informa
healthcare

Molecular mechanism(s) involved in the synergistic induction of CXCL10 by human immunodeficiency virus type 1 Tat and interferon-γ in macrophages

Navneet Dhillon,[1] Xuhui Zhu,[1] Fuwang Peng,[1] Honghong Yao,[1] Rachel Williams,[1] Shannon Callen,[1] Amy O'Brien Ladner,[2] Shilpa Buch,[1] and Jianming Qiu[3]

Departments of [1]Molecular and Integrative Physiology and [2]Pulmonary and Critical Care Medicines and [3]Microbiology, University of Kansas Medical Center, Kansas City, Kansas, USA

Synergistic interactions between viral proteins and soluble host factors released from infected mononuclear phagocytes play a critical role in the pathogenesis of human immunodeficiency virus (HIV)-associated dementia (HAD). The chemokine CXCL10 has been found to be closely associated with the progression of HIV-1–related central nervous system (CNS) disease and its related neuropsychiatric impairment. In this report the authors demonstrate that the HIV-1 protein Tat can interact with the proinflammatory cytokine interferon (IFN)-γ to dramatically induce the expression of CXCL10 in macrophages. Synergistic induction of CXCL10 by both Tat and IFN-γ was susceptible to inhibition by the MEK1/2 inhibitor U0126 and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580. In addition, JAK/STAT pathway plays a major role in Tat/γ-mediated CXCL10 induction in macrophages because pretreatment of stimulated macrophages with JAK inhibitor completely abrogated the synergistic induction of the chemokine. Functionality of the synergistically induced CXCL10 was further demonstrated by its chemotactic activity for peripheral blood lymphocytes. Taken together, these findings demonstrate that the cooperative interaction of Tat and IFN-γ results in enhanced chemokine expression, which in turn can amplify the inflammatory responses within the CNS of HAD patients by recruiting more lymphocytes in the brain. *Journal of NeuroVirology* (2008) **14,** 196–204.

Keywords: HIV; JAK; MAP kinase; STAT

Introduction

It is estimated that almost 25% of untreated human immunodeficiency virus (HIV)-infected individuals and about 7% of HIV-infected patients treated with highly active antiretroviral therapy develop HIV-associated dementia (HAD), a neurodegenerative syndrome that is clinically characterized by progressive cognitive, motor, and behavioral abnormalities (Budka, 1991; Sacktor *et al*, 2001).

Although the primary cell types infected by HIV-1 in the brain are macrophages/microglia, the low numbers of infected cells in the brain do not correlate with the extent of neuropathogenesis observed in HIV encephalitis (HIVE) (Glass *et al*, 1995). Instead, activation of microglia, through direct infection or their interactions with infected cells, their products, or viral products, contribute to the disruption of neuronal homeostasis and development of severe neurological abnormalities, including HAD (Adle-Biassette *et al*, 1999; Garden, 2002). It is thus not the virus load, rather the inflammatory mediators and their interactions, that play a crucial role in the pathophysiology of acquired immunodeficiency syndrome

CXCL10 induction by Tat and IFN-γ
N Dhillon *et al*
197

(AIDS) dementia complex (Glass *et al*, 1993, 1995; Gray *et al*, 2000; Tyor *et al*, 1995).

In our earlier studies aimed at exploring factors contributing to encephalitis caused by simian human immunodeficiency virus (SHIV) in the rhesus macaque models of HIVE, we reported the overexpression of the interferon (IFN)-γ–inducible chemokine CXCL10 in the brains of SIV-infected macaques with encephalitis (Sui *et al*, 2004). CXCL10, a secreted polypeptide of 10 kDa, first identified as an early response gene induced after IFN-γ treatment in a variety of cells, has neurotoxic properties (Sui *et al*, 2006; van Marle *et al*, 2004). There is evidence suggesting increased expression of CXCL10 and its receptor CXCR3 in HIV-1–infected individuals with progressing central nervous system (CNS) disease (Sanders *et al*, 1998; Sasseville *et al*, 1996; Westmoreland *et al*, 1998). CXCL10 has also been detected in the cerebrospinal fluid (CSF) of HIV-1–infected individuals and acts as the major T-cell chemotactic factor in the CSF (Kolb *et al*, 1999). CXCL10 has also been shown to stimulate HIV-1 replication in monocyte-derived macrophages (MDMs) and peripheral blood lymphocytes (PBLs) (Lane *et al*, 2003). Treatment of fetal neuronal cultures with exogenous CXCL10 produced elevations in intracellular calcium and this effect was modulated via the CXCL10 receptor CXCR3. Furthermore, the increased calcium was found to be associated with mitochondrial membrane permeabilization and cytochrome *c* release, followed by activation of initiator caspase-9 and the effector caspase-3, ultimately resulting in apoptosis (Sui *et al*, 2004, 2006).

Based on the premise that virus-host interactions can culminate into the synergistic amplification of the toxic responses in the brain, contributing to disease pathology (Glass *et al*, 2003), we hypothesized that IFN-γ, a cytokine that is both up regulated in HAD (Shapshak *et al*, 2004) and that is known to induce CXCL10, can cooperate with HIV-1 Tat to synergistically enhance the expression of the chemokines.

In the present study we demonstrate the mechanisms by which HIV-1 Tat can synergize with IFN-γ to increase the production of CXCL10 in monocytes and macrophages. We have also evaluated the signaling pathways involved in this synergy and demonstrate the critical roles of mitogen-activated protein kinases (MAPKs) and the JAK/STAT pathways in this process. Understanding the pathways involved in CXCL10 up-regulation is critical for future development of therapeutics aimed at abrogation of neurotoxin expression in the brains of HIV patients with CNS disease.

Results

Synergistic induction of CXCL10 by HIV-1/HIV-1 protein and IFN-γ in macrophages

Because HIV-1 infection of macrophages and peripheral blood mononuclear cells (PBMCs) (Poluektova

et al, 2001; Wetzel *et al*, 2002) is known to induce CXCL10, we first wanted to assess the ability of IFN-γ to synergize with HIV-1 to induce CXCL10 expression in human monocyte-derived macrophages (MDMs). Macrophages infected with $HIV_{NL4.3}$ in the presence of IFN-γ were examined for expression of a battery of chemokines and cytokines such as monocyte chemoattractant protein (MCP)-1, CXCL10, interleukin (IL)-6, IL-1β, and transforming growth factor beta (TGFβ) (all of which are known to be involved in HIV-pathogenesis) using the ribonuclease protection assay (RPA). Cells were inoculated with HIV_{BaL} for 4 h, followed by extensive washing to get rid of the virus and then treated with IFN-γ (1000 U/ml) for 24 h. RNA extracted from these cells was assessed for expression of cytokine/chemokine by RPA. Treatment of MDMs with IFN-γ resulted in modest induction of CXCL10 as expected (Dhillon *et al*, 2007); however, there was negligible chemokine expression in cells infected with HIV_{BaL} alone. On the other hand, CXCL10 expression was significantly up-regulated in HIV-infected MDMs exposed to IFN-γ (Figure 1A). CXCL10 expression in MDMs was more prominent than any of the other factors examined.

To determine the combinatorial effect of HIV-infection and IFN-γ treatment on the expression of CXCL10 protein and for all further studies, we used the established monocytic THP-1 cell line. After stimulation with phorbol 12-myristate 13-acetate (PMA), THP-1 cells are known to differentiate into adherent macrophage-like cells (Tsuchiya *et al*, 1982). Because undifferentiated THP-1 cells are susceptible to infection by HIV isolates that use coreceptor CXCR4 (Muller *et al*, 1992), monocytic THP-1 cells were used to see the effect of X4 virus, $HIV_{NL4.3}$, on the expression of CXCL10. THP-1 cells were infected with $HIV_{NL4.3}$ followed by treatment with IFN-γ and 24 h later. Supernatant fluids were examined for CXCL10 protein by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 1B, levels of CXCL10 protein were found to be dramatically increased in the supernatants from HIV-1 infected THP-1 cells treated with IFN-γ compared with supernatants from either infected but untreated cells or from cells treated with IFN-γ alone.

Because HIV-1 Tat protein is known to be secreted by HIV-infected cells and/or is released due to the cytopathic effect of the virus, we next sought to dissect whether similar to HIV-1, exogenous Tat could synergize with IFN-γ to induce CXCL10. Both differentiated and undifferentiated THP-1 cells were either untreated or treated with Tat (50 ng/ml), and/or IFN-γ. Twenty-four hours later, RNA was extracted and monitored for CXCL10 expression by real-time reverse transcriptase–polymerase chain reaction (RT-PCR). As shown in Figure 2A, treatment with either Tat or IFN-γ alone resulted in identical levels of CXCL10 mRNA expression. Treatment of cells with a combination of both Tat and IFN-γ, however, resulted in a 15-fold enhancement of CXCL10
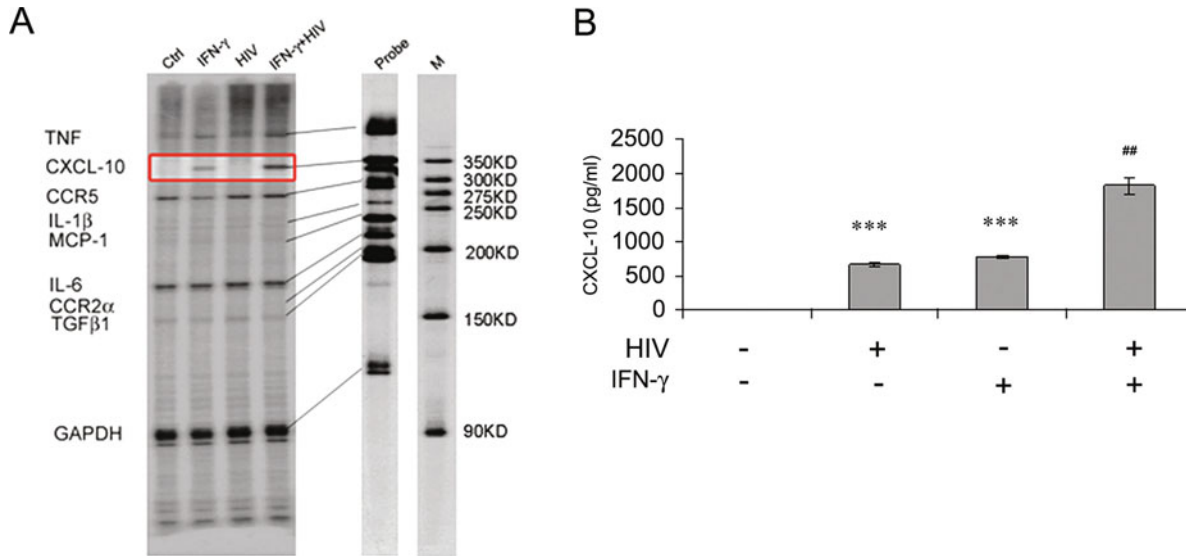
Figure 1 HIV-1 infection enhances IFN-γ–induced CXCL10 RNA expression in human macrophages. (**A**) Overnight serum-starved MDMs were infected with HIV-1$_{BaL}$ and/or treated with IFN-γ (1000 U/ml), followed by total RNA extraction 24 h later and analyses of CXCL10 RNA by ribonuclease protection assay (RPA). (**B**) Quantitative real-time RT-PCR analysis of CXCL10 RNA in THP-1 cells infected with HIV-1$_{NL4.3}$ and/or treated with IFN-γ (1000 U/ml). The average C_T value of the housekeeping gene HPRT was subtracted from that of the CXCL10 gene to give changes in C_T (ΔC_T). The fold-change in gene expression (differences in ΔC_T, or $\Delta\Delta C_T$) was then determined as log$_2$ relative units. The data represent the mean $\pm$ SD from three independent experiments *** $p \leq .001$, IFN-γ/virus versus control; ## $p \leq .01$, IFN-γ versus IFN-γ + HIV.

expression compared to treatment of cells with either Tat or IFN-γ alone.

Similar to primary cultures of macrophages, analyses of culture supernatant fluids from cells treated or untreated with Tat and/or IFN-γ for accumulation of CXCL10 protein by ELISA demonstrated a robust induction of CXCL10 expression in both undifferentiated and differentiated THP-1 cells (Figure 2B and C).

Mechanism(s) involved in the Tat- and IFN-γ–mediated synergistic induction of CXCL10
HIV-1 Tat has been shown to induce the phosphorylation of mitogen-activated protein kinases (MAPKs) to regulate various biological effects (Darbinian-Sarkissian *et al*, 2006; Toschi *et al*, 2006). To dissect the signaling pathways mediated by the combinatorial action of Tat and IFN-γ, lysates from THP-1 cells stimulated with Tat and/or IFN-γ were run on a Western blot and probed for activation of signaling proteins in the MAPK pathway. As shown in Figure 3A, macrophages treated with both Tat and IFN-γ demonstrated time-dependent enhanced activation of excellular signal-regulated kinase 1/2 (Erk1/2) MAPK, with maximal activation observed at 30 min post stimulation. Treatment of monocytes with both Tat and IFN-γ also resulted in activation of p38 MAPK (Figure 3A).

The specificity of these MAPKs in the synergistic induction of CXCL10 was further confirmed by pretreating cells with pharmacological inhibitors specific for the respective cytokines. As shown in Figure 3B, pretreatment of cells with the MEK1/2 in-

hibitor U0126 resulted in abrogation of the synergistic induction of CXCL10 expression to levels that were achieved in cells treated with IFN-γ alone. Both p38 MAPK/SAPK2 inhibitor (SB203580) and c-Jun N-terminal kinase (JNK) inhibitor II (SP600125) also inhibited the synergistic induction of CXCL10 mediated by Tat and IFN-γ, thus underpinning the role of these kinases in the synergy.

Because JAK/STAT signaling is critical, although not sufficient, for the responses mediated by interferons (Yadav *et al*, 2005), we next explored the role of these proteins in CXCL10 induction. STATs are inactive until phosporylated by receptor-activated Janus kinases (JAKs). As shown by Western blot analysis (Figure 4A), treatment of THP-1 cells with both Tat and IFN-γ resulted in phosphorylation of STAT1 at Tyr701 at 15 and 30 min following treatment. No activation of STAT1 was observed in macrophages treated with Tat alone. Treatment of macrophages with inhibitor of JAK (JAK inhibitor 1) completely abrogated CXCL10 induction in cells stimulated with Tat and IFN-γ (Figure 4B), thus highlighting the critical role of the JAK/STAT pathway in this process.

To address the functional role of phosphoinositide 3-kinase (PI3K) in the synergistic induction of CXCL10, cells were pretreated with the PI3K inhibitor LY 294002, followed by stimulation and assessment of CXCL10 expression in the supernatant fluids. As shown in Figure 4B, PI3K inhibitor did not alter Tat-mediated synergistic induction of CXCL10 expression.

Previous studies have implicated activation of the transcription factor nuclear factor kappa B (NFκB),
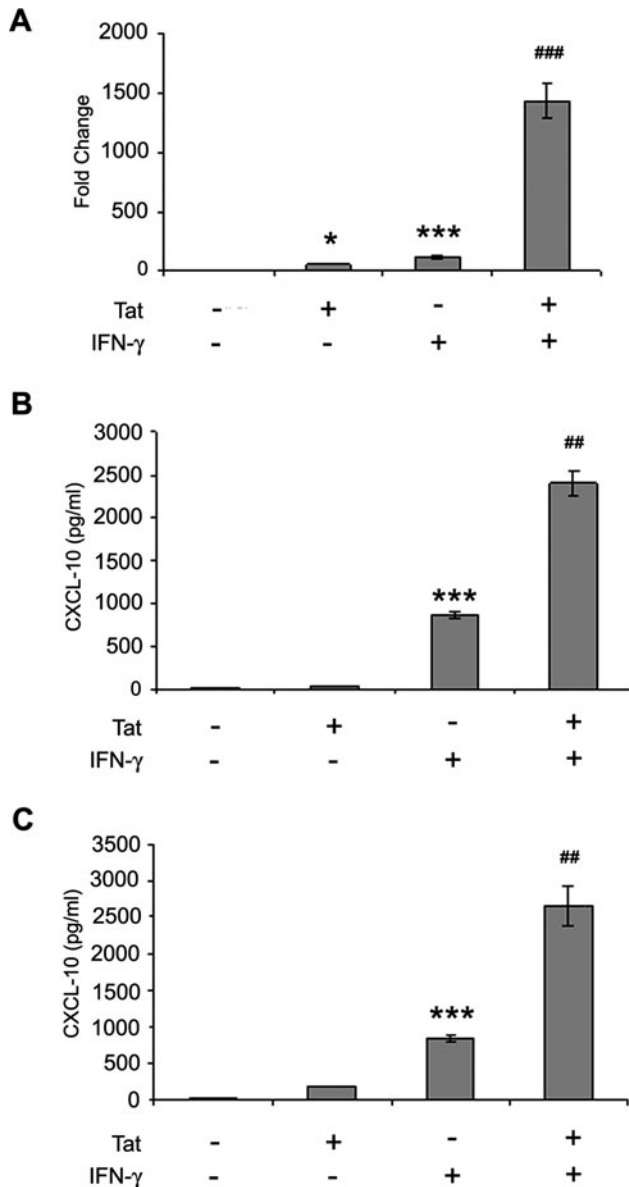
Figure 2 Synergistic induction of CXCL10 by Tat and IFN-γ in THP-1 cells. Real-time RT-PCR analysis of CXCL10 in undifferentiated THP-1 cells treated with Tat (50 ng/ml) with or without IFN-γ (1000 U/ml) at 24 h post treatment (**A**). Supernatants from undifferentiated (**B**) or differentiated (**C**) THP-1 cells treated with Tat in presence or absence of IFN-γ were collected at 24 h post treatment, followed by analysis of CXCL10 protein levels by ELISA. Statistical significance from three independent experiments was calculated. *** $p \leq .001$, * $p \leq .05$ (IFN/virus versus control); ## $p \leq .01$, ### $p \leq .001$ (Tat versus Tat + IFN-γ).

Figure 3 Analyses of Erk1/2 and p38 MAPKs in Tat and IFN-γ treated macrophages. (**A**) Western blot analyses was carried out sequentially using phosphorylated antibodies against Erk1/2 and p38 MAPK on the total cell lysate from THP-1 cultures untreated or treated with Tat (50 ng/ml) and/or IFN-γ (1000 U/ml) for indicated times. Blots were finally reprobed with β-actin antibody for normalization. (**B**) Significant inhibition of the synergistic effect of Tat on the IFN-γ–induced CXCL10 expression by U0126 (MEK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor). Pretreatment of MDMs with the PI3K inhibitor LY294002, on the other hand, had no effect on suppression of CXCL10. Serum-starved MDMs were preincubated with medium alone or with inhibitors at 20 μM for 30 min, followed by incubation with Tat and IFN-γ for 24 h. CXCL10 expression was then analyzed by ELISA in the collected supernatants. Values are mean ± SD from three independent experiments. *** $p \leq .001$, IFN-γ + Tat with inhibitor versus IFN-γ + Tat.

which lies downstream of the MAPK cascades, upon exposure of monocytes to Tat (Kumar *et al*, 1999). It was therefore of interest to examine whether simultaneous treatment of macrophages with Tat and IFN-γ led to enhanced activation of NFκB. As shown in Figure 4C, increased levels of phosphorylated NFκB p65 were present soon after stimulation in n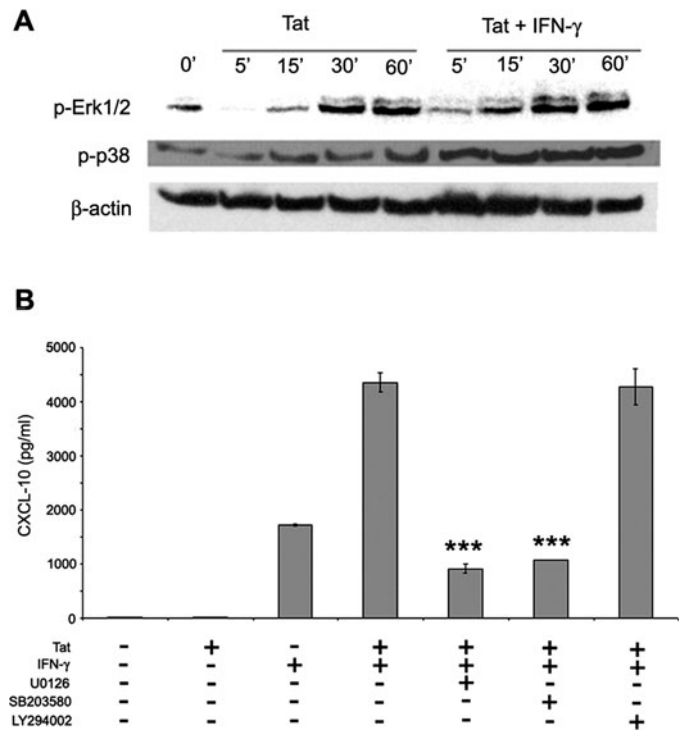uclear extracts of cells treated with Tat and IFN-γ compared to cells treated with Tat alone. Interestingly, synergistic induction of CXCL10 was completely abrogated in the presence of the NFκB p65 inhibitor, TPCK, thus confirming the involvement of NFκB activation in mediating Tat and IFN-γ cooperativity. We also observed increased expression and nuclear translocation (in more than 90% of cells as early as 5 min post treatment) of NFκB p65 in Tat- and IFN-γ–treated cells using polyclonal antibody against p65 subunit of NFκB (Figure 4D).

Functionality of CXCL10 induced by virus/cytokine-stimulated macrophages

The presence of elevated levels of CXCL10 can be related to the migration of T cells into the CNS (Fife *et al*, 2001; Sorensen *et al*, 2004). To test the functionality of CXCL10 released by macrophages following exposure to Tat and IFN-γ, we analyzed the migration of activated peripheral blood mononuclear
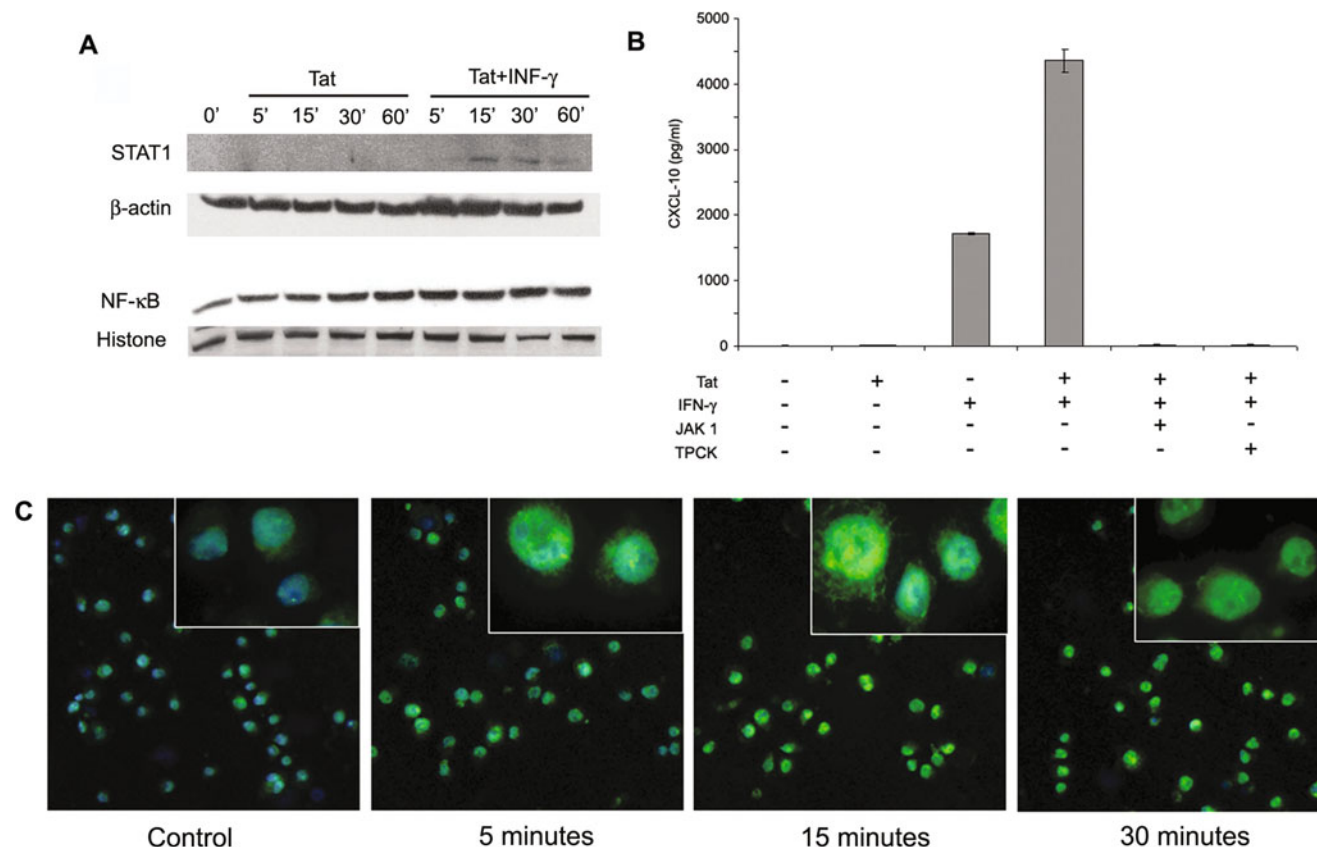
Figure 4 Involvement of JAK/STAT and NF-κB in synergistic induction of CXCL10 by Tat and IFN-γ. (**A**) Western blot analysis of STAT1 and NF-κB p65 subunit in macrophages treated with Tat alone or in combination with IFN-γ. Total cell lysates were immunoblotted with phosphorylated STAT1(Tyr701) antibody and finally reprobed with β-actin antibody for normalization. Nuclear extracts from treated cells were immunoblotted with antibodies specifically directed to the phosphorylated form of NF-κB p65. (**B**) Inhibition of JAK/STAT and NF-κB activity results in down-modulation of Tat- and IFN-γ−mediated induction of CXCL10. Serum-starved MDMs were preincubated in the absence or presence of JAK1 inhibitor (20 μM), TPCK (NF-κB inhibitor; 10 μM) for 30 min, followed by incubation with Tat and IFN-γ for 24 h. Supernatant fluids collected were then analyzed for CXCL10 levels by ELISA. Data represent mean ± SD from three independent experiments. (**C**) Immunocytochemistry of THP-1 cells untreated or treated with Tat plus IFN-γ for different time points. Cells were stained with anti-NF-κB p65 polyclonal antibody followed by treatment with Alexa Fluor 488–conjugated secondary antibody. Slides were mounted in Slow Fade antifade reagent (with DAPI, blue stain) and images were captured by confocal microscopy (magnification 200×). Inset represents high-magnification images at 1000×.

cells (PBMCs) towards the CXCL10 released in the supernatant fluids of stimulated macrophages. Chemotactic assays demonstrated that culture fluids from cells treated with the viral and cytokine factors had a higher chemotactic activity for PBLs than that from the cells treated with IFN-γ alone (Figure 5). Specificity of this response was confirmed by treating the culture fluids with neutralizing antibodies to CXCL10.

Discussion

Pathogenesis of HAD that is accompanied by infiltration of activated T lymphocytes and macrophages in the brain is often characterized by complex interactions of host and viral factors that are released by activated cells in the CNS (Ghafouri *et al*, 2006; Westendorp *et al*, 1995). Interplay of these factors eventually results in amplification of neurotoxic responses. Although neurodegeneration is one of the hallmark features of HAD, neurons themselves do not get infected by the virus. Thus it is not the virus per se, but the viral and cellular protein products released from neighboring infected cells, that can directly or indirectly exert neurotoxicity (Romashkova and Makarov, 1999; Sanders *et al*, 1998; Sasseville *et al*, 1996; Sridhar *et al*, 1999). The chemokine CXCL10, which is IFN-γ inducible and has the ability to attract activated T cells in the CNS, has also been shown to be a potent neurotoxin (Sui *et al*, 2006). Its expression has been found to closely associate with the progression of HIV-1 and SHIV-related CNS infection (Sanders *et al*, 1998; Sasseville *et al*, 1996; Westmoreland *et al*, 1998). It has been shown to be dramatically up-regulated in the brains of humans and macaques with HIVE/SIVE (Sui *et al*, 2003). It is therefore essential to understand the mechanism of induction of this chemokine in the CNS.
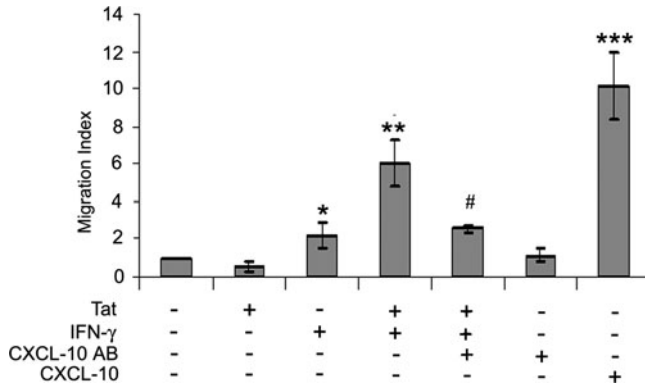
Figure 5 Analysis of chemotactic activity of CXCL10 in supernatants collecrted from THP-1 cells treated with Tat in absence and presence of IFN-γ. Supernatant from unstimulated control cells and from IFN- and/or Tat-treated THP-1 cells were added to the lower chamber of ChemoTX microplate and 2×10^6 PBLs were placed on the top. After 4 h, PBMCs migrating towards supernatant collected from different treatments in lower chamber were counted by using Cell Titer 96 Aqueous One Solution Assay. Migratory response of activated PBMCs to supernatant fluids preincubated with CXCL10-neutralizing antibody and to recombinant CXCL10 (10 ng/ml) are also shown. Migration index was calculated as the number of cells migrating towards the treated supernatants divided by the number of cells migrating toward the medium only. $^{*} p \leq .05$, $^{**} p \leq .01$, $^{***} p \leq .001$, control versus treatment; $^{\#} p \leq .05$, Tat + IFN-γ in presence of neutralizing antibody versus Tat + IFN-γ.

CXCL10 can be induced by a variety of factors, including viral Tat, gp120, and Nef and cellular host factors such as IFN-γ (Kawano *et al*, 2003; Lane *et al*, 2003; Li *et al*, 2000). Additionally, interactions of soluble host factors (IFN-γ and tumor necrosis factor [TNF]-α) can also synergistically induce the expression of CXCL10 (Pellicelli *et al*, 2001; Pistritto *et al*, 1994; Potula *et al*, 2004; Raghavan *et al*, 1997). Because viral proteins can interact with other soluble mediators culminating in enhanced neurotoxicity in the CNS of HAD patients (Flora *et al*, 2003; Maragos *et al*, 2002), we hypothesized that both HIV-1 Tat and IFN-γ, which are released from activated/infected cells and are present in HAD brains, can synergize to further increase the expression of the neurotoxin CXCL10. The present study was thus undertaken to explore the molecular pathways involved in the synergistic induction of CXCL10 in HIV-1–target cells, i.e., macrophages.

IFN-γ is known to induce two parallel but coordinated pathways regulating the expression of genes encoding proinflammatory molecules. Transcript synthesis is regulated by the JAK-STAT pathway and NFκB activation (Boehm *et al*, 1997; Stark *et al*, 1998), whereas transcript stabilization may be regulated by the p38 MAPK pathway (Kanda and Watanabe, 2007; Rani and Ransohoff, 2005). In cells treated with Tat and IFN-γ, there was a significant time-dependent activation of p38 MAPK, suggesting its role in the synergistic effect of Tat and IFN-γ. Activation of Erk1/2 MAPK and STAT1 was also involved in the synergis-

tic induction of IFN-γ–inducible CXCL10 by Tat, as demonstrated by the pharmacological approach using specific inhibitors. D'Aversa *et al* (2004) have earlier reported involvement of p38, Erk1/2, and PI3K pathways in Tat-induced CXCL10 expression in microglia. In our studies, however, we did not find PI3K to be essential for the synergistic induction of CXCL10 by Tat and IFN-γ in macrophages, as demonstrated by inhibitor studies.

Although astrocytes have been shown to express CXCL10 in the human and murine CNS (Asensio *et al*, 2001), very few studies point to the role of macrophages as being the source of CXCL10. Because development of HAD correlates with increased numbers of infiltrating mononuclear cells (Griffin, 1997; Tyor *et al*, 1995), these studies suggest macrophages as yet another source of this pleiotropic chemokine, which is both a chemoattractant and a neurotoxin (Fantuzzi *et al*, 2003; Sui *et al*, 2005). Dramatic upregulation of this chemokine in various neuroinflammatory pathologies could thus be achieved by its induction by various mechanism(s) and by various cells in the CNS. Identification of p38 MAPK and JAK/STATs as important functional intermediary molecules for induction of CXCL10 further paves the way for designing therapeutic strategies that are aimed at these signaling targets.

Materials and methods

Cell culture and treatments
PBMCs from peripheral blood of uninfected humans were obtained by Ficoll-Hypaque (Sigma, St. Louis, MO) gradient centrifugation as described earlier and prepared as suspension cultures grown in RPMI + 10% fetal bovine serum (FBS) + 50 U/ml IL-2 (R10) (Buch *et al*, 2001). MDM cultures were obtained from PBMCs by incubation in macrophage differentiation medium consisting of RPMI medium supplemented with 20% heated human serum (at 56°C for 30 min), 5 U/ml macrophage colony-stimulating factor (M-CSF), 100 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF), and 5% heat-inactivated rhesus monkey serum at 37°C for 7 days to allow adherent monocytes to differentiate into mature macrophages (Buch *et al*, 2001).

Monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C, under 5% CO_2, in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (at 56°C for 30 min), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) (R10 medium). These cells were subcultured (1:4) once a week to obtain a density of 1×10^6 cells/ml. THP-1 cells at a density of 0.5×10^6 cells/ml in R10 were cultured in 24-well plate (2 ml per well), serum-starved overnight, and then treated with Tat (a generous gift from Dr. Avi Nath; 50 ng/ml) and/or IFN-γ (1000 U/ml) for 24 h and supernatant collected for CXCL10

ELISA. Concentrations of Tat and IFN-γ were based on previous publications (Dhillon *et al*, 2007; Hegg *et al*, 2000). For virus infection, cells ($1 \times 10^6/0.2$ ml) were inoculated with either HIV$_{BaL}$ or HIV$_{NL4.3}$ virus at a multiplicity of infection (MOI) of 0.01 for 4 h at 37°C. Cells were then extensively washed with RPMI, resuspended at 0.5×10^6cells/ml in R10 medium containing Tat and/or IFN-γ. For differentiation of THP-1 cells into adherent macrophages, cells were plated at a density of 1×10^6 cells/ml and treated with 160 nM PMA (Sigma, St. Louis, MO) for 24 h at 37°C. Following 6 to 7 days of differentiation, PMA-treated cells were exposed to Tat and/or IFN-γ.

For inhibitor studies, cells were preincubated with various kinase inhibitors for 60 min at 37°C prior to Tat/ IFN-γ treatment. The following inhibitors were used at the final concentration specified: JAK inhibitor 1 (20 μM; Calbiochem, San Diego, CA), LY294002 (20 μM; Calbiochem), U73122 (20 μM; Calbiochem), U0126 (20 μM; Calbiochem), SB203580 (20 μM; Calbiochem), SP600125 (20 μM; Calbiochem), and NFκB (TPCK) inhibitor (10 μM; Sigma).

CXCL10 mRNA analysis
RNA was extracted from tissue samples using Trizol reagent (Life Technologies, Grand Island, NY). Chemokine/cytokine mRNA expression was analyzed using the customized human RPA kit from Pharmingen (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Quantitative analysis of CXCL10 mRNA in cells treated with IFN-γ and/or Tat was done by real-time RT-PCR using the SYBR Green detection method. RT-PCR primer pair set for CXCL10 was obtained from SuperArray Bioscience (Frederick, MD) and amplification of CXCL10 from first-strand cDNA was performed as described earlier using ABI Prism 7700 sequence detector (ABI, Foster City, CA). Data were normalized using C_T values for the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) in each sample. In order to calculate relative amounts of CXCL10, the average C_T value of the HPRT was subtracted from that for each target gene to provide changes in C_T (ΔC_T) value. The fold-change in gene expression (differences in ΔC_T, or $\Delta\Delta C_T$) was then determined as $\log_2$relative units.

CXCL10 protein analysis by ELISA
Supernatant fluids collected from MDMs/THP-1 cells treated with or without Tat and/or IFN-γ were examined for CXCL10 protein accumulation using CXCL10 ELISA kit (R&D Systems, Minneapolis, MN). Samples were analyzed for CXCL10 expression in three independent experiments in three to four replicates.

Western blot analysis
Cells with or without Tat and/or IFN-γ treatment for different time intervals were lysed in Lysis Buffer (Sigma) containing phosphatase and protease inhibitors. Equal amount of protein samples were run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel in reducing conditions, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane. The blots were blocked with 5% nonfat dry milk in phosphate-buffered saline. Western blots were then probed with antibodies recognizing phosphorylated forms of STAT1(Tyr701) (Cell Signaling Technology, Danvers, MA; 1:200), p38 (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:500), JNK (Santa Cruz Biotechnologies; 1:200), Erk1/2 (Cell Signaling; 1:200), NFκB p65 rabbit polyclonal antibody (1:500, Abcam, Cambridge, MA), and $\beta\beta$-actin (Sigma;1:4000). The secondary antibodies used were horseradish peroxidase–conjugated anti-mouse or anti-rabbit (1:5000; Pierce Chemical) and detection was performed using the enhanced chemiluminescence system (Pierce Chemical).

Chemotactic assays
Migration assays were performed in a Disposable ChemoTX microplate (NeuroProbe, Cabin John, MD) with a pore size of 8 μm. Supernatant fluids from macrophages treated with or without Tat and/or IFN-γ were added in each lower compartment and 2 million activated PBLs resuspended in 30-μl volume of RPMI 1640 were added to the top chamber. The microplate was then incubated at 37°C with 5% CO$_2$ for 3 h, following which the cells in the upper chamber were removed by washing with PBS. Cells that had migrated to the lower chamber were counted by using Cell Titer 96 Aqueous One Solution Assay (Promega, Madison, WI). All assays were performed in triplicate. Migration index was calculated as the number of cells migrating toward the supernatant from Tat-and/or IFN-γ–exposed cells divided by the number of cells migrating toward RPMI medium only.

Immunocytochemistry
Immunocytochemical analysis for NFκB was done on Tat cells were fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 0.5% Triton X-100 in PBS. The cells were then incubated with a blocking buffer containing 5% BSA in PBS for 1 h at room temperature. Following blocking, anti-human NFκB p65 rabbit polyclonal antibody (1:500; Abcam) was added to each coverslip and incubated 2 h at room temperature. Finally, the secondary antibody AlexaFluor 488–conjugated goat anti-rabbit immunoglobular G (IgG) was used at a 1:1000 dilution for 2 h to view NFκB activation in cells by Eclipse TE2000 Inverted fluorescence microscope. 4'-6-Diamino-2-phenylindole (DAPI) was used to stain the cell nuclei (blue).

Statistical analysis
All statistical analyses were performed by using a one-tail, independent, *t* test. Results were judged as statistically significant at *p* values $\leq$.05.

CXCL10 induction by Tat and IFN-γ
N Dhillon *et al*
203

References

Adle-Biassette H, Chretien F, Wingertsmann L, Hery C, Ereau T, Scaravilli F, Tardieu M, Gray F (1999). Neuronal apoptosis does not correlate with dementia in HIV infection but is related to microglial activation and axonal damage. *Neuropathol Appl Neurobiol* **25:** 123–133.

Asensio VC, Maier J, Milner R, Boztug K, Kincaid C, Moulard M, Phillipson C, Lindsley K, Krucker T, Fox HS, Campbell IL (2001). Interferon-independent, human immunodeficiency virus type 1 gp120-mediated induction of CXCL10/IP-10 gene expression by astrocytes in vivo and in vitro. *J Virol* **75:** 7067–7077.

Boehm U, Klamp T, Groot M, Howard JC (1997). Cellular responses to interferon-gamma. *Annu Rev Immunol* **15:** 749–795.

Buch S, Pinson D, King CL, Raghavan R, Hou Y, Li Z, Adany I, Hicks A, Villinger F, Kumar A, Narayan O (2001). Inhibitory and enhancing effects of IFN-gamma and IL-4 on SHIV(KU) replication in rhesus macaque macrophages: correlation between Th2 cytokines and productive infection in tissue macrophages during late-stage infection. *Cytokine* **13:** 295–304.

Budka H (1991). Neuropathology of human immunodeficiency virus infection. *Brain Pathol* **1:** 163–175.

Darbinian-Sarkissian N, Czernik M, Peruzzi F, Gordon J, Rappaport J, Reiss K, Khalili K, Amini S (2006). Dysregulation of NGF-signaling and Egr-1 expression by Tat in neuronal cell culture. *J Cell Physiol* **208:** 506–515.

D'Aversa TG, Yu KO, Berman JW (2004). Expression of chemokines by human fetal microglia after treatment with the human immunodeficiency virus type 1 protein Tat. *J NeuroVirol* **10:** 86–97.

Dhillon NK, Peng F, Ransohoff RM, Buch S (2007). PDGF synergistically enhances IFN-gamma-induced expression of CXCL10 in blood-derived macrophages: implications for HIV dementia. *J Immunol* **179:** 2722–2730.

Fantuzzi L, Belardelli F, Gessani S (2003). Monocyte/macrophage-derived CC chemokines and their modulation by HIV-1 and cytokines: a complex network of interactions influencing viral replication and AIDS pathogenesis. *J Leukoc Biol* **74:** 719–725.

Fife BT, Kennedy KJ, Paniagua MC, Lukacs NW, Kunkel SL, Luster AD, Karpus WJ (2001). CXCL10 (IFN-gamma-inducible protein-10) control of encephalitogenic CD4+ T cell accumulation in the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol* **166:** 7617–7624.

Flora G, Lee YW, Nath A, Hennig B, Maragos W, Toborek M (2003). Methamphetamine potentiates HIV-1 Tat protein-mediated activation of redox-sensitive pathways in discrete regions of the brain. *Exp Neurol* **179:** 60–70.

Garden GA (2002). Microglia in human immunodeficiency virus-associated neurodegeneration. *Glia* **40:** 240–251.

Ghafouri M, Amini S, Khalili K, Sawaya BE (2006). HIV-1 associated dementia: symptoms and causes. *Retrovirology* **3:** 28.

Glass JD, Fedor H, Wesselingh SL, McArthur JC (1995). Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. *Ann Neurol* **38:** 755–762.

Glass JD, Wesselingh SL, Selnes OA, McArthur JC (1993). Clinical-neuropathologic correlation in HIV-associated dementia. *Neurology* **43:** 2230–2237.

Glass WG, Rosenberg HF, Murphy PM (2003). Chemokine regulation of inflammation during acute viral infection. *Curr Opin Allergy Clin Immunol* **3:** 467–473.

Gray F, Adle-Biassette H, Brion F, Ereau T, le Maner I, Levy V, Corcket G (2000). Neuronal apoptosis in human immunodeficiency virus infection. *J NeuroVirol* **6(Suppl 1):** S38–S43.

Griffin DE (1997). Cytokines in the brain during viral infection: clues to HIV-associated dementia. *J Clin Invest* **100:** 2948–2951.

Hegg CC, Hu S, Peterson PK, Thayer SA (2000). Beta-chemokines and human immunodeficiency virus type-1 proteins evoke intracellular calcium increases in human microglia. *Neuroscience* **98:** 191–199.

Kanda N, Watanabe S (2007). Prolactin enhances interferon-gamma-induced production of CXC ligand 9 (CXCL9), CXCL10, and CXCL11 in human keratinocytes. *Endocrinology* **148:** 2317–2325.

Kawano H, Kim S, Ohta K, Nakao T, Miyazaki H, Nakatani T, Iwao H (2003). Differential contribution of three mitogen-activated protein kinases to PDGF-BB-induced mesangial cell proliferation and gene expression. *J Am Soc Nephrol* **14:** 584–592.

Kolb SA, Sporer B, Lahrtz F, Koedel U, Pfister HW, Fontana A (1999). Identification of a T cell chemotactic factor in the cerebrospinal fluid of HIV-1-infected individuals as interferon-gamma inducible protein 10. *J Neuroimmunol* **93:** 172–181.

Kumar A, Dhawan S, Mukhopadhyay A, Aggarwal BB (1999). Human immunodeficiency virus-1-tat induces matrix metalloproteinase-9 in monocytes through protein tyrosine phosphatase-mediated activation of nuclear transcription factor NF-kappaB. *FEBS Lett* **462:** 140–144.

Lane BR, King SR, Bock PJ, Strieter RM, Coffey MJ, Markovitz DM (2003). The C-X-C chemokine IP-10 stimulates HIV-1 replication. *Virology* **307:** 122–134.

Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U (2000). PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. *Nat Cell Biol* **2:** 302–309.

Maragos WF, Young KL, Turchan JT, Guseva M, Pauly JR, Nath A, Cass WA (2002). Human immunodeficiency virus-1 Tat protein and methamphetamine interact synergistically to impair striatal dopaminergic function. *J Neurochem* **83:** 955–963.

Muller WE, Schroder HC, Ushijima H, Dapper J, Bormann J (1992). gp120 of HIV-1 induces apoptosis in rat cortical cell cultures: prevention by memantine. *Eur J Pharmacol* **226:** 209–214.

Pellicelli AM, Palmieri F, Cicalini S, Petrosillo N (2001). Pathogenesis of HIV-related pulmonary hypertension. *Ann N Y Acad Sci* **946:** 82–94.

Pistritto G, Ventura L, Mores N, Lacal PM, D'Onofrio C (1994). Regulation of PDGF-B and PDGF receptor expression in the pathogenesis of Kaposi's sarcoma in AIDS. *Antibiot Chemother* **46:** 73–87.

Poluektova L, Moran T, Zelivyanskaya M, Swindells S, Gendelman HE, Persidsky Y (2001). The regulation of alpha chemokines during HIV-1 infection and leukocyte activation: relevance for HIV-1-associated dementia. *J Neuroimmunol* **120:** 112–128.

Potula R, Dhillion N, Sui Y, Zien CA, Funa K, Pinson D, Mayo MS, Singh DK, Narayan O, Buch S (2004).

Association of platelet-derived growth factor-B chain with simian human immunodeficiency virus encephalitis. *Am J Pathol* **165:** 815–824.

Raghavan R, Stephens EB, Joag SV, Adany I, Pinson DM, Li Z, Jia F, Sahni M, Wang C, Leung K, Foresman L, Narayan O (1997). Neuropathogenesis of chimeric simian/human immunodeficiency virus infection in pig-tailed and rhesus macaques. *Brain Pathol* **7:** 851–861.

Rani MR, Ransohoff RM (2005). Alternative and accessory pathways in the regulation of IFN-beta-mediated gene expression. *J Interferon Cytokine Res* **25:** 788–798.

Romashkova JA, Makarov SS (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401:** 86–90.

Sacktor N, Lyles RH, Skolasky R, Kleeberger C, Selnes OA, Miller EN, Becker JT, Cohen B, McArthur JC (2001). HIV-associated neurologic disease incidence changes: Multicenter AIDS Cohort Study, 1990–1998. *Neurology* **56:** 257–260.

Sanders VJ, Pittman CA, White MG, Wang G, Wiley CA, Achim CL (1998). Chemokines and receptors in HIV encephalitis. *AIDS* **12:** 1021–1026.

Sasseville VG, Smith MM, Mackay CR, Pauley DR, Mansfield KG, Ringler DJ, Lackner AA (1996). Chemokine expression in simian immunodeficiency virus-induced AIDS encephalitis. *Am J Pathol* **149:** 1459–1467.

Shapshak P, Duncan R, Minagar A, Rodriguez dlV, Stewart RV, Goodkin K (2004). Elevated expression of IFN-gamma in the HIV-1 infected brain. *Front Biosci* **9:** 1073–1081.

Sorensen TL, Roed H, Sellebjerg F (2004). Optic neuritis: chemokine receptor CXCR3 and its ligands. *Br J Ophthalmol* **88:** 1146–1148.

Sridhar P, Liu Y, Chin LD, Borja CE, Mann M, Skopicki HA, Freter RR (1999). Platelet-derived growth factor-stimulated expression of the MCP-1 immediate-early gene involves an inhibitory multiprotein complex. *Mol Cell Biol* **19:** 4219–4230.

Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD (1998). How cells respond to interferons. *Annu Rev Biochem* **67:** 227–264.

Sui Y, Li S, Pinson D, Adany I, Li Z, Villinger F, Narayan O, Buch S (2005). Simian human immunodeficiency virus-associated pneumonia correlates with increased expression of MCP-1, CXCL10, and viral RNA in the lungs of rhesus macaques. *Am J Pathol* **166:** 355–365.

Sui Y, Potula R, Dhillon N, Pinson D, Li S, Nath A, Anderson C, Turchan J, Kolson D, Narayan O, Buch S (2004). Neuronal apoptosis is mediated by CXCL10 over-expression in simian human immunodeficiency virus encephalitis. *Am J Pathol* **164:** 1557–1566.

Sui Y, Potula R, Pinson D, Adany I, Li Z, Day J, Buch E, Segebrecht J, Villinger F, Liu Z, Huang M, Narayan O, Buch S (2003). Microarray analysis of cytokine and chemokine genes in the brains of macaques with SHIV-encephalitis. *J Med Primatol* **32:** 229–239.

Sui Y, Stehno-Bittel L, Li S, Loganathan R, Dhillon NK, Pinson D, Nath A, Kolson D, Narayan O, Buch S (2006). CXCL10-induced cell death in neurons: role of calcium dysregulation. *Eur J Neurosci* **23:** 957–964.

Toschi E, Bacigalupo I, Strippoli R, Chiozzini C, Cereseto A, Falchi M, Nappi F, Sgadari C, Barillari G, Mainiero F, Ensoli B (2006). HIV-1 Tat regulates endothelial cell cycle progression via activation of the Ras/ERK MAPK signaling pathway. *Mol Biol Cell* **17:** 1985–1994.

Tsuchiya S, Kobayashi Y, Goto Y, Okumura H, Nakae S, Konno T, Tada K (1982). Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res* **42:** 1530–1536.

Tyor WR, Wesselingh SL, Griffin JW, McArthur JC, Griffin DE (1995). Unifying hypothesis for the pathogenesis of HIV-associated dementia complex, vacuolar myelopathy, and sensory neuropathy. *J Acquir Immune Defic Syndr Hum Retrovirol* **9:** 379–388.

van Marle G, Henry S, Todoruk T, Sullivan A, Silva C, Rourke SB, Holden J, McArthur JC, Gill MJ, Power C (2004). Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology* **329:** 302–318.

Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V (1995). HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. *EMBO J* **14:** 546–554.

Westmoreland SV, Rottman JB, Williams KC, Lackner AA, Sasseville VG (1998). Chemokine receptor expression on resident and inflammatory cells in the brain of macaques with simian immunodeficiency virus encephalitis. *Am J Pathol* **152:** 659–665.

Wetzel MA, Steele AD, Henderson EE, Rogers TJ (2002). The effect of X4 and R5 HIV-1 on C, C-C, and C-X-C chemokines during the early stages of infection in human PBMCs. *Virology* **292:** 6–15.

Yadav A, Kalita A, Dhillon S, Banerjee K (2005). JAK/STAT3 pathway is involved in survival of neurons in response to insulin-like growth factor and negatively regulated by suppressor of cytokine signaling-3. *J Biol Chem* **280:** 31830–31840.