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Expression of chemokines by human fetal microglia after treatment with the human immunodeficiency virus type 1 protein Tat

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Chemokines are important mediators of inflammation. It has been demonstrated that there is an increase in chemokine expression in both the sera and brain of individuals infected with human immunodeficiency virus type 1 (HIV-1). The HIV-1 viral protein, Tat, a transcriptional regulator, has been detected in the central nervous system (CNS) of infected individuals, and has been demonstrated to induce chemokines from various cells within the brain. The authors now show that the interaction of human microglia, the resident phagocytes of the brain, with Tat leads to dramatic increases in the secretion of the chemokines CCL2, CXCL8, CXCL10, CCL3, CCL4, and CCL5. Treatment of microglia with Tat plus specific inhibitors of signal transduction pathways demonstrated that the induction of each chemokine is regulated differently. Tat-induced expression of CCL2 and CCL4 was mediated by the activation of the extracellular regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway, whereas the induction of CXCL8 and CCL3 was mediated only by the p38 MAPK pathway. Tat-induced CXCL10 expression was mediated, to some extent, by activation of the ERK1/2 MAPK pathway, phosphatidylinositol 3-kinase pathway, and the p38 MAPK pathway, whereas CCL5 expression was not mediated by any pathway tested. Western blot analysis demonstrated phosphorylation of ERK 1/2 and Akt upon stimulation of microglia with Tat. These data suggest that a soluble HIV-1 viral protein can alter the chemokine balance in the brain, which can then lead to an influx of inflammatory cells and contribute to the neuropathogenesis of HIV-1 infection. *Journal of NeuroVirology* (2004) **10,** 86–97.

Keywords: CCL2; CCL3; CCL4; CXCL8; HIV encephalitis; MAP kinases

Introduction

An early consequence of infection with human immunodeficiency virus type 1 (HIV-1) is entry of the virus into the central nervous system (CNS) (Epstein *et al*, 1986; Wiley *et al*, 1986; Berger *et al*, 1987; Grant *et al*, 1987). Before the use of highly active antiretroviral therapy (HAART), almost half of children and a quarter of adults infected with HIV-1 eventually developed dementia and/or encephalitis (McArthur *et al*, 1993; Lipton and Gendelman, 1995). Although HAART has decreased dementia by 50%, HIV-1–associated neurological impairment persists among HIV-1–infected individuals (Maschke *et al*, 2000; Sacktor *et al*, 2002). HIV-1 dementia presents as a "subcortical dementia" and is associated with cognitive, behavioral, and motor changes. HIV-1 dementia does not usually appear before the onset of acquired immunodeficiency syndrome (AIDS), suggesting that once in the brain, HIV-1 may remain quiescent for a period of time. It has been shown that

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CNS dysfunction can occur without ongoing infection (Spencer and Price, 1992), and that HIV-1 encephalitis and dementia do not correlate with viral load, but rather with the amount of monocytic infiltration and activation of microglia (Glass *et al*, 1993, 1995; Tyor *et al*, 1993; Gray *et al*, 2000). Therefore, one hypothesis to explain encephalitis in the absence of abundant HIV-1 infection is through the actions of viral proteins.

Among the various viral proteins, Tat, a regulatory protein that promotes viral replication (Wiley *et al*, 1996), can be secreted by infected cells and taken up by, or act upon, other cells (Haseltine and Wong-Staal, 1988; Helland *et al*, 1991; Marcuzzi *et al*, 1992; Ensoli *et al*, 1993; Thomas *et al*, 1994; Chirmule *et al*, 1995). Tat has been found in the CNS and sera of HIV-1–infected individuals (Westendorp *et al*, 1995), as well as in mononuclear cells of individuals with HIV encephalitis (Hofman *et al*, 1994). Transgenic mice expressing Tat in astrocytes under an inducible promoter develop neuropathologies similar to those seen in the brains of individuals with AIDS (Kim *et al*, 2003).

Tat can activate many cell types in the brain, including neurons (New *et al*, 1998; Nath *et al*, 2000), astrocytes (Kutsch *et al*, 2000), and microglia (Polazzi *et al*, 1999; McManus *et al*, 2000; Sheng *et al*, 2000). Immunohistochemical analysis of HIV-1–infected brains has localized Tat to the extracellular matrix of microglia nodules (Kruman *et al*, 1998, 1999), as well as to cells themselves.

Microglia serve as the resident macrophages of the brain (Ehrlich *et al*, 1998; Stoll and Jander, 1999). Microglia have been shown to be the primary cell type of the CNS that can maintain productive infection (Dickson *et al*, 1991; Kolson *et al*, 1998), whereas astrocytic infection is still controversial (Dewhurst *et al*, 1987; Nath *et al*, 1995). Despite the fact that microglia can become infected, we and others have previously demonstrated that Tat can induce proinflammatory mediators from these cells even in the absence of infection (Bonwetsch *et al*, 1999; Mengozzi *et al*, 1999; Mayne *et al*, 2000; McManus *et al*, 2000; Sheng *et al*, 2000; Bruce-Keller *et al*, 2001; Nicolini *et al*, 2001).

Chemokines are low-molecular-weight cytokines that mediate recruitment of inflammatory cells to sites of damage, including the CNS (Bornemann *et al*, 1997; Hesselgesser and Horuk, 1999; and reviewed in Rollins, 1997). Chemokines have been classified into four families based upon the position of their amino-terminal cysteine residues. The CC family includes CCL2 (monocyte chemoattractant protein-1; MCP-1), CCL3 (macrophage inflammatory protein-1α; MIP-1α), CCL4 (macrophage inflammatory protein-1β; MIP-1β), and CCL5 (regulated upon activation, normal T cell expressed and secreted; RANTES). These chemokines chemoattract monocytes as well as activated T cells. The C-X-C family is comprised of CXCL8 (interleukin-8; IL-8), which attracts neutrophils and T cells, and CXCL10 (interferon inducible protein of 10 kDa; IP-10), which is chemotactic for monocytes and activated T cells (Taub *et al*, 1993).

Chemokines play an important role in HIV-1 encephalitis and dementia (Bonwetsch *et al*, 1999; McManus *et al*, 2000; Xiong *et al*, 2003) as chemotactic agents for inflammatory cells, both infected and uninfected. They can also activate resident cells, and several are chemotactic for microglia (Cheeran *et al*, 2001; Rezaie *et al*, 2002; and unpublished data, E.A. Eugenin and J.W. Berman). The chemokines CCL3, CCL4, CCL5, CXCL8, and CXCL10 have all been shown to be up-regulated in the brains of macaques with simian immunodeficiency virus (SIV) encephalitis (Matsumoto *et al*, 1993; Sasseville *et al*, 1996). CXCL8 is expressed early after both SIV infection of rhesus monkeys (Sopper *et al*, 1996), and HIV-1 infection of monocytes (Esser *et al*, 1996). Increased CCL3 and CCL4 mRNA have been detected in microglia and astrocytes by reverse transcriptase–polymerase chain reaction (RT-PCR) (Schmidtmayerova *et al*, 1996). CXCL8 was detected in the brains of individuals with HIV-1 encephalitis, and CCL3, CCL4, and CCL5 were demonstrated in microglial nodules of infected tissue (Sanders *et al*, 1998; McManus *et al*, 2000). CCL2 was also detected in the brains and cerebrospinal fluid (CSF) of individuals with HIV-1 dementia (Conant *et al*, 1998). We and others have previously demonstrated that in response to Tat, microglia, as well as astrocytes, secrete CCL2, CCL3, and CCL4 (Conant *et al*, 1998; McManus *et al*, 2000; Sheng *et al*, 2000), and that astrocytes cocultured with endothelial cells in a model of the human blood-brain barrier (BBB) and treated with Tat secrete CCL2, which was found to be chemotactic for monocytes and lymphocytes (Weiss *et al*, 1999). Astrocytes treated with Tat also secrete CXCL8 and CXCL10 (Kutsch *et al*, 2000).

Given the importance of these chemokines in the pathogenesis of neuroAIDS, we expanded upon our previous findings to determine whether the viral protein Tat can induce these chemokines in human fetal microglial cells, and what signaling pathways are contributing to Tat-induced chemokine expression. We demonstrated that CCL2, CXCL8, CXCL10, CCL3, CCL4, and CCL5 are all up-regulated in human fetal microglia after Tat treatment, and that different transduction pathways contribute to the expression of individual chemokines.

Results

Tat treatment of microglia induces chemokine protein secretion and mRNA expression

CCL2, CXCL8, CXCL10, CCL3, CCL4, and CCL5 expression has been demonstrated in HIV-1 encephalitis and/or dementia. Therefore, we determined whether purified human microglia express these

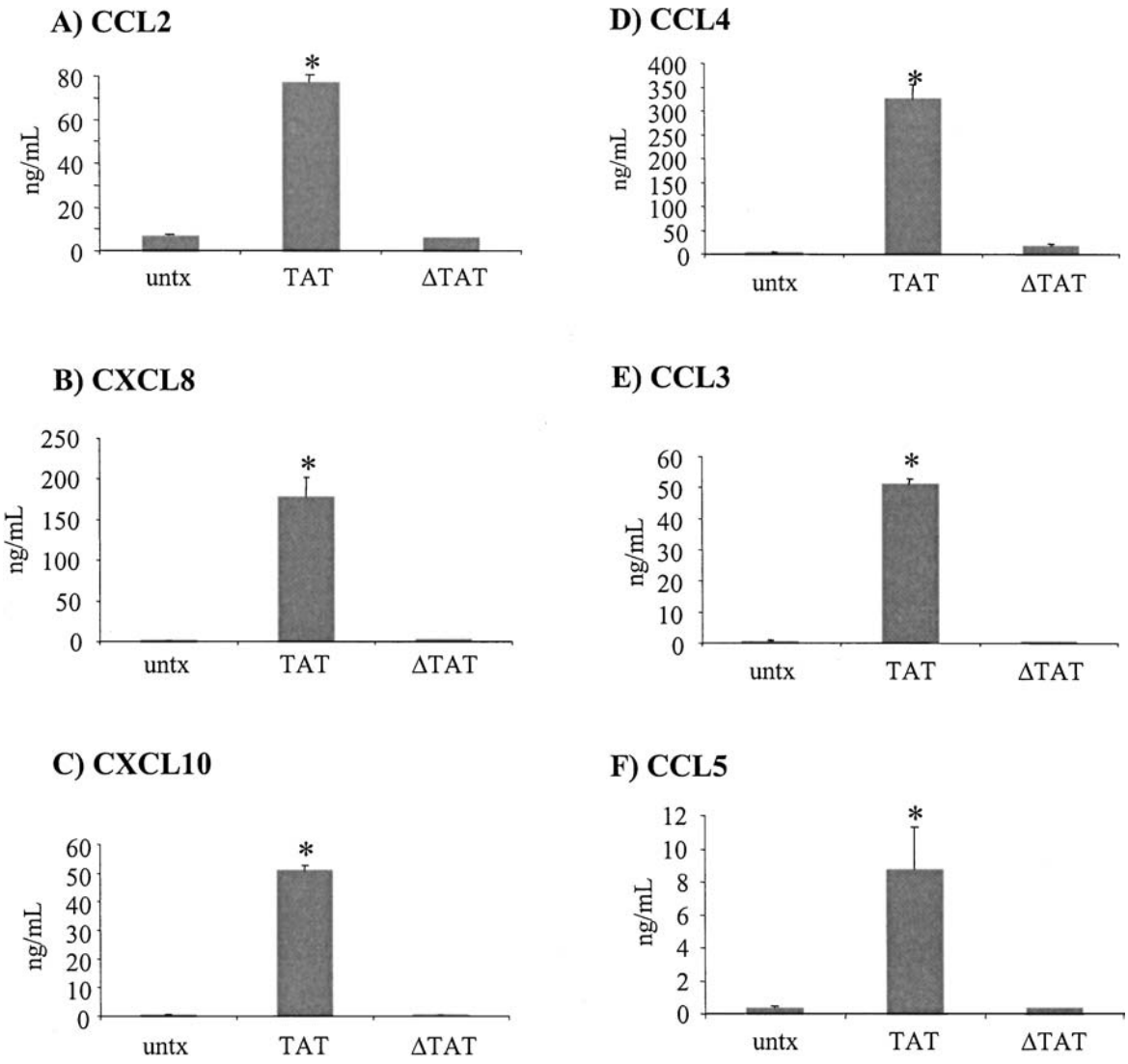

Figure 1 Tat treatment of microglia induces chemokine protein secretion. Microglia were treated with Tat (100 ng/ml), a Tat mutant (ΔTat; 100 ng/ml), or untreated (untx) for 24 h, after which the supernatants were collected and ELISAs were performed for (**A**); CCL2, (**B**) CXCL8, (**C**) CXCL10, (**D**) CCL4, (**E**) CCL3, and (**F**) CCL5 as described in Materials and Methods. Shown are the means of five separate experiments ±SEM. $*P < .007$ compared to either untreated or ΔTat.

chemokines in response to the HIV-1 protein Tat. Treatment of microglia with Tat induced protein secretion of all the chemokines tested (Figure 1A–F). To demonstrate that this protein induction was specific to the effects of Tat, microglia were treated with a mutant of Tat that has a deletion in amino acids 31 to 61 (ΔTat), a region which is important for many of the activities of Tat, including glial cell activation and neurotoxicity (Dr. Avindra Nath, personal communication; Weeks *et al*, 1995; Nath *et al*, 1996; Prendergast *et al*, 2002). As seen in Figure 1, ΔTat did not induce chemokine production. It is interesting to note that we detected high levels of all chemokines tested except for CCL5, as will be addressed in Discussion. We also determined the effects of Tat treatment of microglia at the mRNA level using ribonuclease protection assay (RPA). We detected transient mRNA after 12 h of treatment (Figure 2), with a loss of message expression by 24 h (data not shown). Densitometric analyses of these data show that Tat treatment increases CCL2, CXCL8, CXCL10, CCL3, CCL4, and CCL5 mRNA expression compared to untreated (data not shown).

Tat activates different signal transduction pathways

Many different signal transduction pathways can regulate chemokines, including the mitogen-activated protein kinase (MAPK) pathways. It has been demonstrated that Tat can activate the extracellular regulated kinase (ERK)1/2 MAPK pathway in a murine microglial cell line (Bruce-Keller *et al*, 2001) and the p38 MAPK pathway in human adult astrocytes as

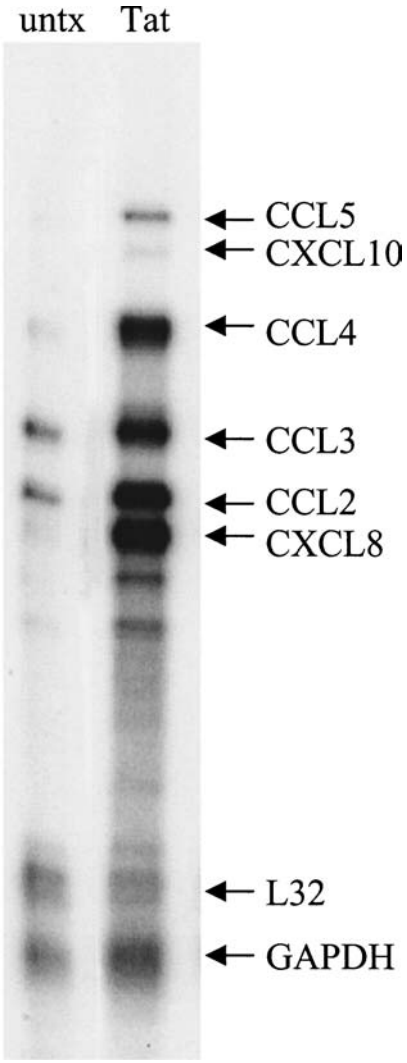

Figure 2 Tat induces chemokine mRNA expression from microglia. RNA from microglia treated with Tat for 12 h was analyzed by RPA. Tat treatment increased mRNA for each chemokine tested. (*Left to right*) Lane 1: untreated; lane 2: Tat 100 ng/ml. Shown is a representative RPA of two experiments.

well as a human astrocytic cell line (Kutsch *et al*, 2000). It has also been shown that Tat treatment of Jurkat cells can stimulate the catalytic activity of the phosphatidylinositol 3-kinase (PI3K) pathway (Borgatti *et al*, 1997). Therefore, microglial cells were analyzed for activation of the MEK1/2 (MAPK kinase) MAPK pathway, p38 MAPK pathway, and PI3K pathway using specific inhibitors. Figure 3 shows the results for each chemokine. Both CCL2 (Figure 3A) and CCL4 (Figure 3D) protein expression were significantly decreased by the MEK1/2 MAPK pathway inhibitor U0126 at 10 μM. CCL2 was also significantly decreased by the PI3K inhibitor LY294002 at 10 μM, whereas CCL4 was only modestly decreased. The p38α and p38β inhibitor SB203580 at 10 μM is the only inhibitor that significantly decreased CXCL8 (Figure 3B) and CCL3 (Figure 3E) protein expression. Interestingly, U0126, LY294002, and SB203580 all significantly decreased CXCL10 protein expression (Figure 3C), but none of the inhibitors tested affected CCL5 protein expression (Figure 3F). These data indicate that there are both overlapping and divergent signaling pathways for Tat-induced chemokine secretion.

Tat treatment of microglia causes phosphorylation of ERK1/2 and Akt

Our inhibitor data indicated that MAPK pathways are involved in Tat-induced chemokine expression. Because the activity of these signal transduction pathways is mediated, in part, by phosphorylation of MAPK proteins, we used Western blot analysis to demonstrate that these MAPK proteins were activated. Tat treatment resulted in phosphorylation of ERK1/2 after 10 min (Figure 4A, lane 2). This phosphorylation decreased after 30 min (Figure 4A, lane 8). Pretreatment with U0126 at 10 μM completely abolished this induction (Figure 4A, lanes 3, 6, 9). Akt is a target for the PI3K pathway. It has been demonstrated that Tat treatment of Kaposi sarcoma cells can enhance the phosphorylation of Akt (Deregibus *et al*, 2002), and that a substrate of p38, MAPKAP kinase 2, can phosphorylate Akt *in vitro* (Alessi *et al*, 1996; Hemmings, 1997). We therefore investigated the activation of Akt in microglia after Tat treatment. We found that Tat treatment induced the phosphorylation of Akt after 45 min (Figure 4B, lane 2). Treatment with the inhibitor LY294002 for 1 h prior to Tat treatment abrogated Tat-induced Akt phosphorylation (Figure 4B, lane 3).

Discussion

HIV-1 infection of the CNS results in significant neurological impairment and neuropathological changes, often resulting in encephalitis and/or dementia. HIV-1 dementia manifests as cognitive, motor, and/or behavioral disorders (Marder *et al*, 1996). HIV-1 encephalitis is characterized by the presence of HIV-1–infected macrophages and microglia in the brain. Multinucleated giant cells, the fusion of microglia with perivascular and parenchymal brain macrophages, are the hallmark of CNS HIV-1 infection. Perivascular infiltrates of inflammatory cells, diffuse myelin pallor, microgliosis, astrogliosis, and neuronal damage are all evident pathologies found in the brains of individuals with HIV-1 encephalitis (Price *et al*, 1988; Budka *et al*, 1991; Wiley *et al*, 1991; Masliah *et al*, 1992, 1997). With the use of HAART, the occurrence of HIV-1 dementia has been reduced by approximately 50% (Maschke *et al*, 2000; Sacktor *et al*, 2002); but despite this, neurological dysfunction remains present in many infected individuals. It is still unclear whether HAART will diminish or just delay the neurological sequelae associated with HIV-1 infection.

It has been shown that both HIV-1 encephalitis and dementia correlate with the degree of monocyte

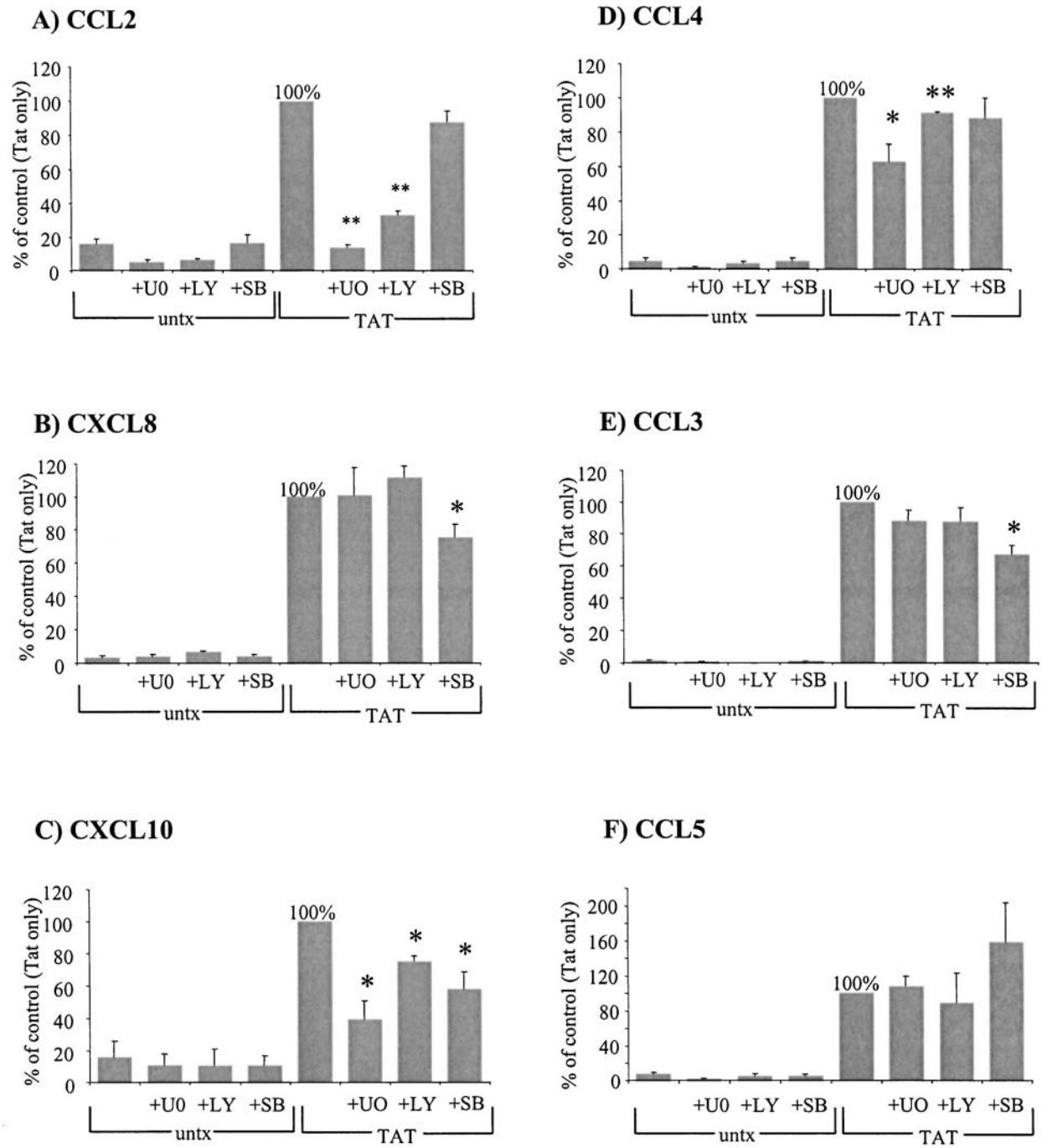

Figure 3 Tat-induced chemokine protein expression is mediated by different signal transduction pathways. (**A**) CCL2, (**B**) CXCL8, (**C**) CXCL10, (**D**) CCL4, (**E**) CCL3, and (**F**) CCL5 chemokine proteins were expressed as percent of Tat treatment (set to 100%). Microglia were pretreated with inhibitors for 1 h, and then treated with or without Tat for an additional 24 h. Shown are the means of four separate experiments ±SEM. $^{*}P < .05$, $^{**}P < .005$ compared to Tat treatment. U0 = U0126 at 10 μM; LY = LY294002 at 10 μM; SB = SB203580 at 10 μM.

infiltration and microglial activation in the brain rather than with viral load in the CNS (Glass *et al*, 1993, 1995). Therefore, even a low presence of virus may cause neurological impairment. Tat is a viral protein that is secreted from infected cells and taken up by uninfected cells (Helland *et al*, 1991; Marcuzzi *et al*, 1992; Ensoli *et al*, 1993; Chirmule *et al*, 1995). Tat has been shown to have several effects. Tat by itself is chemotactic and induces the influx of inflammatory cells (Lafrenie *et al*, 1996a, 1996b; Mitola *et al*, 1997; Benelli *et al*, 1998). We and others demonstrated that Tat induces proinflammatory mediators

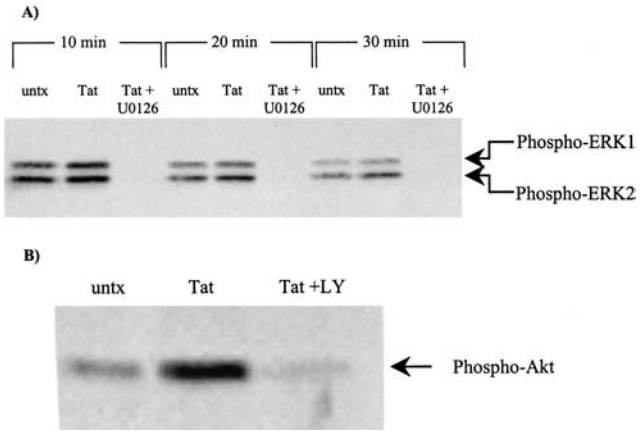

Figure 4 Tat induces phosphorylation of ERK1/2 and Akt. (**A**) Western blot analysis of Tat-treated microglia shows phosphorylation of ERK1/2 starting at 10 min and lasting until 30 min after treatment. (*Left to right*) Lane 1: untreated at 10 min; lane 2: Tat treated at 10 min; lane 3: Tat + 10 μM U0126 at 10 min; lane 4: untreated at 20 min; lane 5: Tat treated at 20 min; lane 6: Tat + 10 μM U0126 at 20 min; lane 7: untreated at 30 min; lane 8: Tat treated at 30 min; lane 9: Tat + 10 μM U0126 at 30 min. (**B**) Western blot analysis of Tat-treated microglia shows phosphorylation of Akt at 45 min. Shown is a representative blot of two separate experiments. (*Left to right*) Lane 1: untreated; lane 2: Tat 100 ng/ml; lane 3: Tat + LY294002 at 10 μM.

from CNS cells, including chemokines (Chen *et al*, 1997; Conant *et al*, 1998; McManus *et al*, 2000), that may participate in the initiation and amplification of the inflammatory cascade that is evident in HIV-1 encephalitis. We previously demonstrated that Tat induces the transmigration of mononuclear cells across a model of the BBB through the induction of CCL2 in astrocytes (Weiss *et al*, 1999). Tat is also neurotoxic, and therefore may contribute to the neuronal dropout that is observed in HIV-1 dementia. Therefore, having Tat-secreting inflammatory cells in the CNS may contribute to the pathology seen in neuroAIDS.

Recently it has been demonstrated that transgenic mice that express Tat under the control of an inducible glial fibrillary acidic protein (GFAP) promoter exhibit inflammation and similar neuropathology as that seen in the brains of HIV-1–infected individuals (Kim *et al*, 2003). Tat gene expression in the brain resulted in several developmental and behavioral abnormalities. These mice had atrophic and apoptotic neurons, which were in close proximity to Tat-positive areas, and there was a significant increase in monocytic and lymphocytic infiltration. Thus, *in vivo* expression of Tat mediates inflammatory cell influx into the CNS, contributing to significant CNS pathology.

Chemokines are chemotactic cytokines that can mediate leukocyte infiltration into the CNS. As described above, we had previously demonstrated that Tat induces chemokine expression by astrocytes (Weiss *et al*, 1999), and, in preliminary studies, that Tat induces chemokines from microglia (McManus *et al*, 2000). We expanded upon these studies to examine chemokines that have been shown to be important in HIV-1 infection of the CNS: CCL2, CCL3, CCL4, CCL5, CXCL8, and CXCL10. We now demonstrate that each of these chemokines is expressed by human microglial cells after treatment with Tat. We also investigated the signaling pathways of these chemokines, and determined their regulation.

The importance of chemokines in HIV-1 encephalitis and dementia is well established. Dementia (Conant *et al*, 1998; Kelder *et al*, 1998) and encephalitis (Cinque *et al*, 1998) are associated with CCL2 in the CSF and serum of HIV-1–infected individuals. CCL3 and CCL4 mRNA (Schmidtmayerova *et al*, 1996) and proteins (Sanders *et al*, 1998; Bonwetsch *et al*, 1999; McManus *et al*, 2000) were expressed in glial cells in the CNS of individuals with HIV-1 dementia or encephalitis, and not in normal brains. CCL3, CCL4, CCL5, and CXCL10 are all increased in the brain of macaques with SIV encephalitis (Sasseville *et al*, 1996; Westmoreland *et al*, 1998). Although their function as chemotactic proteins is well established, CCL3, CCL4, and CCL5 also may serve a protective role against HIV-1 infection because they bind to the same receptor as does the virus (Cocchi *et al*, 1995; Arenzana-Seisdedos *et al*, 1996; Trkola *et al*, 1996; Amara *et al*, 1997). Thus, they may inhibit HIV-1 infection of receptor expressing cells.

CXCL8 has been demonstrated to be expressed in HIV-1 infection. Not only does CXCL8 chemoattract neutrophils (Schroder *et al*, 1987; Walz *et al*, 1987; Yoshimura *et al*, 1989), but it has also been shown to chemoattract T lymphocytes (Larsen *et al*, 1989) and monocytes (Gerszten *et al*, 1999), as well as to enhance the adhesion of monocytes to endothelial cells (Gerszten *et al*, 1999). CXCL8 is up-regulated in HIV-1 and SIV, and is elevated in the serum of HIV-1–infected individuals (Matsumoto *et al*, 1993). CXCL8 is expressed early after both SIV infection of rhesus monkeys (Sopper *et al*, 1996), and HIV-1 infection of monocytes (Esser *et al*, 1996). In one study of HIV-1 encephalitic tissue, CXCL8 expression was localized to astrocytes (Sanders *et al*, 1998), but whether these cells secreted or bound CXCL8 was not determined. In addition, CXCL8 is able to stimulate HIV-1 replication in macrophages and T cells (Lane *et al*, 2001).

We have previously demonstrated in an *in vitro* model of the BBB that 2 ng/ml of CCL2 is sufficient to induce the transmigration of monocytes, with peak transmigration of cells at a concentration of 100 ng/ml of CCL2 (Weiss *et al*, 1998). Our unpublished data also demonstrate that 100 ng/ml of CCL2, CCL3, CCL4, and CCL5 are sufficient to obtain transmigration of HIV-1–infected cells, as well as activated leukocytes, across this model. In this study, we demonstrate that after Tat treatment of human fetal microglia, there is sufficiently high induction of CCL2, CCL3, CCL4, CXCL8, and CXCL10 to mediate transmigration *in vitro*. The chemokine protein levels

of all but CCL5 correlate to the optimal amounts that induce transmigration of cells across our BBB model. How this would translate to transmigration of cells across the BBB *in vivo* is not known, but high amounts of chemokines have been detected in the CNS parenchyma of individuals with neuroAIDS and would likely mediate chemotaxis. Tat-induced CCL5, although significant, is low (8.77 ng/ml) compared to the levels of the other chemokines studied. It has been demonstrated that there is an increase in viral replication following CCL5-mediated signal transduction in T cells (Kinter *et al*, 1998) and monocytes/macrophages (Kelly *et al*, 1998). It has also been shown that high concentrations of CCL5 can enhance the infectivity of certain HIV-1 strains. This is achieved through the cross-linking of virions to the cell surface by CCL5 oligomers, which form after binding to glycosaminoglycans on the virion and cell membranes (Gordon *et al*, 1999; Trkola *et al*, 1999). Therefore, low levels of CCL5 after Tat treatment may suggest a mechanism by which cells are responding to Tat by producing levels of CCL5 that may be sufficient to compete for the CCR5 receptor and therefore inhibit infection of microglia by R5 isolates, but are not high enough to enhance viral replication.

Although well recognized as chemotactic factors, chemokines participate in additional cellular responses, such as proliferation, migration, and the induction of inflammatory mediators (Rollins, 1997; Luo *et al*, 2000; McManus *et al*, 2000; Tarzami *et al*, 2002). As described above, they may also be protective against HIV-1 infection by competing for the same receptor; alternatively, they may enhance replication. We recently demonstrated a novel function for CCL2 and CCL5. These chemokines protect neurons and astrocytes from Tat-induced apoptosis. The effects of CCL5 were mediated through the induction of CCL2 (Eugenin *et al*, 2003). Thus chemokines may play multiple roles in the pathogenesis of neuroAIDS, depending upon when and where during the course of infection they are expressed. Therefore, an understanding of the signaling pathways that mediate the different roles that chemokines play in neuroAIDS may facilitate the development of interventional strategies that target these pathways in a relevant and appropriate time frame.

It has been demonstrated that Tat treatment of a murine microglial cell line increased several markers of microglial activation and resulted in the activation of multiple signal transduction pathways (Bruce-Keller *et al*, 2001). Tat activation of the ERK1/2 MAPK pathway resulted in superoxide production, tumor necrosis factor α (TNFα) release, and phagocytosis, all indicators of activated microglia (Bruce-Keller *et al*, 2001). We studied the signaling pathway for Tat-induced chemokine expression in human microglia. We found that regulation of chemokine expression is different for each chemokine. CCL2 and CCL4 are regulated through the ERK1/2 MAPK and PI3K pathways; CXCL8 and CCL3 are regulated through p38 MAPK; CXCL10 is regulated, to some extent, by all three pathways; and CCL5 production was independent of the three pathways tested. Although Tat-induced CCL4 protein expression was consistently and significantly reduced after treatment with the p38 inhibitor, this decrease was modest, and therefore suggests that pathways other than those examined are involved in CCL4 secretion. After treatment with inhibitor, Tat-induced protein expression of CXCL8, CCL3, and CXCL10 is significant, but not decreased to baseline levels. Alternate signaling pathways are particularly evident for CCL5 protein secretion, as none of the inhibitors tested reduced Tat-induced CCL5 production. CCL2 is the only chemokine that was clearly regulated by only the ERK1/2 MAPK pathway, as the MEK1/2 inhibitor reduced protein levels to baseline. These data demonstrate that Tat-induced chemokine expression is complex, and that more than one pathway may be necessary for their expression.

Our studies demonstrate a role for different signaling pathways in Tat-induced chemokine production by human microglia. Tat treatment of human astrocytes also resulted in the activation of different signaling pathways. One study demonstrated that CCL2 and CXCL8 expression was mediated through the ERK1/2 MAPK pathway and CXCL10 was mediated through the p38 MAPK pathway (Kutsch *et al*, 2000). Although we also determined that Tat-induced CCL2 expression is ERK1/2 mediated, our data for CXCL8 and CXCL10 are different than that found in the astrocyte studies. The differences observed could be attributed to the different cell types studied. The observed differences in signaling in both cell types may indicate that Tat may not be acting through a single receptor-mediated mechanism, and that its means of entry may contribute to the effects of Tat, because Tat is able to enter a cell through multiple means.

The possible mechanisms by which Tat is taken up by cells include the binding of Tat to integrins (Barillari *et al*, 1993), to the vascular endothelial growth factor (VEGF) receptor, KDR (Albini *et al*, 1996; Ganju *et al*, 1998; Morini *et al*, 2000), to the low-density lipoprotein receptor–related protein (LRP) on neurons (Liu *et al*, 2000) and microglia, or by endocytosis. Because Tat may be taken into a cell by a number of mechanisms, this could contribute to the differences in regulation of chemokine secretion we observed in microglia. It has not yet been determined if microglia express KDR, but they do express LRP on their surface (Moestrup *et al*, 1992; Wolf *et al*, 1992; Marzolo *et al*, 2000). It is interesting to note that ΔTat is deleted in the region that includes the LRP binding site. Perhaps, in our system, Tat is mediating its effects through binding of LRP on microglia, and because ΔTat has been rendered unable to bind LRP, it cannot induce chemokine secretion from microglia. However, because the glial cell activation region is also deleted in this mutant and has not yet been fully

defined, Tat may be exerting its effects through this site.

In conclusion, our data demonstrate that the HIV-1 protein, Tat, is sufficient for the activation of microglia. This may have significant implications for HIV-1 encephalitis and dementia. HIV-1 proteins in the brain can interact with microglia, causing this elaboration of chemokines. These chemokines chemoattract inflammatory cells into the brain, and these inflammatory cells may elaborate additional proinflammatory mediators or viral proteins. If the inflammatory cells entering the CNS are infected, they can then infect microglia. Also, because CCL3, CCL4, CCL5, and CCL2 are chemotactic for microglia, HIV-1 infection and the secretion of these chemokines can facilitate the migration of microglia to sites of damage, and perhaps assist in the formation of multinucleated giant cells that are seen in HIV-1 encephalitis. Thus, interactions of viral proteins with microglia may initiate a cascade of events that contribute to CNS inflammation and the progression of neuroAIDS.

Materials and methods

Cell culture and reagents
Human fetal CNS tissue (16 to 24 weeks) was obtained at the time of elective abortuses from healthy females. The tissue was used as part of an ongoing research protocol approved by the Albert Einstein College of Medicine. Microglia were obtained as previously described (D'Aversa *et al*, 2002). Briefly, the tissue was minced and digested, after which it was passed through a 250-μm nylon mesh filter followed by a 150-μm filter, and washed. Cells were resuspended in complete Dulbecco's modified Eagle medium (DMEM) (DMEM plus 25 mM Hepes, 10% fetal calf serum [FCS], 1% penicillin-streptomycin, 1% nonessential amino acids), seeded at 9×10^7 per 150 cm^2 flask, and maintained at 5% CO_2, 37°C. After 12 days, the medium (containing microglia) was removed and centrifuged for 5 min at 220 $\times$ *g*. The microglia were resuspended in complete DMEM and seeded at 5×10^5 per well of a 24-well plate. Cells were analyzed and shown to be greater than 95% HAM56 (a microglial marker) positive (McManus *et al*, 2000). The medium was changed after 4 h. Twenty-four h after plating, microglia were either treated with 200 μl DMEM, without FCS, and containing 100 ng/ml HIV-1 Tat protein and 100 ng/ml ΔTat, or left untreated for 24 h, after which supernatants were collected and analyzed for chemokine secretion. For inhibitor studies, microglia were pretreated with 10 μM of the MEK1/2 MAPK inhibitor, U0126 (Sigma-Aldrich, St. Louis, Missouri), 10 μM of the p38α and p38β MAPK inhibitor, SB203580 (Tong *et al*, 1997) (Sigma-Aldrich), or 10 μM of the PI3K inhibitor, LY294002, for 1 h, followed by treatment with 100 ng/ml of Tat for 24 h. Supernatants were then collected and analyzed for chemokine protein expression. Each microglial culture was derived from an independent cortex, each resulting in a single experiment.

Tat and ΔTat
The Tat protein was a generous gift from Dr. Avindra Nath (Johns Hopkins University School of Medicine) and was prepared as previously described (Ma and Nath, 1997). The mutant Tat (ΔTat) contains a deletion in amino acids 31 to 60 (Prendergast *et al*, 2002). This deleted region corresponds to the long terminal repeat (LTR) binding site, the neurotoxic domain, the glial cell activation region, and the LRP binding site (Dr. Nath, personal communication, and Prendergast *et al*, 2002). No detectable levels of endotoxin in the Tat preparation were detected.

Chemokine ELISA
Supernatants were analyzed for chemokine proteins using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. CCL2, CXCL8, CCL3, CCL4, and CCL5 ELISA antibody pairs were purchased from R&D Systems. The antibody pairs for CXCL10 were from Pharmingen (San Diego, California). The sensitivities for these assays are 4 pg/ml, 2.6 pg/ml, 8 pg/ml, 1 pg/ml, 3 pg/ml, and 5 pg/ml, respectively.

RNA extraction and analysis
Microglia were plated at 1×10^6 cells/100-mm dish for 12 h. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio). Chemokine mRNA expression was analyzed using the human chemokine RPA kit hCK5 from Pharmingen. Densitometry was performed using Ambis QuantProbe software, with values normalized to GAPDH.

Western blot analysis
Twenty-four h after plating, cells were pretreated with or without inhibitor for 1 h, after which some cells were left untreated, and others were treated with Tat for 10, 20, 30, and 45 min. Microglia were washed with 1$\times$ phosphate-buffered saline (PBS) and lysed (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO4, 1 mg/ml leupeptin, 100 nM phenylmethylsulfonyl fluoride [PMSF]; Cell Signaling, Beverly, Massachusetts). The slurry was passed through an 18-G needle five times and protein was quantitated using the Bradford assay. Protein was heated at 95°C for 5 min and 20 μg was loaded onto each lane of an sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (12% for ERK1/2 and 7% for Akt). Proteins were transferred electrophoretically to Protran nitrocellulose (Schleicher & Schuell, Keene, New Hampshire). Membranes were

blocked with 5% nonfat dry milk in 0.1% Tween-20/Tris-buffered saline (TBS) and incubated with primary antibodies (phospho-p44/42 MAP kinase antibody or phospho-Akt Ser 473 antibody; Cell Signaling) at a concentration of 1:1000 overnight at 4°C. After washing, membranes were incubated with anti–rabbit horseradish peroxidase (HRP) secondary antibody (1:2000, Cell Signaling) for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia, Piscataway, New Jersey).

Statistical analysis

The paired Student's *t* test (one-tailed) was used to determine statistical significance. A value of $P < .05$ was considered to be significant.

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