HIV replication and enhances neurotoxin production

Thapsigargin treatment results in multiple cellular effects, including robust activation of the UPR signaling pathways that respond to the accumulated misfolded proteins in the ER. However, thapsigargin treatment also results in the depletion of ER Ca^{2+} stores and potential alterations to Ca^{2+} dependent kinases in the cytosol. In order to better define the role of the UPR in affecting HIV replication and macrophage-mediated neurotoxicity, more specific pharmacological modulators were used. Clotrimazole (CLT) disrupts ER calcium stores by inhibiting calcium-activated potassium (K_{Ca2+}) channels, resulting in calcium release from intracellular stores of the ER. In contrast to thapsigargin, however, clotrimazole treatment of human macrophages does not induce the UPR, as assessed by Western blotting for phosphorylated eIF2α and BiP (Figure 5.6C). Sal003, a derivative of salubrinal with enhanced cell permeability, indirectly inhibits the dephosphorylation of eIF2, resulting in robust activation of the UPR (Figure 5.5C) without directly affecting ER calcium levels or Ca2+ dependent kinases. As shown in Figure 5.6A, clotrimazole has no major effect on HIV replication when added to HIV/MDM for 24 hours during an established infection. In contrast, Sal003 treatment inhibited HIV replication by approximately 25%, as compared to vehicle control. This suggests that HIV replication is moderately sensitive to phosphorylated eIF2α levels, but not to disruptions in ER calcium homeostasis. While Sal003 most likely mediates the attenuation of HIV replication by inhibiting translation via induction of phosphorylated eIF2α, further experiments are required to confirm the mechanism of action.

While clotrimazole treatment had minimal impact on HIV replication, supernatant collected from macrophages that were pulsed with clotrimazole 48 hours previously were more neurotoxic than the vehicle treated control (Figure 5.6B). Sal003 treatment, which attenuated HIV replication, also caused HIV-infected macrophages to produce more neurotoxins (Figure 5.6B). These results confirm that attenuation of HIV infection does not necessarily result in a concomitant decrease in neurotoxin production by macrophages. Furthermore, both the activation of the UPR via induction

of phosphorylated eIF2α and disruption of ER calcium homeostasis are capable of enhancing neurotoxin production in HIV-infected macrophages.

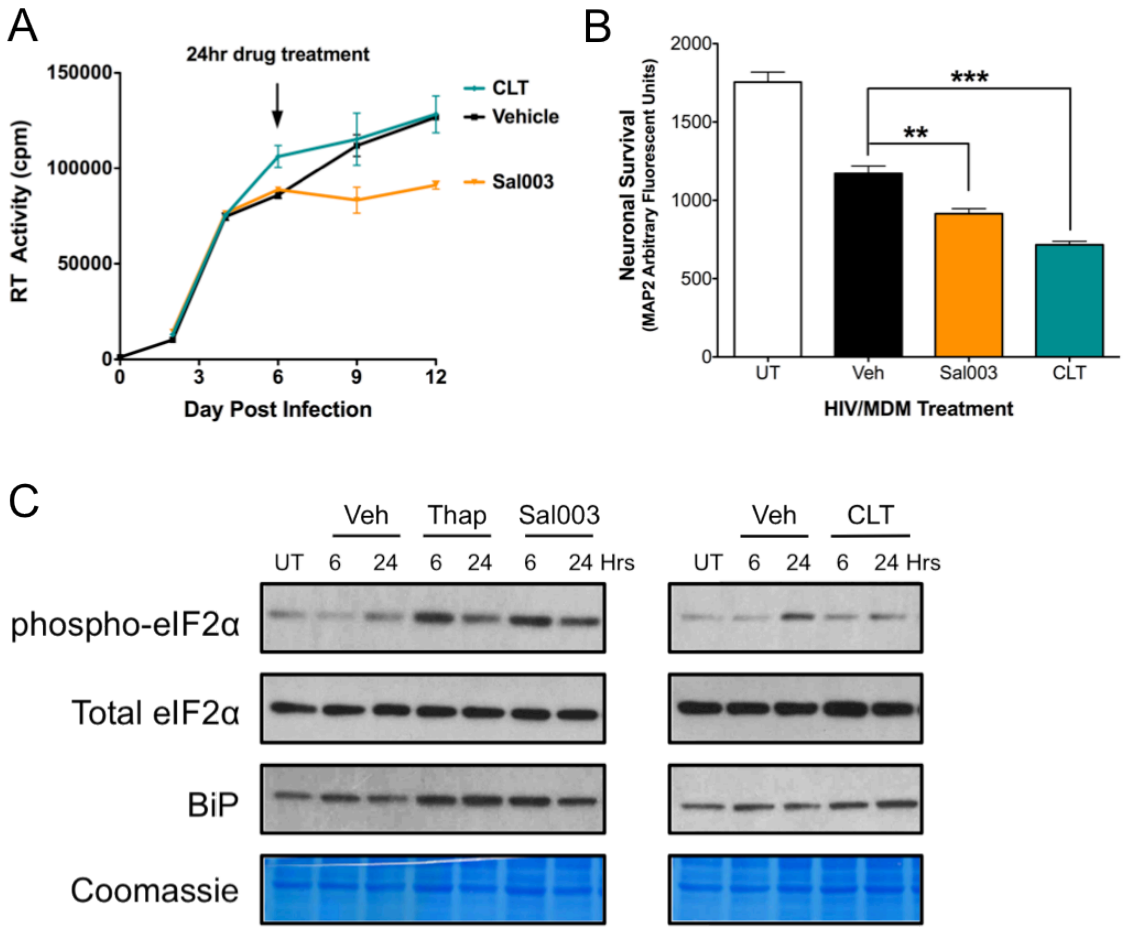

Figure 5.6 Pharmacological induction of phosphorylated eIF2α and disruption of Ca^{2+} stores in the endoplasmic reticulum enhances neurotoxin release in human MDM. A) Sal003, a specific inhibitor of eIF2α dephosphorylation, but not clotrimazole (CLT), an inducer of Ca^{2+} release from the endoplasmic reticulum, attenuates HIV replication in human macrophages. MDM infected with HIV-Jago were pulse-treated with Sal003 or clotrimazole for 24 hours (day 6-7 post infection). Following drug exposure, cells were washed and put into fresh drug-free media. Culture supernatants were collected every 2-3 days, as indicated, and assessed for RT activity. Values indicate the mean ± SEM. **B)** Sal003 and clotrimazole enhance neurotoxin production in HIV-infected human macrophages. Neuronal survival was assessed by MAP2 ELISA following exposure to macrophage supernatants from day 9-post infection, which had been treated with Sal003 or clotrimazole 48-hours previously. MAP2 data is expressed in arbitrary fluorescent units (n = 6). All statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing, **p<0.01 and ***p<0.001. **C)** 0.5μM thapsigargin and 50μM Sal003 treatment (6 or 24-hours) results in increased levels of phosphorylated eIF2α and BiP, while 10μM clotrimazole has little effect on these markers of the unfolded protein response (UPR), as assessed by Western blotting in uninfected human macrophages.

Activation of the antioxidant response reduces levels of phosphorylated eIF2α in HIV-infected macrophages

In addition to misfolded protein accumulation in the ER, the UPR can also respond to oxidative stress (Holtz et al., 2006; Yan et al., 2008). Reactive oxygen species (ROS) can target ER calcium channels and protein folding enzymes to exacerbate ER calcium release and ER stress. As a consequence of ROS, unfolded or misfolded proteins can accumulate in the ER and induce the activation of the classic UPR pathways (Ron and Walter, 2007; Zhang and Kaufman, 2008). We have previously demonstrated that HIV infection results in the dysregulation of components of the antioxidant response in macrophages and that specifically, reductions in HO-1 levels contribute to macrophage-mediated neurotoxicity (Cross et al., 2011). We first sought to determine how restoration of the antioxidant response, through DMF and MMF treatment, affected levels of phosphorylated eIF2α in HIV-infected macrophages. As shown in Figure 5.7, DMF and MMF treatment inhibited HIV replication and induced components of the antioxidant response (HO-1 and NQO1) in a dose-dependent manner. DMF and MMF treatment caused substantial decreases in phosphorylated eIF2α in a dose-dependent manner, approaching levels seen in uninfected (mock) MDM with 30μM of DMF/MMF (Figure 5.7E and F). Additionally, DMF and MMF treatment also resulted in moderate decreases in BiP and GRP94, while total levels of eIF2α remained unchanged. Since DMF/MMF treatment results in both the restoration of the antioxidant response (Figure 4.4) and attenuation of HIV replication in HIV/MDM, the observed decreases in phosphorylated eIF2α could be a consequence of either effect.

In uninfected macrophages, MMF treatment results in the activation of the antioxidant response, as detected by increased levels of Nrf2 and NQO1 (Figure 5.8A). MMF treatment also decreases levels of GRP94 and BiP, without substantial alterations to levels of phosphorylated eIF2α or total eIF2α (Figure 5.8B). These findings suggest that DMF and MMF activate the antioxidant response and cause the concomitant down-regulation in expression of ERSE regulated genes, including BiP and GRP94, without significantly affecting levels of phosphorylated eIF2α.

Therefore, in HIV-infected macrophages, DMF/MMF treatment likely decreases levels of phosphorylated eIF2α secondary to attenuation of HIV replication. However, further experiments are necessary to demonstrate that phosphorylated eIF2α levels are not varying with the redox state of the cell, since induction of HO-1 and NQO1 can correlate with a substantial decrease in phosphorylated eIF2α levels without significant changes in the level of HIV replication (5μM versus 15μM DMF treatment in Figure 5.7).

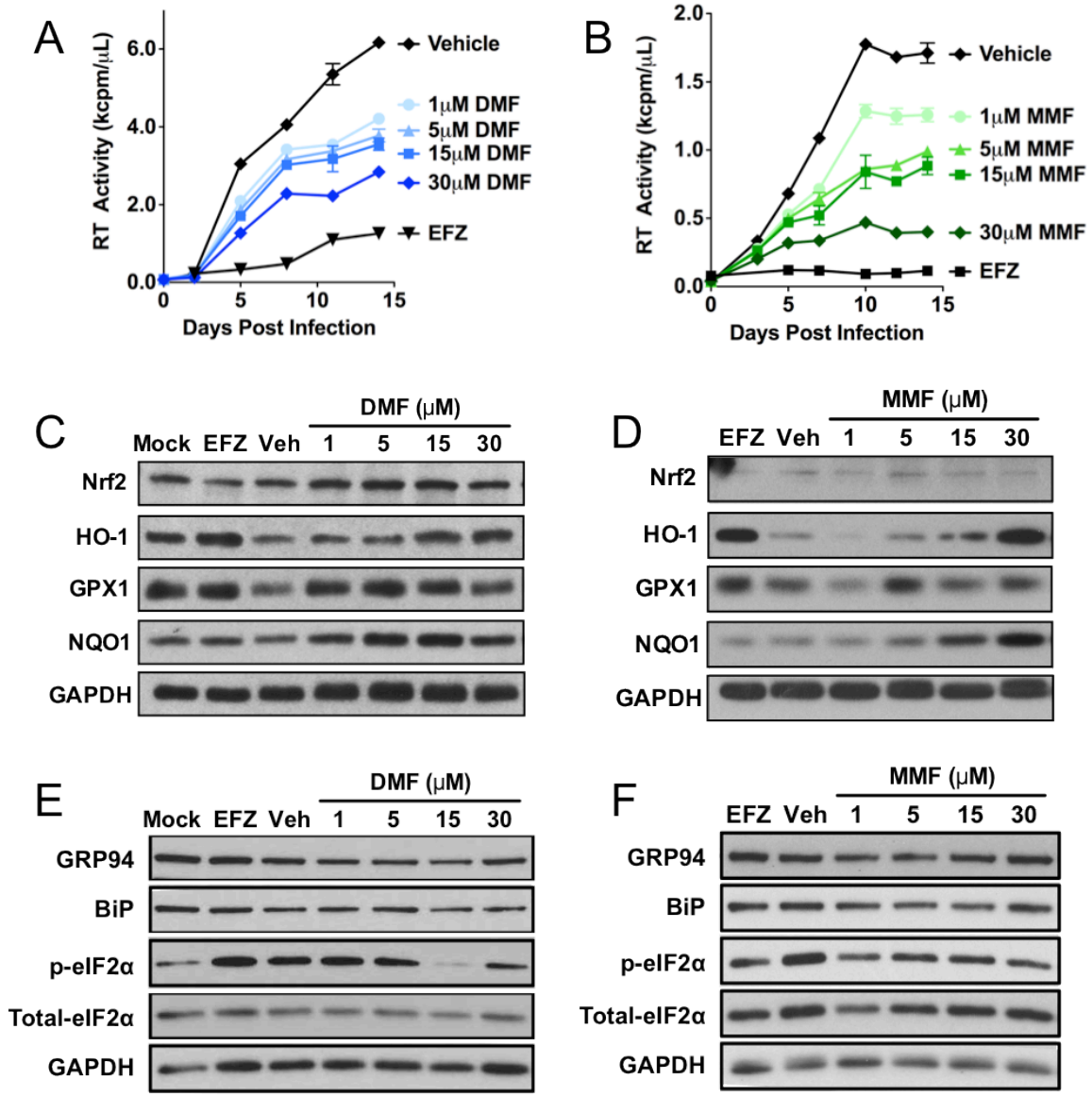

Figure 5.7 DMF and MMF activate the antioxidant response and decrease levels of phosphorylated eIF2α in HIV-infected human macrophages. A) DMF and **B)** MMF attenuate HIV replication in human MDM. MDM were infected with HIV-Jago and treated with DMF, MMF or the non-nucleoside reverse transcriptase inhibitor, efavirenz (EFZ), over the course of infection. Culture supernatants were collected every 2-3 days, as indicated, and assessed for reverse transcriptase (RT) activity. Values indicate the mean ± SEM. RT curves are representative of 3 independent experiments, with each replicate performed on cell preparations from a different donor. **C)** DMF and **D)** and MMF activate the Nrf2-dependent antioxidant response in HIV/MDM and restore HO-1 and GPX1 levels to that found in uninfected Mock cells. **E)** DMF and **F)** MMF decrease levels of phosphorylated eIF2α, which is induced with HIV replication, as assessed by Western blotting. DMF and MMF treatment also decreases Grp94 and BiP levels in a dose-dependent manner. Blots are representative of 2-4 independent experiments, with each replicate performed on cell preparations from different donors.

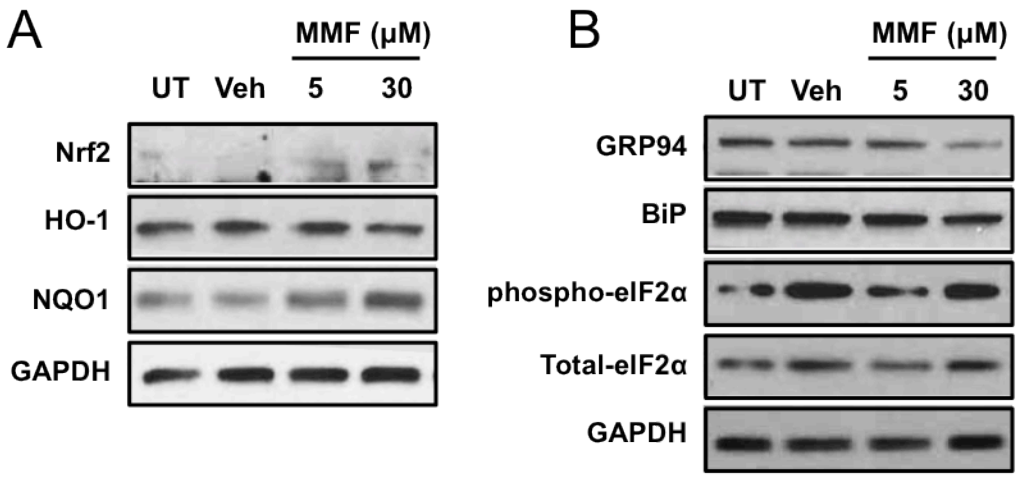

Figure 5.8 MMF activates the antioxidant response and decreases levels of Grp94 and BiP in uninfected human macrophages. A) MMF activates the Nrf2-dependent antioxidant response in uninfected human macrophages, as demonstrated by increased levels of NQO1 and Nrf2. **B)** MMF treatment decreases Grp94 and BiP levels in a dose-dependent manner in uninfected human macrophages, without consistently affecting levels of phosphorylated eIF2α. Blots are representative of 2 independent experiments, with each replicate performed on cell preparations from different donors.

Levels of phosphorylated eIF2α do not predict the extent of HIV replication or neurotoxin production in HIV-infected macrophages

Components of the UPR and antioxidant response are both altered during HIV infection of macrophages. As we have previously demonstrated that decreased expression of HO-1 is associated with high levels of HIV replication and neurotoxin production in HIV/MDM (Cross et al., 2011), we examined levels of phosphorylated eIF2α in conditions of high viral replication but altered neurotoxin production. As shown in Figure 5.9, restoration of HO-1 levels with CoPP, an inducer of HO-1 expression, decreases macrophage-mediated neurotoxicity without altering levels of replication. In contrast, enzymatic inhibition of HO-1 with SnMP results in enhanced neurotoxin production in the absence of alterations to HIV replication. Interestingly, both SnMP and CoPP treatment decreased levels of phosphorylated eIF2α, relative to vehicle controls, without causing significant alterations to GRP94, BiP or total eIF2α levels (Figure 5.9D). This

suggests that phosphorylated eIF2α levels do not necessarily correlate with HIV replication or with neurotoxin production in HIV-infected macrophages. Furthermore, these results suggest that pharmacological modulation of the antioxidant response, even in the setting of high viral replication and enhanced neurotoxin production, is sufficient to decrease levels of phosphorylated eIF2α. CoPP and SnMP are relatively specific for their actions on HO-1, however, both drugs also moderately increase NQO1 levels, suggesting that they have off-target effects on other components of the antioxidant response element (ARE). SnMP treatment also causes a reflexive increase in total HO-1 protein levels (Figure 5.9E), and while the enzymatic activity of HO-1 is inhibited, HO-1 may alter the redox state of the cell or dampen activation of phosphorylated eIF2α via a non-enzymatic mechanism. In fact, total HO-1 levels may specifically correlate with phosphorylated eIF2α since unlike DMF and MMF treatment, CoPP and SnMP had no effect on levels of the ERSE regulated chaperones, BiP and GRP94. The relationship between the antioxidant response and the UPR is complex and has not been fully elucidated for any cell type or paradigm. However, these experiments suggest that induction of the antioxidant response dampens the UPR in HIV-infected macrophages, although the underlying mechanisms and consequences of this interaction require more study.

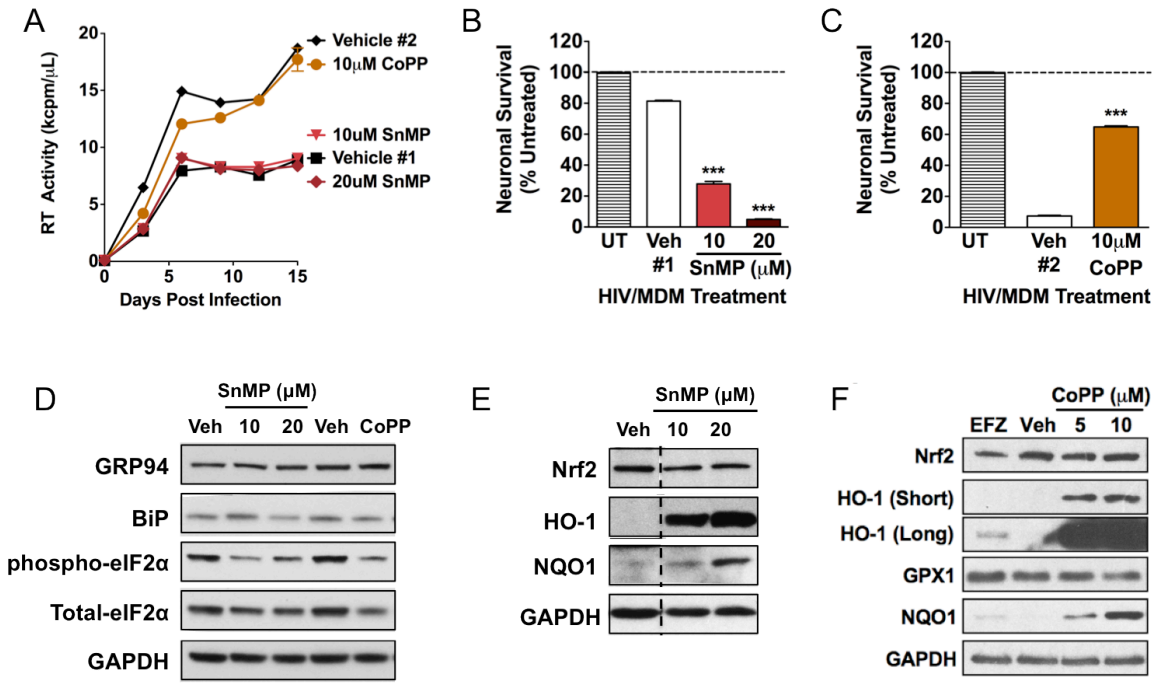

Figure 5.9 Levels of phosphorylated eIF2α do not correlate with HIV replication or neurotoxin production in HIV-infected human macrophages. A) SnMP, an enzymatic inhibitor of HO-1, and CoPP, an inducer of HO-1, do not alter levels of HIV replication. MDM were infected with HIV-Jago and then treated with SnMP or CoPP from day 6 post infection onwards and culture supernatants were collected every 3 days and assessed for RT activity. RT curves are representative of 3 independent experiments, with each replicate performed on cell preparations from different donors. **B)** SnMP enhances neurotoxin production while (**C**) CoPP decreases neurotoxin production in HIV-infected macrophages. For neuronal survival assays, survival was assessed by MAP2 ELISA and expressed as a percentage of untreated (UT) cultures (n = 6; ***p<0.001 vs. Vehicle). Statistical comparisons were made by one-way ANOVA plus Newman-Keuls post hoc testing. **D)** SnMP and CoPP decrease levels of phosphorylated eIF2α, as assessed by Western blotting. **E)** SnMP inhibits HO-1 enzymatic activity but exponentially increases total levels of HO-1 and modestly increases NQO1, as assessed by Western blotting. **F)** CoPP treatment exponentially increases HO-1 levels and modestly increases NQO1 levels, as assessed by Western blotting. Western blots are representative of 2-3 independent experiments, with each replicate performed on cell preparations from different donors. Two film exposures (short and long) of HO-1 are presented in order to demonstrate the extent of HO-1 induction over basal levels.

Discussion

Viral infection can induce the activation of host cell survival pathways, which may have direct antiviral effects and/or promote cell survival during sustained ER stress, alterations to the cellular redox state, and viral infection. We have demonstrated that HIV infection of human macrophages results in the activation of phosphorylated eIF2α and BiP, two critical components of the UPR, and that activation of the UPR correlates with macrophage-mediated neurotoxicity. In our *in vitro* model system, HIV infected macrophages have elevated levels of phosphorylated eIF2α during times of high viral replication but expression of the ERSE regulated genes, BiP and GRP94, is not increased. However, we found a significant increase in the amount of macrophagic-BiP in HAND frontal cortex, demonstrating that the UPR and ERSE regulated genes are activated in HIV infected brain tissue, suggesting as association with human pathology. Both our *in vitro* and *in vivo* data demonstrate activation of the UPR during HIV infection of the macrophage. Importantly, in our *in vitro* model system the majority of macrophages are robustly infected with HIV, while less than 5% of macrophages in the CNS are estimated to be infected during HAND. Furthermore, HAND pathology is mediated by both infected and non-infected, activated macrophages with both populations driving neuroinflammation and producing excitatory neurotoxins. Therefore, primary HIV infection of the macrophage may increase levels of phosphorylated eIF2α without activation of ERSE regulated genes. In contrast, immune activation of non-infected (or chronic, low-level replicating) macrophages may induce expression of ERSE genes, which may be more sensitive to upregulation in inflammatory states. In order to address these hypotheses, we plan to assess for the activation of phosphorylated eIF2α and ERSE regulated genes in uninfected macrophages that have been exposed to virus-free HIV/MDM supernatant in our *in vitro* model system. In this way, we can determine how the UPR is modulated in uninfected macrophages exposed to a pro-inflammatory environment induced by HIV-infection. We are also continuing evaluate how the three signaling pathways comprising the UPR (PERK, IRE1 and ATF6) respond to HIV infection and correlate to macrophage-mediated

neurotoxicity. Given limitations to available antibodies and the difficulty of performing efficient genetic manipulations in primary human macrophages, future studies will utilize qPCR.

Importantly, we have demonstrated that conditions that induce robust activation of the UPR, with alterations in ERSE gene expression, are associated with the enhancement of neurotoxin production in macrophages (Figure 5.5 and 5.6). This suggests that activation of ER stress pathways can augment neurotoxin production pathways, even in uninfected macrophages. Interestingly, we have noted that in our *in vitro* model system, HIV-infected macrophages produce neurotoxins late in the course of infection. The extent of neurotoxin production varies with the donor and as previously noted, increases with robust HIV replication. However, macrophage-mediated neurotoxicity also correlates with the appearance of increased phosphorylated eIF2α and decreased levels of HO-1, as assessed by Western blotting. While this may be a coincidental observation, it is consistent with the hypothesis that neurotoxin production in the macrophage is enhanced under states of extreme cellular stress, as indicated by dysregulation of the antioxidant response and activation of the UPR. Furthermore, these findings have important implications for the development of adjunctive therapeutics for HAND. Reduction of HIV replication has been the principal target for ameliorating the pathological consequences of HIV-infection. And while ART has greatly improved clinical outcomes for patients infected with HIV, our data demonstrate that drugs that activate the UPR may attenuate HIV replication, but will also enhance macrophage-mediated neurotoxicity. In addition to physiological stressors like viral infection and inflammation, there have been reports that ART, specifically protease inhibitors, can activate the UPR in macrophages (Zhou et al., 2005). The role of ART-induced activation of the UPR in macrophages and the consequence to macrophage-mediated neurotoxicity has not yet been studied and remain a focus for future research.

Activation of the UPR, especially phosphorylated eIF2α, during viral infection may be part of a cellular antiviral defense that serves to limit virus replication in the host cell. We have

demonstrated that HIV infection activates components of the UPR and that pharmacological induction of the UPR can attenuate HIV replication. This suggests that during HIV infection, the induction of phosphorylated eIF2α likely has some antiviral effect, but that HIV replication is relatively efficient despite this. In fact, selective induction of phosphorylated eIF2α with the pharmacological agent Sal003 (Figure 5.6) resulted in only a 25% inhibition of viral replication when applied for 24 hours. In contrast, a 6 hour treatment with thapsigargin resulted in a 70% inhibition of viral replication. These results suggest that high levels of phosphorylated eIF2α can attenuate HIV replication, to an extent, but that robust activation of all components of the UPR has a more substantial effect on viral replication. Perhaps, thapsigargin treatment induces the IRE1-XBP1 signaling pathway to upregulate EDEM and ERAD, thereby inducing an antiviral component of the UPR that HIV infection does not. It also remains possible that HIV is capable of escaping the translational inhibition normally imposed by activation of phosphorylated eIF2α.

In order to understand how induction of the UPR alters HIV replication, there are several key experiments that need to be undertaken. One of the outstanding questions for this work is whether the level of phosphorylated eIF2α induced with HIV infection is sufficient to attenuate host cell protein translation. And, to determine how sensitive uninfected macrophages are to phosphorylated eIF2α-mediated inhibition of protein translation. In HIV-infected macrophages, we have seen no evidence of protein instability by Western blotting to suggest that the levels of phosphorylated eIF2α induced with infection have a substantial effect on protein translation. However, we must determine if this is due to viral modulation of the UPR and/or cellular translational machinery or an intrinsic property of the macrophage. To address this question, we are planning to perform metabolic labeling experiments (^{35}S-methionine and ^{35}S-cysteine) on uninfected and HIV-infected macrophages, both in the presence and absence of pharmacological modulators of the UPR (thapsigargin, Sal003 and cyclohexamide). We will also use qPCR to determine if HIV-induced induction of phosphorylated eIF2α results in the transcriptional upregulation of ATF4, and if ATF4 transcriptional targets and CHOP are upregulated.

If we find that HIV-infected macrophages are less sensitive to the inhibition of protein translation as mediated by phosphorylated eIF2α, we must then consider the mechanism by which HIV modulates this component of the UPR. The 5' leader of the genomic RNA of HIV has an IRES (Brasey et al., 2003), and it is possible that HIV replication is capable of efficient translation even in conditions of phosphorylated eIF2α-mediated inhibition of protein translation, in a mechanism similar to other stress-responsive mRNAs (Fernandez et al., 2002). Independent of these findings, the relationship between UPR-activation following HIV infection and neurotoxin production in the macrophage will also be explored.

Following HIV infection of the CNS, infected and non-infected/activated macrophages and glia release neurotoxins that result in neuronal dendritic damage, apoptosis and necrosis (Adamson et al., 1996; Dawson et al., 1993; Gelbard et al., 1995; Kaul et al., 2001; O'Donnell et al., 2006; Petito and Roberts, 1995). These neurotoxins include HIV viral proteins, prominflammatory cytokines, chemokines, and excitatory amino acids (Chen et al., 2002; Dawson et al., 1993; Kaul et al., 2001; Power et al., 1998; Tardieu et al., 1992). Excitatory neurotoxins that mediate neuronal damage via NMDA receptor activation are the major mediators of HIV-induced neurotoxicity. These low-molecular weight excitatory neurotoxins include glutamate, quinolinic acid, platelet activating factor, reactive oxygen species and NTox. In our *in vitro* system, glutamate has been determined to mediate a significant component of HIV/MDM-mediated neurotoxicity, with other low-molecular weight heat resistant species also contributing (O'Donnell et al., 2006). This correlates to *in vivo* findings, where HIV-infected patients have been found to have significantly higher concentrations of glutamate in their plasma and CSF, compared to uninfected controls (Droge et al., 1987; Ferrarese et al., 2001; Ollenschlager et al., 1988). Glutaminase, which is localized to the inner membrane of the mitochondria, is the primary enzyme involved in converting glutamine into glutamate by macrophages in the CNS (Curthoys and Watford, 1995; Holcomb et al., 2000; Nicklas et al., 1987; Wurdig and Kugler, 1991). HIV

infection results in the upregulation of the GAC isoform of glutaminase and its release into the cytosol of macrophages, presumably due to HIV-induced destabilization of the mitochondrial membrane (Erdmann et al., 2009). Interestingly, sustained activation of the UPR results in caspase-12 activation and cellular apoptosis mediated by mitochondrial outer membrane permeabilization via BAX and/or BAK. Therefore, activation of the UPR by HIV-infection or pharmacological treatment may result in enhanced macrophage-mediated neurotoxicity by compromising the mitochondria membrane and increasing glutaminase-mediated glutamate production. It is also possible for ER stress to directly affect the mitochondria through calcium and reactive oxygen species, thereby altering mitochondrial processes without inducing apoptosis pathways (Zhang and Kaufman, 2008).

In addition to investigating glutamate production during UPR activation in HIV-infected macrophages, we will also examine quinolinic acid (QUIN). QUIN is a neurotoxin derived from tryptophan through the kynurenine metabolic pathway. HIV infection increase levels and the activity of indoleamine-2,3-dioxygenase (IDO), the rate-limiting enzyme of the kynurenine pathway, which leads to increased metabolism of tryptophan and the enhanced generation of neurotoxins such as QUIN (Guillemin et al., 2003; Sardar et al., 1995). Generation of QUIN by IDO is limited to activated macrophages and microglia in the CNS and IDO is induced by exposure to interferon-γ or during infection in order to deplete cellular tryptophan and limit metabolic processes (Carlin et al., 1989). Furthermore, TNFα levels can alter IDO activity, suggesting that any process that alters the inflammatory state and TNFα production can ultimately modulate IDO and QUIN production in the macrophage (Werner-Felmayer et al., 1989). UPR stress can directly increase inflammation and the production of proinflammatory mediators (Zhang and Kaufman, 2008). Activation of IRE1 can induce a signaling cascade involving JNK and the transcription factor AP1, resulting in the transcription of genes involved in the inflammatory response. Additionally, phosphorylated eIF2α-mediated inhibition of protein

translation can result in the destabilization of IκB, promoting the nuclear translocation of NF-κB proteins and inducing the expression of TNFα and other pro-inflammatory genes.

Interactions between ER stress, oxidative stress and the inflammatory response are necessary and crucial for cell types with high metabolic and secretory demands, such as macrophages (Gregor and Hotamisligil, 2007; Schenk et al., 2008; Zhang and Kaufman, 2008), in order to cope with the cellular consequences of high levels of protein translation. Numerous stimuli such as glucose, lipids, and cytokines can activate both the UPR and the inflammatory pathways through calcium, reactive oxygen species, and/or nitric oxide mediators. We have demonstrated that HIV-infection of human macrophages results in the suppression of components of the antioxidant response and activation of the UPR and that alterations to these pathways affect neurotoxin production following HIV infection. Interestingly, restoration of the antioxidant response attenuates HIV replication, decreases neurotoxin production and dampens induction of phosphorylated eIF2α (Figure 5.7). However, decreased neurotoxin production is associated with restoration of HO-1 levels and not to levels of phosphorylated eIF2α or the ERSE regulated genes, Bip and GRP94. Phosphorylated eIF2α levels can be dampened by restoring the antioxidant response in HIV-infected macrophages, even in the setting of robust HIV replication. These data suggest that reducing phosphorylated eIF2α will not necessarily attenuate HIV replication or neurotoxin production. In fact, phosphorylated eIF2α and activation of the UPR in HIV-infected macrophages may better correlate to overall cellular health, redox state or levels of inflammatory signaling. In HIV-infected macrophages, activation of the UPR likely enhances neurotoxin production, but our studies suggest that treatments that dampen activation of phosphorylated eIF2α will not be effective as adjunctive therapies in HAND. However, more study is needed to understand the role of phosphorylated eIF2α during HIV infection, especially as it intersects with inflammatory pathways and neurotoxin production in macrophages.

CHAPTER 6

DISCUSSION

Summary of Work

Despite antiretroviral therapy (ART), HIV infection of the central nervous system (CNS) promotes cognitive dysfunction and neurodegeneration through persistent inflammation and the release of neurotoxic factors from infected and/or activated macrophages/microglia. The persistence of HAND in individuals effectively controlled for systemic viral replication is incompletely explained, although prolonged inflammation in both the CNS and periphery may be responsible (Ancuta et al., 2008; Brenchley et al., 2006b; Eden et al., 2007). Because markers of inflammation and immune activation correlate with disease progression and morbidity in ART-treated individuals, drugs targeting these pathological processes are needed for effective, adjunctive therapy. This work begins to address the roles of two adaptive stress pathways, the antioxidant response and the unfolded protein response (UPR), in modulating macrophage-mediated neurotoxicity following HIV infection.

Using our *in vitro* model of HIV-mediated neurotoxicity, we have shown that HIV infection dysregulates the macrophage antioxidant response, with profound reductions in levels of heme oxygenase-1 (HO-1). Induction of the antioxidant response with DMF, MMF or tBHQ attenuates HIV replication in a dose-dependent manner. Our data suggest that decreased levels of the HIV co-receptors, CXCR4 and CCR5, mediate attenuation of viral replication following the induction of the antioxidant response. We have also demonstrated that activation of the antioxidant response and restoration of HO-1 expression, specifically, reduces HIV-induced macrophage-mediated neurotoxicity. Importantly, we demonstrate that therapeutics that increase HO-1 levels, even during times of robust HIV replication, can suppress neurotoxin production. In addition to effects on macrophage-mediated neurotoxicity, inducers of the antioxidant response can also modulate inflammatory pathways and may decrease the immune activation state of HIV-infected macrophages. We have demonstrated that induction of the antioxidant response with DMF, MMF and tBHQ inhibits of NF-κB nuclear translocation, signaling and the production of the proinflammatory mediator, TNFα. Finally, we found that DMF and MMF treatment attenuates

CCL2-induced monocyte chemotaxis, suggesting that therapeutic inducers of the antioxidant response could decrease recruitment of activated monocytes to the CNS in response to inflammatory mediators. We propose that dysregulation of the antioxidant response during HIV infection drives macrophage-mediated neurotoxicity and that inducers of the antioxidant response, such as DMF, could serve as adjunctive neuroprotectants and HIV disease modifiers in ART-treated individuals.

In addition to investigating the antioxidant response in HIV-infected macrophage, we also assessed the consequence of HIV infection on the unfolded protein response (UPR). Using our *in vitro* model system, we found that HIV infection increases levels of phosphorylated eIF2α in human macrophages. We also demonstrate that the chaperone BiP, considered the master regulator of the UPR, is increased in the macrophages of HAND frontal cortex. HIV-infected macrophages are capable of responding to pharmacological induction of the UPR and induction of the UPR attenuates HIV replication. These results suggest that induction of phosphorylated eIF2α during HIV replication has antiviral consequences, although activation of the UPR it is not sufficient to considerably attenuate HIV replication during the normal course of infection in macrophages. Interestingly, pharmacological induction of the UPR, which attenuates viral replication, is associated with increased macrophage-mediated neurotoxicity. This finding has important implications for the development of adjunctive therapies for HAND. Therapeutics or processes that induce the UPR in macrophages, regardless of the effect on HIV replication, could enhance neurotoxin production and contribute to the pathological processes underlying HAND.

As we have also described the effect of HIV replication on the antioxidant response, we assessed how restoration of the antioxidant response altered the UPR in HIV-infected macrophages. Activation of the antioxidant response with DMF and MMF attenuated HIV replication and decreased levels of phosphorylated eIF2α and BiP in HIV/MDM. Reductions in phosphorylated eIF2α trend with increased levels of HO-1, but do not depend upon HO-1 enzymatic activity. In

contrast, BiP levels decreased with induction of the antioxidant response, but these alterations were independent of HO-1. These findings suggest that while dysregulation of the antioxidant response reflects levels of HIV replication and HO-1 levels modulate neurotoxin production, levels of phosphorylated eIF2α and BiP do not predict the extent of HIV replication or macrophage-mediated neurotoxicity. Ongoing studies are assessing the link between UPR activation, inflammation pathways and the antioxidant response as they relate to HIV infection and macrophage-mediated neurotoxicity.

Developing adjunctive therapies for HAND requires an understanding of the pathological processes underlying the disease. The macrophage is a critical mediator of HAND neuropathology and is a critical mediator of inflammation and neurotoxin production within the CNS. This body of work focused on the roles of the antioxidant response and the UPR in modulating HIV replication, macrophage-mediated neurotoxicity and inflammatory processes. These experiments have demonstrated that induction of the antioxidant response, but not the UPR, should be considered as a potential target for adjunctive therapies for HAND. Our lab is continuing its focus on the role of the antioxidant response in modulating the neurological consequences of HIV infection and is planning to apply this work to animal model systems and hopefully, human clinical trials in the future.

Implications

Despite highly active antiretroviral therapies (ART) and effective virological control, approximately 50% of HIV-infected individuals will develop neurological consequences of HIV infection. In order to develop effective adjunctive therapies for HAND, we must consider and target the pathological consequences of HIV infection that persist in the CNS despite ART. Chronic immune activation, inflammation and viral persistence all contribute to neuronal damage and one of the principal cell types mediating these processes is the macrophage. While previous studies have explored the pathways responsible for neurotoxin production, little is known about the mechanisms linking HIV infection to these pathways. We hypothesized that HIV infection alters many components of cellular metabolism and signaling within the infected macrophage. Specifically, we focused on adaptive stress pathways, which have been implicated in the modulation of viral replication and viral-mediated pathology in recent years. We concentrated on the antioxidant response, due to clinical data demonstrating alterations to the oxidative state in HIV-infected patients, and the unfolded protein response (UPR), because of its clear link to inflammation pathways and its involvement during infection by many other viruses. The effect of HIV infection on the antioxidant response, the UPR and the interactions between these adaptive stress responses and inflammation had not yet been explored.

This work has begun to address the roles of the antioxidant response and UPR in modulating HIV-induced macrophage mediated neurotoxicity and has implications for the development of adjunctive therapy for HAND. Namely, we demonstrate that restoration of the antioxidant response in HIV-infected macrophages can attenuate many of the key pathways contributing to the neuropathology of HAND including HIV replication, neurotoxin production, inflammatory signaling and chemotaxis. In order to develop more targeted therapies, we are continuing to explore how the antioxidant response affects neurotoxin production pathways and are concentrating on the role of HO-1 and its enzymatic activity. HO-1 reduces neurotoxin production in macrophages, even when viral replication is robust, suggesting that it may have a direct effect

on or modulate a critical component of neurotoxin production pathways. There are also reports that HO-1 plays a role in determining the immune activation state of cells, although this is still at a rather speculative stage and will require more experimentation. However, when considering how the antioxidant response, inflammation and neurotoxin production pathways modulate and are influenced by one other, we will need to consider the immune activation state, or profile, of the HIV-infected macrophages in our *in vitro* system.

The microbial and cytokine milieu drives macrophages, T-cells, and other cell types of the immune system to express specialized and polarized functional properties. Interestingly, it has been reported that MMF shifts CD4+ T-cells towards a Th2 phenotype, which inhibits proinflammatory signaling (de Jong et al., 1996; Litjens et al., 2004). DMF and MMF likely also influence the polarization of activated and HIV-infected macrophages. TNFα exposure elicits a classic M1 form of macrophage activation, which results in the secretion of high levels of proinflammatory cytokines (TNFα, IL-1 and IL-6), and reactive oxygen and nitrogen intermediates (Mantovani, 2006). In contrast, M2 is a generic name for various forms of macrophage activation, which encompass all but M1-activated cells. The various versions of M2 cells generally dampen proinflammatory cytokine levels (IL-4Rα, IL-10, IL-1 receptor antagonist^{high}, decoy IL-1 type II receptor^{high}) and have high levels of scavenger, mannose, and galactose-type receptors. DMF may promote a shift from M1 activated macrophages towards a M2 phenotype. M2 HIV-infected macrophages would produce less TNFα, have less NF-κB signaling and may have decreased transcription from the HIV-LTR. It is also highly likely that this immune activation state and alterations to inflammation pathways and cell surface receptors will influence signaling events related to neurotoxin production pathways. The mechanism by which DMF and MMF shift the activation state of immune cells towards an anti-inflammatory state and the impact of activation state on neurotoxin production remains to be determined.

With this body of work, we have also demonstrated the antioxidant response is a better therapeutic target in regards to reducing macrophage-mediated neurotoxicity than targeting the UPR. It has been well established that activation of the UPR can drive inflammatory pathways and the production of proinflammatory mediators (Ron and Walter, 2007; Zhang and Kaufman, 2008). And although pharmacological activation of the UPR attenuates HIV replication, it also enhances neurotoxin production, making it a poor therapeutic target for HAND. These data have implications for how we think about continued inflammation and macrophage-mediated neurotoxicity in patients experiencing neurological complications of HIV. Patients who are well controlled for viral replication through ART can still have neuronal damage and neurological impairment. We demonstrate that HAND patients have evidence of UPR activation in the frontal cortex, as evidenced by elevated levels of macrophagic BiP. And as we have demonstrated that UPR activation enhances neurotoxin production, even if HIV replication is low or absent, these results suggest that activation of the UPR may be driving neurotoxin production in macrophages even in the setting of ART. Even more importantly, our data demonstrate that simply controlling HIV replication does not guarantee a concomitant decrease in macrophage-mediated neurotoxicity, a concept that has not been previously proposed. Furthermore, we need to consider how potential therapies impact the UPR of the macrophage when we consider adjunctive therapies for HAND, since controlling viral replication is not sufficient to prevent neurological sequelae or ensure a reduction in neurotoxicity.

This body of work has proposed many future avenues of research in regards to the role of adaptive stress responses in macrophage-mediated neurotoxicity. One of the key questions that remains in this work is how the antioxidant response, UPR and neurotoxin pathways intersect. It has been demonstrated that intracellular calcium signals and free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO), act as messengers in coordinating adaptive stress responses. However, we have yet to determine if HIV infection directly impacts the antioxidant response or UPR, or if the markers of modulation/activation that we detect are an indirect

consequence of infection or mediated by other signaling pathways. It is possible that the antioxidant response drives the activation of the UPR, increases inflammation and directly modulates neurotoxin production pathways. However, it is also a possibility that activation of the UPR or inflammation drives the antioxidant response and neurotoxin production. In order to develop targeted and effective adjunctive therapies for HAND, we must continue to explore these pathways and identify critical mediators of the pathology.

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