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Research Report

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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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-γ (IFN-γ) 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-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 derived 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*. Taken together, these data suggest EGCG attenuates the neurotoxicity of IFN-γ augmented neuronal damage from HIV-1 proteins gp120 and Tat both *in vitro* and *in vivo*. Thus EGCG may represent a novel natural copound for the prevention and treatment of HAD.

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Abbreviations:
EGCG, (–)-epigallocatechin-3-gallate
HIV-1, human immunodeficiency virus type 1
gp120, HIV envelope glycoprotein 120
Tat, HIV transactivator protein
JAK, Janus associated kinase
STAT1, signal transducer and activator of transcription 1
HAART, highly active antiretroviral therapy

1. Introduction

Epidemiologic studies indicate that some 60% of HIV-1-infected patients suffer some form of related neuropsychiatric impairment (Ozdener, 2005; Stephanou, 2004) characterized by cognitive, motor, and/or behavioral symptoms. HIV-associated dementia (HAD; Goodkin et al., 2001; Melton et al., 1997; 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). Later in the symptomatic phase of HAD, commonly coinciding with CD4+ T cell depletion to levels lower than 200 cells/mm^3, 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 etal., 2003; Shapshak et al., 2004; 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 etal., 1986; Wiley et al., 1986; 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 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; Shapshak et al., 2004). Pervious 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-γ-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.

2. Results

2.1. *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 (Figs. 1A, B).

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. 1C). 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-

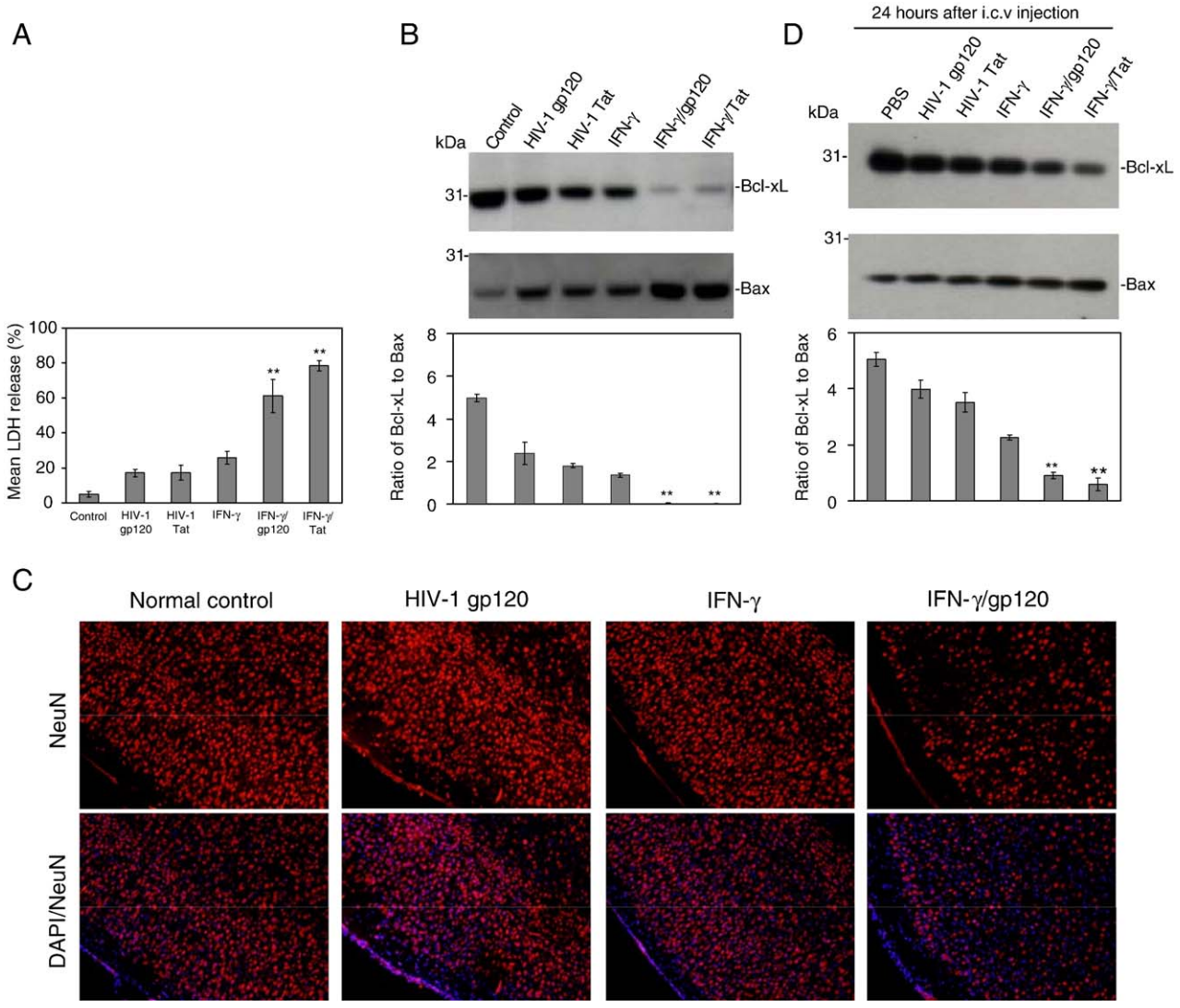

Fig. 1 – IFN-γ enhances neuronal injury induced by HIV-1 proteins gp120 or Tat *in vitro* and *in vivo*. Primary cultured neuronal cells were treated with gp120 (250 ng/ml), Tat (250 ng/ml), IFN-γ alone or gp120 (250 ng/ml), Tat (250 ng/ml) in combination with IFN-γ (100 U/ml; IFN-γ/gp120/or IFN-γ/Tat) 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 result 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).**

xL and Bax protein expression. Consistently, a significant reduction in the ratio of Bcl-xL to Bax (Fig. 1D) 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.

2.2. *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. 2A, 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. 2C, 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.

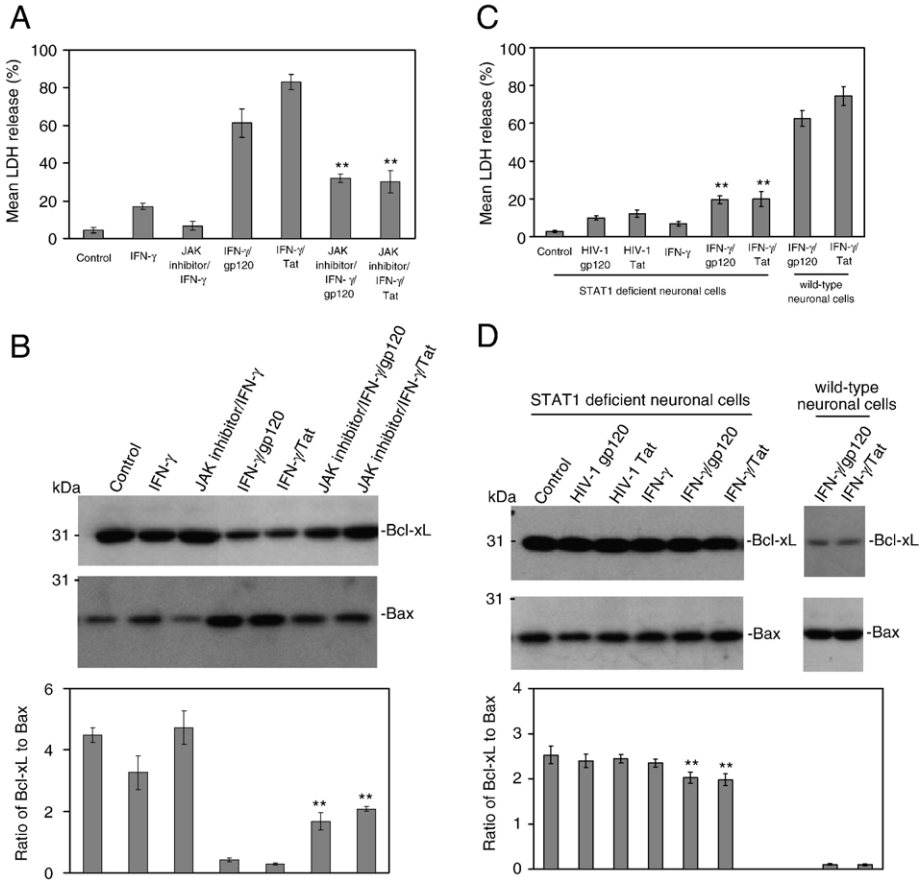

Fig. 2 – JAK/STAT1 signaling is critically involved in the IFN-γ mediated enhancement of HIV-1 gp120 and Tat-induced neuronal damage. Primary cultured neuronal cells were cotreated with IFN-γ (100 U/ml) and gp120 or Tat at 250 ng/ml in the presence of JAK inhibitor (50 nM) for 12 h. Cell cultured media 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 cultured 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 (**$P<0.001$).**

2.3. *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. 3A) and STAT1 (Fig. 3C) 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. As shown in Figs. 3B, D, the presence of EGCG resulted in dose-dependent inhibition of JAK1/STAT1 phosphorylation.

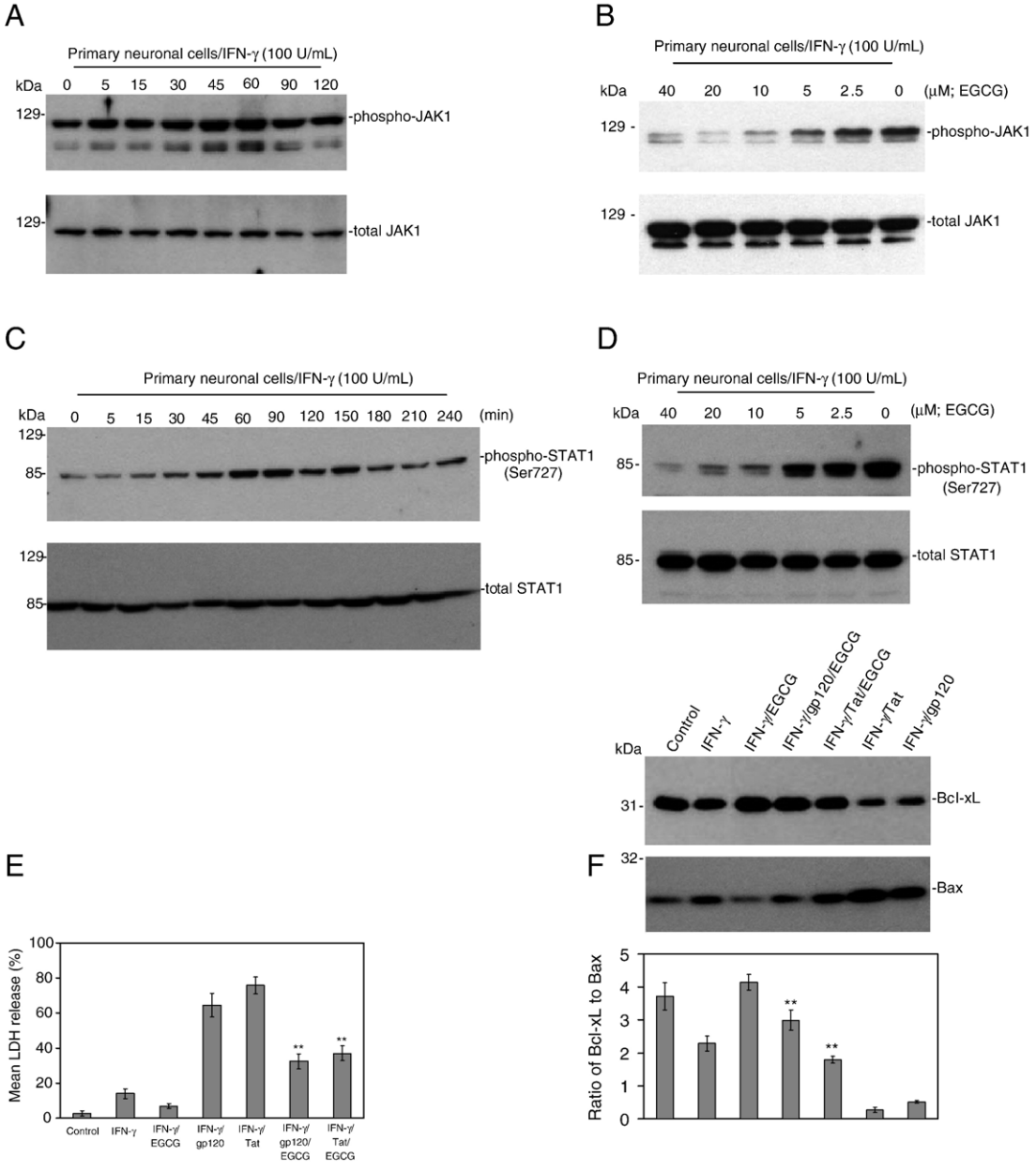

Fig. 3 – EGCG inhibits IFN-γ-induced JAK/STAT1 phosphorylation; protecting neurons from injury induced by IFN-γ/gp120 or IFN-γ/Tat *in vitro*. JAK1 and STAT1 protein phosphorylations were examined by Western blot (A, C). Cell lysates were prepared from primary cultured neurons treated with IFN-γ (100 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; Tedeschi et al., 2002) and *in vivo* (Stephanou, 2004; 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. 3E) and increased ratio of Bcl-xL to Bax (Fig. 3F). 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.

2.4. *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

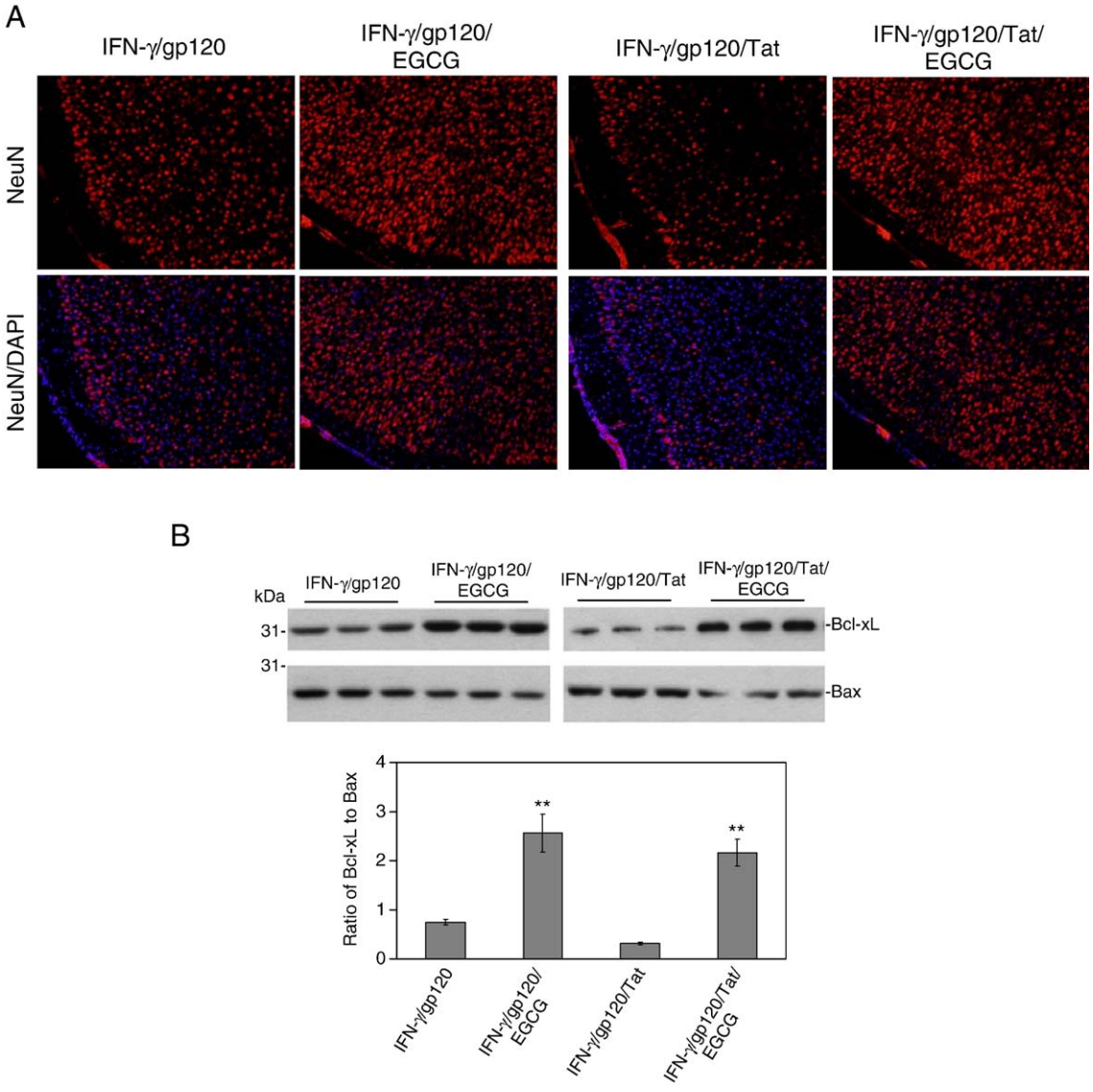

Fig. 4 – Mice i.p. injected with EGCG demonstrate significant reductions in neuronal injury after i.c.v. injection of IFN-γ/gp120, IFN-γ/Tat or IFN-γ/gp120/Tat. (A) Coronal, frozen mouse brain sections were stained with NeuN (top panels) and NeuN/DAPI (lower panels) and analyzed for neuron injury/loss. 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 ($P<0.001$).**

(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. 4A). 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. 4B) 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/gp120and IFN-γ/gp120/Tat in Western blot band density ratio of Bcl-xL to Bax (Fig. 4B).

3. 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-1 proteins 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; 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. 1C, 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. 1D). 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; Nath et al., 1999). Consistent with these findings clinical reports detail correlations between HIV-1 proteins, IFN-γ and neuron cell loss resulting in cognitive decline in HAD patients (Kumar et al., 2003; Mattson et al., 2005; 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; 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; 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; 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. 2A–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. 3A, D). JAK1 inhibitor mitigated neuron damage, inflicted by combinations of IFN-γ and gp120 and Tat proteins, *in vitro* (Figs. 2A 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 neuron 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. 2C, 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. 3E, 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. 4B), and reductions in neuron loss in cortical sections by immunohistochemistry (Fig. 4A).

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; 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. Experimental procedures

4.1. 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_{CN54} gp120) and Tat were obtained from The National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (Rockville, MD).

4.2. 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.

4.3. 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 2×10^5 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 LDH assay while cell lysates were used for Western blot analysis of Bcl-x and Bax proteins.

4.4. 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 NeuN staining, Western blot, and LDH analysis.

4.5. 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 HRP-conjugated IgG secondary antibody as a tracer. After being washed in TBS, the membranes were incubated in the luminol reagent and exposed to film.

4.6. *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.

4.7. *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 PVDF membranes (Bio-Rad), washed in dH_2O, 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 dH_2O 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 Multi-ImagerTM 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.

4.8. *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 cryostate 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-neuronal nuclei 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.

4.9. *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.

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