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The Role of HIV-1 Proteins in Alzheimer's Disease Pathology

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The Role of HIV-1 Proteins in Alzheimer’s Disease Pathology

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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ABSTRACT

Prevalence of HIV-associated cognitive impairment is rising, the worst form of which is HIV-associated dementia (HAD). The disease is fuiled by a chronic innate type pro-inflammatory response in the brain which is highly dependent upon the activation of microglia. We first created an in vitro model of HAD composed of cultured microglial cells synergistically activated by the addition of IFN-γ and the HIV-1 coat glycoprotein, gp120. This activation, as measured by TNF-α and NO release, is synergistically attenuated through the α7nAChR and p44/42 MAPK system by pretreatment with nicotine, and the cholinesterase inhibitor, galantamine. As these medications have been FDA approved, and over time, have shown only minor improvement in neurodegenerative disease for a limited period, we next sought to explore natural compounds that may attenuate HAD mediated inflammation and related pathology. This inflammation is a key moderator of Aβ plaque deposition in the brain. Indeed it is likely a contributing factor as epidemiological data suggests significant numbers of HIV survivors are at elevated risk of developing Alzheimer's disease (AD). HIV-1 Tat-induced Aβ deposition, tau phosphorylation, and subsequent neuronal death could be risk factors for subsequent AD and/or HAD. Recent reports suggest green tea-derived (-)-epigallocatechin-3-gallate (EGCG) can attenuate neuronal damage mediated by conditions such as brain ischemia. In order to investigate the therapeutic potential of EGCG to mitigate the neuronal damage characteristic of HAD, IFN-γ was evaluated for its ability to enhance well-known neurotoxic properties of HIV-1 proteins gp120 and Tat
in primary neurons and mice. Indeed, IFN-γ enhanced the neurotoxicity of gp120 and Tat via increased JAK/STAT signaling. Additionally, primary neurons pretreated with a JAK1 inhibitor, or those from STAT1-deficient mice, were largely resistant to the IFN-γ-enhanced neurotoxicity of gp120 and Tat. Moreover, EGCG treatment of primary neurons from normal mice reduced IFN-γ-enhanced neurotoxicity of gp120 and Tat by inhibiting JAK/STAT1 pathway activation. EGCG was also found to mitigate the neurotoxic properties of HIV-1 proteins in the presence of IFN-γ in vivo. To explore the mechanism by which HIV may augment AD-like pathology, we found HIV-1 Tat protein inhibits microglial uptake of Aβ1-42 peptide, a process enhanced by IFN-γ and rescued by EGCG. To mimic the HAD clinical condition, we generated mice with HIV-1 Tat-induced AD-like pathology by cross-breeding HIV-1 Tat expressing mice (expressed under control of GFAP, Doxycycline inducible promoter) with the PSAPP mouse model of AD. To simulate chronic Tat secretion over we used an optimized dose of 54 mg/kg/day on a biweekly basis over three months Tat significantly induced neuron degeneration and tau phosphorylation in Tat transgenic mice, dox dependently (P<0.001). Similar effects at the chronic 54 mg/kg/day dose were observed in PSAPP/Tat mice induced with dox. These mice also showed significantly more Aβ deposition (P < 0.05), neurodegeneration, neuronal apoptotic signaling, and phospho-tau than PSAPP mice (P < 0.05). In conclusion, HIV-1 Tat significantly promotes AD-like pathology in PSAPP/Tat mice. This model may provide a framework in which to identify new mechanisms involved in cognitive impairment in the HIV infected population, and possible treatments. Additional works will be needed to fully characterize the mechanism(s) of HIV-induced amyloid deposition, and to uncover viral mechanisms promoting AD-like pathology in general.
CHAPTER ONE
INTRODUCTION

1.1 The Impact of HIV-associated neurocognitive disorders (HAND) including HIV

According to the Centers for Disease Control and Prevention greater than 1 million individuals in the United States are infected with the human immunodeficiency virus (HIV). Worldwide, some 33 million people are infected. HIV-1 enters the central nervous system (CNS) early after primary infection, and commonly in HIV-1 associated neurologic disease (HAND) (McArthur et al., 2010). HAND may include neurocognitive and behavioral impairments, motor deficits, or dementias (Kaul and Lipton, 2006, Clark et al., 2010); the most extreme form being HIV-associated dementia (HAD).

Combination antiretroviral therapies (cART) are widely available in developed countries and have led to efficacious reduction of viral load however, HAND including HAD remain a major public health concern (Gupta et al., 2010). Indeed, HIV is the most common cause of neurocognitive impairment in patients under 50 years of age (Ances and Ellis, 2007).

Although cART has reduced the incidence of HAD, it cannot always prevent it (Masliah et al., 2000) or reduce its severity when administered to cART-naive patients (Chang et al., 2003). With cART-treated HIV infected patients living longer, the prevalence of HAND continues to increase, pointing to the importance of understanding HIV neuropathogenetic mechanisms as they may synergize with, or increase susceptibility to, comorbidly evolving neurological disease in aging HIV
infected populations, including (PD) (Gelbard et al., 2010) and Alzheimer’s Disease (AD) (Clifford et al., 2009; Rempel and Pulliam, 2005; Green et al., 2005, Alisky et al., 2007, Esiri et al., 1998).

1.2 HIV life cycle

Because of the variability of HIV-1 phenotypes, strains are defined by their co-receptor usage of either (chemokine receptor type 5) CCR5 or c-x-c chemokine receptor type 4 (CXCR4), and termed R5- or X4- viruses respectively (Wu and Marsh, 2001). Among the human chemokine receptors, CXCR4 appears most important for HIV-1 entry into lymphocytes while CCR5 is more crucial for monocytes, macrophages and microglia (Moore et al., 2004). After cell entry, reverse transcription of the HIV RNA genome occurs; generating a double-stranded DNA, a pre-integration complex of viral DNA with integrase, and other viral protein such as Vpr. This pre-integration complex facilitates integration of the HIV DNA into the host cell chromatin; generating the provirus and eventually mature HIV-1. High levels of viral DNA remain non-integrated within the nucleus; directing expression of further viral transcripts as well (Wu and Marsh, 2001; Kilzer et al., 2003). Production of infectious virus particles is facilitated by the generation of viral transcripts, proteins such as Nef, Tat and Rev, viral assembly, release, and maturation. RNA genomes are produced in a later phase. During the assembly phase, Gag and Gag-Pol Polyproteins, envelope proteins, and viral RNA are assembled into immature virus particles at the cell membrane where they are released. Finally, mature virus results after the cleavage of Gag and Gag-Pol polyproteins by HIV-1 protease (Nielsen et al., 2005; Seelamgari et al., 2004).
1.2.1 HIV infection of CNS inflammatory cells

HIV-1, through various techniques, has been shown to be present in cells of the spinal cord, brain, and peripheral nerves in vitro and in vivo (Dunfee et al., 2006). Neurons are not productively infected by HIV (Corasaniti et al., 1998). The virus does however productively infect T cells, macrophages, microglial cells, and astrocytes (Corasaniti et al., 1998; Kaul et al., 2001). Tissue macrophages and microglia, which express lower levels of CD4 and CCR5 than CD4+ T cells in periphery, serve as the initial major targets for HIV-1 infection (Dunfee et al., 2006; Levy et al., 1998). Indeed, HAD is believed to be sustained by viral replication in these cell types (Persidsky et al., 1997).

HIV-1 invades the CNS by infecting T cells and monocytes (Gonzalez-Scarano and Martin-Garcia, 2005) which express the HIV-1 receptors, CD4 and CD8, and several chemokine receptors that function as co-receptors; most prominently CXCR4. These receptors aid in docking of the virus to the cell and membrane fusion; resulting in viral entry into the cell (Moore et al., 2004). The well characterized dichotomy between the distribution and number of HIV infected cells and brain parenchymal damage point to an indirect mechanism for CNS damage (Epstein, 1993; Geleziunas et al., 1992). Thus it is believed neuron cell damage occurs due to the action of soluble factors including viral proteins, cytokines, and chemokines released by HIV-1-infected macrophages, astrocytes, and microglial cells. These soluble factors activate uninfected macrophages, microglia, and astrocytes and perpetuate CNS damage (Kaul et al., 2001, Okamoto et al., 2001).
1.2.2 HIV-1 replication by cell type in CNS

HIV-1 infected cells are classified as "productive" or "restricted" producers of viruses and both types occur in the CNS. Perivascular macrophages and microglia are the resident brain immunocompetent cells and considered “productive” (Anderson et al., 2002; Kaul et al., 2001). In vitro works have suggested long-lived human brain-derived mixed microglial cultures infected with R5 HIV-1, maintain replication competent HIV-1 for up to two and a half months (Vallat et al., 1998; Albright et al., 1999). Viral production in glial cells as a whole can be modulated by the level of inflammatory signaling within these cells (Trkola et al., 2004) or the addition of activating compounds such as cytokines (Albright et al., 2004). For example, an inflammatory cell environment characterized by high levels of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β), can reactivate viral production (Kramer-Hammerle et al., 2005; Gorry et al., 1998) in both restricted and productively infected cells (Gorry et al., 1998; Sabri et al., 1999).

1.2.3 Microglial HIV-1 replication

The pathoneumonic signature of HIV infection in the brain is productive viral replication in brain macrophages, microglia and multinucleated giant cells (Koenig et al., 1986; Wiley et al., 1986; Gabuzda et al., 1986). These immune cells secrete viral and cell expressed neurotoxins that cause to neuronal injury/death. Further inflammatory cell proliferation, and myelin pallor, during the later stages of HAD (Giulian et al., 1990,
1993; Pulliam et al., 1991). Microglia, like macrophages, express the CD4/CCR5 receptors and co-receptors used by HIV-1 (Albright et al., 1999) and are productive producers of virus (Anderson et al., 2002; Kaul et al., 2001). These “productive” immune cells play a critical role in the transmission of the infection and rapid evolution of viral genome and eventually die. However, peripheral macrophage populations are replenished quickly which allows for migration of more infected monocytes into the CNS where they can then take up residency and differentiate into microglia. In-vitro studies demonstrated HIV-1 replication takes place in primary microglia grown from adult (Albright et al., 2000; Watkins et al., 1990), infant (Ioannidis et al., 1995), and fetal brain (McCarthy et al., 1998; Sundar et al., 1995). Many reports have demonstrated HIV-1 in macrophages and microglial cells by double labeling immunohistochemical and/or in situ hybridization techniques in brain and spinal cord (Koenig et al, 1986; Koyanagi et al., 1987; Gendelman et al., 1989; Kure et al., 1990). Investigations of the progression of HIV-1 infection in purified primary human microglial cultures have demonstrated productive infection was more efficient by R5-tropic strains of HIV-1 compared to X4-tropic strains (Ioannidis et al., 1995). It has been suggested the genetic heterogeneity of HIV-1 in patients results in strains of HIV-1 that are selected for replication in macrophages. Then macrophage spread HIV-1 to the tissues, including the CNS, where HIV-1 can have its neurocognitive effects.
1.2.4 Astrocyte HIV-1 replication

Astrocytes are restricted producers; resisting efficient viral production (Anderson et al., 2002; Kaul et al., 2001). A large proportion of astrocytes are actively or latently infected with HIV-1; likely resulting in neuronal dysfunction via loss of supporting growth factors, excitotoxicity due to dysregulation of neurotransmitter reuptake, and decreased integrity of the blood-brain barrier (BBB) permitting further penetration of HIV-1 into the brain. Infection of astrocytes is characterized by near-exclusive synthesis of early regulatory gene products of HIV-1 (Blumberg et al., 1996). These glial cells lack CD4 receptor although they do express CXCR4 and possibly other HIV-1 co-receptors including CCR5 (Gorry et al., 2003). Immunopositivity of astrocytes for HIV-1 structural proteins has also been reported (Brack-Werner, 1999). In situ hybridization or in situ polymerase chain reaction (PCR) also showed the presence of HIV-1-specific nucleic acids in astrocytes (Takahashi et al., 1996). Other studies reported the presence of HIV-1 DNA and Nef protein in astrocytes as well. Astrocytes typically continue to survive as a viral reservoir (Gorry et al., 2003). Restricted infection is thought to result from inhibition of HIV-1 replication at various stages from entry, to maturation of progeny virions. Although only 1% of astrocytes may be potentially infected, the total number of infected astrocytes contributing to neuropathology has been suggested to be substantial in multiple studies (Takahashi et al., 1996).
1.2.5 Neuronal HIV-1 replication

Although HIV does not productively infect neurons (Nuovo et al., 1994; Bagasra et al., 1996), neural damage/death, and brain atrophy are readily observed in the HAD brain and have been correlated with severity of cognitive symptoms (Everall et al., 1991, Everall et al., 1993 a-c, Masliah et al., 1994; Petito and Roberts, 1995). Investigations have largely demonstrated an absence of any in vivo infection of neurons, although some works have reported the presence of HIV-1 DNA and proteins in neuronal cells (Nuovo et al., 1994; Bagasra et al., 1996). In vitro studies indicated restricted infection of primary neurons (Ensoli et al., 1995), and neuronal cell lines by X5 and R4 strains of HIV-1 (Obregon et al., 1999; Mizrachi et al., 1994). Neural loss in the neocortex has been estimated between 18 and 38% (Everall et al., 1991, Everall et al., 1993 a-c, Ketzler et al., 1990). It has been suggested that mild dendritic injury may lead to behavioral alterations in HIV-associated minor cognitive/motor disorder, and that this injury precedes the loss of neural loss typically associated with HAD (Thompson et al., 2005). Clinical correlations utilizing magnetic resonance imaging (MRI), confirm HIV infection is associated with progressive cortical atrophy of the gray and white matter in the brain, particularly in the later stage of the disease (Dal Pan et al., 1992; Hall et al., 1996). Several works demonstrated a correlation between the deterioration of neurocognitive function and reduction in volume of certain brain structures including the basal ganglia and caudate nucleus. Volumetric MRI scans have indicated cortical atrophy associated with HIV infection may be secondary to neuronal loss and demyelination. Both cross-sectional and longitudinal cohorts have indicated the degree of atrophy to be correlated with the degree
of cognitive motor dysfunction (Stout et al., 1998; Dal Pan et al., 1992; Hall et al., 1996). In addition quantitative MRI shows a correlation between cerebral atrophy and the degree of neuropsychological deficits. Further, neuropsychiatric impairment has been related specifically to white matter damage in HAD (Grassi et al., 2002). Specifically, a variable degree of white matter neuropathology with myelin damage ranging from pallor to widespread breakdown and loss leading to accumulation of lipid macrophages and axonal damage has been correlated with HIV-1 in the cerebral spinal fluid (CSF) in multiple works (Banks et al., 2006; Santosh et al., 1995; Lawrence et al., 2002; Gonzalez-Scarano et al., 2005). Neuron cell damage and death may occur ultimately as a direct result of the release of cytokines from infected microglial cells (Everall et al, 1991). Indeed it has been suggested that the major neuropathological and symptomatic hallmarks of HAD including vacuolar myelopathy, and sensory neuropathy (Wesselingh et al., 1994), are directly or indirectly related to the increased numbers of macrophages found in brain, spinal cord, and peripheral nerve (Tyor et al., 1995). HIV-1-infected macrophages begin inflammatory processes are amplified through interactions with activated astrocytes which then interact with microglia and macrophages to produce arachidonic metabolites and neurotoxic cytokines, which in high levels, lead to more astroglial activation and proliferation (Blumberg et al., 1996) and ultimately neuronal disfunction/death.

1.3 The Role of Microglia in HAD

Macrophages and microglia compose some 12% of the cells in the central nervous system (CNS) (Beneviste, 1993). Their roles include phagocytosis, antigen presentation, as well as generation and excretion of cytokines, eicosanoids, complement components,
and excitatory amino acids (EAA) including, glutamate, oxidative radicals, and nitric oxide (NO) (Banati et al., 1993). At least three phenotypic states of microglia exist based on developmental and pathophysiologic studies: (i) resting, ramified; (ii) activated non-phagocytic (or antigen presentine cell [APC]- like) found in areas involved in central nervous system (CNS) inflammation; and (iii) reactive, phagocytic microglia observed in areas of trauma or infection (Walker et al., 1995; Panek et al., 1995; Frei et al., 1987; Suzumura et al., 1987; Williams et al., 1992).

In regard to activation, macrophages and microglia are able to polarize into two major subtypes, categorized as M1 or M2. The “classical” or M1 subtype over-produces pro-inflammatory cytokines and promotes cell-mediated immunity. It is marked by production of high levels of interferon–gamma (IFN-γ) tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-12, and low levels of IL-10. The M1 phenotype may be activated when microglia contact HIV proteins (Moser, 2003) or bind toll-like receptors 3 or 4 (Gordon and Taylor, 2005). “Alternatively activated” or M2 microglia tend to dampen inflammation, clear cellular debris (including amyloid plaques), and produce very low levels of TNF-α, IL-1, IL-12 and high amounts of anti-inflammatory IL-10 and transforming growth factor (TGF)-β, and SOCS (suppressor of cytokine signaling) (Bruce-Keller et al., 2001; Suh et al., 2009). These two phenotypes, respectively, correspond to the type ii or iii microglial states described in the preceding paragraph. Further, the factors which cause polarization to M1 or M2, reinforce the maintenance of that phenotype in a cycle-like manner. Increased M1 polarization is consistent with increased TNF-α observed in plasma and brain specimens in HAD and AD, and may play a role in the pathophysiology of both diseases (Akhtar et al., 2003).
Stimulation of Th1 (T-helper 1) and Th2 immune response by microglia is dependent upon the expression of specific molecules including major histocompatibility complex (MHC) II and CD40 (Ma and Streilein, 1999). Microglia expressing MHC II induce CD4+ T cells to generate IFN-\(\gamma\) and TNF-\(\alpha\) (Ford et al., 1996). In the case of both HAD and AD, this response is considered harmful to the brain and in both diseases TNF-\(\alpha\) is elevated to neurotoxic levels while only in HAD is IFN-\(\gamma\) is prominently elevated (Minagar et al., 2002).

In HAD, microglia and macrophages are productively infected by HIV-1 and show diffuse inflammatory activation, which ultimately leads to neuronal damage, death, CNS dysfunction (Genis et al., 1992; Persidsky et al., 2003). A clinical trial using a small number of post-mortem HIV-infected individuals showed a direct correlation between microglial activation/infection and cognitive decline (Adle-Biasette et al., 1999). Studies have found microglial HIV infection as central in exacerbating HIV dementia (Genis et al., 1992; Persidsky and Gendelman, 1992). Importantly, neuronal dysfunction and death in HIV infection results from cytokine stimulation, but especially several cytokine-mediated apoptotic mechanisms emanating from microglia. Thus microglial cytokine production is central to the pathogenesis of HAD (Minagar et al., 2002).

Indeed, viral infection and/or immune activation of microglia fuels HAD pathogenesis ending in neuronal-injury and death Avison et al., 2004; Thind and Sabbagh et al., 2007). Microglia are the main target for the HIV-1 infection in the brain. The virus infiltrates the CNS via infected monocytes (Sulkava et al., 1985; Consenza et al., 2002). Once infected or activated by HIV-proteins such as gp120 or Tat, microglia begin to excrete endogenous pro-inflammatory cytokines of the M1 subtype (D’aversa et al.,
Histopathologically, activated microglia represent a highly accurate correlate to neuronal death and damage in CNS (Avison et al., 2004). Severity of dementia in persons with HAD is strongly correlated with the number of activated macrophages and microglia within the basal ganglia and frontal lobes (Schneider et al., 2007; Hachiya et al., 2008). Moreover, activation of microglial cells by HIV is associated with astrogliosis, myelin pallor, and severe neuronal loss (Avison et al., 2004).

TNF-α is released by HIV-1-infected microglia, and oligodendrocytes are particularly sensitive to its effects (Wilt et al., 1995). Steady-state levels of TNF-α mRNA are higher in the subcortical regions of the CNS of patients with HAD than in HIV-1-infected patients without CNS involvement (Wesselingh; 1997). QUIN is a highly excitotoxic marker most well known in HIV neurological disease which may reflect the extent of immune activation in both blood and the brain and correlates with systemic and neurological disease status (Ma et al., 1999).

During immune activation, particularly while levels of IFN-γ are increased, induction of the enzyme indoleamine 2,3-dioxygenase occurs, increasing the synthesis of quinolinic acid (QUIN) (Byrne et al., 1986; Fuchs et al., 1991). HIV-infected microglia also release chemokines (Schmidt Mayerova et al., 1996), which may enhance infiltration and recruitment of both infected and uninfected microglia (Guillemin et al., 2002).

HAD pathology is typically marked by the presence of multinucleated giant cells and microglial nodules by immunohistochemistry or in situ hybridization. The presence of microglia in the CNS is strongly associated with severe neurobehavioral
complications. Microglia, as a major target of HIV-1 infection in the CNS, are typically a viral reservoir and are also key in HIV-1 neuroinvasiveness-penetration into the CNS by the virus (Persidsky et al., 1999). Most importantly, a discrepancy between the localization of HIV-infected cells and the severity of neurocognitive symptoms has been described (Ferrando, 2000). Thus, other mechanisms secondary to virus infection, such as passage of monocytes and lymphocytes into the brain, activation of astrocytes/microglia, and production and release of inflammatory cytokines, all participate in the pathogenesis of HAD. This is a key concept which makes the neuropathogenesis of HAD, in many ways, similar to that of AD.

β-amyloid is a potent and direct neurotoxic agent (Lorenzo and Yankner, 1994), much like the HIV-1 proteins gp120 and Tat, and it induces a cascade of cellular mechanisms including activation of microglia, which leads to neuronal damage (Giulan et al., 1995). Indeed, reactive microglia are closely associated with neuritic and β-amyloid plaques, just as they are with HIV-1 Tat protein (Rozemuller et al., 1989). Research by our group and others of the microglia signal transduction pathways mediating the neurotoxic response of Aβ demonstrated that mitogen-activated protein-kinase (MAPK) superfamily members extracellular signal regulated kinases 1 and 2 (ERK1/2) and p38 MAPK act as mediators (McDonald et al., 1998; Combs et al., 1999). Furthermore, several lines of evidence indicate the nuclear factor kappa beta (NF-κB in microglia is stimulated by β-amyloid (Yankner et al., 1990). Activation of NF-κB can stimulate transcription of genes expressing TNF-α, IL-1, IL-6, monocytes chemo-attractant protein-1(MCP-1), and nitric oxide synthase (NOS).
Adding biological “insult to injury,” in the HIV-1 infected brain, microglial phagocytosis of Aβ1-42 peptide appears inhibited (Keblesh et al., 2008). The deposition of Aβ plaques in the HIV-1 infected brain is likely caused by several factors including the effects of cytokines and HIV-1 proteins on microglial phenotype, activation and activity. IFN-γ is hypothesized to enhance the effects of HIV-1 Tat by promoting the switch from a microglial phagocytic phenotype to one that is an antigen presenting cell (APC) phenotype (Giunta et al., 2008).

1.4 The HIV Transactivator (Tat) protein

The viral and/or host factor(s) responsible for HAD have yet to be fully identified and characterized, but reports that mRNA levels of HIV viral regulatory transactivator protein “Tat” are abnormally high in patients with dementia (Wiley et al., 1996), and that Tat is actively secreted by infected cells (Ensoli et al., 1993) points to a role for this protein in the progression of HAND. Additionally, Tat has pro-inflammatory and neurotoxic properties in astrocytes (Giunta et al., 2009) microglia (Giunta et al., 2006) and macrophages (Turchan-Cholewo et al., 2009 a, b; Minghetti et al., 2004). Most importantly, Tat levels positively correlate with development of HIV- and chimeric simian-human immunodeficiency virus (SHIV)-induced encephalitis (Hudson et al., 2000).

As the virus productively infects microglia and perivascular macrophages without productively infecting neurons, the brain pathology caused by HIV is indirect (Kiebala et
al., 2010). Thus, neurologic deficits in HAD are more closely correlated with the presence of activated macrophages and microglia than with the amount of viral RNA (Avison et al., 2004). Soluble viral toxic proteins such as Tat and the glycoprotein gp120 can be released from infected microglia and macrophages (Rumbaugh and Nath 2006). Tat protein has been detected in blood plasma, serum, and cerebral spinal fluid (CSF) from HIV+ individuals, at levels ranging from 1–40 ng/ml (Westendorp et al., 1995; Xiao et al., 2000), thus local extracellular concentrations in the CNS may be significantly higher, especially proximal to HIV+ pervascular cells (Hyashi et al., 2006).

Furthermore, HIV-1 Tat activates neighboring, uninfected cells including microglia, astrocytes and neurons. Both infected and activated microglia and astrocytes over-produce the pro-inflammatory cytokines TNFα and IL-1β, which further activate neighboring cells in feed forward cycle. Infected and activated cells also produce chemokines such as MCP-1, attracting even more inflammatory monocytes and macrophages (D’Aversa et al., 2002; Eugenin et al., 2005). Therefore, circulating Tat has a high propensity to trigger this vicious pro-inflammatory cycle, leading to neuronal death and neurologic deficits (Westendorp et al., 1995).

1.5 Clinical and histological association of Alzheimer’s disease (AD) like- pathology with HAD associated dementia (HAD)

Because of the prolonged survival in the aging HIV population, HAD occurs with increasing prevalence (Sacktor et al., 2002) and is commonly characterized by (a). AD-like neuropathology (Xu et al., 2009; Everall et al., 1993a-b, 1995, Green et al., 2005; Alisky et al., 2007) b) syncitial activated macrophages/microglia (Navia et al., 2005) (c)
dendritic pruning, (d) synaptic density loss (Nath et al., 2000), and (e) neuron loss (Everall et al., 1993 a and b). Recent neuropathologic reports of severe white matter damage (i.e. leukoencephalopathy) in patients with HIV-1 infection and on cART (Oberdorfer et al., 2009; Muller-Oehring et al., 2009; Major, 2009; Cardenas et al., 2009; Letendre et al., 2009; Chen et al., 2009; Gongvatana et al., 2009), including significant frontostriatal and prefrontal cortex involvement in HAND (Pfefferbaum et al., 2009; Nguyen et al., 2009; Melrose et al., 2008; Wohlschlaeger et al., 2009), suggest additional patterns of primary brain disease are emerging, due to either changes in host cell signaling, or as yet undiscovered interactions between virus, vulnerable neurons, and cART (Langford et al., 2002; 2003)

Several studies identified AD-like changes in HIV patients. Aβ plaques formed at an earlier age and in greater amounts in an acquired immunodeficiency syndrome (AIDS) group with a significantly greater prevalence of plaques in the AIDS group as a whole and in those in the fourth decade than in controls (Esiri et al., 1998). Also, significant deposition of amyloid occurred in the frontal cortex, hippocampus, and basal ganglia of almost half of 162 autopsied AIDS brains studied neuropathology (Everall et al., 1993a-b, 1995, Green et al., 2005; Alisky et al., 2007). In postmortem human brain sections from HIV-infected patients’ significant increases in Aβ, compared to controls, was found (Rempel and Pulliam, 2005). Further, compared to HIV negative cases, in HIV positive cases, there was abundant intracellular Aβ in pyramidal neurons and along axonal tracts. Further HAD cases had higher levels of Aβ immunoreactivity compared to HIV+ cases without HAD (Achim et al., 2009). Finally, Aβ measurements in CSF of cognitively impaired patients with HIV are similar to those in patients with mild AD. Normal or
slightly depressed CSF tau and p-tau measurements distinguished these patients with HAND from patients with AD (Clifford et al., 2009).

Recently, with the advent of highly active antiretroviral therapy (HAART; also known as combination antiretroviral therapy or “cART) patients with HIV have been living significantly longer lives. While HAART has been increasing the lifespan of those infected with HIV, it has also led to an increased prevalence of HAD (Alisky 2007; Simone et al., 2008; Repetto and Petitto, 2008; Keblesh et al., 2008; Hult et al., 2008; Giunta et al., 2008; Valcour et al., 2004). As the pathology of HAD, like AD, is commonly characterized by an increase in the amount of Aβ peptide in the brain (Green et al., 2005) evidence suggesting microglia modulate the clearance of potentially neurotoxic Aβ species from the brain is of special importance (Rogers et al., 2002; Rogers and Lue, 2001).

1.6 Current mouse models of HAD and AD

Mutations in the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) genes have all been implicated in increased risk for AD. The mouse mutant APP (K670N,M671L) transgenic line, Tg2576, demonstrates markedly elevated Aβ loads by 3 months of age and, by 9-12 months, develops extracellular AD-type Aβ deposits in the cortex and hippocampus (Hsiao et al., 1996). Mutant PS1 transgenic mice do not show abnormal pathology, but do display subtly elevated levels of the highly amyloidogenic 42- or 43-amino acid peptide (Duff et al., 1996). The doubly transgenic progeny from a cross between line Tg2576 and a mutant PS1M146L transgenic line develop a large load of fibrillar Aβ deposits in cerebral cortex and hippocampus much earlier than the singly transgenic Tg2576 model. Indeed prior to overt Aβ deposition, PSAPP mice demonstrate
a selective 41% increase in Aβ_{42(43)} in their brains. Progressive cognitive impairment is exhibited in PSAPP mice for water maze acquisition, radial arm water maze, circular platform performance, standard water maze retention, visible platform recognition, and Y-maze working memory(Arendash et al., 2001; Holcomb et al., 1999a). Regarding sensorimotor tasks, these mice show a progressive increase in open field activity, a progressive impairment in string agility, and an early-onset impairment in balance beam. Given the age-related cognitive impairments demonstrated by PSAPP mice and their progressive Aβ deposition/neuroinflammation, PSAPP transgenic mice represent a model to develop therapeutics to treat or prevent AD-like pathology through modulation of Aβ deposition/Neuroinflammation (Arendash et al., 2001; Holcomb et al., 1999a).

A transgenic mouse model of HAD has been developed whereby Tat expression is regulated by both the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter and a doxycycline (Dox)-inducible promoter. These inducible Tat transgenic mouse colonies (GT-tg) were created by cross breeding of two separate transgenic lines, Teton-GFAP mice (G-tg) and TRE-Tat86 mice (T-tg) (Kim et al., 2003). Both in vitro and in vivo assays previously show Tat expression occurs exclusively in astrocytes and is Dox-dependent; leading to degeneration of neuronal dendrites, and neuronal apoptosis reflected by behavioral impairment (Kim et al., 2003). Thus Tat expression without HIV-1 infection (Kim et al., 2003) is sufficient in to cause brain damage mimicking that of HAD (Everall et al., 1993; Everall et al., 1991; Everall et al., 1993; Everall et al., 2005a; Ketzler et al., 1990) and provide evidence in the context of a whole organism to support a the role of Tat in HAD.
CHAPTER TWO

GALANTAMINE AND NICOTINE HAVE A SYNERGISTIC EFFECT ON
INHIBITION OF MICROGLIAL ACTIVATION INDUCED BY HIV-1 GP120

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2.1 Abstract

Chronic brain inflammation is the common final pathway in the majority of neurodegenerative diseases and central to this phenomenon is the immunological activation of brain mononuclear phagocyte cells, called microglia. This inflammatory mechanism is a central component of HIV-associated dementia (HAD). In the healthy state, there are endogenous signals from neurons and astrocytes, which limit excessive central nervous system (CNS) inflammation. However, the signals controlling this process have not been fully elucidated. Studies on the peripheral nervous system suggest that a cholinergic anti-inflammatory pathway regulates systemic inflammatory response by way of acetylcholine acting at the α7 nicotinic acetylcholine receptor (α7nAChR) found on blood-borne macrophages. Recent data from our laboratory indicates that cultured microglial cells also express this same receptor and that microglial anti-inflammatory properties are mediated through it and the p44/42 mitogen-activated protein kinase (MAPK) system. Here we report for the first time the creation of an in vitro model of HAD composed of cultured microglial cells synergistically activated by the addition of IFN-γ and the HIV-1 coat glycoprotein, gp120. Furthermore, this activation, as measured by TNF-α and nitric oxide (NO) release, is synergistically attenuated through the α7nAChR and p44/42 MAPK system by pretreatment with nicotine, and the cholinesterase inhibitor, galantamine. Our findings suggest a novel therapeutic combination to treat or prevent the onset of HAD through this modulation of the microglia inflammatory mechanism.
2.2 Introduction

HIV-associated dementia (HAD) is present in approximately 20% of AIDS patients late in the course of HIV infection (Bansal et al., 2000). Central to its pathologic mechanism is the prolonged activation of brain mononuclear phagocytes, called microglia (Polazzi, 2002). They provide structural and functional support to neurons as well as serving as the primary source of productive infection by HIV-1 in the CNS (Schuenke et al., 2003).

Postmortem studies have suggested the brain is a viral reservoir in both demented and non-demented HIV patients and that only those individuals with immune activation in the brain will likely develop HAD (Anderson et al., 2002). Early in HIV infection, the infected monocyte-derived cells infiltrate the brain and HIV is observed to gain access to T-cells, microglia, and other cells primarily through the interaction of gp120 binding to CD4 and CCR5. The risk, however, of developing HAD increases late in HIV infection, as the CD4 cell count reaches 200 and below (Llano et al., 2001). At this time the HIV typically displays greater usage of the CXCR4 coreceptors rather than CCR5. A CD4 cell count, which has declined to approximately 200 cells/μL, is believed to also allow or facilitate autonomous brain infection by HIV. Brain inflammation then becomes a self-potentiating cycle once the initial CD4 nadir is reached and immune mediators are released. For example under the influence of IFN-γ, expression of CD40 is dose-dependently enhanced by as much as 20-fold (Aloisi et al., 2000; Matyszak et al., 1999; Nguyen and Beneviste, 2000, Tan et al., 1999); greatly enhancing the ability of brain microglia to become activated. Additionally the HIV-1 proteins, such as gp120, have been shown in vivo to be toxic to neurons via independent, direct activation of microglia (Haughey and Mattson, 2002).
Nicotine binding at microglial or neuronal nicotinic acetylcholine receptors (nAChR) has shown anti-inflammatory properties. Nicotine and acetylcholine pretreatment inhibit LPS-induced TNF-α release in murine derived microglia (Tan et al., 1999). In nicotine precultured neurons, binding at the α7 nAChR provides neuroprotection from the excitatory amino acid (EAA) glutamate as well as other inflammatory factors (Kaneko et al., 1997).

Galantamine is a potent allosteric potentiating ligand (APL) of nAChRs (Samochocki et al., 2003) and cholinesterase inhibitor (Shytle et al., 2004). Galantamine up-regulates agonist responses of nAChR receptors at concentrations between 0.1 and 1 µM while concentrations greater than 10 µM result in nAChR inhibition. In vivo studies have shown that galantamine, acting as an APL on presynaptic and tonically active nAChRs, potentiates glutameric or GABAergic transmission whereas the non-APL cholinesterase inhibitors lack this therapeutic effect on synaptic transmission (Santos et al., 2002).

While many studies have used live HIV-1 to produce microglial activation, here we sought to investigate a novel in vitro model simulating HAD-like microglial activation after introduction of HIV-1gp120, and IFN-γ. Using this model we then examined the anti-inflammatory mechanism of galantamine, and nicotine.
2.3 Materials and Methods

2.3.1 Murine primary cell culture

Murine primary culture microglial cells were isolated from mouse cerebral cortices and were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 2mM glutamine, 100 U/mL penicillin, 0.1μg/mM streptomycin, and 0.05μM 2-mercaptoethanol according to previously described methods (Tan et al., 2000). Briefly, cerebral cortices from newborn mice (1-day-old) were insolated under sterile conditions and were kept at 4°C before mechanical dissociation. Cells were plated in 75 cm² flasks (NunclonTM, Roskilde, Denmark), and complete medium was added. Primary cultures were kept for 2 weeks so that only glial cells remained, and microglial cells were isolated. More than 98% of these cells were stained positive for CD11b (Boehringer Mannheim, Indianapolis, IN).

2.3.2. TNF-α ELISA and nitric oxide release assay

Primary cultured microglial cells were plated in 24-well tissue culture plates (NunclonTM, Roskilde, Denmark) at 1× 10⁵ cells/well. Some of these cells were treated with either HIV-1CN54 gp120 (2μg/mL; provided by NIH AIDS Research & Reference Reagent Program) or murine recombinant IFN-γ (100 ng/mL; R&D systems, Minneapolis, MN) or a combination of HIV-1CN54 gp120 (2 μg /mL) and IFN-γ (100 ng/mL) in the presence or absence of anti-CXCR4 antibody (2 μg/mL; Clone, 44717; provided by NIH AIDS Research & Reference Reagent Program) or control antibody
(PharMingen, San Diego, CA) for 24 h. Some of these cultured cells were pretreated with 0.05 μM galantamine (Sigma) and/or 5 μM nicotine (Sigma) for 30 min and then co-challenged with HIV-1 gp120 (2 μg/mL) and IFN-γ (100 ng/mL) for 8 h in the presence or absence of α-bungarotoxin (10 nM, Sigma). Cell-free supernatants were then collected and assayed by a TNF-α ELISA kit (R&D systems) or by NO assay kit (Calbiochem, La, Jolla, CA) in strict accordance with the manufacturer’s instructions. The Bio-Rad protein assay (Hercules, CA) was performed to measure total cellular protein from each of the cell groups under consideration just prior to quantification of TNF-α release by ELISA or NO secretion by NO assay.

2.3.3 Western immunoblotting

Murine primary culture microglial cells were plated in six-well tissue culture plates (NunclonTM) at a density of 1 × 10^6 cells/well. As previously described (Shytle et al., 2004), for examining phosphorylation of p44/42 MAPK, these cells were co-pretreated with galantamine (0.05μM) and nicotine (5μM) for 30 min in the presence or absence of α- bungarotoxin (10 nM) and then challenged with HIV-1 gp120 (2μg/mL) and IFN-γ (100 ng/mL) for various timepoints. Immediately following culturing, microglial cells were washed in ice-cold PBS, and lysed in an ice-cold lysis buffer. After incubating for 30 min on ice, samples were centrifuged at high speed for 15 min, and supernatants were collected. Total protein content was estimated using the Bio-Rad protein assay. An aliquot corresponding to 50 μg of total protein of each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Immun-BlotTM
polyvinylidene difluoride membranes. Nonspecific antibody binding was blocked with 5% nonfat dry milk in Tris-buffered saline (20mM Tris, 500mM NaCl, pH 7.5) for 1 h at room temperature. Membranes were hybridized with phosphospecific p44/42 MAPK monoclonal antibody, stripped with β-mercaptoethanol stripping solution (62.5mM Tris–HCl, pH 6.8; 2% SDS, and 100mMβ-mercaptoethanol), and then re-probed with an antibody that recognizes total p44/42. Alternatively, membranes with identical samples were probed with either phosphor-specific p44/42 with an antibody that recognizes total p44/42 MAPK. Immunoblotting was carried out with a primary antibody followed by an anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated IgG secondary antibody as a tracer. The Immun-StarTM chemiluminescence substrate was used to develop the blots.

2.3.4. Statistical analysis

Data were analyzed using ANOVA followed by post hoc comparisons of means by Bonferroni’s or Dunnett’s T3 method, for which Levene’s test for homogeneity of variances was used to determine the appropriate method of post hoc comparison. In instances of single-mean comparison, t test for independent samples was used to assess significance. The p levels were set at 0.05 for each analysis. All analyses were performed using SPSS for Windows release 9.0.
2.4 Results

2.4.1 IFN-γ synergistically enhances HIV-1 gp120-induced microglial activation

It has been previously reported that IFN-γ has a synergistic effect on cytokine production secreted by cultured microglial cells treated with stimuli (such as amyloid β peptides) (Tan et al., 1999). In order to test whether IFN-γ could synergistically enhance HIV-1 gp120-induced microglial activation, we cotreated primary cultured microglial cells with IFN-γ and HIV-1 gp120 in the presence or absence of anti-CXCR4 antibody or control antibody for 24 h. Cell-free supernatants were collected from each of the cell groups and assayed by a TNF-α ELISA kit and by a NO release assay in strict accordance with the manufacturer’s instructions. The Bio-Rad protein assay was performed to measure total cellular protein from each of the cell groups under consideration just before quantification of TNF-α production and NO release. Data show that co-treatment of cultured microglial cells with IFN-γ and HIV-1 gp120 results in a synergistic effect on microglial activation as evidenced by increased TNF-α production (Fig. 1A) and elevated level of NO secretion (Fig. 1B). Furthermore, these effects are significantly attenuated by the presence of anti-CXCR4 antibody, but not control antibody, suggesting that HIV-1 gp120 specifically has an effect on microglial activation.
Fig. 1. A synergistic effect of HIV-1 gp120 and IFN-γ on microglial activation.

Primary cultured microglial cells (1 × 10^5 per well in 24-well tissue cultureplate) were treated with HIV-1 gp120 (2 μg/mL) or IFN-γ (IFN-γ; 100 ng/mL), or HIV-1 gp120/IFN-γ in the presence or absence of anti-CXCR4 antibody (2 μg/mL) or these cells went with no treatment (control) for 24 h. Microglial activation was measured by TNF-α production (mean ± S.D., picograms per milligram of total protein) in cultured media by TNF-α ELISA (A), NO release (mean ± S.D., micromolar concentration per milligram of total protein) in cultured media by NO assay (B). For (A and B), ANOVA revealed that a significant interaction between gp120 and IFN-γ (P < 0.001) compared with either gp120 or IFN-γ treatment alone. Further, there is a significant difference between gp120/IFN-γ and anti-CXCR4/IFN-γ/gp120 (P < 0.005).
2.4.2. Co-treatment with galantamine and nicotine synergistically oppose HIV-1 gp120/IFN-γ-induced microglial activation

Our recent studies have shown that the cholinergic pathway is involved in negative regulation of microglial activation through α7 nicotinic acetylcholine receptor (nAChR). In addition, it is well known that galantamine is a potent allosteric potentiating ligand (APL) of nAChRs (Shytle et al., 2004). In order to determine whether galantamine and/or nicotine could inhibit HIV-1 gp120/IFN-γ-induced TNF-α production and NO release in cultured microglial cells, we pre-incubated microglial cells with galantamine, nicotine, or a combination of galantamine and nicotine for 30 min followed by challenging with HIV-1 gp120/IFN-γ for 8 h. As shown in Fig. 2A and B, data indicate that the pretreatment with either galantamine or nicotine results in a slight reduction of HIV-1 gp120/IFN-γ-induced TNF-α production and NO release. Most importantly, the pretreatment with a combination of galantamine and nicotine for 30 min and then challenged these cells with HIV-1 gp120/IFN-γ for 8 h. As shown in Fig. 2A and C, data indicate that the pretreatment with either galantamine or nicotine results in a slight reduction of HIV-1 gp120/IFN-γ-induced TNF-α production and NO release. Most importantly, the pretreatment with a combination of galantamine and nicotine produces an even greater marked reduction of HIV-1 gp120/IFN-γ-induced TNF-α production and NO release (Fig. 2A and B). Furthermore, in order to test if this effect is specifically to the action of α7 nAChR, we co-pretreated these cells with galantamine and nicotine in the presence or absence of α-bungarotoxin, a selective α7 nAChR antagonist, for 30 min and then challenged them with HIV-1 gp120/IFN-γ for 8 h. Data show that
this co-pretreatment of these cells with α-bungarotoxin significantly attenuates the effect of galantamine/nicotine on inhibition of microglial TNF-α production and NO release induced by HIV-1 gp120 and IFN-γ challenge (Fig. 2A and B).

**Figure. 2.** Galantamine and nicotine synergistically inhibit microglial activation induced by HIV-1 gp120 and IFN-γ co-treatment, which is attenuated by the presence of α-bungarotoxin (alpha-Bgt), a selective α7 nicotinic antagonist. As described in Fig. 1, primary cultured microglial cells were pretreated with galantamine (gal; 0.05μM) and/or nicotine (5 μM) in the presence or absence of α-bungarotoxin (10 nM) for 30 min and then challenged with HIV-1 gp120 (2μg/mL) and IFN-γ (100 ng/mL) for 8 h. Co-treatment of these cells with galantamine and nicotine markedly inhibits microglial activation as evidenced by decreased TNF-α production (A, mean ± S.D., picograms per milligram of total protein) and reduced NO release (B, mean ± S.D., micromolar concentration per milligram of total protein) in cultured media. These effects are
significantly blocked by co-pretreatment of microglial cells with α-bungarotoxin (A and B). For (A and B), ANOVA revealed that significant main effects of co-treatment of these cells with galantamine and nicotine compared with control (gp120/IFN-γ challenge alone) and gp120/IFN-γ challenge in the presence of either galantamine or nicotine ($P < 0.005$). Furthermore, ANOVA revealed that a significant main effect of co-pretreatment of these cells with α -bungarotoxin compared with pretreatment of these cells with galantamine/nicotine in the absence of α-bungarotoxin ($P < 0.005$).

2.4.3. Co-pretreatment with galantamine/nicotine suppresses HIV-1 gp120/IFN-γ-induced microglial activation through inhibiting p44/42 MAPK phosphorylation

Previous studies have shown that activation of mitogen activated protein kinase (MAPK) p44/42 is involved in TNF-α production in macrophages, monocytes and microglia after activation of these cells with a variety of stimuli, including LPS and CD40 ligand (Tan et al., 1999). Given that the combination of galantamine and nicotine at low doses greatly opposes microglial activation as evidenced by a reduction of TNF-α production and NO release, we wished to determine whether reduced phosphorylation of p44/42 could be responsible for these effects. Thus, we analyzed p44/42 phosphorylation status in microglial cell lysates after pretreatment with the combination of galantamine (0.05 μM) and nicotine (5 μM) for 30 min and then challenge with HIV-1 gp120/IFN-γ at a variety of time points. As shown in Fig. 3A and B, results show that pretreatment with the combination of galantamine and nicotine significantly inhibits phosphorylation of p44/42 MAPK induced by HIV-1 gp120/IFN-γ compared with controls (HIV-1 gp120/IFN-γ
challenge alone); suggesting the functionality of the combination of galantamine and nicotine co-stimulated cholinergic signaling on reduction of p44/42 MAPK activation. To further evaluate this functionality, we pretreated microglial cells with the combination galantamine/nicotine in the presence of α-bungarotoxin. Thirty minutes later, these cells were challenged with HIV-1 gp120/IFN-γ. Phosphorylation status of p44/42 MAPK was examined by western blot. As shown in Fig. 3C, this pretreatment leads to attenuating the effects of the combination galantamine and nicotine on inhibition of phosphorylation of p44/42 MAPK induced by HIV-1 gp120/IFN-γ. Finally, to semi-quantify these data, we carried out the Densitometric analysis as previously described (Shytle et al., 2004). As shown in Fig. 3D and E, the co-pretreatment of these cells with galantamine and nicotine markedly inhibits phosphorylation of p44/42 MAPK compared with gp120/IFN-γ challenge alone. However, this effect was significantly attenuated by the presence of α-bungarotoxin.
Primary cultured microglial cells (1×10^6 per well in six-well tissue culture plate) were co-pretreated with galantamine (gal) and nicotine (nico) in the presence (C) or absence (B) of α-bungarotoxin (α-Bgt) for 30 min and then challenged with HIV-1 gp120 (2 μg/mL) and IFN-γ (100 ng/mL) or control (A; HIV-1 gp120/IFN-γ challenge alone) for various time points as indicated. The phosphorylation of p44/42 MAPK was measured by western blot using the antibodies specifically against phopho-p44/42 and total p44/42. Data presented here are representative of three independent experiments. Histogram represents the mean band density ± S.D. (D, ratio of phospho-p44 MAPK to total p44 MAPK at 30 min; E, ratio of phospho-p42 MAPK to total p42 MAPK at 30 min). ANOVA revealed significant main effect of co-pretreatment of these cells with galantamine and nicotine in inhibition of phosphorylation of p44/42 MAPK induced by HIV-1 gp120/IFN-γ.
galantamine and nicotine compared with control (gp120/IFN-γ challenge alone) ($P < 0.001$). However, there is not a significant effect of co-pretreatment of these cells with galantamine and nicotine compared with gp120/IFN-γ challenge in the presence of α bungarotoxin ($P > 0.05$).

### 2.5 Discussion

The TNF-α released upon this microglial activation plays a central and multifaceted role in affected individuals. Along with IL-1β, it feeds back to upregulate microglial release of the excitatory amino acid (EAA), l-cysteine. In turn l-cysteine binds neuronal n-methyl-d-aspartic acid (NMDA) receptors thereby lowering the threshold of activation in synergy with HIV-1 gp120. This then proceeds to a state of excititotoxicity in which uncontrolled amounts of calcium enter the cell with ensuing activation of the apoptotic cycle (for review see Garden, 2002). Another function of the microglial release of TNF-α is to act in synergy with stromal derived factor-1 (SDF-1) to potentiate glutamate release from neighboring microglia and astrocytes (for review see Garden, 2002). Studies have indicated that Platelet-activating factor (PAF) is also released from microglia in response to TNF-α. In addition, TNF-α acts in synergy with the HIV protein Tat, to signal apoptosis in neurons (Garden, 2002). Finally, TNF-α can directly activate the neuronal apoptotic pathway by promoting the aggregation of TNF-α receptor-1 (TNFR1) subsequently leading to the activation of caspase-8. TNFR1 is found on a portion of neurons, making the TNF-α induced apoptosis scenario quite likely in the setting of
elevations of this cytokine as is seen in HAD (Garden, 2002). Neutralization of TNF-α prevents HIV-1 gp120-induced neurotoxicity in mixed cerebrocortical cultures.

Nitric oxide is also important in the pathophysiology of HAD. It is thought to be related to: impairment of antiviral defense mediated by T-helper-1 immune response by suppressing T-helper-1 functions; inducement of cytotoxic effects by oxidative injury with cellular and organ dysfunctions; and inducement of oxidative stress leading to rapid viral evolution with production of drug-resistant and immunologically tolerant mutants (Torre et al., 2002). Our findings also strongly suggest a synergistic attenuation of microglial NO and TNF-α release by pretreatment with galantamine and nicotine. Release of cytokines was significantly less when both medications were added than each was added individually (Fig. 2A and C).

The mechanism of this attenuation relies on α7 nAChR signaling. Data show that co-pretreatment of these cells with α-bungarotoxin (a specific inhibitor of the α7 nAChR) significantly attenuates the effect of galantamine/nicotine on inhibition of microglial TNF-α production and NO release induced by HIV-1 gp120 and IFN-γ challenge (Torre et al., 2002) (Fig. 2 B and D). The α7 nAChR attenuates cytokine release intracellularly through negative modulation of p44/42 MAPK phosphorylation. This is evidenced by and concurrent decreased attenuation of cytokine release in the presence of α-bungarotoxin as well as band density ratio with and without the addition of α-bungarotoxin. As indicated in Fig. 3D, band density ratio of phospho-p44 to total p44 increased from approximately 0.45 to 0.90 in the presence of α-bungarotoxin. This is nearly equal to previous levels when only HIV-1 gp120 and IFN-γ were added to microglia. This increased release of
cytokines in the presence of HIV-1 gp120, IFN-γ, and selective α 7 nAChR blockade strongly suggests the protective effects mediated by activation of this receptor by galantamine and nicotine. This is in concordance with previous unpublished data indicating that microglial α 7 nAChR are responsible for attenuation of cytokine release when they are bound by acetylcholine (Shytle et al., 2004).

Further support comes in the form of numerous other studies showing the neuroprotective effects of this receptor when expressed by neurons (Kaneko et al., 1997). Microglial modulation by nAChRs may represent a novel physiological mechanism for the reported neuroprotective properties of nicotinic drugs in animal models of neurodegenerative disease. In the peripheral nervous system, a non-neuronal cholinergic system is strongly expressed within different components of the immune system and is likely involved in the regulation of host inflammation. An example has been provided by Wang et al. (2003) who have shown that efferent vagus nerve stimulation attenuates the systemic inflammatory response to LPS in blood-borne macrophages and this is mediated by acetylcholine acting at α 7 nAChRs (Wang et al., 2003). Our findings provide evidence suggesting a similar role for nicotine and galantamine at the same receptor; this time involved in regulation of inflammation in the brain. It is thought that microglia can serve both neurotrophic and neurotoxic functions in the brain and factors determining which function microglia carry out depend on a combination of signals received from nearby astrocytes and neurons (Polazzi and Contestabile, 2002). Our results are in agreement with this hypothesis and suggest that signals related to suppression of the immunological cytokine release may involve neuronal cholinergic communication via α7 nAChR’s.
In summary, microglial release of TNF-α and NO is positively regulated by the addition of HIV-1 gp120 and IFN-γ. The CXCR4 receptor is instrumental in modulating the intensity of the synergistic relationship such that there it is negatively regulated in the presence of a selective CXCR4 blockade. This provides a novel in vitro model for the study of HAD. We also suggest that galantamine and nicotine, acting through α7 nAChR’s p44/42 MAPK system, is a novel combination for synergistically reducing HIV mediated microglial activation. Future studies will be needed to characterize the mechanism of synergy between HIV-1 gp120 and IFN-γ in the presence of selective CXCR4 blockade.

Although this *in vitro* model has used a central mediator of inflammation in HAD, TNF-α and NO, future studies will be needed to characterize the full spectrum of inflammatory mediators in the presence of galantamine/nicotine in an *in vivo* model as well.
CHAPTER THREE

EGCG MITIGATES NEUROTOXICITY MEDIATED BY HIV-1 PROTEINS GP120 AND TAT IN THE PRESENCE OF IFN-γ: ROLE OF JAK/STAT1 SIGNALING AND IMPLICATIONS FOR HIV ASSOCIATED DEMENTIA.

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3.1 Abstract

Human immunodeficiency virus (HIV)-1 infection of the central nervous system occurs in the vast majority of HIV-infected patients. HIV-associated dementia (HAD) represents the most severe form of HIV-related neuropsychiatric impairment and is associated with neuropathology involving HIV proteins and activation of proinflammatory cytokine circuits. Interferon-gamma (IFN-\(\gamma\)) activates the JAK/STAT1 pathway, a key regulator of inflammatory and apoptotic signaling, and is elevated in HIV-1-infected brains progressing to HAD. Recent reports suggest green tea-derived (-)-epigallocatechin-3-gallate (EGCG) can attenuate neuronal damage mediated by this pathway in conditions such as brain ischemia. In order to investigate the therapeutic potential of EGCG to mitigate the neuronal damage characteristic of HAD, IFN-\(\gamma\) was evaluated for its ability to enhance well-known neurotoxic properties of HIV-1 proteins gp120 and Tat in primary neurons and mice. Indeed, IFN-\(\gamma\) enhanced the neurotoxicity of gp120 and Tat via increased JAK/STAT signaling. Additionally, primary neurons pretreated with a JAK1 inhibitor, or those derived from STAT1-deficient mice, were largely resistant to the IFN-gamma-enhanced neurotoxicity of gp120 and Tat. Moreover, EGCG treatment of primary neurons from normal mice reduced IFN-\(\gamma\) -enhanced neurotoxicity of gp120 and Tat by inhibiting JAK/STAT1 pathway activation. EGCG was also found to mitigate the neurotoxic properties of HIV-1 proteins in the presence of IFN-\(\gamma\) in vivo. Taken together, these data suggest EGCG attenuates the neurotoxicity of IFN-\(\gamma\) augmented neuronal damage from HIV-1 proteins gp120 and Tat both in vitro and in vivo. Thus EGCG may represent a novel natural compound for the prevention and treatment of HAD.
3.2 Introduction

Epidemiologic studies indicate that some 60% of HIV-1-infected patients suffer some form of related neuropsychiatric impairment (Ozdener, 2005 and Stephanou, 2004) characterized by cognitive, motor, and/or behavioral symptoms. HIV-associated dementia (HAD; Goodkin et al., 2001, Melton et al., 1997 and Fujimura et al., 1996), represents the most severe form of HIV-related neuropsychiatric impairment (Shapshak et al., 2004) and the average survival after diagnosis is six months (Nath et al., 1999). In the early phases of HIV infection, virus invades the central nervous system (CNS) tissues from peripheral cell populations including infected: T cells, monocytes, and macrophages. Through this process HIV effectively establishes a viral reservoir in the CNS early after primary infection which is resistant to highly active antiretroviral therapy (HAART; Melton et al., 1997), also known as combine ART or “cART”. Later in the symptomatic phase of HAD, commonly coinciding with CD4+ T cell depletion to levels lower than 200 cells/mm³, the virus is sustained in the CNS primarily by resident microglia and macrophages that have invaded from peripheral tissues. These cells seemingly serve as both viral factories and as mediators for inflammatory events resulting in neuropathology and related neuropsychiatric impairment (Aquaro et al., 2005, Kumar et al., 2003, Shapshak et al., 2004 and Xiong et al., 2000). Indeed, pathologic CNS immune dysfunction has been widely explored in many past studies of microglia; the primary host cells for HIV-1 in the CNS (Garden et al., 2002, Koenig et al., 1986, Wiley et al., 1986 and Yoshioka et al., 1992). In addition, considering HIV-1 rarely infects neurons (Li et al., 2005), many investigations have focused on
the neurotoxic effects of viral proteins including HIV-1 gp120 and Tat, acting in concert with proinflammatory soluble factors released from reactive immune cells; inducing neuron death in the HAD brain (Xiong et al., 2000). Able to directly induce neuron damage through apoptosis (Kaul et al., 2001), the actions of HIV-1 proteins gp120 and Tat may be enhanced by cytokine-mediated signaling. For example in HAD, cytokines including IFN-γ, TNF-α, and IL-1β augment the neurotoxic properties of HIV-1 gp120 (Peruzzi et al., 2005). A similar role has been suggested to be at work in Alzheimer's disease (AD) where IFN-γ has been demonstrated to augment neuronal death in response to amyloid-beta (Bate et al., 2006). Indeed several studies have implicated this Th1 cytokine in the pathophysiology of HAD (Benveniste et al., 1994). IFN-γ binding to its receptor causes Janus associated kinases (JAKs) to phosphorylate tyrosine residues on the intracytoplasmic side of the IFN-γ receptor leading to signal transducer and activator of transcription (STAT) proteins activation and migration to the nucleus; a system known collectively as the JAK/STAT pathway (Heitmeier et al., 1999). In normal cells, IFN-γ-mediated JAK/STAT1 activation is a transient, lasting from several minutes to several hours.

It has been suggested this key regulatory system of proinflammatory and apoptotic signaling is dysfunctional in patients with HAD such that it is in a recurring state of inflammatory, cytokine-mediated apoptotic signaling; leading to widespread neuron damage (Kim and Maniatis, 1996, Lee et al., 1999, Peruzzi et al., 2005 and Shapshak et al., 2004). Previous studies support a role for JAK/STAT activation in the mediation of neuronal damage in HAD (Bovolenta et al., 1999) as well as stroke (Stephanou et al., 2000). Given the major constituent of green tea, (−)-epigallocatechin-3-
gallate (EGCG), can inhibit neuronal JAK/STAT-regulated neuronal damage (Townsend et al., 2004), we tested whether EGCG could modulate HAD-like neuronal damage by inhibition of JAK1/STAT1 activation. Thus the ability of IFN-γ to enhance neuronal damage inflicted by HIV-1 proteins gp120 and Tat in mice was first investigated. We report HIV-1 protein-induced neuronal damage was enhanced by IFN-γ in vitro and in vivo; an effect associated with increased JAK/STAT1 signaling. Primary neurons treated with JAK1 inhibitor or STAT1-deficient neurons were accordingly resistant to IFN-enhanced neurotoxicity of gp120 and Tat. Importantly, EGCG treatment attenuated HAD-like neuronal injury mediated by HIV-1 proteins gp120 and Tat in the presence of IFN-γ in vitro and in vivo through JAK/STAT1 inhibition.

3.3 Materials and Methods

3.3.1 Reagents

Green tea-derived EGCG (> 95% purity by HPLC) was purchased from Sigma (St. Louis, MO) and murine recombinant IFN-γ was obtained from R&D systems (Minneapolis, MN). Monoclonal mouse anti-neuronal nuclei antibody was obtained from Chemicon (Temecula, CA). Donkey anti-mouse IgG Alexa Fluor 594 was purchased from Molecular Probes (Eugene, OR). Tris-buffered saline was obtained from Bio-Rad (Hercules, CA) and luminol reagent was obtained from Pierce Biotechnology. Anti-phospho-STAT1/anti-phospho-JAK1, anti-total-STAT1/anti-total-JAK1, anti-Bcl-xL, and anti-Bax antibodies were purchased from Upstate (Lake Placid, NY). Anti-actin antibody was obtained from Roche. JAK inhibitor I was purchased from EMD Biosciences, Inc.(San Diego, CA). Recombinant HIV-1 proteins gp120 (HIV-1 gp120) and Tat were
obtained from The National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD).

3.3.2 Mice

Breeding pairs of C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and STAT1-deficient mice were purchased from Taconic (Hudson, NY). Animals were housed and maintained at the College of Medicine Animal Facility of the University of South Florida, and all experiments were in compliance with protocols approved by the University of South Florida Institutional Animal Care and Use Committee.

3.3.3 In vitro neurotoxicity analysis

Primary cultures of mouse cortical neurons were prepared as described previously (Chin et al., 1997). Briefly, neuronal cells were isolated from newborn C57BL/6 mice and seeded in 6-well tissue-culture plates at $2 \times 10^5$ cells/well for 48 h. Cells were then treated with gp120 (250 ng/ml) or Tat (250 ng/ml) in the presence or absence of IFN-γ (100 U/ml; Pierce Endogen) for 12 h. In addition, to test whether EGCG could inhibit JAK/STAT1 signaling and neuronal damage induced by gp120 or/and Tat in the presence of IFN-γ, EGCG was also employed as the cotreatment. Cell culture supernatants were used for lactate dehydrogenase (LDH) assay while cell lysates were used for Western blot analysis of Bcl-x and Bax proteins.
3.3.4 In vivo neurotoxicity analysis

Animals were anesthetized using isoflurane (chamber induction at 4–5% isoflurane, intubation and maintenance at 1–2%). After reflexes were checked to ensure that mice were unconscious, they were positioned on a stereotaxic frame (Stoelting Lab Standard) with ear-bars positioned and jaws fixed to a biting plate. The axis coordinates were taken from a mouse brain atlas, and a 5-mm sterile plastic guide cannula (21 GA; Plastic One, Inc., Roanoke, VA) was implanted into the left lateral ventricle delimited from the stereotaxic coordinates (coordinates relative to bregma: −0.6 mm anterior/posterior, +1.2 mm medial/lateral, and −3.0 mm dorsal/ventral) using the stereotaxic device (Stoelting Lab Standard) and an attached probe (cannula) holder. IFN-γ (200 U/mouse) with HIV-1 protein gp120 (500 ng/mouse) or Tat (500 ng/mouse) or PBS (10 µl) was administered at the rate of 1 µl/min using a Hamilton syringe (modified with a solder stop to prevent over insertion of the needle) through the implanted cannula. Correctness of the injection was confirmed by trypan blue dye administration and histological examination. The wounds were closed with 1–2 staples and mice were all observed until anesthesia had cleared. For testing EGCG effect on inhibiting Tat or/and gp120/IFN-γ neurotoxicity, the EGCG (50 mg/kg) or vehicle was intraperitoneally (i.p.) administered immediately after intracerebroventricular (i.c.v.) injection. Twenty-four hours after the i.c.v. injections animals were sacrificed with isofluorane and brain tissues collected. All dissected brain tissues were rapidly frozen for subsequent neuronal nuclear (NeuN) staining, Western blot, and LDH analysis.
3.3.5 JAK/STAT1 signaling analyses

Normal C57BL/6 primary cultured neuronal cells as well as STAT1-deficient primary neuronal cells were isolated and cultured as described previously (Chin et al., 1997). Normal cells were cotreated with either gp120 or Tat (250 ng/ml) with or without IFN-γ (100 U/ml) and/or JAK inhibitor (50 nM). STAT1-deficient cells were treated with HIV-1 gp120 or HIV-1 Tat (250 ng/ml) in the presence or absence of IFN-γ (100 U/ml) for 12 h. At the end of the treatment period, neuronal cells were washed in ice-cold PBS three times and lysed in ice-cold lysis buffer. After incubation for 30 min on ice, samples were centrifuged at high speed for 15 min, and supernatants were collected. Total protein content was estimated by using the Bio-Rad protein assay. For phosphorylation of JAK1, membranes were first hybridized with phosphospecific Tyr1022/1023 JAK1 antibody (Cell Signaling Technology, Beverly, MA) and then stripped and finally analyzed by total JAK1. For STAT1 phosphorylation, membranes were probed with a phospho-Ser727 STAT1 antibody (Cell Signaling Technology), stripped with stripping solution, and then re-probed with an antibody that recognizes total STAT1 (Cell Signaling Technology). Alternatively, membranes with identical samples were probed either with phospho-JAK or STAT1 or with an antibody that recognizes total JAK or STAT1. Immunoblotting was performed with a primary antibody, followed by an anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody as a tracer. After being washed in tris-buffered saline (TBS), the membranes were incubated in luminol reagent and exposed to film.
3.3.6 LDH assay

LDH release assay (Promega, Madison, WI) was performed as previously described (Tan et al., 2002). Briefly, after treatment at a variety of conditions, cell cultured media were collected for LDH release assay. Total LDH release was represent maximal lysis of target cells with 5% Triton X-100. Data are presented as mean ± SD of LDH release.

3.3.7 Western blot analysis

Western blot was performed as described previously (Tan et al., 2002). Briefly, for the in vivo studies left hemispheres of mouse brains were lysed in ice-cold lysis buffer and an aliquot corresponding to 50 µg of total protein was electrophoretically separated using 16.5% Tris–tricine gels. Electrophoresed proteins were then transferred to polyvinlyidene fluoride (PVDF) membranes (Bio-Rad), washed in dH2O, and blocked for 1 h at ambient temperature in Tris-buffered saline containing 5% (w/v) non-fat dry milk. After blocking, membranes were hybridized for 1 h at ambient temperature with various primary antibodies. Membranes were then washed three times (5 min each) in dH2O and incubated for 1 h at ambient temperature with the appropriate HRP-conjugated secondary antibody (1:1000). All antibodies were diluted in TBS containing 5% (w/v) non-fat dry milk. Blots were developed using the luminol reagent. Densitometric analysis was done using the Fluor-S MultiImagerTM with Quantity OneTM software (Bio-Rad). Antibodies used for Western blot included: anti-Bcl-xL antibody (1:1000), anti-Bax antibody (1:1000), anti-phospho-STAT1 (1:500), anti-total-STAT1 (1:500), anti-phospho-JAK1 (1:500), anti-total-JAK1 (1:500), and anti-actin antibody (1:1500). Similar
procedures were followed for the *in vitro* studies using cell culture supernatant aliquots corresponding to 50 µg of total protein.

3.3.8 NeuN immunochemistry analysis

At sacrifice, mice were anesthetized with isofluorane and transcardially perfused with ice-cold physiological saline containing heparin (10 U/ml). Brains were rapidly isolated and separated into left and right hemispheres using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). The right hemispheres were used for cryostat sectioning and subsequent NeuN immunochemistry analysis. NeuN staining was performed under standard immunofluorescence-labeling procedures. Briefly, frozen tissue sections were washed in PBS and blocked in 10% horse serum for 1 h, then incubated overnight in primary antibody, monoclonal mouse anti-NeuN antibody (1:100). The following day, sections were washed in PBS 3 times (10 min each), and then incubated for 1 h in the dark with secondary antibody, donkey anti-mouse IgG Alexa Fluor 594 at 1:100. After another cycle of washing, floating sections were mounted onto slides, dehydrated and coverslipped with Vectashield fluorescence mounting media (Vector Labs., Burlingame, CA). Slides were visualized under dark field using an Olympus BX-51 microscopy.

3.3.9 Statistical analysis

All data were normally distributed; therefore, in instances of single mean comparisons, Levene's test for equality of variances followed by *t*-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by *post hoc* comparison using
Bonferonni's method. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, Illinois) was used for all data analysis.

3.4 Results

3.4.1. IFN-γ enhances neuronal injury induced by gp120 and Tat in vitro and in vivo

It has been reported that neurons express IFN-γ receptor (Bate et al., 2006). In support, we also found IFN-γ receptor mRNA and protein expressed by both neuron-like cells (N2a cells) and primary cultured neurons (data not shown). To test whether IFN-γ plays a role in the modulation of HIV-1 proteins gp120 and Tat-induced neuronal injury, primary cultured neuronal cells were first isolated from newborn mice (1- to 2-day-old, wild-type C57BL/6) according to a method previously described (Tan et al., 2002). These cells were treated with gp120 or Tat (250 ng/ml) in the presence or absence of IFN-γ (100 U/ml) for 12 h. Cell cultured media were collected for LDH assay and cell lysates were prepared for neuronal injury examination by Western blot analysis (Tan et al., 2002). The presence of IFN-γ significantly increased LDH release and reduced the band density ratio of Bcl-xL to Bax in primary neurons challenged with HIV-1 proteins gp120 or Tat (4A, B).
Figure 4. IFN-γ enhances neuronal injury induced by HIV-1 proteins gp120 or Tat in vitro and in vivo.

Primary cultured neuronal cells were treated with gp120 (250 ng/ml), Tat (250 ng/ml), IFN-γ alone or gp120 (250 ng/ml), Tat (250 ng/ml) in combination with IFN-γ (100 U/ml; IFN-γ/gp120/or IFN-γ/Tat) for 12 h. Cell cultured media were collected for LDH assay (A) and cell lysates were prepared for neuronal injury examination by Western blot analysis (B). Data are presented as the mean ± SD of LDH release and Western blot band density ratio of Bcl-xL to Bax (n = 3). One-way ANOVA followed by post hoc comparison.
revealed significant differences between gp120 or Tat and HIV-1 gp120 or Tat plus IFN-γ (**P < 0.001) for both LDH release and band density ratio of Bcl-xL to Bax. (C) Mouse coronal, frozen brain sections were stained with NeuN and NeuN/DAPI. Marked neuronal damage was observed in the gp120 plus IFN-γ condition compared to controls. Similar results were also observed in the Tat plus IFN-γ condition (data not shown).

(D) Bcl-xL and Bax protein levels in mouse brain homogenates were analyzed by Western blot. Data are presented as the mean ± SD of Western blot band density ratio of Bcl-xL to Bax (n = 8; 4 male/4 female). One-way ANOVA followed by post hoc comparison revealed significant differences between gp120 or Tat compared to gp120 or Tat plus IFN-γ for band density ratio of Bcl-xL to Bax (**P < 0.001).

Furthermore, to test whether IFN-γ could enhance neuronal injury induced by gp120 and Tat in vivo, we treated C57BL/6 mice (n = 8; 4 male/4 female) with gp120 or Tat (500 ng/mouse) in the presence of IFN-γ (200 U/mouse) via the intracerebroventricular (i.c.v) route. Twenty-four hours after i.c.v. injection, these mice were sacrificed and then brain tissues were collected. All dissected brain tissues were rapidly frozen for subsequent biochemical and immunohistochemical analyses. Mouse brain sections from cortical regions were stained with NeuN and NeuN/DAPI. Results indicated a marked increase in neuronal damage in cortical brain regions from mice i.c.v injected with gp120 plus IFN-γ compared to controls, IFN alone, or gp120 alone (Fig. 4C). A similar result was also observed in the Tat plus IFN-γ condition (data not shown). In addition, brain homogenates from these mice were prepared for Western blot analysis of Bcl-xL and Bax protein expression. Consistently, a significant reduction in the ratio of Bcl-xL to Bax (Fig. 4D) in IFN-γ/gp120 or IFN-
γ/Tat conditions was observed. One-way ANOVA followed by post hoc comparison revealed significant differences between gp120 or Tat compared to gp120 or Tat plus IFN-γ for Western blot band density ratio of Bcl-xL to Bax.

3.4.2 Critical involvement of JAK/STAT1 signaling in neuronal damage induced by gp120 or Tat in the presence of IFN-γ

To further investigate IFN-γ-enhanced neuronal injury induced by gp120 and Tat, IFN-γ-activated JAK/STAT1 signaling was analyzed. Primary cultured neurons were treated with PBS, gp120 (250 ng/ml), Tat (250 ng/ml), IFN-γ (100 U/ml), and/or JAK inhibitor (50 nM) for 12 h. Importantly, neuronal injury was significantly inhibited by the presence of JAK inhibitor (Figs. 5A, B). One-way ANOVA followed by post hoc comparison revealed significant differences between IFN-γ/gp120 or IFN-γ/Tat compared to JAK inhibitor/IFN-γ/gp120 or JAK inhibitor/IFN-γ/Tat for both LDH release and Western blot band density ratio of Bcl-xL to Bax.

Furthermore, we isolated and cultured primary neurons from STAT1-deficient mice. These cells were treated with gp120 or Tat (250 ng/ml), respectively in the presence or absence of IFN-γ (100 U/ml) for 12 h. Cell cultured media and cell lysates from these cells were then subjected to LDH and Western blot analyses. Results demonstrated neuronal injury was largely attenuated in the STAT1-deficient neurons treated with IFN-γ/gp120 or IFN-γ/Tat (Figs. 5C, D). One-way ANOVA followed by post hoc comparison revealed significant differences between STAT1-deficient neurons compared to wild-
type neurons following treatment with IFN-γ/gp120 or IFN-γ/Tat for both LDH release and Western blot band density ratio of Bcl-xL to Bax.

Figure 5. JAK/STAT1 signaling is critically involved in the IFN-γ mediated enhancement of HIV-1 gp120 and Tat-induced neuronal damage.

Primary cultured neuronal cells were cotreated with IFN-γ (100 U/ml) and gp120 or Tat at 250 ng/ml in the presence of JAKinhibitor (50 nM) for 12 h. Cell cultured media were collected for LDH assay (A) and cell lysates were prepared for
neuronal injury examination by Western blot analysis (B). Data are presented as mean ± SD of LDH release and Western blot band density ratio of Bcl-xL to Bax (n = 3). One-way ANOVA followed by post hoc comparison revealed significant differences between IFN-γ/gp120 or IFN-γ/Tat compared to JAK inhibitor/IFN-γ/gp120 or JAK inhibitor/IFN-γ/Tat (**P < 0.001). Primary neuronal cells derived from STAT1-deficient mice were treated with gp120 or Tat at 250 ng/ml in the presence or absence of IFN-γ (100 U/ml) for 12 h. Cell culture media and cell lysates from these cells were subjected to LDH assay (C) and Western blot analysis (D). Data are presented as the mean ± SD of LDH release and Western blot band density ratio of Bcl-xL to Bax (n = 5). One-way ANOVA followed by post hoc comparison revealed significant differences between STAT1-deficient neurons compared to wild-type neurons following treatment with IFN-γ/gp120 or IFN-γ/Tat for both LDH release and the band density ratio of Bcl-xL to Bax (**P < 0.001).

3.4.3. EGCG inhibits JAK/STAT1 signaling; attenuating neuronal damage induced by gp120 or Tat in the presence of IFN-γ in vitro

Primary cultured neurons were treated with IFN-γ (100 U/ml) for different time points as indicated. Results demonstrated IFN-γ stimulates phosphorylation of JAK1 (Fig. 6A) and STAT1 (Fig. 6C) time-dependently. To test whether EGCG could modulate this phosphorylation in neuronal cells, we co-incubated them with IFN-γ (100 U/ml) and EGCG at a range of doses as indicated for 60 min. JAK1/STAT1 phosphorylation was analyzed by Western blot.
Figure 6. EGCG inhibits IFN-γ-induced JAK/STAT1 phosphorylation; protecting neurons from injury induced by IFN-γ/gp120 or IFN-γ/Tat in vitro.

JAK1 and STAT1 protein phosphorylations were examined by Western blot (A, C). Cell lysates were prepared from primary cultured neurons treated with IFN-γ (100 U/ml) for various time points as indicated (A, C). Cell lysates were prepared from primary
cultured neurons cotreated with IFN-γ (100 U/ml) and EGCG at different doses as indicated for 60 min (B, D). To examine the putative role of EGCG in opposing neuronal injury induced by IFN-γ/gp120 or IFN-γ/Tat, primary neurons were cotreated with gp120 or Tat at 500 ng/ml in the presence of IFN-γ (100 U/ml) and EGCG (20 µM) for 12 h. Cell cultured supernatants were collected for LDH assay (E) and cell lysates were prepared for Bcl-xL/Bax Western blot analysis (F). Data are presented as the mean ± SD of LDH release and Western blot band density ratio of Bcl-xL to Bax (n = 3). One-way ANOVA followed by post hoc comparison revealed significant differences between IFN-γ/gp120 or IFN-γ/Tat compared to EGCG/IFN-γ/gp120 or EGCG/IFN-γ/Tat for both LDH release and band density ratio of Bcl-xL to Bax (**P < 0.001). It has been reported that EGCG modulates STAT1 activation in vitro (de Prati et al., 2005, Magro et al., 2005 and Tedeschi et al., 2002) and in vivo (Stephanou, 2004 and Townsend et al., 2004). To examine the putative role of EGCG in opposing neuronal injury induced by HIV-1 gp120 or Tat in the presence of IFN-γ, we cotreated primary neurons with gp120 or Tat (500 ng/ml) in the presence of IFN-γ (100 U/ml) and EGCG (20 µM) for 12 h. Cell cultured supernatants were collected for LDH assay and cell lysates were prepared for Bcl-xL/Bax Western blot analysis. Results show EGCG cotreatment markedly attenuates neuronal injury; as evidenced by decreased LDH release (Fig. 6E) and increased ratio of Bcl-xL to Bax (Fig. 6F). One-way ANOVA followed by post hoc comparison revealed significant differences between IFN-γ/gp120 or IFN-γ/Tat compared to EGCG/IFN-γ/gp120 or EGCG/IFN-γ/Tat for both LDH release and Western blot band density ratio of Bcl-xL to Bax.
3.4.4. EGCG inhibits neuronal damage mediated by gp120 or Tat in the presence of IFN-γ in vivo

To evaluate the ability of EGCG to inhibit neuronal damage induced by HIV-1 proteins in combination with IFN-γ in vivo, C57BL/6 mice (n = 8; 4 male/4 female) were treated with HIV-1 proteins gp120 or Tat (500 ng/mouse) in the presence of IFN-γ (200 U/mouse) via an i.c.v. injection. EGCG (50 mg/kg) or vehicle was intraperitoneally (i.p.) administered immediately after the i.c.v. injection. Twenty-four hours after EGCG treatment, mice were sacrificed and brain tissues were and rapidly frozen for subsequent biochemical and immunohistochemical analyses. Mouse brain sections from cortical regions were stained with NeuN and NeuN/DAPI. Results demonstrated a marked reduction of neuronal damage in cortical regions of the brains from mice i.c.v injected with IFN-γ/gp120 or IFN-γ/gp120/Tat in the presence of EGCG compared to controls (Fig. 7A). Similar reductions in neuronal injury were also observed in mice treated with IFN-γ/Tat in the presence of EGCG compared to mice treated with IFN-γ/Tat alone (data not shown). In addition, brain homogenates were prepared from these mice for Western blot analysis of Bcl-xL and Bax protein expressions. Consistently, significant increases in the ratio of Bcl-xL to Bax for both IFN-γ/gp120/EGCG and IFN-γ/gp120/Tat/EGCG (Fig. 7B) compared to IFN-γ/gp120 and IFN-γ/Tat conditions were observed, respectively. One-way ANOVA followed by post hoc comparison revealed significant differences between IFN-γ/gp120/EGCG or IFN-γ/gp120/Tat/EGCG compared to IFN-γ/gp120 and IFN-γ/gp120/Tat in Western blot band density ratio of Bcl-xL to Bax (Fig. 7B).
Figure. 7. Mice i.p. injected with EGCG demonstrate significant reductions in neuronal injury after i.c.v. injection of IFN-γ/gp120, IFN-γ/Tator IFN-γ/gp120/Tat. (A) Coronal, frozen mouse brain sections were stained with NeuN (top panels) and NeuN/DAPI (lower panels) and analyzed for neuron injury/loss. A marked reduction of neuronal damage was observed when EGCG was added to either IFN-γ/gp120 or IFN-γ/gp120/Tat. Similar effects of EGCG were also observed in IFN-γ/Tat condition (data not shown). (B) Bcl-xL and Bax protein levels in mouse brain homogenates were analyzed by Western blot.
Data are presented as mean ± SD of Western blot band density ratio of Bcl-xL to Bax (n = 8; 4 female/4 male). One-way ANOVA followed by post hoc comparison revealed significant differences in the band density ratio of Bcl-xL to Bax observed between gp120/IFN-γ or gp120/Tat/IFN-γ compared to gp120/IFN-γ/EGCG or gp120/Tat/IFN-γ/EGCG conditions, respectively (**P < 0.001).

3.5 Discussion

Neuronal damage and cognitive impairment found in HAD occurs in the later stages of infection whereas a CNS viral reservoir is initiated early after infection. The specific components leading to neurological dysfunction in HAD remains unclear. However, current studies aim to differentiate and characterize individual disease mechanisms involved in this complex process comprising chronic inflammatory activation of immune effector cells, and HIV protein-induced dysfunction of neurons; ultimately resulting in neuronal cell death.

In HAD, neurons are not killed by direct viral infection but rather viral proteins released from infected CNS mononuclear cells may directly kill neurons or render them susceptible to death signaling. Clearly viral proteins can bind to cell surface receptors such as CXCR4 and N-methyl-D-aspartate receptors. Thus HIV-1 proteins gp120 and Tat may trigger neuronal apoptosis and excitotoxicity resulting from altered cellular intracellular calcium concentrations and mitochondrial dysfunction (Mattson et al., 2005). Inflammation and proinflammatory soluble factors also play important roles in the pathogenesis of HAD. Increasingly, studies point to the central roles played by reactive immune cells including macrophages and microglia in the
generation and progression of many disease mechanisms implicated in the pathology of 
HAD (Aquaro et al., 2005), as well as other neurodegenerative diseases. To effectively 
investigate components of HAD-like neuronal damage we developed a multifaceted 
approach involving HIV-1 proteins gp120 and Tat in combination with the 
proinflammatory cytokine, IFN-γ. Collaboration of proinflammatory cytokines with HIV-
1proteins in the induction of neuronal injury/death appears to be an important component 
of the pathogenesis of HAD (Aquaro et al., 2005, Fujimura et al., 1996, Kaul et al., 
2001, Koenig et al., 1986, Speth et al., 2005 and Xiong et al., 2000). Here it has been 
demonstrated in vitro that IFN-γ enhances HAD-like neuronal damage mediated 
by gp120 and Tat (Figs. 1A, B). Moreover, normal mice i.c.v. injected with gp120, Tat, 
or IFN-γ display neuron loss and pro-apoptotic signaling. Importantly, 
combining gp120 or Tat with IFN-γ resulted in a dramatic rise in neuron loss in the 
cortical regions examined (Figs. 4C, D). Indeed we found a synergistic, pro-apoptotic 
effects when IFN-γ was combined with a challenge of HIV-1 gp120 or Tat proteins (Fig. 
4D). Previous investigations have demonstrated cause and effect relationships between 
production of HIV-1 proteins gp120 and Tat, and neuronal damage (Li et al., 
2005, Mattson et al., 2005 and Nath et al., 1999). Consistent with these findings clinical 
reports detail correlations between HIV-1proteins, IFN-γ and neuron cell loss resulting in 
cognitive decline in HAD patients (Kumar et al., 2003, Mattson et al., 2005 and Shapshak 
et al., 2004).

Further, previous studies investigating the neurotoxic effects of IFN-γ implicated 
members of the JAK and STAT families (Heitmeier et al., 1999, Kim and Maniatis, 
1996 and Lee et al., 1999). The JAK1/STAT1 interaction is extensively described in
studies investigating apoptosis induced by ischemia/reperfusion in cardiovascular, CNS, and other tissues (Chin et al., 1997, Kumar et al., 1997, Stephanou, 2004 and Takagi et al., 2002). In neurons, STAT1 appears to be primed by ischemia/reperfusion and thus rendered more sensitive to IFN-γ receptor activation (Stephanou, 2004 and Takagi et al., 2002). Occlusion of the middle cerebral artery resulted in rapid co-localization of STAT1 with TUNEL-positive neurons, thereby suggesting a role for STAT1 in cell apoptosis/death (Takagi et al., 2002). Since HIV infection of the CNS induces marked increases in IFN-γ expression in CNS tissues (Shapshak et al., 2004) the involvement of the JAK/STAT pathway in neuronal damage associated with HIV dementia is likely. Thus we investigated the involvement of JAK1 and STAT1 (Figs. 5A–D) in the IFN-γ-enhanced, gp120/Tat-induced neuronal damage in primary culture neurons. Clearly activation of JAK1 and STAT1 is markedly evident after treatment with IFN-γ in primary cultured neurons from wild-type mice (Figs. 6A, D). JAK1 inhibitor mitigated neuron damage, inflicted by combinations of IFN-γ and gp120 and Tat proteins, in vitro (Figs. 5A B). Additionally both LDH and Bcl-xL/Bax ratios were greatly reduced by addition of JAK1 inhibitor. However, these indicators of cell death and apoptosis were not returned to baseline levels of the control treatment group when combination of gp120 and Tat were included in the treatment; indicating an alternate pathway/mechanism utilized by these proteins to induce cell damage. Thus, primary neurons from STAT1-deficient mice were examined and found to be highly resistant to IFN-γ-enhanced neuronal damage. However, in combination with gp120 or Tat greater neuronal damage ensued; albeit dramatically less than the
damage observed in wild-type neurons treated with gp120 or Tat in combination with IFN-γ (Figs. 5C, D).

Previous studies investigating the properties of the green tea-derived polyphenol, EGCG, indicated this compound was able to attenuate cell death induced by ischemia/reperfusion through downregulation of the JAK/STAT1 pathway (Townsend et al., 2004). Thus whether EGCG could effectively down-regulate IFN-γ-mediated JAK/STAT1 signaling; a process that enhanced gp120/Tat-induced neuron damage. Our in vitro studies utilizing primary culture neurons from wild-type mice demonstrated marked reductions in both LDH release and in Bcl-xL/Bax ratios when EGCG was added to Tat/IFN-g or gp120/IFN-g compared to these conditions in the absence of EGCG (Figs. 6E, F). These data suggest that EGCG's ability to reduce JAK/STAT1 signaling in primary culture neurons is protective against IFN-γ-enhanced gp120/Tat-induced HAD-like neuronal damage in vitro.

To evaluate the effects of EGCG on inhibition of neuronal damage induced by HIV-1 proteins gp120 and Tat in the presence of IFN-γ in vivo, control mice were administered i.p. injections of EGCG or PBS (vehicle control) and then i.c.v. injected with HIV-1 proteins, gp120 or Tat, in the presence of IFN-γ. Consistent with our above-mentioned results, EGCG was protective against neuron loss induced by i.c.v injected IFN-γ and/or gp120/Tat in cortical regions examined. This was evidenced by increased Bcl-xL/Bax ratios in brain homogenates of mice cotreated with EGCG plus IFN-g/gp120 or IFN-g/Tat/gp120, respectively (Fig. 7B), and reductions in neuron loss in cortical sections by immunohistochemistry (Fig. 7A).
Several reports investigating EGCG's ability to block JAK/STAT1 signaling have reported protective effects of the compound against: proinflammatory activation of immune cells, epithelial barrier dysfunction, and neuronal apoptosis after ischemia/reperfusion injury. Thus, JAK/STAT1 interaction may be an important therapeutic target for a variety of CNS disorders (Tedeschi et al., 2002 and Townsend et al., 2004). Taken together, our data suggest the JAK/STAT1 pathway may be an important therapeutic target for opposing the neuronal death and injury seen in the HAD brain. Indeed inhibition of the JAK/STAT pathway by green tea-derived EGCG or analogous compounds may provide an effective therapeutic intervention as an adjunct to HAART for the treatment of HAD.
4.1 Abstract

Human immunodeficiency virus (HIV)-associated dementia (HAD) is a subcortical neuropsychiatric syndrome that has increased in prevalence in the era of highly active antiretroviral therapy (HAART). Several studies demonstrated increased amyloidosis in brains of HIV patients and suggested that there may be a significant number of long-term HIV survivors with co-morbid Alzheimer's disease (AD) in the future. We show HIV-1 Tat protein inhibits microglial uptake of Aβ_{1-42} peptide, a process that is enhanced by interferon-gamma (IFN-γ) and rescued by the STAT1 inhibitor (-)-epigallocatechin-3-gallate (EGCG). It is hypothesized that reduced Aβ uptake occurs through IFN-γ mediated STAT1 activation. This process promotes a switch from a phagocytic to an antigen presenting phenotype in microglia through activation of class II transactivator (CIITA). Additionally, we show that HIV-1 Tat significantly disrupts apolipoprotein-3 (Apo-E3) promoted microglial Aβ uptake. As Tat has been shown to directly interact with the low density lipoprotein (LRP) receptor and thus inhibit the uptake of its ligands including apolipoprotein E4 (Apo-E4) and Aβ peptide in neurons, we further hypothesize that a similar inhibition of LRP may occur in microglia. Future studies will be required to fully characterize the mechanisms underlying IFN-γ enhancement of HIV-1 Tats disruption of microglial phagocytosis of Aβ and Apo-E3.
4.2 Introduction

Marked by impairment in cognition, affect, emotion, and motor skills, HIV-associated dementia (HAD) represents the most severe form of HIV related neuropsychiatric impairment (Shapshak et al., 2004). It is a relatively common sequela of advanced HIV disease, occurring in some 20% of patients in the era preceding highly active antiretroviral therapy (HAART) (McArthur et al., 1993). Although the incidence in the HAART era has halved relative to pre-HAART, the prevalence has roughly doubled, due to the life-extending effect of HAART (Sacktor et al., 2002; Neuenburg et al., 2002).

Moreover, HAD is commonly characterized by amyloid-beta (Aβ) accumulation and other associated Alzheimer's disease (AD)-like neuropathology (Everall et al., 2005; Green et al., 2005). This will likely complicate management of HIV by requiring greater provisions for long-term care of HIV-infected patients with dementia (Alisky et al., 2007).

Amyloid deposition in the brain occurs with aging and is an important pathological finding in AD and HAD. Aβ peptide is neurotoxic and its accumulation in brain has been implicated in the associated neurodegeneration (Nath and Hersh, 2005). The first study to identify AD-like changes in HIV patients was reported by Esiri and colleagues who compared prevalence of argyrophilic amyloid plaques in 97 AIDS cases dying at ages 30–69 years with that in 125 age matched, non-HIV infected controls. They found that Aβ plaques formed at an earlier age and in greater amounts in the AIDS group. Moreover, there was a significantly greater prevalence of plaques in the AIDS group as a whole (29%) and in those in the fourth decade (18%) than in control subjects (13% and
0% respectively; Esiri et al., 1998). In a recent study, 4G8 and 6E10 antibody staining demonstrated that significant deposition of amyloid occurred in the frontal cortex of almost half of 162 autopsied AIDS brains studied (Green et al., 2005). Similar but less abundant deposition was detected in the hippocampus and basal ganglia (Green et al., 2005). In accord, another study of postmortem human brain sections from patients with HIV-1 infection (n = 14; 31–58 years old) demonstrated a significant increase in Aβ, compared to controls (n = 5; 30–52 years old) (Rempel and Pulliam, 2005).

Several factors could produce this increasing prevalence of AD pathology in the HIV population. Because of HAART, more HIV patients will live to an age where AD commonly presents (Brew et al., 2005; Weiler, 1986; Borjesson-Hanson et al., 2004). Additionally, a recently characterized phenomenon in patients receiving HAART, known as immune reconstitution syndrome (IRS), may increase the incidence of AD in long-term HIV survivors (Alisky, 2007). IRS is an autoimmune condition occurring when reconstituted T cell populations attack opportunistic pathogens which proliferated during T cell suppression by HIV. It is characterized by connective tissue disease symptoms and vasculitis (Stoll and Schmidt, 2003; Gray et al., 2005; Townsend et al., 2005). As inflammation has been linked to AD (Townsend et al., 2005; Tan et al., 2002; Sastre et al., 2006) patients with IRS would seem to be at an elevated risk of developing AD pathology (Alisky, 2007). The same would apply to patients who demonstrate lipodystrophic and metabolic effects of HAART, which cause hyperlipidemia, alterations in distribution of body fat to metabolically inactive regions, insulin resistance and coronary artery disease; all known AD risk factors (Brew et al., 2005; Barbaro, 2006; Luchsinger et al., 2005; Newman et al., 2005). Furthermore, HIV itself produces
neurotoxicity from chemokines, cytokines such as interferon-gamma (IFN-γ) (Shapshak et al., 1992), and from excitotoxic effects of the secreted proteins including HIV-1 transactivator of transcription (Tat) protein (Shapshak et al., 2004; Giunta et al., 2004, 2006; Navia and Rostasy, 2005; Diesing et al., 2002; Ryan et al., 2002). Here we examined the effects of two these factors, specifically IFN-γ inflammatory signaling and HIV-1 Tat protein.

HIV-infected cells secrete Tat protein which is taken up by adjacent uninfected cells (Sabatier et al., 1991; Kruman et al., 1998; Chang et al., 1997; Ensoli et al., 1993). It is uncertain, however, whether Tat is present within CNS of HIV-infected patients at sufficient concentrations to directly produce acute neurotoxicity (Liu et al., 2000). It has been shown that nanomolar concentrations of Tat protein induce gene expression, cell proliferation, differentiation, adhesion and morphological changes without detectable cytotoxic effects (Ensoli et al., 1993; Marcuzzi et al., 1992; Milani et al., 1993; Kolson et al., 1993). Importantly, increasing evidence suggests that HIV-1 Tat protein may directly lead to increased Aβ deposition in the HIV infected brain. Pulliam and colleagues demonstrated neprilysin interacts with the cysteine-rich domain of Tat. Neprilysin functions as a type II plasma membrane zinc metallopeptidase; representing one mechanism for its clearance (Rempel and Pulliam 2005; Daily et al., 2006). It has also been demonstrated that binding of HIV-1 Tat protein to low-density lipoprotein receptor-related protein (LRP) resulted in substantial inhibition of neuronal binding, uptake and degradation of physiological ligands for LRP, including apolipoprotein E4 (Apo-E4), and Aβ (Liu et al., 2000). In the following experiments we tested the hypothesis that Tat inhibition of Aβ uptake occurred in microglia, a principle mechanism for the removal of
Aβ from the brain parenchyma (Bard et al., 2000).

Previous investigations have demonstrated a critical role of microglia in Aβ plaque clearance (Rogers and Lue, 2001; Rogers et al., 2002). Microglia, functioning as resident brain macrophages, represent the initial defense against invading pathogens and are a critical link between the central nervous system (CNS) and the immune system (Townsend et al., 2005). In normal adult brain, microglia are quiescent, but in reaction to CNS injury they actively phagocytose cellular debris and apoptotic cells (Kreutzberg, 1996). This has the effect of reducing pro-inflammatory cytokine production and minimizes injury in the inflamed brain (Magnus et al., 2002).

In AD clinical cases as well as animal models, increased activation and recruitment of microglia to areas of cerebral amyloidosis are observed. However, these activated microglia are unable to clear Aβ deposits in AD mice (Stalder et al., 1997). Strategies which augment microglial phagocytic activity are able to ameliorate cerebral Aβ load in AD mouse models as evidenced by a report demonstrating that transforming growth factor-β over-expression promotes phagocytic clearance of cerebral Aβ (Wyss-Coray et al., 2001). Thus augmentation of this capacity for microglia to clear amyloid plaques, by opposing the effects of HIV-1 Tat protein, may prove therapeutic.

4.3 Materials and Methods

4.3.1 Reagents

Aβ<sub>1-42</sub> and FITC-conjugated Aβ<sub>1-42</sub> were obtained from Biosource International (Camarillo, CA). Recombinant HIV-1 Tat protein was obtained from the NIH AIDS Research and Reference Reagent program. Recombinant IFN-γ was purchased from
R&D Systems. Purified rat anti-mouse major histocompatibility (MHC) class II antibody was obtained from PharMingen (San Diego, CA). (-)-Epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich.

4.3.2 Mouse Primary Cell Culture

Breeding pairs of C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in the animal facility at the University of South Florida Health Science Center. Mouse primary cultured microglia were isolated from mouse cerebral cortices and grown in complete Roswell Park Memorial Institute (RPMI) 1640 medium according to previously described methods (Tan et al., 2000). Briefly, cerebral cortices from newborn mice (1–2 days old) were isolated under sterile conditions and were kept at 4°C prior to mechanical dissociation. Cells were plated in 75-cm² flasks, and complete medium was added. Primary cultures were kept for 14 days so that only glial cells remained, and microglial cells were isolated by shaking flasks at 200 rpm in a Lab-Line™ Incubator-Shaker. More than 98% of these glial cells stained positive for MAC-1 (Boehringer Mannheim, Indianapolis, IN). Additionally, between 85% and 95% of microglia stained positive for CD45 by fluorescence-activated cell sorter (FACS) analysis as described previously (Tan et al., 2000).

4.3.3 Microglial Phagocytosis Assays

4.3.3.1 Fluorimetric analysis

Primary mouse microglia were seeded at 1×10⁵ cells/well (n=6 for each condition) in 24-well tissue culture plates containing 0.5 mL of complete RPMI-1640 medium. These cells were treated for 60 minutes with “aged” Aβ₁₋₄₂ conjugated with fluorescein.
Isothiocyanate (FITC; Biosource International) (Chung et al., 1999). In the presence of FITC- Aβ₁₋₄₂, microglia were then co-treated with HIV-1 Tat or control (heat-inactivated Tat protein) in the presence or absence of IFN-γ and/or EGCG. Microglia were then rinsed three times in Aβ-free complete medium, and the media were exchanged with fresh Aβ-free complete medium for 10 minutes both to allow for removal of non-incorporated Aβ and to promote concentration of the Aβ into phagosomes.

Extracellular and cell-associated FITC-Aβ was quantified using a SpectraMax multi-detection reader (Molecular Devices) with an emission wavelength of 538 nm and an excitation wavelength of 485 nm. A standard curve from 0 to 500 nM of FITC-Aβ was run for each plate. Total cellular proteins were quantified using the Bio-Rad protein assay. The mean fluorescence values for each sample at 37°C and 4°C at the indicated time points were determined by fluorimetric analysis. Relative fold change values were calculated as: mean fluorescence value for each sample at 37°C/mean fluorescence value for each sample at 4°C. In this manner, both extracellular and cell-associated FITC-Aβ was quantified. Considering nonspecific adherence of Aβ to plastic surface of cultured plates, an additional control without cells was carried out through all of experiments above. An incubation time of less than 4 hours did not change the amount of Aβ peptide detected in the supernatant, which is consistent with a previous report (Mitrasinovic and Murphey, 2002). In order to determine the extent to which cell death might have influenced the phagocytic activity in the various treatment groups, we performed the LDH assay on the relevant supernatant. Data showed that there was no significant cell death occurring over the 3-h time frame in any of the treatment groups (data not shown, p>0.05).
4.3.3.2 Fluorescence microscope examination

“Aged” FITC-Aβ₁₋₄₂ was prepared according to methods described above. In the presence of FITC-Aβ₁₋₄₂, microglia were then co-treated with HIV-1 Tat or control (heat-inactivated Tat protein) in the presence or absence of IFN-γ, Apo-E3, and/or EGCG at 37°C for 60 min. In addition, in parallel 24-well tissue culture plates, microglia were incubated at 4°C with FITC-conjugated Aβ (50 nM) in the presence or absence of IFN-γ, Apo-E3, and/or EGCG for 60 min. Following treatment, these cells were washed 5 times with ice-cold phosphate buffered saline (PBS) to remove extracellular Aβ and then stained with 4′, 6-Diamidino-2-phenylindole (DAPI) at 4°C for 15 min. After washing three times with ice-cold PBS, these cells were fixed in 4% paraformaldehyde, mounted, and then viewed under an Olympus IX71/IX51 microscope equipped with a digital camera system.

4.4 Results

4.4.1 HIV-1 Tat Inhibits Microglial Phagocytosis of Aβ₁₋₄₂ Peptide

HIV-1 Tat protein has been positively associated (Rempel and Pulliam 2005; Liu et al., 2000; Daily et al., 2006) with increased brain deposition of Aβ/β-amyloid; a common pathologic feature in HIV-positive patients (Green et al., 2005; Nath and Hersh, 2005; Esiri et al., 1998; Brew et al., 2005; Mankowski et al., 2002; Izycka-Swieszewska et al., 2000; Cozzi et al., 1992). Since microglial phagocytosis of Aβ has been considered a principle mechanism for the removal of Aβ from the brain parenchyma (Bard et al., 2000), we evaluated whether Tat could inhibit microglial uptake of Aβ. “Aged”, FITC-tagged Aβ₁₋₄₂ was added to primary cultured microglia for 60 min in the presence of
varying concentrations of recombinant HIV-1 Tat at varying concentrations or heat inactivated HIV-Tat (negative control). As shown in Figures 8 and 9, addition of HIV-1 Tat protein significantly and dose-dependently decreased microglial uptake of Aβ1-42 compared to heat inactivated Tat (negative control; p<0.05) for each of the doses of Tat tested.

**Figure 8** HIV-1 Tat protein inhibits microglial phagocytosis of Aβ1-42 peptide. Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with “aged” FITC-tagged Aβ1-42 (300 nM) in complete medium for 60 min in the presence of either recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent Program; 32, 63, 125, 250 ng/mL), or heat inactivated recombinant Tat protein (32, 63, 125, 250 ng/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4°C with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular (top) and cell-
associated (bottom) FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between HIV-Tat compared to heat inactivated HIV-1 Tat (control) dose dependently from 63–250 ng/mL (p<0.001), but no significant difference between HIV-Tat compared to heat inactivated HIV-1 Tat (control) at the 32 ng/mL concentrations (p>0.05).

Figure 9 HIV-1 Tat protein inhibits microglial phagocytosis of Aβ 1-42 peptide: In order to examine microglia Aβ co-localization on the microglia surface, primary cultured microglia were treated with "aged" FITC- Aβ 1-42 peptide (50 nM) in the presence of either recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent
Program; 63, 125, 250 ng/mL), or heat inactivated recombinant Tat protein (250 ng/mL) for 60 min and then stained these cells with DAPI. HIV-1 Tat dose-dependently inhibited microglia – Aβ co-localization as examined by fluorescence microscopy (a, b, c). As noted, green indicates Aβ1-42-positive; blue indicates microglial nuclei. Addition of HIV-1 Tat protein at 32 ng/mL yielded similar results as heat inactivated Tat (data not shown; d). Original magnification = 20×.

4.4.2 IFN-γ Enhances HIV-1 Tat-mediated Reduction in Microglial Phagocytosis of Aβ1-42 Peptide

A pro-inflammatory environment characterizes the HIV-infected and AD brain, respectively (Everall et al., 2005; Alisky, 2007; Mahfoud et al., 2002; Minagar et al., 2002; Smits et al., 2000; Stanley et al., 1994; Dickson et al., 1993; Dou et al., 2006; Corder et al., 1998; Avison et al., 2004; Anderson et al., 2002; Persidsky et al., 2000; Petito et al., 1999). Importantly, there is evidence of microglial cell loss of functional capacity to phagocytose Aβ associated with inflammation (Townsend et al., 2005; von Bernhardi et al., 2007). Considered a pro-inflammatory cytokine, IFN-γ is elevated in the brains of HIV-infected patients as well as those patients who go one to develop HAD (Shapshak et al., 2004). This holds clinical relevance as positive associations between severities of cognitive dysfunction in HAD with expression of inflammatory cytokines have been demonstrated (Navia and Rostasy, 2005). This microglial loss of function has been associated with co-stimulatory cytokine signaling which shifts the functional state of microglia from being primarily phagocytes to antigen presenting cells (APC) (Townsend et al., 2005). In the phagocytic phenotype, microglia engulf and degrade Aβ peptide, preventing accumulation of Aβ peptide to neurotoxic levels in the brain.
Conversely, switching to the APC phenotype inhibits this phagocytic ability and occurs in the presence of pro-inflammatory cytokines, leading to chronic microgliosis (Townsend et al., 2005; Gregerson and Yang, 2003; Mack et al., 2003; Aloisi et al., 1999). This may act to exacerbate both AD and HAD neuropathology (Everall et al., 2005; Navia and Rostasy, 2005; Rumbaugh and Nath, 2006; Mattson et al., 2005; Frackowiak et al., 1992).

To investigate whether IFN-\(\gamma\) augments the inhibited phagocytic ability of microglia in the presence of FITC-A\(\beta_1-42\), and HIV-1 Tat, IFN-\(\gamma\) (50U/mL) or its denatured form (negative control) were added in the presence or absence of Tat (63 ng/mL [minimal dose which significantly inhibited A\(\beta\) uptake, Fig. 10) for 60 min at 37°C. Denatured Tat was not used in this paradigm as the denatured form was shown to be inactive in preventing microglial A\(\beta\) uptake (Figure 1). Data shows IFN-\(\gamma\) significantly augmented HIV-1 Tat-mediated decreased microglial phagocytosis of A\(\beta\) 1-42 (Figure 3, left panel) compared to control.
**Figure 10.** HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ1-42 peptide is enhanced by IFN-γ and opposed by EGCG.

**Left panel:** IFN-γ significantly enhances HIV-1-Tat mediated reduction in microglial phagocytosis of Aβ1-42 peptide (left panel). Primary cultured microglia (1×10⁵ cells/well in 24-well tissue culture plates) were treated with “aged” FITC-tagged Aβ 1-42 (300 nM) in complete medium for 60 min in the absence (control) or presence of either HIV-1 Tat (63 ng/mL, minimal effective concentration), active or denatured IFN-γ (50 U/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4°C with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD),
calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between Tat alone, IFN-γ alone, or denatured IFN-γ + Tat compared to the IFN-γ + Tat control (p<0.001). **Right panel:** EGCG significantly inhibits IFN-γ enhancement of HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ 1-42 peptide. Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with “aged” FITC-tagged Aβ 1-42 (300 nM) in complete medium for 60 min with addition of HIV-1 Tat (63 ng/mL) and IFN-γ (50 U/mL) in the presence of EGCG (0 [control], 2.5, 5, 10, or 20 µM). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4°C with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as mean fluorescence/each sample at 37°C divided by mean fluorescence at 4°C (n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed a significant difference between EGCG (10 or 20 µM) compared to control (p<0.001), but no significant difference between EGCG at the 2.5 and 5.0 µM dose compared to control (p>0.05).
4.4.3 EGCG Inhibits IFN-γ Enhancement of Tat-mediated Reduction in Microglial Phagocytosis of Aβ1-42 Peptide

We have previously shown IFN-γ synergistically enhances neuronal injury induced by Tat in vitro and in vivo via activation of the JAK/STAT1 signaling pathway which is specifically attenuated by EGCG inhibition of STAT1 activation (Giunta et al., 2006). Other investigations have also shown that EGCG inhibits IFN-γ mediated STAT1 signaling as well (Jeong et al., 2007; de Prati et al., 2005; Watson et al., 2004). Thus we evaluated whether EGCG could oppose the IFN-γ mediated enhancement of Tat down-regulation of microglial uptake of Aβ. Again, “aged” FITC-tagged Aβ 1-42 was added to primary cultured microglia with IFN-γ (50 U/mL) and Tat (63 ng/mL) for 60 min at 37°C in the presence of varying concentrations of EGCG (0, 2.5, 5, 10, and 20μM). As shown in Figure 10 (right panel), data indicated EGCG dose-dependently increased microglial cell associated Aβ 1-42 in the presence of IFN-γ and HIV-1 Tat. The minimal effective concentration was 10μM. As shown in Figure 11, HIV-1 Tat + IFN-γ (c) significantly inhibited microglial phagocytosis of Aβ peptide as compared to HIV-1 Tat (a) or IFN-γ alone (b) as examined by fluorescence microscopy.
Figure 11 HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ1-42 peptide is enhanced by IFN-γ and opposed by EGCG: EGCG significantly inhibits IFN-γ enhancement of HIV-1 Tat-mediated reduction in microglial phagocytosis of Aβ 1-42 peptide. In order to examine microglial Aβ co-localization on the microglial surface, primary cultured microglia were treated with “aged” FITC-tagged Aβ 1-42 (50 nM) in complete medium for 60 min with addition of HIV-1 Tat (63 ng/mL), IFN-γ (50 U/mL), or HIV-1 Tat (63 ng/mL) + IFN-γ (50 U/mL) in the presence or absence of EGCG (20 μM) and then stained these cells with DAPI. HIV-1 Tat + IFN-γ (c) significantly inhibited microglia – Aβ co-localization as compared to HIV-1 Tat (a) or IFN-γ alone (b) as examined by fluorescence microscopy. As noted, green indicates Aβ1-42-positive; blue indicates microglial nuclei. Addition of EGCG alone yielded similar results as addition of PBS alone (data not shown). Original magnification = 20×.
4.4.3 HIV-1 Tat Opposes Apo-E3-promoted Aβ1-42 Microglial Phagocytosis

Apolipoprotein E (Apo-E) is a constituent of amyloid plaques in the brains of AD patients and is genetically polymorphic. Three frequent alleles exist at the Apo-E gene locus: E2, E3, and E4 (Davignon et al., 1988). The E4 polymorphism strongly affects the risk of developing both HAD and AD (Corder et al., 1998). Several hypotheses have been put forward to account for the association. When Aβ and Apo-E are co-incubated, E4 forms denser monofibrillar structures than E3 (Wisniewski et al., 1993). Further, Apo-E4 is more effective than E3 in enhancing the rate and amount at which fibrils are generated from soluble amyloid in vitro (Castano et al., 1995). In addition, Apo-E4, but not E3, is neurotoxic; reducing neurite sprouting (Nathan et al., 1994) and enhancing depolymerization of microtubule (Nathan and Chang, 1995; Mahley et al., 1995). Conversely, under normal conditions, Apo-E3 promotes microglial clearance of Aβ (Manelli et al., 2004; Ladu et al., 1994). In support, cell associated Aβ is approximately half that observed in the presence of heat-inactivated Tat (control; Figure 8) compared to Apo-E3 conditions where cell associated Aβ is nearly doubled (Figure 11). Moreover, other groups have shown native E3 has been shown to form an stable complex with Aβ that is more abundant than the Apo-E4:Aβ complex (Manelli et al., 2004; Ladu et al., 1994). Additionally, exogenous Apo-E3 but not E4 prevents Aβ-induced neurotoxicity by a process that requires Apo-E receptors (Manelli et al., 2004).

Thus to investigate whether Tat could inhibit this Apo-E3 promoted microglial uptake of Aβ, recombinant Apo-E3 protein (Sigma; 2µg/mL) was added to primary cultured microglia for 60 min in the presence of varying concentrations of recombinant Tat protein (0, 32, 63, 125, and 250 µg/mL) and “aged” FITC-tagged Aβ1-42. As shown
in Figure 11, the Tat protein significantly and dose-dependently decreased Apo-E3 promoted microglial uptake of FITC-tagged Aβ\textsubscript{1-42}. As shown in Figure 12, HIV-1 Tat significantly inhibited Apo-E3 enhanced microglial phagocytosis of Aβ as compared to control (a), Apo-E3 alone (b), or heat inactivated HIV-1 Tat + Apo-E3 (d) conditions as examined by fluorescence microscopy.

**Figure 12 HIV-1 Tat protein opposes Apo-E3 promoted microglial phagocytosis of Aβ\textsubscript{1-42} peptide.**

Primary cultured microglia (1×10^5 cells/well in 24-well tissue culture plates) were treated with "aged" FITC-tagged Aβ\textsubscript{1-42} (300 nM) in complete medium for 60 min in the presence of recombinant HIV-1 Tat protein (NIH AIDS Research and Reference Reagent Program; 0, 32, 63, 125, 250 ng/mL) as well as Apo-E3 (2µg/mL). As a control for nonspecifically incorporated Aβ, microglia were incubated at 4°C with the same treatment as above in parallel 24-well tissue culture plates. Cell supernatants and lysates were analyzed for extracellular and cell-associated FITC- Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C.
(n=6 for each condition presented). When measuring FITC-tagged Aβ 1-42 in cell supernatants or lysates, one-way ANOVA followed by post-hoc comparison showed HIV-1 Tat dose-dependently inhibited Apo-E3 enhanced microglial phagocytosis of Aβ peptide (p<0.05).

Figure 13 HIV-1 Tat protein opposes Apo-E3 promoted microglial phagocytosis of Aβ1-42 peptide.

In order to examine microglia Aβ co-localization on the microglia surface, primary cultured microglia were treated with "aged" FITC- Aβ 1-42 peptide (50 nM) in the presence PBS (a; control), Apo-E3 (b; 2 µg/mL), Apo-E3 (2 µg/mL) + HIV-1 Tat (125 ng/ml; c) or heat inactivated HIV-1 Tat (250 ng/mL) + Apo-E3 (2 µg/mL; d) and then stained with DAPI. HIV-1 Tat significantly inhibited Apo-E3 enhanced microglial phagocytosis of Aβ (c) as compared to control (a), Apo-E3 alone (b), or heat inactivated
HIV-1 Tat + Apo-E3 (d) conditions as examined by fluorescence microscopy. As noted, green indicates Aβ_{42}-positive; blue indicates microglial nuclei. Addition of HIV-1 Tat protein at 32 ng/mL yielded similar results as heat inactivated Tat (data not shown). Original magnification = 20×.

4.5 Discussion

Interactions between HIV and mechanisms underlying AD may cause accelerated and severe dementias (Nath and Hersh, 2005). Such interactions may occur at several levels. First, it has been most recently demonstrated that the cysteine-rich domain of Tat interacts with neprilysin, a type II plasma membrane zinc metallopeptidase which cleaves the toxic secreted Aβ peptide (Rempel and Pulliam, 2005; Daily et al., 2006; Nikolic et al., 2007; Obregon et al., 2006). Second, Tat can directly inhibit uptake of Aβ and Apo-E. In the case of neurons, this occurs through inhibition of the LRP receptor (Liu et al., 2000). As shown in Figures 8 and 9, Tat protein dose-dependently decreased uptake of Aβ_{42} in microglia as well. We suggest extracellular enzymatic degradation of the peptide by neprilysin does not account for this increased clearance of Aβ as endopeptidase activity is generally non-existent or minimal at 4°C (Oliveira et al., 2002). Indeed microglia incubated at 4°C in parallel cell culture plates under the same treatment conditions described above displayed similar results to those incubated at 37°C. In further support, our system involved whole recombinant Tat protein whereas Daily and colleagues in 2006 reported that peptides derived from the Tat protein, but not Tat protein itself, inhibit neprilysin (Daily et al., 2006). Additionally, we suggest cell death due to the neurotoxic properties of Tat (Giunta et al., 2006; Sui et al., 2007; Bruce-Keller et al., 2003, Kim et al., 2003) did not significantly influence the phagocytic activity in the
various treatment groups as determined by LDH assay on the relevant supernatants. Indeed results showed that there was no significant cell death occurring over the 3-h time frame in any of the treatment groups (data not shown, p>0.05). Therefore our data point to a possible role for a receptor-mediated inhibition of Aβ uptake by Tat. As will be discussed below, these phenomena may be occurring through inhibition of microglial LRP by HIV-1 Tat, as has been previously demonstrated with neuronal LRP (Liu et al., 2000). Further studies using the specific antagonist of LRP, receptor associated protein (RAP), are required to test this hypothesis.

IFN-γ may enhance inhibitory effect of HIV Tat through a microglial switch to the non-phagocytic APC phenotype. This remains to be validated in future works focusing on microglial APC markers. However, several past reports strongly indicate that IFN-γ promotes the APC phenotype in a variety of immune cells including microglia and astrocytes through activation of class II transactivator (CIITA). Indeed both constitutive and inducible MHC class II gene expression requires CIITA (Reith et al., 2005; Steimle et al., 1993; LeibundGut-Landmann et al., 2004 a, b; a scaffolding protein which interacts with itself and other transcription factors to activate the MHC class II promoter (Masternak et al., 2000; Sisk et al., 2001). CIITA transcription is modulated by at least three promoters: pI, pIII and pIV each of which generates unique CIITA transcripts (LeibundGut-Landmann et al., 2004 a, b; Muhlethaler-Mottet et al., 1997). All three promoters can be induced in macrophages by IFN-γ although the major form is transcribed from pIV (LeibundGut-Landmann et al., 2004 a, b; Muhlethaler-Mottet et al., 1997). In turn, pIV is regulated by three major cis-acting elements: an IFN-γ activation sequence (GAS), an E box, and an interferon regulatory factor (IRF) element, which bind
the transcription factors, among them are signal transducer and activator of transcription
(STAT1) and interferon regulatory factor-1 (IRF-1). IFN-γ is known to activate tyrosine
Janus-associated kinase (JAK)1 and JAK2, yielding phosphorylated STAT1 in many
cells including microglia (Takagi et al., 2002; Ramana et al., 2002). Phosphorylated
STAT1 then dimerizes and migrates to the nucleus, where it binds to the GAS element in
pIV (Shuai, 1994a, b; Vinkemeier et al., 1996; Mowen and David, 2000). STAT1 also
regulates IRF-1 expression, which in turn activates the CIITA promoter (Piskurich et al.,
1999; Morris et al., 2002). In further support, Aloisi and colleagues demonstrated IFN-γ
induces functional expression of MHC class II I-A and I-E molecules (Aloise et al.,
1998). Additionally astrocytes display IFN-γ-induced MHC class II expression upon
IFN-β stimulation (Zeinstra et al., 2006; Satoh et al., 1995). Moreover, IFN-γ mediates
induction of MHC class II molecules in cultured rat astrocytes (Frohman et al., 1998).
Therefore it is our hypothesis that IFN-γ mediated STAT1 activation leads to pIV
activation in turn causing induction of CIITA and the APC phenotype. This yields a
minimal number of cells acting as functional phagocytes. To further explore STAT1
activation in these observed results, we next examined the effect of a specific STAT1
inhibitor, the Green tea-derived flavonoid, EGCG.

IFN-γ exerts effects in many cells including microglia (Balabanov et al., 2006;
Simon et al., 2006) via phosphorylation of JAKs which then go on to activate STAT
proteins which then migrate to the nucleus where they can activate CIITA. Although
STATs are not exclusively activated by JAKs, one of the best studied pathways for STAT
activation is through the JAK; a system termed as the JAK/STAT pathway. Importantly,
chronic HIV-1 infection among individuals with progressive disease has been correlated
with a 6 to 10 fold increase in levels of activated STATs in peripheral blood mononuclear cells after exposure to virions (Kohler et al., 2003). Our results suggest EGCG, a specific STAT1 inhibitor, attenuates the IFN-γ mediated augmentation of the anti-phagocytic properties of HIV-1 Tat in microglia. We therefore hypothesize that STAT1 mediated activation of CIITA and thus the APC phenotype, is attenuated. Importantly, EGCG has been suggested to be safe since administration of EGCG to rats or dogs for 13 weeks was not toxic at doses up to 500 mg/kg/day (Isbrucker et al., 2006). Additionally the compound does not confer genotoxicity in rats (Isbrucker et al., 2006). Furthermore, EGCG has been suggested safe in humans in doses up to 800 mg daily (Ullman et al., 2004).

Uptake of Aβ by microglia is mediated by various receptors, including class A scavenger receptors (SR-A) (Paresce et al., 1996), Fc receptors (Bard et al., 2000), and LRP, provided that Aβ is complexed to α2-macroglobulin (α2M), lactoferrin (Qiu et al., 1999), or Apo-E (Yang et al., 1999), which are ligands of LRP. Of these ligands, the epsilon-4 allele of Apo-E is the primary risk factor for AD pathology (Aleshkov et al., 1997; Manelli et al., 2004). We hypothesize that LRP is inhibited by HIV-1 Tat, resulting in elevated Aβ/β-amyloid deposition. This is supported by several lines of evidence. First, it has previously been shown that HIV-1 Tat inhibits binding and uptake of Aβ and Apo-E at the LRP receptor in neurons (Liu et al., 2000). Second, Aβ binds to heparan sulfate proteoglycans (HSPG) on the cell surface from where they are subsequently transferred to LRP for cellular uptake (Ji et al., 1993, 1994; Snow et al., 1995; Gupta-Bansal et al., 1995; Lindahl et al., 1999; Buee et al., 1993; Brunden et al., 1993; Bellosta et al., 1995). Third, genetic and biochemical investigations demonstrated that cell membrane HSPG
are the receptors for extracellular Tat internalization as well. Indeed Tyagi and colleagues in 2000 demonstrated Tat uptake is inhibited by heparin and that cell treatment with lyases specific for HSPG, but not for chondroitin sulfates, blocks Tat internalization, and that cell lines genetically deficient in the cellular pathway involved in the production of sulfated HSPG fail to internalize Tat (Tyagi et al., 2001). It has been suggested this phenomenon underlies the ability of HIV-1 Tat to enter into a wide array of human, rodent, and simian cell lines and that extracellular Tat enters most of the exposed cells (Tyagi et al., 2001). Finally, among the above named receptors for uptake of Aβ by microglia, it seems that LRP is more important than SR-A or Fc receptors. In studies of microglia using competitive ligands of SR-A, it has been shown that SR-A was not involved in the recognition of amyloid peptide deposits, whereas LRP specifically recognized deposits of Aβ1-42 and mediated their uptake (Laporte et al., 2004).

Taken together, these experiments suggest HIV-1 Tat inhibits microglial uptake of Aβ peptide, a phenomenon which is exacerbated by IFN-γ and attenuated by green tea derived EGCG. Our data may partially explain a past report indicating HIV-infected subjects with the E4 allele for Apo-E have excess dementia (Corder et al., 1998). Specifically, it has been shown that twice as many E4(+) subjects were demented (30% compared with 15%; P < 0.0001) compared to E3(+) subjects by Corder and colleagues (1998). Given that the other Apo-E isoforms are more efficient at promoting microglial uptake of Aβ via LRP, and that the promotion conferred by E3 is dampened in vitro by HIV-1 Tat (Figures 12 and 13), it is plausible that having the double risk of both HIV infection and the E4 allele leads to a very severe deficiency in the ability of microglia to uptake and degrade Aβ, which may in turn result in a more severe dementia. In
conclusion, we have shown that HIV-1 Tat inhibits microglial phagocytosis of Aβ peptide in vitro, a process which is enhanced by IFN-γ, and opposed by the STAT1 inhibitor EGCG. Secondarily these data point to the microglial LRP and HSPG as sites of HIV-1 Tat inhibition of microglial Aβ phagocytosis. Future investigations are required to fully characterize the receptor(s) and full intracellular signaling mechanisms responsible for these observed phenomena.
CHAPTER FIVE

HIV-1 TAT CONTRIBUTES TO ALZHEIMER’S LIKE PATHOLOGY IN PSAPP MICE.

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5.1 Abstract

Prevalence of HIV-associated cognitive impairment is rising. Amyloid-beta (Aβ) plaque deposition in the brain may be a contributing factor as epidemiological data suggests significant numbers of long-term HIV survivors are at elevated risk of developing Alzheimer's disease (AD). HIV-1 Tat-induced Aβ deposition, tau phosphorylation, and subsequent neuronal death could be risk factors for subsequent AD and/or HIV-related cognitive impairment. To mimic this clinical condition, we generated mice with HIV-1 Tat-induced AD-like pathology. We first performed a short-term Doxycycline (dox) dosing (54, 108, and 216 mg/kg/day) study in transgenic mice whose astrocytes express HIV-1 Tat via activation of a GFAP/dox-inducible promoter. After one week, mouse brains were examined histologically and the expression of Bcl-xL, Bax, and phospho-tau was investigated by Western blotting. We next cross-bred these mice with the PSAPP mouse model of AD. To simulate chronic Tat secretion over periods longer than one week, we used an optimized dose of 54 mg/kg/day on a biweekly basis over three months; based on the initial dose ranging study in the Tat transgenic mice. This was followed by antisera detection of Aβ, and Western blot for phospho-tau, Bcl-xL, and Bax. Tat significantly induced neuron degeneration and tau phosphorylation in Tat transgenic mice, dox dependently (P<0.001) with the most robust effects at the 216 mg/kg/day dose. In the long term study, similar effects at the chronic 54 mg/kg/day dose were observed in PSAPP/Tat mice induced with dox. These mice also showed significantly more Aβ deposition (P < 0.05), neurodegeneration, neuronal apoptotic signaling, and phospho-tau than PSAPP mice (P < 0.05). In conclusion, HIV-1 Tat
significantly promotes AD-like pathology in PSAPP/Tat mice. This model may provide a framework in which to identify new mechanisms involved in cognitive impairment in the HIV infected population, and possible treatments. Additional works will be needed to fully characterize the mechanism(s) of HIV-induced amyloid deposition, and also to uncover viral mechanisms promoting AD-like pathology in general.

5.2 Introduction
The natural history of HIV infection is changing. Advanced age will be an important moderator of clinical outcomes associated with the disease, particularly dementia. Historically there was not much need to consider age-related neurodegenerative disorders, such Alzheimer's disease (AD), as contributing to affective or cognitive disorders in HIV-infected patients, as infection existed virtually only in young adults (Valcour and Paul., 2006). This paradigm no longer holds true in the era of highly active antiretroviral therapy (HAART), raising new issues regarding the diagnosis and treatment of HIV-related neurocognitive disorders. Indeed it has been suggested this new course in the HAART era may also signify a phenomenon whereby HIV lowers the threshold (cerebral reserve hypothesis) for the clinical presentation of other neurodegenerative diseases such as AD expanding risk to younger, middle-aged patients (Valcour and Paul., 2006).

Because of long-term survival in the HIV-infected population, the epidemic is extending into older age brackets and is commonly characterized by AD-like pathology. Currently, some 60,000 HIV-infected individuals are over the age of 50. The number of HIV+ patients over the age of 65 increased exponentially from some 1,000 to more than
10,000 in the past ten years (Levy-Dweck, 2005; Stoff et al., 2004). Furthermore, it is predicted that 50% of prevalent AIDS cases in this country will fall into this older age group by 2015. Several post-mortem studies have revealed a significant incidence of AD-like pathology in the HIV-infected brain including increased brain beta-amyloid (A-beta) deposition (Green et al., 2005), increased extracellular amyloid plaques (Achim et al., 2004), and decreased cerebrospinal fluid (CSF) A-beta levelsn (Brew et al., 2005).

Particularly, the HIV-1 transactivator protein (Tat) has been implicated in this AD-like pathology through a variety of mechanisms. However the chronicity of neuropathologies in humans is uncertain due to the fact that all histological data comes from post-mortem samples. Using an in vitro system, we have recently shown HIV-1 Tat inhibits microglial phagocytosis of Aβ peptide in vitro and that this dysfunction is augmented by the pro-inflammatory cytokine, IFN-γ (interferon-gamma) (Giunta et al., 2008), and opposed by the natural STAT1 (signal transducer and activator of transcription-1) inhibitor from Green Tea, epigallocatechin-3-gallate (EGCG). To date, no in vivo model has been created to study HIV-1 Tat-induced AD-like pathology. Such a model is necessary to generate both novel therapeutic targets and also a pharmacological screening platform.

In an effort to generate such a model, we crossed transgenic PSAPP (APPswe, PSEN1dE9) mice (Holcomb et al., 1998) and HIV-1 Tat-transgenic mice (GT-tg mice). The latter demonstrates HIV-1 Tat expression from astrocytes under the control of both the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter and a doxycycline (dox)-inducible promoter (Kim et al., 2003). We found HIV-1 Tat significantly induced neuron degeneration and tau phosphorylation in Tat transgenic mice dox dependently.
(P<0.001). Compared to both PSAPP mice + dox, and/or PSAPP/Tat mice without dox, PSAPP/GT-tg mice + dox (54 mg/kg/day) showed significantly more Aβ deposition in brain regions examined (P<0.05). Western blot analysis confirmed these results indicating dox-induced PSAPP/Tat inducible mice demonstrated significantly more Aβ deposition quantitatively (P<0.05). Additionally PSAPP/Tat mice induced with dox displayed significantly more neurodegeneration, neuron apoptosis, and tau phosphorylation than either PSAPP mice + dox or PSAPP/GT-tg mice not induced with dox (P<0.05).

5.3 Materials and Methods

5.3.1 Mice

The creation and genotyping of the inducible and brain-targeted HIV-1 Tat transgenic (GT-tg) mice was previously described (Kim et al., 2003). A founder pair of GT-tg mice was generously provided by Dr. Johnny He (Indiana University). Transgenic PSAPP (APPswe, PSEN1dE9) mice were obtained from the Jackson Laboratory. These animals were housed and maintained at the College of Medicine Animal Facility of the University of South Florida (USF) Health Sciences Center, and all experiments were in compliance with protocols approved by the USF Institutional Animal Care and Use Committee (IACUC).
5.3.2 Short Term Dose Ranging Study in Tat-Transgenic Mice

To express Tat for the initial dose-ranging study, a total of 18 GT-tg mice were used divided evenly between males and females and administered one of three doses of dox (Sigma, Louis, MO; 54, 108, or 116 mg dox/mg/day) in drinking water based on the previous works of Kim et al 2003. After 7 days mice were euthanized with overdose of isofluorane and transcardially perfused with ice-cold physiological saline containing heparin (10 U/ml). Brains were rapidly isolated and quartered using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). The 1\textsuperscript{st} and 2\textsuperscript{nd} anterior quarters were homogenized for Western blot analysis and ELISA, and the 3\textsuperscript{rd} and 4\textsuperscript{th} posterior quarters were fixed in 4\% paraformaldehyde in PBS at 4°C overnight and routinely processed for hematoxylin/eosin (H/E) staining as immunohistochemistry. Empty vehicle treatment was included as a control (data not shown).

5.3.3 Long Term HIV-1 Tat Expression in PSAPP Mice

PSAPP mice with administered dox (PSAPP/dox) were compared to PSAPP/Tat mice without dox administration, and PSAPP/Tat mice administered with dox (PSAPP/Tat/Dox). The first two groups are both negative controls as little to no HIV-1 Tat expression occurs in the absence of dox. The third group, PSAPP/Tat/Dox, represents the novel model of HIV-1 Tat-induced, AD-like pathology. Twelve mice (6 males and females) at 10 months of age were used per group. The mice were divided evenly between males and females and administered this minimum dose of dox (54 mg/kg/day, every-other-week) in drinking water. In order to mimic a chronic secretion of Tat in the brain, as seen in clinical cases of HIV-1 infection, we chose to lower the dose of dox to
54 mg/kg, daily, on a biweekly basis (administered every other week) for three months based on our short term dose ranging study. We find that longer periods of Tat expression, at this lower dose, closely mimics the HAD-like damage seen in the 1 week dose ranging study at the higher dox dose of 216 mg/kg/day. After three months mice were euthanized with overdose of isofluorane and transcardially perfused with ice-cold physiological saline containing heparin (10 U/ml). Brains were prepared for pathologic examination as above.

5.3.4 Histological Examination

Five coronal sections from each brain (10 µm thickness) were cut with a 150 µm interval. Sections were routinely deparaffinized and hydrated in a graded series of ethanol before pre-blocking for 30 min at ambient temperature with serum-free protein block (Dako Cytomation, Carpinteria, CA). Nuclear pyknosis suggestive of chromatin condensation was examined by H&E stain. Ten micrometer sections were fixed with 4% paraformaldehyde in phosphate-buffered saline solution (PBS: 10 mM KH2PO4, 37 mM Na2HPO4, 87 mM NaCl, 53 mM KCl, pH 7.4) for 1 hr, washed with distilled water for 2 min, then incubated with Mayer's hematoxylin (diluted 1:10) for 4 min at room temperature. The slices were then rinsed with ethanol for 2 min, followed by a dH2O rinse for 2 min, covered with a 0.5% eosin solution for 5 min, and then rinsed twice with water. Sections were then examined under light microscopy.
5.3.5 Immunohistochemistry

Immunohistochemical staining was performed using anti-human Aβ antibody (clone 4G8; Sigma) in conjunction with the VectaStain Elite ABC kit (Vector Laboratories, Burlingame, CA) coupled with diaminobenzidine substrate. 4G8-positive Aβ deposits were examined under bright field using an Olympus (Tokyo, Japan) BX-51 microscope. To study neurofibrillary tangle-like structures, conventional immunofluorescence staining using mouse PHF-1 monoclonal antibody, which recognizes the dually phosphorylated Ser396 and Ser404 epitope of tau peptides (Otvos et al., 1994) was utilized. The PHF-1 antibody was kindly provided by Dr. Peter Davies (Albert Einstein College of Medicine; Bronx, NY). Incubation with PHF-1 antibody (1:60) took place overnight at 4°C, followed by 1 hr at RT. One control section was processed identically except that the primary antibody was omitted from the incubation buffer. After rinsing, sections were incubated in the presence of Cy3-conjugated goat, anti-rabbit secondary antibody (1:200) or goat anti-mouse antibody (1:100) (Sigma; St. Louis, MO) for 3 hrs at room temperature. Sections were then thoroughly rinsed in PBS. For the same-section comparisons in this study, this step was immediately followed by the thioflavin-S staining procedure as previously described (Rezai-Zadeh et al., 2005). Sections were stained by immunohistochemistry for Aβ using 4G8 monoclonal antibody.

5.3.6 Western Blot Analysis and Immunoprecipitation

Mouse brains were lysed in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM
α-glycerolphosphate, 1 mM NaVO₃, 1 μg/ml leupeptin, 1 μM PMSF), and an aliquot corresponding to 50 g of total protein was electrophoretically separated using 10% Tris-gels. Electrophoresed proteins were then transferred to polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), washed in dH₂O, and blocked for 2 hrs at room temperature in Tris-buffered saline (TBS; Bio-Rad) containing 5% (w/v) nonfat dry milk. After blocking, membranes were hybridized for 2 hrs at room temperature with various primary antibodies. Membranes were then washed 3 × for 5 min each in dH₂O and incubated for 1 hr at ambient temperature with the appropriate HRP-conjugated secondary antibody (1:1000; Pierce Biotechnology, Rockford, IL). All antibodies were diluted in TBS containing 5% (w/v) nonfat dry milk. Blots were developed using the luminol reagent (Pierce Biotechnology). Densitometric analysis was done using a FluorS Multiimager with Quantity One software (Bio-Rad). Immunoprecipitation was performed for detection of phosphorylated Tau by incubating 200 µg of total protein of each sample with monoclonal antibody AT270 (1:400; Pierce Biotechnology) or anti-phosphotau antibody that recognizes human phosphorylated tau at Ser199/202 overnight with gentle rocking at 4 °C. Then 10 L of 50% protein A-Sepharose beads were added to the sample (1:10; Sigma) before gentle rocking for an additional 4 hrs at 4°C. After washes with 1 × cell lysis buffer, samples were subjected to Western blot analysis as described above. Antibodies used for Western blot analysis included anti-Bcl-xL antibody (1:1,000, AnaSpec), anti-Bax antibody (1:1,000, AnaSpec), and anti-actin antibody (1:1,500 an internal reference control; Roche).
5.3.7 ELISA

Mouse brains were isolated under sterile conditions on ice and placed in ice-cold lysis buffer. Brains were then sonicated on ice for a total of 1.5 min (30 second sonification separated by 30 second rest period, cycled over three minutes), allowed to stand for 15 min at 4°C, and centrifuged at 15,000 rpm for 15 min. Total A (including Aβ\textsubscript{1-40,42}) species were detected by acid extraction of brain homogenates in 5 M guanidine buffer, followed by a 1:10 dilution in lysis buffer. Soluble total A-beta was directly detected in brain homogenates prepared with lysis buffer described above by a 1:4 or 1:10 dilution, respectively. A-beta was quantified in these samples using the total A-beta ELISA kits (Rezai-Zadeh et al., 2005). Total Aβ species were detected by acid extraction of brain homogenates according to our previous methods (Rezai-Zadeh et al., 2005). Data are expressed as pg/mg A1-x, mean ± SD.

5.3.8 Image Analysis

Quantitative image analysis (conventional “Aβ burden” analysis) was performed for A immunohistochemistry in PSAPP mice receiving dox, and all PSAPP/Tat groups. Images were obtained using an Olympus BX-51 microscope and digitized using an attached MagnaFire imaging system (Olympus). Briefly, images of five 5 µm sections (150 µm apart) through each anatomic region of interest (hippocampus or cortical areas) were captured, and a threshold optical density was obtained that discriminated staining from background. Manual editing of each field was used to eliminate artifacts. Data are reported as a percentage of immunolabeled area captured (positive pixels) divided by the
full area captured (total pixels). Quantitative image analysis was performed by a single examiner (M.T.) blinded to sample identities.

5.3.9 Statistical Analysis

All data were normally distributed; therefore, in instances of single mean comparisons, Levene's test for equality of variances followed by t-test for independent samples was used to assess significance. To examine for gender differences, we employed analysis of variance (ANOVA) followed by pairwise comparison of the means using a post hoc Newman-Keuls test. In instances of multiple mean comparisons, analysis of variance was used, followed by post hoc comparison using Bonferroni's method. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, Illinois) was used for all data analysis.

5.4 Results

5.4.1 HIV-1 Tat Dose Dependentlty Increases Neuronal Damage and tau Phosphorylation in Tat Transgenic Mice (GT-tg)

Administration of dox is positively associated with increased nuclear H&E staining, suggesting that HIV-1 Tat protein expression induces increased neurodegeneration (Figure 14A). As an additional confirmatory measure, Western blotting analysis for the apoptotic repressor protein, Bcl-xL, shows that dox dose dependently decreases its ratio to actin (Figures 14B and 14C)(P<0.005). As shown in Figure 1D, tau phosphorylation was significantly and positively correlated with the dosage of dox administration. As a
validation, we examined HIV-1 Tat expression in the mice after dox administration using Western blotting analysis and found that expression of HIV-1 Tat protein occurred dox dependently (data not shown) as has also been demonstrated in previous studies (Kim et al., 2003). In addition, a minimum dosage of dox, 54 mg/kg/day, was determined for the following study where PSAPP and GT-tg transgenic mice were cross-bred and subjected to a long term study of brain HIV-1 Tat secretion. Furthermore, these data suggest that dox-induced HIV-1 Tat expression significantly results in neurodegeneration in the mice.
Figure 14 Oral administration of dox results in increased neuronal damage and tau phosphorylation with decreased Bcl-xL expression dose dependently in GT-tg mice.

A. Mouse brain coronal frozen sections were stained with H&E. Left column indicates PSAPP mice receiving dox, middle column indicates PSAPP/Tat mice without dox and
right column indicates PSAPP/Tat mice receiving dox. B. Bcl-xL expression was analyzed by Western blotting analysis of the mouse brain homogenates with anti-Bcl-xL antibody. C. Densitometry analysis shows the band density ratio of Bcl-xL to actin. D. Western blot analysis by antibody AT270 (left) or Ser199/202 (right) shows phosphorylated tau.

5.4.2 Oral Administration of Dox Increases A-beta Plaque Formation in PSAPP/Tat Mice

Most interestingly, as determined by chronic dox administration, the PSAPP/Tat mice shows significantly increased A-beta deposits in the brain regions examined (Figure 15A) (P<0.05). Image analysis of micrographs from A-beta antibody stained sections reveals that plaque burdens are significantly increased in the cingulate cortex (CC), hippocampus (H), and entorhinal cortex (EC) by 47%, 55%, and 30%, respectively (Figure 15B). To verify the findings from these coronal sections we found dox-induced HIV-1 Tat expression markedly increases both in soluble and insoluble forms of Aβ_{1-40}, 42 by ELISA (Figure 2C) (P<0.05). No significant differences in pathology were noted between males and females of each group (P>0.05).
Figure 15  PSAPP/Tat mice receiving oral dox show increased A-beta plaques compared to PSAPP mice A. Mouse brain coronal frozen sections were stained with rabbit polyclonal anti-uman A antibody. Left column indicates PSAPP mice receiving dox, middle column indicates PSAPP/Tat mice without dox and right column indicates PSAPP/Tat mice receiving dox. As indicated, the top panels are from the cingulate cortex (CC), the middle panels are from the hippocampus (H), and bottom panels are from the entorhinal cortex (EC). B. Percentages of A antibody-immunoreactive A plaque (mean ± SD) were calculated by quantitative image analysis. C. Soluble Aβ_{1-40, 42} (left) and
insoluble Aβ1-40, 42 (right) prepared with 5 M guanidine were analyzed by ELISA. Data are presented as (pg/mg protein) of Aβ1-40 or Aβ1-42 separately.

5.4.3 Oral Administration of Dox Increases tau Hyperphosphorylation and Neurodegeneration in PSAPP/Tat Mice

Given that the induced HIV-1 Tat expression results in increased Aβ deposition, we wondered whether or not HIV-1 Tat could also contribute to other Aβ associated pathology. Since it has been shown that A-beta plaques and neuronal damage are associated with phosphorylated tau-immunoreactive structures in AD transgenic mice (Noda-Saita et al., 2004), we investigated neuronal injury (Figure 16A) and tau hyperphosphorylation (Figure 16B and 16D). Clearly, in the PSAPP/Tat mice receiving dox, significantly higher number of positive cortical neuronal cells for anti-phospho-tau antibody (AT270) was found. To further determine if tau hyperphosphorylation would be involved in this Tat-induced neuronal damage, we examined Bcl-xL and Bax expression in these mice using Western blot. As shown in Figure 16, the ratio of Bcl-xL to Bax was significantly decreased (P<0.001). No significant differences in pathology were noted between males and females of each group (P>0.05). Similar results were obtained in the same samples using Ser199/202 antibody (data not shown).
Figure 16  PSAPP/Tat mice receiving oral dox show increased neuronal degeneration and tau-phosphorylation after oral administration of dox compared to PSAPP mice. Left column indicates PSAPP mice receiving dox, middle column indicates PSAPP/Tat mice without dox and right column indicates PSAPP/Tat mice receiving dox. Mouse brain coronal frozen sections were stained with H&E (A) or with phospho-tau antibody.
(AT270) (B). PSAPP/Tat mice receiving oral dox show increased neuronal degeneration and tau-phosphorylation after oral administration of dox compared to PSAPP mice. Mouse brain homogenates were prepared from these mice and subjected to Western blot analysis for Bcl-xL, Bax; and phosphor-Tau. Compared to PSAPP mice receiving dox or PSAPP/Tat mice without dox, PSAPP/Tat mice receiving dox demonstrate a significantly increased ratio of Bax to Bcl-xL (C) as well as Tau protein (phospho-

AT270) to actin ratio by densitometry analyses (D).
5.5 Discussion

HIV-infected patients are either aging with HIV infection or becoming newly infected at older ages. This, compounded by the amyloidogenic effects of Tat protein, and possibly the lipodystrophic effects of HAART medications seem to promote the AD-like pathology seen in some 50% of the HIV-1 infected population (Green et al., 2005). Given that this type of pathology may contribute to the neurobehavioral morbidity of HIV-1 infection, we created a mouse model of HIV-1 Tat-induced AD-like pathology by crossing PSAPP and HIV-1 Tat (GT-tg) transgenic mice. This effectively mimics age-related changes in A-beta deposition (Holcomb et al., 1998) with superimposed brain targeted Tat expression in the PSAPP/Tat mouse model (Kim et al., 2003).

As expected in our initial dose ranging study in Tat transgenic mice, brain-targeted expression was sufficient to cause a dose dependent increase in neuronal degeneration (Figure 1A), and tau phosphorylation (Figure 14D) while causing a decrease in the anit-apoptotic protein, Bcl-xL (Figures 14B and14C). The microtubule-associated protein tau is abundantly expressed in neurons, and is deposited in cells in an abnormally phosphorylated state as fibrillar lesions in many neurodegenerative diseases, particularly AD. In neurons, the protein plays a crucial role in binding and stabilizing microtubules, and regulating axonal transport. Both are controlled by site-specific phosphorylations. There is growing evidence that disruption in the normal phosphorylation state of tau followed by conformational changes plays a key role in the pathogenic events that occur in AD (Mi and Johnson, 2006). HAD patients have been shown to have significantly increased total and phosphorylated-tau concentrations similar to AD (Brew et al., 2005). Aberrant phosphorylation of tau protein might be responsible for the breakdown of
microtubules in affected neurons classically seen in AD not only because the altered protein has greatly reduced microtubule-promoting ability but also because it interacts with normal tau, making the latter unavailable for promotion of tubulin assembly into microtubules (Mi and Johnson, 2006). Past in vitro studies demonstrated HIV-1 Tat directly modulates polymerization of microtubules; a process which positively correlates with apoptosis. Thus the decrease in the anti-apoptotic Bcl-xL which positively correlated dose dependently with dox administration (and thus Tat expression) is in accord with previous studies. This effect of Tat on the self-association of tubulin may be of interest for studies on the mechanism of microtubule formation in the HIV-infected brain and could be used in the design of new agents aimed at protecting neuronal microtubules (deMareuil et al., 2005). Furthermore Campbell and colleagues demonstrated this Tat-mediated effect on microtubules occurs without the full-length protein. Indeed peptides from its central glutamine-rich and basic regions are involved in Tat-mediated apoptosis (Campbell et al., 2004).

Based on previous studies (Giunta et al., 2006) as well as the dox dose-dependent decrease in Bcl-xL and increase in neurodegeneration, we hypothesize that HIV-1 Tat is able to induce apoptosis through both the release of cytochrome c, removal of the Bcl-xL suppression of Bax, and/or an increase in intracellular Ca^{2+} via the phospholipase C pathway (Haughey et al., 1999). First, it has previously been shown that Tat is able to directly trigger cytochrome c release, a central event in the mitochondrial apoptotic pathway. A recent study also implicates Ca^{2+} uptake into mitochondria in Tat-dependent toxicity (Langford et al., 2004). Macho et al (1999) show that, in lymphocyte cultures under low serum conditions, Tat accumulates at the mitochondria and positively
correlates with disruption of the mitochondrial membrane potential (Macho et al., 1999). This then leads to the release of pro-apoptotic factors such as cytochrome c. Regarding excitotoxic effects and Tat-mediated cell death, induction of the phospholipase c pathway by Tat confers a rapid increase in neuronal intracellular Ca2+ in primary neuron cultures, presumably causing phospholipase C and inositol phosphate-3 release, and a subsequent response via a plasma membrane glutamate receptor (Haughey et al., 1999; Jajoo et al., 2008). In this novel PSAPP/GT-tg model of Tat-induced AD-like pathology, amyloid burden, neurodegeneration, and apoptotic signaling were significantly enhanced by Tat expression. Moreover, superimposed Tat expression appears to over-ride gender differences seen in plaque loads between male and female PSAPP mice. Previous investigations (Kim et al., 2003) have shown that neurodegeneration and apoptosis are positively correlated with the level and spatial distribution of Tat mRNA and/or protein expression (Kim et al., 2003) and occurred only when dox is administered but not without dox, or in dox-treated wild-type normal mice. Therefore, these findings did not result from an insertional mutation, or other unknown nonspecific effects.

Several studies have focused on Tat-mediated induction of amyloidosis in vitro. We previously demonstrated Tat inhibits microglial uptake of Aβ1-42 peptide, a process that is enhanced by interferon-gamma (IFN-γ) and rescued by the STAT1 inhibitor (-)-epigallocatechin-3-gallate (EGCG) (Giunta et al., 2008). It is hypothesized that reduced A-beta uptake occurs through IFN-γ mediated STAT1 activation, which promotes a phenotype switch from a phagocytic to an antigen presenting phenotype in microglia. Additionally, we showed that HIV-1 Tat significantly disrupts apolipoprotein-3 (Apo-E3) promoted microglial Aβ uptake (Giunta et al., 2008). As microglial are a primary means
for A-beta removal from brain parenchyma, this process may be involved in the enhanced amyloid burden in the PSAPP/GT-tg model compared to the PSAPP model. Tat protein has also been shown to directly interact with the neuronal low density lipoprotein (LRP) receptor and thus inhibit the uptake of its ligands including apolipoprotein E4 (Apo-E4) and A peptide (Liu et al., 2000). A similar inhibition of LRP may occur in microglia. Moreover Daily and colleagues (Daily et al., 2006) have demonstrated that Tat competitively and reversibly inhibits the extracellular A-beta degrading enzyme, neprilysin. However it was also found that both Tat peptides and Tat protein were slowly hydrolyzed by neprilysin. Thus although the accumulation of Tat-derived proteolytic fragments may serve to inhibit neprilysin and increase amyloid beta peptide levels, one would suspect that there is also some level of compensatory destruction of Tat protein also, by neprilysin. Therefore the mechanisms involved in enhanced amyloid deposition mentioned above may be acting in concert in this model.

Future studies using this model will be required to delineate the molecular steps most important in HIV-1 Tat-induced AD-like pathology including amyloid burden and tau phosphorylation. Combined with behavioral testing this should lay the foundation to isolate new mechanistic underpinnings and treatment targets. Additionally the model may simulate some prognostic implications for newly HIV-infected patients in terms of brain pathology and future development of neurobehavioral deficits.
CHAPTER SIX
DISCUSSION

The natural history of HIV infection is changing. Advanced age will be an important moderator of clinical outcomes associated with the disease, particularly dementia. Historically there was not much need to consider age-related neurodegenerative disorders, such as AD, as contributing to affective or cognitive disorders in HIV-infected patients, as infection existed virtually only in young adults (Valcour and Paul., 2006). This paradigm no longer holds true in the era of highly active antiretroviral therapy (HAART), raising new issues regarding the diagnosis and treatment of HIV-related neurocognitive disorders. Indeed it has been suggested this new course in the HAART era may also signify a phenomenon whereby HIV lowers the threshold (cerebral reserve hypothesis) for the clinical presentation of other neurodegenerative diseases such as AD, expanding risk to younger, middle-aged patients (Valcour and Paul., 2006).

Because of long-term survival in the HIV-infected population, the epidemic is extending into older age brackets and is commonly characterized by AD-like pathology. Currently, some 60,000 HIV-infected individuals are over the age of 50. The number of HIV+ patients over the age of 65 increased exponentially from some 1,000 to more than 10,000 in the past ten years (Levy-Dweck, 2005; Stoff et al., 2004). Furthermore, it is predicted that 50% of prevalent AIDS cases in this country will fall into this older age group by 2015. Several post-mortem studies have revealed a significant incidence of AD-like pathology in the HIV-infected brain including increased brain beta-amyloid (Aβ)
deposition (Green et al., 2005), increased extracellular amyloid plaques (Achim et al., 2004), and decreased cerebrospinal fluid (CSF) Aβ levels (Brew et al., 2005).

### 6.1 Mechanism of neurodegeneration in HAD and AD

Microglia play a major role in the neuropathogenesis of HAD and AD in somewhat similar ways, although the etiology of these diseases differ greatly (Minagar et al., 2002). Neuropathological similarities between HAD and AD include cortical neuronal loss and amyloid plaque deposition (Green et al., 2005; Esiri et al., 1998; Everall et al., 2005). Indeed, most forms of dementia are accompanied by a widespread degeneration in the cerebral cortex - such as the plaques in AD brain. AD is thus considered a "cortical dementia." HAD is also considered to be a cortical dementia however there is also targeted damage to regions lying under the cortex. Some authors consider HAD to be a subcortical dementia however this terminology may be somewhat misleading. HAD can cause damage to both cortical and subcortical areas. The resulting brain damage is often visualized by magnetic resonance imaging (MRI) as generalized brain atrophy and also visibly damaged subcortical areas (Bell, 1998; Adle-Biassette et al., 1995).

Amyloid plaques in AD result from the deposition of Aβ which is a putative pathogenic molecule in AD. Aβ is the cleavage product of the amyloid precursor protein (APP) and APP mutations are associated with inherited forms of AD. The clinical implication or pathogenic consequences of brain amyloid deposition are still controversial in the AD field; although, the finding of Aβ deposition in both AD and HAD strongly suggests parallel pathways of chronic inflammation-mediated change that
eventually yields cortical dysfunction characterized by identical “biomarkers”. For example, decreased cerebrospinal fluid (CSF) Aβ and increased tau (a component of the neurofibrillary tangle, a second AD neuropathological hallmark) have been proposed as sensitive and specific markers of AD in several studies (Galasko et al., 1998; Motter et al., 1995). It has also been found that changes in CSF Aβ and tau are comparable to those observed in AD and HAD patients (Brew et al., 2005). The pathogenic significance of these biomarkers is not well established but is has been hypothesized that decreased CSF Aβ indicates increased aggregation of insoluble Aβ and sequestration into amyloid plaques (Andreasen et al., 1999).

The mechanisms of neurodegeneration, which are highly microglia-dependent, in AD and HAD are similar in many ways as well (Minagar et al., 2002). Cascades of inflammatory processes lead to neurodegeneration in both dementias. The initial step in each disease differs. HAD is secondary to infection with HIV-1, while the exact cause of AD remains to be established. A common feature among both diseases is the interactions of microglia which promote a neurotoxic inflammatory environment. These interactions play significant roles in the initiation and continuation of the neurodegenerative process in each disease (Minagar et al., 2002).

In both diseases, whether activation is by HIV itself, its proteins, or Aβ peptides, microglia release cytokines, ROS, and several neurotoxins that impair cellular function, neurotransmitter action, and induce neuronal loss (Guillemin and Brew, 2002). Some of these neurotoxins in both forms of dementia include TNF-α, arachidonic acid, platelet activating factors (PAF), NO, and quinolinic acid (QUIN) (Snyder et al., 1992; Heyes et al., 1992). Nitric oxide is synthesized by endothelial cells, neurons, and
macrophages and is thought to be associated with NMDA-type glutamate-initiated neurotoxicity (Snyder et al., 1992).

Tat protein has been detected in blood plasma, serum, and CSF from HIV+ individuals, at levels ranging from 1–40 ng/ml (Westendorp et al., 1995; Xiao et al., 2000), thus local extracellular concentrations in the CNS may be significantly higher (Hyashi et al., 2006). Furthermore, HIV-1 Tat activates neighboring, uninfected cells including microglia, astrocytes and neurons. Both infected and activated microglia and astrocytes over-produce pro-inflammatory cytokines which further activate neighboring cells in feed forward cycle. Infected and activated cells also produce chemokines attracting even more inflammatory monocytes and macrophages (D’Aversa et al., 2002; Eugenin et al., 2005). The viral and/or host factor(s) responsible for HAD have yet to be fully identified and characterized, but reports that mRNA levels of HIV viral regulatory transactivator protein “Tat” are abnormally high in patients with dementia (Wiley et al., 1986), and that Tat is actively secreted by infected cells (Ensoli et al., 1993) points to a role for this protein in the progression of HAND. Additionally, Tat has pro-inflammatory and neurotoxic properties in astrocytes (Giunta et al., 2009) microglia (Giunta et al., 2004) and macrophages (Turchan-Cholewo et al., 2009 a,b; Minghetti et al., 2004) and Tat levels positively correlate with development of HIV- and chimeric simian-human immunodeficiency virus (SHIV)-induced encephalitis (Hudson et al., 2000). Therefore, Tat has a high propensity to trigger this vicious pro-inflammatory cycle, leading to neuronal death and neurologic deficits (Westendorp et al., 1995).

This, compounded by possibly the lipodystrophic effects of HAART medications, seem to promote the AD-like pathology seen in some 50% of the HIV-1 infected
population (Green et al., 2005). Given that this type of pathology may contribute to the neurobehavioral morbidity of HIV-1 infection, we created a mouse model of HIV-1 Tat-induced AD-like pathology by crossing PSAPP and HIV-1 Tat (GT-tg) transgenic mice. This effectively mimics age-related changes in A\(\beta\) deposition (Holcomb et al., 1998) with superimposed brain targeted Tat expression in the PSAPP/Tat mouse model (Kim et al., 2003).

In this novel PSAPP/GT-tg model of Tat-induced AD-like pathology, amyloid burden, neurodegeneration, and apoptotic signaling were significantly enhanced by Tat expression. Moreover, superimposed Tat expression appears to over-ride gender differences seen in plaque loads between male and female PSAPP mice. Previous investigations (Kim et al., 2003) have shown that neurodegeneration and apoptosis are positively correlated with the level and spatial distribution of Tat mRNA and/or protein expression (Kim et al., 2003) and occurred only when dox is administered but not without dox, or in dox-treated wild-type normal mice. Therefore, these findings did not result from an insertional mutation, or other unknown nonspecific effects.

6.2 EGCG as a therapeutic strategy for HAD

The major constituent of Green Tea, epigallocatechin-3-gallate (EGCG), confers a specific and strong anti-inflammatory (de Prati et al., 2005; Rezai-Zadeh et al., 2005; Tedeschi et al., 2002) and anti-STAT1 effect (Tedeschi et al., 2002; Townsend et al., 2004). It reduced expression of a known STAT-1 pro-apoptotic target gene, Fas receptor, in cardiac myocytes thereby preventing ischemic/repurfusion induced apoptosis (Townsend et al., 2004) and also attenuated stroke-induced neuronal death via STAT
suppression in multiple studies (Suzuki et al., 2004).

Moreover EGCG dramatically suppressed clinical severity of experimental autoimmune encephalitis (EAE) via suppression of brain inflammation and accompanying neural injury/death (Aktas et al., 2004). Importantly, EGCG has been suggested to be safe (Isbrucker et al., 2006a; Isbrucker et al., 2006b; Isbrucker et al., 2006c). Dietary administration of EGCG to rats or dogs for 13 weeks was not toxic at doses up to 500 mg/kg/day (Isbrucker et al., 2006c). Additionally EGCG is not genotoxic in rats (Isbrucker et al., 2006a).

Although introduction of (highly active antiretroviral therapy) HAART since 1996 has reduced the incidence rates of HAD (Sacktor et al., 2001) new cases of HAD continue due to development of drug resistance. Also many patients experience difficulty in following rigorously the complex HAART medication regimens. Neurocognitive impairment correlates with medication non-compliance. Thus new strategies to prevent the development, or treat HAND are required.

Previous studies investigating the properties of the green tea-derived polyphenol, EGCG, indicated this compound was able to attenuate cell death induced by ischemia/reperfusion through downregulation of theJAK/STAT1 pathway (Townsend et al., 2004). Thus whether EGCG could effectively down-regulate IFN-γ-mediated JAK/STAT1 signaling; a process that enhanced gp120/Tat-induced neuron damage. Our in vitro studies utilizing primary culture neurons from wild-type mice demonstrated marked reductions in both LDH release and in Bcl-xL/Bax ratios when EGCG was added to Tat/IFN-g or gp120/IFN-g compared to these conditions in the absence of EGCG. These data suggest that EGCG's ability to
reduce JAK/STAT1 signaling in primary culture neurons is protective against IFN-γ-enhanced gp120/Tat-induced HAD-like neuronal damage in vitro.

To evaluate the effects of EGCG on inhibition of neuronal damage induced by HIV-1 proteins gp120 and Tat in the presence of IFN-γ in vivo, control mice were administered i.p. injections of EGCG or PBS (vehicle control) and then i.c.v. injected with HIV-1 proteins, gp120 or Tat, in the presence of IFN-γ. Consistent with our above-mentioned results, EGCG was protective against neuron loss induced by i.c.v injected IFN-γ and/or gp120/Tatin cortical regions examined. This was evidenced by increased Bcl-xL/Bax ratios in brain homogenates of mice cotreated with EGCG plus IFN-g/gp120 or IFN-g/Tat/gp120, respectively and reductions in neuron loss in cortical sections by immunohistochemistry.

Several reports investigating EGCG's ability to block JAK/STAT1 signaling have reported protective effects of the compound against: proinflammatory activation of immune cells, epithelial barrier dysfunction, and neuronal apoptosis after ischemia/reperfusion injury. Thus, JAK/STAT1 interaction may be an important therapeutic target for a variety of CNS disorders (Tedeschi et al., 2002 and Townsend et al., 2004). Taken together, our data suggest the JAK/STAT1 pathway may be an important therapeutic target for opposing the neuronal death and injury seen in the HAD brain. Indeed inhibition of the JAK/STAT pathway by green tea-derived EGCG or analogous compounds may provide an effective therapeutic intervention as an adjunct to HAART for the treatment of HAD. Other investigations have also shown that EGCG inhibits IFN-γ mediated STAT1 signaling as well (Watson et al., 2004, Jeong et al., 2007).
Thus we next evaluated whether EGCG could oppose the IFN-γ mediated enhancement of Tat down-regulation of microglial uptake of Aβ. EGCG dose-dependently increased microglial cell associated Aβ 1-42 in the presence of IFN-γ and HIV-1 Tat. To further explore STAT1 activation in these observed results, we next examined the effect of a specific STAT1 inhibitor, the Green tea-derived flavonoid, EGCG.

IFN-γ exerts effects in many cells including microglia (Balabanov et al., 2006; Simon et al., 2006) via phosphorylation of JAKs which then go on to activate STAT proteins which then migrate to the nucleus where they can activate CIITA. Although STATs are not exclusively activated by JAKs, one of the best studied pathways for STAT activation is through the JAK; a system termed as the JAK/STAT pathway. Importantly, chronic HIV-1 infection among individuals with progressive disease has been correlated with a 6 to 10 fold increase in levels of activated STATs in peripheral blood mononuclear cells after exposure to virions (Kohler et al., 2003). Our results suggest EGCG, a specific STAT1 inhibitor, attenuates the IFN-γ mediated augmentation of the anti-phagocytic properties of HIV-1 Tat in microglia. We therefore hypothesize that STAT1 mediated activation of CIITA and thus the APC phenotype, is attenuated. Importantly, EGCG has been suggested to be safe since administration of EGCG to rats or dogs for 13 weeks was not toxic at doses up to 500 mg/kg/day (Isbrucker et al., 2006b). Additionally the compound does not confer genotoxicity in rats (Isbrucker et al., 2006a) Furthermore, EGCG has been suggested safe in humans in doses up to 800 mg daily.

In conclusion, we have shown that microglial activation, disrupted microglial phagocytosis of Aβ, and neuronal death/damage in the face of HIV-1 Tat protein in
models of HAD are synergistically enhanced by the presence of IFN-γ. It seems that HIV Tat protein thus plays a multi-facted and central role in the classical neuropathology of HAD as well as the AD-like pathology commonly found. Future works will be required to explore the effect of EGCG with or without addition of galantamine/nicotine, to find an optimized treatment for HIV-1 Tat inhibition of microglial phagocytosis of Aβ peptide.
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