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Induction of monocyte chemoattractant protein-1 (MCP-1/CCL2) gene expression by human immunodeficiency virus-1 Tat in human astrocytes is CDK9 dependent

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Human immunodeficiency virus-1 (HIV-1) invades the brain early in infection and may cause HIV-associated dementia (HAD), which is characterized by reactive astrocytes, and macrophage and T-cell infiltrates. HIV-1 Tat protein is thought to contribute to HAD by transactivating host genes, such as that encoding monocyte chemoattractant protein-1 (MCP-1/CCL2), although its mechanisms of action are not fully understood. We investigated the molecular pathways involved in Tat-induced MCP-1/CCL2 gene expression in human astrocytes. We found that Tat induced MCP-1/CCL2 synthesis in human astrocytes infected with a lentivirus carrying the gene encoding Tat or treated with a biologically active synthetic Tat protein. The induction of MCP-1/CCL2 was independent of the nuclear factor κB (NF-κB) classical pathway, but was significantly inhibited by specific cyclin-dependent kinase 9 (cdk9) inhibitors, such as a dominant-negative mutant or siRNA. By contrast, broader-spectrum cdk inhibitors, such as roscovitine, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and flavopiridol, inhibited MCP-1/CCL2 induction by Tat. We also analyzed the effects of roscovitine, DRB, and flavopiridol on Tat-induced HIV-1 long terminal repeat (LTR) expression following the infection of astrocytes and HeLa cells. Astrocytes showed no inhibition by roscovitine, 59% inhibition by DRB, and 80% inhibition by flavopiridol. In control HeLa cells, high levels of inhibition were observed with roscovitine, DRB, and flavopiridol. We have ascertained the direct implication of cdk9 in Tat-induced MCP-1 expression by performing ChIP assay. These results demonstrate that cdk9 is involved in Tat-induced HIV-1 LTR, MCP-1/CCL2 gene expression. *Journal of NeuroVirology* (2010) **16,** 150–167.

Keywords: cdk9; glial cells; HIV-1; MCP-1/CCL2; roscovitine; transcription regulation

Introduction

Human immunodeficiency virus-1 (HIV-1) invades the central nervous system early in infection (Kolson, 2002). It has been estimated that 10% to 20% of HIV-1–infected patients in the United States and Western Europe go on to develop neurological complications associated with acquired immunodeficiency syndrome (AIDS); these complications are known as HIV-associated dementia (HAD)

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(Nath *et al*, 2008). Before the advent of highly active antiretroviral therapy, the incidence of HAD in AIDS patients was reported to be more than 30% in adults, and 50% in children (Seth and Major, 2005). Characteristic astrogliosis, with macrophage and T-cell infiltrates, has been found to be closely correlated with HAD in the brains of patients (Petito *et al*, 2003). A major factor contributing to HAD development is the chronic activation of brain microglia/macrophages and astrocytes associated with an increase in the release of chemokines by these cells (Kaul *et al*, 2001). Monocyte chemoattractant protein-1 (MCP-1/CCL2) is thought to be a major chemokine in neuropathogenesis, facilitating the migration of infected and/or activated monocytes into the brain and activating macrophages, microglia, and astrocytes, leading to the release of potent neurotoxins (Asensio and Campbell, 1999). High levels of MCP-1/CCL2 are found in the cerebrospinal fluid (CSF) of patients with HAD and are correlated with CSF viral load, suggesting a role for this protein in the aggravation of HAD (Cinque *et al*, 1998; Conant *et al*, 1998; Kelder *et al*, 1998). HIV-1 productively infects brain macrophages and microglial cells, killing them (Watkins *et al*, 1990). Astrocytes may also become infected, resulting in restricted, chronic, persistent infection. This restriction is unique, because it leads to an initial burst of low-level HIV replication, followed by the accumulation of multiply spliced mRNAs encoding viral proteins, such as Tat—a major HIV-1 regulatory protein—but without completion of the viral life cycle (Gorry *et al*, 1999; Janabi *et al*, 1998). Tat, an 86- to 101-amino acid protein, is produced in infected astrocytes and may be secreted and taken up by neighboring cells. Tat may therefore affect both infected and uninfected cells (Chang *et al*, 1997; Nath *et al*, 2000). HIV-1 Tat protein has been identified as a key factor in HAD, due to its contribution to molecular pathways involving the modulation of host gene expression (Langford and Masliah, 2002). Tat mRNA levels in the brain have been linked to HIV- and simian immunodeficiency virus (SIV)-induced encephalitis (Hudson *et al*, 2000). *In vivo* studies in rodents have shown that Tat induces lymphocyte and macrophage infiltration similar to that seen in patients with HAD (Jones *et al*, 1998; Kim *et al*, 2003). Tat can also induce oxidative stress and causes neuronal death by activating the intrinsic apoptotic pathway (Kruman *et al*, 1998).

The transactivation of HIV-1 long terminal repeat (LTR) by Tat is regulated by a complex interaction between cellular transcription factors and the basal transcription machinery. The transcription factors nuclear factor κB (NF-κB) and the positive transcription elongation factor (P-TEFb), consisting of cellular protein cyclin-dependent kinase 9 (cdk9) and cyclin T, have been implicated in the transactivation of HIV-1 LTR by Tat. Several compounds are known to abolish HIV-1 gene expression and replication by inhibiting NF-κB activation (Baba, 2004; Pande and Ramos, 2003). Other studies have shown that the treatment of cells with cdk9-specific inhibitors or the inhibitors of P-TEFb, such as roscovitine, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), and flavopiridol, decreases HIV-1 LTR transcription and viral replication in HIV-1–infected cells (Agbottah *et al*, 2005; Biglione *et al*, 2007; Mancebo *et al*, 1997; Richter and Palu, 2006; Wang *et al*, 2001). Expression of the MCP-1/CCL2 gene has been shown to be directly transactivated by HIV-1 Tat protein in human astrocytes (Abraham *et al*, 2003; Conant *et al*, 1998; Lim and Garzino-Demo, 2000; Sheng *et al*, 2003). In astrocytes, Tat was shown to induce several inflammatory cytokines (Borjabad *et al*, 2009). The mechanisms by which Tat transactivates these genes are not fully understood. Only a few cellular genes are known to be activated by P-TEFb (Garriga *et al*, 2009). In particular, the effect of P-TEFb inhibitors on Tat-induced MCP-1/CCL2 gene expression in astrocytes remains unknown. The aim of this study was to evaluate the effect of various transcription inhibitors, including P-TEFb inhibitors in particular, on Tat-induced MCP-1/CCL2 gene expression in human astrocytes *in vitro*, and to characterize the mechanisms of action of these inhibitors.

Results

Tat expression in human astrocytes, using HIV-based vectors

For the expression of Tat in human astrocytes, we used an HIV-based vector (LTR-Tat-IRES-GFP) in which both the *tat* and green fluorescent protein (*gfp*) open reading frames are transcribed under control of the HIV promoter, to generate a single transcript (Jordan *et al*, 2001). As a control, we used an HIV-based vector encoding *gfp* under control of the EF-1alpha promoter (Wiznerowicz and Trono, 2003). We first checked that the Tat produced was functional, by infecting HeLa P4C5 cells, which carry an HIV-inducible β-galactosidase reporter gene (LTR-lacZ) in their genomes. We observed a dose-dependent increase in β-galactosidase expression, and estimated the titer of the virus at 2×10^7 colony-forming units per ml (CFU/ml). For studies of the effect of Tat, we used primary cultures of human embryonic astrocytes that were about 99% pure according to glial fibrillary acidic protein filament (GFAP) staining. We first determined the infection efficiencies of the lentiviruses, by quantifying GFP levels in the lentivirus-Tat-infected cells by flow cytometry. The percentage of live cells positive for GFP 48 h after infection with a multiplicity of infection (MOI) of 2 CFU/cell was 50% ± 15% ($n = 6$), as determined by fluorescence-activated cell sorting (FACS) analysis (Figure 1A). We then evaluated Tat protein levels in infected primary cells by Western blot analysis.

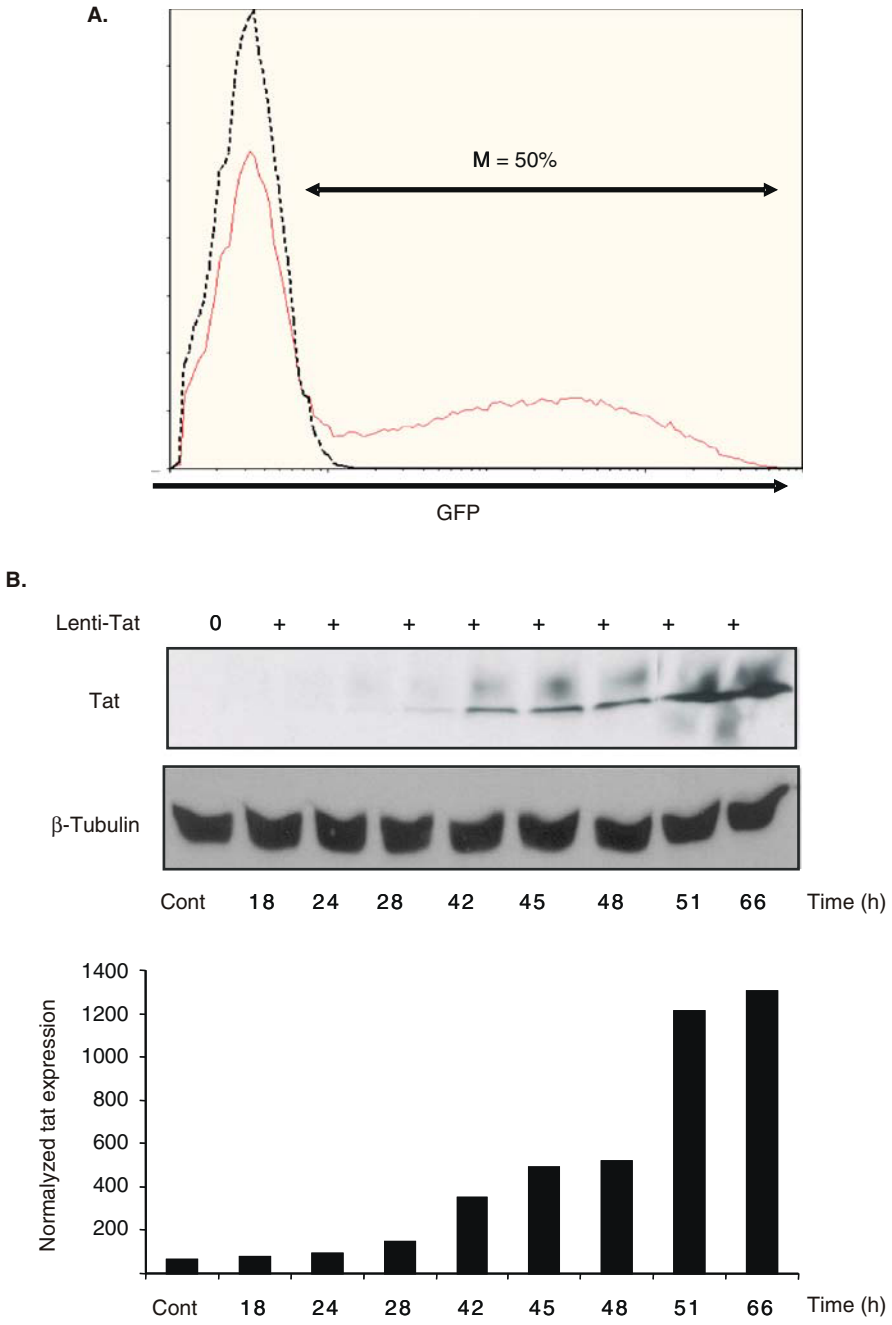

Figure 1 Analysis of *tat* gene expression. (**A**) Flow-cytometric detection of GFP expression 48 h after infection. GFP expression in noninfected control (dotted line) and infected astrocytes (thin line) is shown. M indicates the percentage of cells displaying fluorescence. One experiment representative of six is shown. (**B**) Western blot for Tat. Astrocytes (7×10^5) were infected with lentivirus-Tat, as described in Materials and Methods. Cell lysates were Western blotted for Tat. The blot was stripped and reprobed for β-tubulin, used as a loading control. Tat levels on Western blots were quantified after normalization with respect to β-tubulin, using Image J. One experiment representative of three is shown.

Tat was detectable 28 h post infection and its levels increased to a maximum at 51 h (Figure 1B). As HIV infection induces the up-regulation of GFAP, a hallmark of reactive astrocytes (Anderson *et al*, 2003), we first studied the effects of lentivirus-Tat and Tat protein on GFAP gene expression in human primary astrocytes. We found that neither the lentivirus-Tat nor the Tat protein induced GFAP in human astrocytes. However, infection with a vesicular stomatitis virus G (VSV-G)–pseudotyped HIV-1 virus induced GFAP in these cells, suggesting that a viral gene other than that encoding Tat was involved in up-regulating GFAP in human astrocytes (data not shown).

Tat induces MCP-1/CCL2 in human astrocytes

We assessed the ability of Tat to induce MCP-1/CCL2 expression in human astrocytes by infecting the cells with lentivirus-Tat to allow the intracellular expression of the *tat* gene. The astrocytes were serum-starved 36 h later and culture medium was replaced 48 h after infection as described in Materials and Methods. Both cells and supernatant were collected 6 h later. We then quantified MCP-1/CCL2 mRNA in cells and protein levels in the supernatant by real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. We found that lentivirus-Tat increased 2.5-fold MCP-1/CCL2 mRNA levels, as assessed by rRT-PCR, and approximately doubled MCP-1/CCL2 levels from baseline secretion (20 ng/ml), as detected by ELISA (data not shown). The induction of MCP-1/CCL2 mRNA production was observed 48 h after infection, suggesting that Tat transactivates the MCP-1/CCL2 gene after its expression, which was first detected at 28 h. We investigated whether extracellular Tat could also induce MCP-1/CCL2 in human astrocytes, by studying the effect of a highly pure and biologically active 86-amino acid Tat protein synthesized by solid-phase chemistry. We first evaluated the dose and time dependence of Tat-mediated MCP-1/CCL2 gene expression in primary astrocytes. Significant increases in MCP-1/CCL2 mRNA levels were observed at a concentration of 5 μg/ml Tat, and these mRNA levels were maximal at 6 h. Under these optimal conditions, Tat-induced MCP-1/CCL2 mRNA production by a factor of about 5 (data not shown). Controls were performed by treatment of Tat with 5 μg of trypsin at 37°C for 60 min or heat denaturation at 100°C for 30 min as described (Albini *et al*, 1998), abrogating the Tat response (data not shown).

Thus, in our system, human primary astrocytes were efficiently infected with lentivirus-Tat and both lentivirus infection and the extracellular application of Tat induced MCP-1/CCL2 gene expression.

Study of NF-κB involvement in Tat-induced cytokine expression in astrocytes

It has been shown that, in astroglioma, a form of NF-κB containing the classical alpha complex directly or indirectly interacts with Tat (Taylor *et al*, 1994) and that Tat proteins activate NF-κB and induce IκBα phosphorylation (Mahlknecht *et al*, 2008). We therefore investigated whether NF-κB transcription factor was activated by Tat in human astrocytes, by assessing IκBα phosphorylation by Western blot analysis. The astrocytes were infected with lentivirus-Tat and cell proteins were extracted for the analysis of IκBα phosphorylation. This study was performed 48 h after infection. The levels of IκBα phosphorylation observed were similar to those in control astrocytes (Figure 2A). We then timed the effect of Tat by analyzing IκBα phosphorylation in astrocytes after the extracellular application of Tat protein (5 μg/ml) at various time points during astrocyte culture. As shown in Figure 2B, Western blot analysis demonstrated the presence of intracellular Tat in astrocytes that were treated with the Tat protein. Similarly, as for Tat-infected astrocytes, levels of IκBα phosphorylation did not differ from those in control astrocytes after stimulation with extracellular Tat at various times from 30 min to 3 h (data not shown). Even when MCP-1/CCL2 induction was strongest, Tat did not increase the level of IκBα phosphorylation 6 h after treatment (Figure 2B).

We used the selective IKK2 inhibitor SC-514, which inhibits the transcription of NF-κB–dependent genes, and studied its effect on MCP-1/CCL2 gene expression in Tat-stimulated primary astrocytes, to confirm the absence of NF-κB activation. At the highest dose used (25 μM), SC514 did not significantly decrease Tat-induced MCP-1/CCL2 mRNA levels, after treatment for 2 to 25 h (Figure 2C). Thus, Tat did not increase IκBα phosphorylation, suggesting that NF-κB was not involved in Tat-induced MCP-1/CCL2 gene expression in serum-starved cultured astrocytes.

Effect of dn-cdk9 and siRNA-cdk9 on MCP-1/CCL2 induction by Tat

As Tat protein is known to strongly increase the processivity of RNA polymerase II, by recruiting the cyclin T1/cdk9 (P-TEFb), we decided to evaluate the possible role of cdk9 in the induction of MCP-1/CCL2 gene expression by Tat. We investigated the role of cdk9, using a specific dominant-negative mutant (dn-cdk9) and an siRNA specific for cdk9. We transfected astrocytes with a plasmid encoding dn-cdk9, using the Primary Mammalian Neurons Nucleofector Kit for Nucleofection, as described in Materials and Methods. We assessed the efficiency of primary astrocyte transfection, by measuring GFP expression after transfection with pMaxGFP DNA, by flow cytometry. We obtained 80% ± 5% ($n = 3$) GFP expression in astrocytes 48 h after transfection (Figure 3A).

We transfected astrocytes with pFlag-CMV2-hCdk9-D167N, containing a human dn-cdk9 gene. We treated the transfected cells with extracellular Tat protein for 6 h and analyzed MCP-1/CCL2 mRNA levels by rRT-PCR. MCP-1/CCL2 mRNA levels in GFP-transfected astrocytes were not significantly different ($P > .05$) from those in control untransfected astrocytes. MCP-1/CCL2 mRNA levels were significantly 2-fold lower in dn-cdk9–transfected cells than in control astrocytes. Tat induced MCP-1/CCL2 mRNA in control astrocytes, but not in dn-cdk9–transfected astrocytes (Figure 3B). ELISA showed that the decrease in MCP-1/CCL2 mRNA levels induced by dn-cdk9 resulted in a parallel decrease

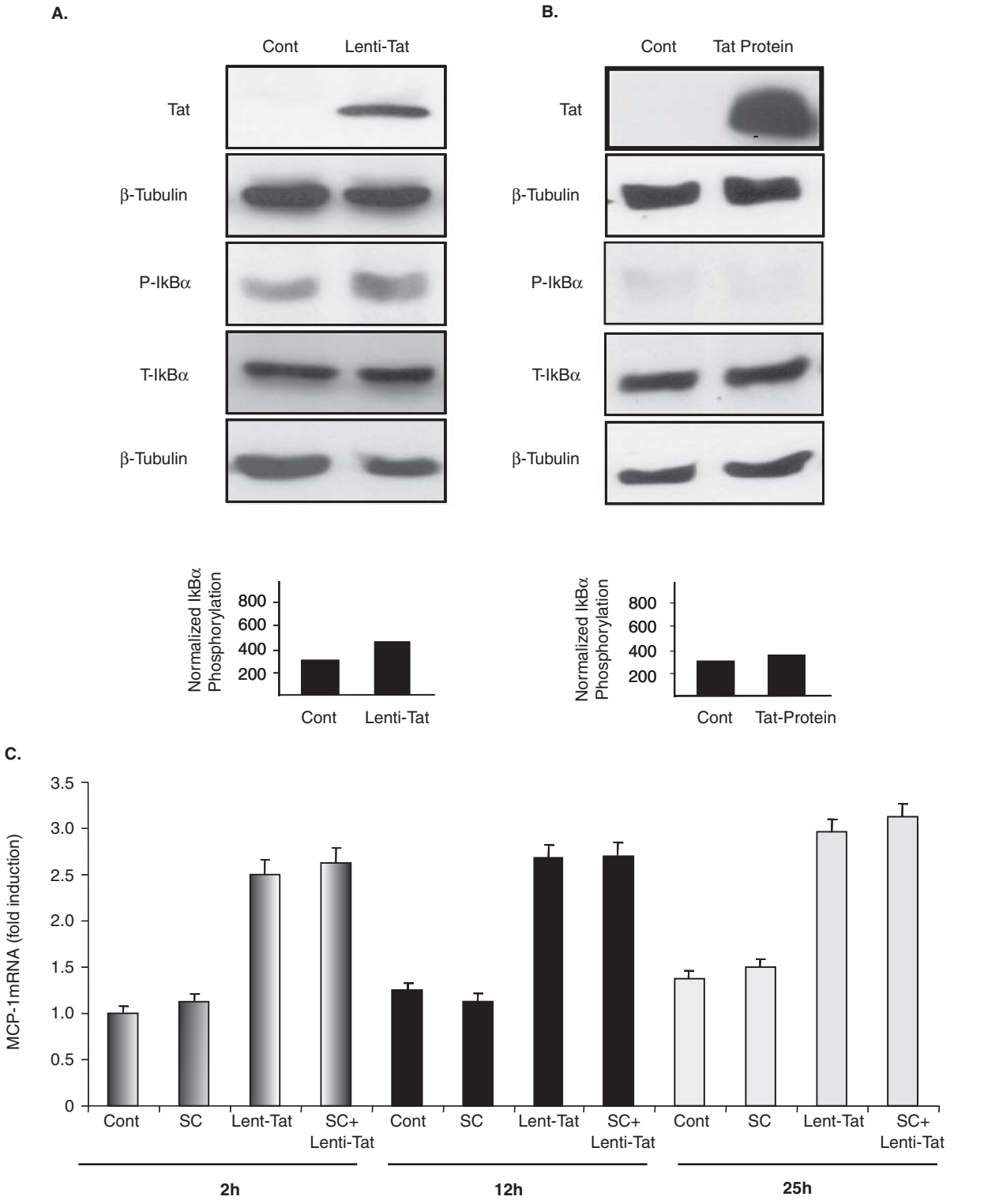

Figure 2 Western blot analysis of IκBα phosphorylation levels in Tat-treated or Tat-infected astrocytes. Astrocytes (7×10^5) were infected with lentivirus-Tat (**A**) or treated with Tat (**B**). Western blots for Tat, phospho-IκBα (P-IκBα), and IκBα were performed on cell lysates. The blot was stripped and reprobed for β-tubulin, used as a loading control. Western blots were quantified for phospho-IκBα after normalization with respect to β-tubulin, using Image J. One experiment representative of five is shown. (**C**) Effect of SC-514 on MCP-1/CCL2 mRNA. Cultured astrocytes were infected with lentivirus-Tat as described in Materials and methods. These cells were treated for 2, 12 and 25 h with SC-514 (25 μM, SC). MCP-1/CCL2 mRNA levels were determined by rRT-PCR analysis. Results were normalized with respect to 18 S rRNA levels and are expressed as fold induction with respect to basal conditions at 2 h, set at 1. Each point represents the mean ± SEM of n = 3 independent determinations performed in duplicate.

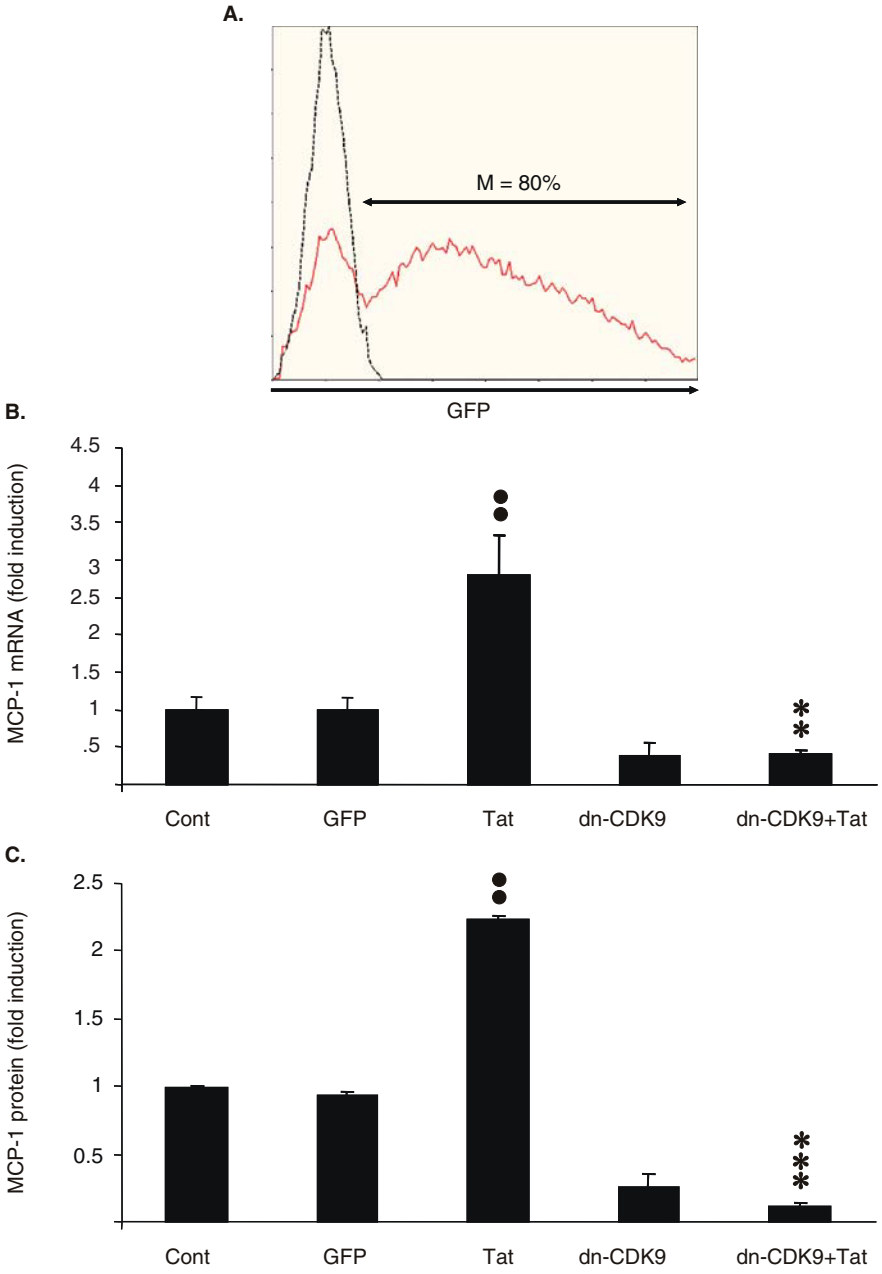

Figure 3 Effects of dn-cdk9 on expression of the MCP-1/CCL2 genes. (**A**) Flow cytometric detection of GFP expression 48 h after transfection. GFP expression on nontransfected control (dotted line) or pMaxGFP-transfected astrocytes (thin line) are shown. M indicates the percentage of cells displaying fluorescence. Effect of dn-cdk9 on MCP-1/CCL2 mRNA (**B**) and MCP-1/CCL2 protein (**C**). Astrocytes were transfected with dn-cdk9 by nucleofection, 48 h before treatment with Tat. Tat protein was added to the cell culture medium (5 µg/ml) for 6 h. Data for the rRT-PCR analysis of MCP-1/CCL2 mRNA levels were normalized to the levels of 18 S rRNA and are expressed as fold induction with respect to basal conditions, set at 1. MCP-1/CCL2 concentrations in the culture supernatant were analyzed by ELISA and are expressed as fold induction with respect to basal conditions, set at 1. Data are mean ± SEM of $n = 3$ independent determinations performed in duplicate. $^{\bullet\bullet}P < .01$ versus basal values; $^{**}P < .01$ and $^{***}P < .001$ versus Tat-induced values.

in MCP-1/CCL2 secretion (Figure 3C). These results suggest that cdk9 is involved in both Tat-induced and basal transcription of the MCP-1/CCL2 gene in human astrocytes.

We then decided to study the effect of down-regulating cdk9 mRNA specifically with siRNA, to confirm that cdk9 inhibition regulated Tat-induced MCP-1/CCL2 gene expression. For transfection, we used the same Nucleofection method as for the transfection of astrocytes with DNA. We determined the optimal siRNA concentration for cdk9 mRNA degradation by quantifying cdk9 mRNA levels by rRT-PCR after transfection with various concentrations of a smart pool of siRNA (100 nM, 250 nM, and

1 μM). A concentration of 250 nM induced a maximal (80%) decrease in cdk9 mRNA levels. Time-course studies for identifying the optimal time point at which to knock down cdk9 mRNA and cytokine levels were also performed. We assessed the degradation of cdk9 mRNA, by estimating percentage of cdk9 mRNA levels. Cdk9 degradation was strongest (83% ± 4%) 48 h after transfection with siRNA cdk9 (Figure 4A). A control smart pool of nontargeting siRNA had no significant effect on cdk9 mRNA levels. We also evaluated the decrease in cdk9 protein levels by Western blot analysis. We detected the two known isoforms of cdk9 (42 and 55 kDa) and found that they were equally abundant in primary astrocytes. There was a 87% decrease in both isoforms 48 h after transfection with cdk9 siRNA (Figure 4B). We then studied the effect of the cdk9 siRNA on Tat-induced MCP-1/CCL2 60 h after transfection. An analysis of MCP-1/CCL2 mRNA in cells transfected with the cdk9 siRNA revealed a significant decrease in the mean concentration of MCP-1/CCL2, 6 h after Tat treatment (Figure 4B). ELISA showed that the decrease in MCP-1/CCL2 mRNA levels induced by cdk9 degradation resulted in a parallel decrease in MCP-1/CCL2 secretion after transfection with siRNA (Figure 4C). Cdk9 knockdown with siRNA confirmed that Tat-induced MCP-1/CCL2 levels depended on cdk9 activity levels in human astrocytes.

Effect of pharmacological inhibitors of cdk on LTR promoter transcription and MCP-1/CCL2 induction by Tat

Roscovitine, DRB, and flavopiridol pharmacological inhibitors of cdk have been used to block the phosphorylation of RNA polymerase II by the cyclin T/cdk9–Tat complex and the elongation of transcription resulting in inhibition of HIV-1 replication. We therefore studied the impact of these cdk inhibitors on LTR promoter transcription and the induction of MCP-1/CCL2 by Tat in primary human astrocytes. We first analyzed the effects of roscovitine, DRB, and flavopiridol on Tat-induced HIV-1 LTR expression. HeLa cells, in which roscovitine, DRB, and flavopiridol have anti-HIV activity, were used as a control (Biglione *et al*, 2007). The cells were infected with both lentivirus carrying the HIV-1 LTR luciferase reporter gene and lentivirus-Tat and were treated with roscovitine, DRB, and flavopiridol. As a control, we used a self-inactivating (SIN) lentiviral vector, in which the luciferase gene was under the control of the internal human cytomegalovirus (CMV) immediate-early promoter. Coinfection with lentivirus-Tat increased luciferase activities to levels 43 ± 5 times higher than those obtained with lentivirus LTR-luciferase alone in HeLa cells, and 225 ± 15 times higher than those obtained with lentivirus LTR-luciferase alone in astrocytes. The application of roscovitine, DRB, or flavopiridol at concentrations known to inhibit cdk9 (10 μM, 25 μM, and 100 nM, respectively) (Biglione *et al*, 2007; Shan *et al*, 2005), at the time of infection, inhibited luciferase activities in HeLa cells, 55% inhibition observed for roscovitine, 73% for DRB, and 50% for flavopiridol (Figure 5A). By contrast, DRB gave 59% inhibition in astrocytes, flavopiridol 80%, whereas roscovitine gave no inhibition (Figure 5B). CMV-luciferase activity was not increased by coinfection with lentivirus-Tat, and was not affected by roscovitine, DRB, or flavopiridol (Figure 5C). In conclusion, treatment with DRB, roscovitine, and flavopiridol inhibited Tat induction of the HIV-1 LTR promoter in HeLa cells, whereas only DRB and flavopiridol affected that in astrocytes.

We then studied the effects of roscovitine, DRB, and flavopiridol on levels of Tat-induced cytokines. MCP-1/CCL2 levels in control astrocytes were not significantly different ($P > .05$) from those in roscovitine-, DRB-, and flavopiridol-treated astrocytes. An analysis of MCP-1/CCL2 mRNA levels in cells transfected with lentivirus-Tat or treated with extracellular Tat revealed a significant 2- to 3-fold decrease in the mean concentration of MCP-1/CCL2, 6 h after the application of cdk inhibitors (Figure 6A). This decrease persisted over the 25 h of analysis in cells transfected with lentivirus-Tat (data not shown). ELISA analysis showed that the decrease in MCP-1/CCL2 mRNA levels induced by cdk inhibitors resulted in a parallel decrease in MCP-1/CCL2 secretion levels (Figure 6B).

Tat enhances cdk9 binding to the MCP-1/CCL2 promoter

Our results strongly suggest the implication of cdk9 in Tat-induced MCP-1/CCL2 expression. To demonstrate that cdk9 is directly involved in the induction of MCP-1/CCL2 by Tat, we performed ChIP assays. Binding of cdk9 to MCP-1/CCL2 promoter was measured as described in Materials and Methods. We compared the fold-increase in signal obtained by using anti-cdk9 antiserum versus control isotype immunoglobulin G (IgG). We observed a 4-fold increase in cdk9 binding to the MCP-1/CCL2 promoter in lentivirus-Tat–infected astrocytes but not in control astrocytes infected with lentivirus LTR-luciferase alone (Figure 7A). A 7-fold increase in cdk9 binding to the HIV-1 LTR in lentivirus-Tat–infected astrocytes was observed, but not in astrocytes infected with lentivirus LTR-luciferase alone (Figure 7B). We conclude from these experiments that cdk9 bind to MCP-1/CCL2 promoter and to HIV-1 LTR in human astrocytes in the presence of Tat but not in absence of Tat.

Discussion

We have shown that the molecular mechanisms of the induction of MCP-1/CCL2 gene transcription by

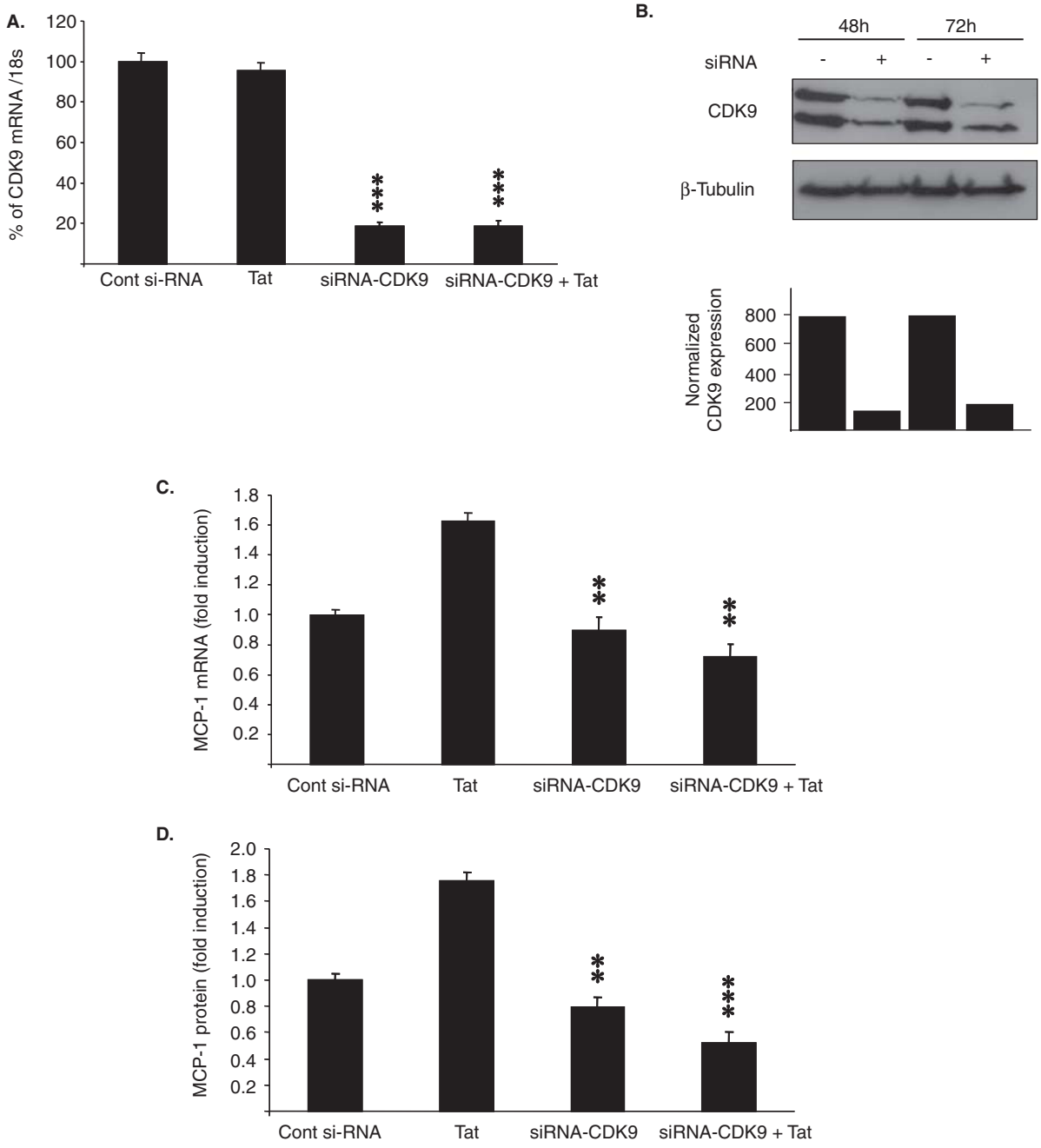

Figure 4 Effects of siRNA-cdk9 on MCP-1/CCL2 gene expression. The effect of siRNA-cdk9 on cdk9 mRNA levels (**A**) and cdk9 protein level (**B**) were analyzed. Western blots were quantified for cdk9 after normalization with respect to β-tubulin, using Image J. One experiment representative of three is shown (**B**). The effect of siRNA-cdk9 on cdk9 protein levels was analyzed (**B**). Effects of siRNA-cdk9 on Tat-induced MCP-1/CCL2 mRNA production (**C**) and MCP-1/CCL2 secretion (**D**). Cultured astrocytes were transfected with siRNA-cdk9 or a control nontargeting siRNA (Cont siRNA) by nucleofection 48 h before treatment with Tat at 5 µg/ml. The data obtained in rRT-PCR analyses of cdk9 and MCP-1/CCL2 mRNA levels were normalized to the levels of 18 S rRNA and are expressed as fold induction with respect to basal conditions, set at 1. MCP-1/CCL2 concentrations in culture supernatant were analyzed by ELISA and are expressed as fold induction with respect to basal conditions, set at 1. Data are means ± SEM of $n = 3$ independent determinations performed in duplicate. $^{**}P < .01$ and $^{***}P < .001$ versus Tat-induced values.

Tat in human astrocytes involve cdk9. Several studies have shown that the elongation of transcripts of the HIV genome requires the cyclin T/cdk9 complex. Our results suggest that the specific inhibition of cdk9 blocks not only HIV replication, but also the production of MCP-1/CCL2, a pathophysiologically important chemokine linked to the effects of viral proteins, such as Tat. We evaluated the effects of inhibiting cdk9 in primary astrocytes on Tat-induced cytokine levels, using two specific inhibitors, i.e., a

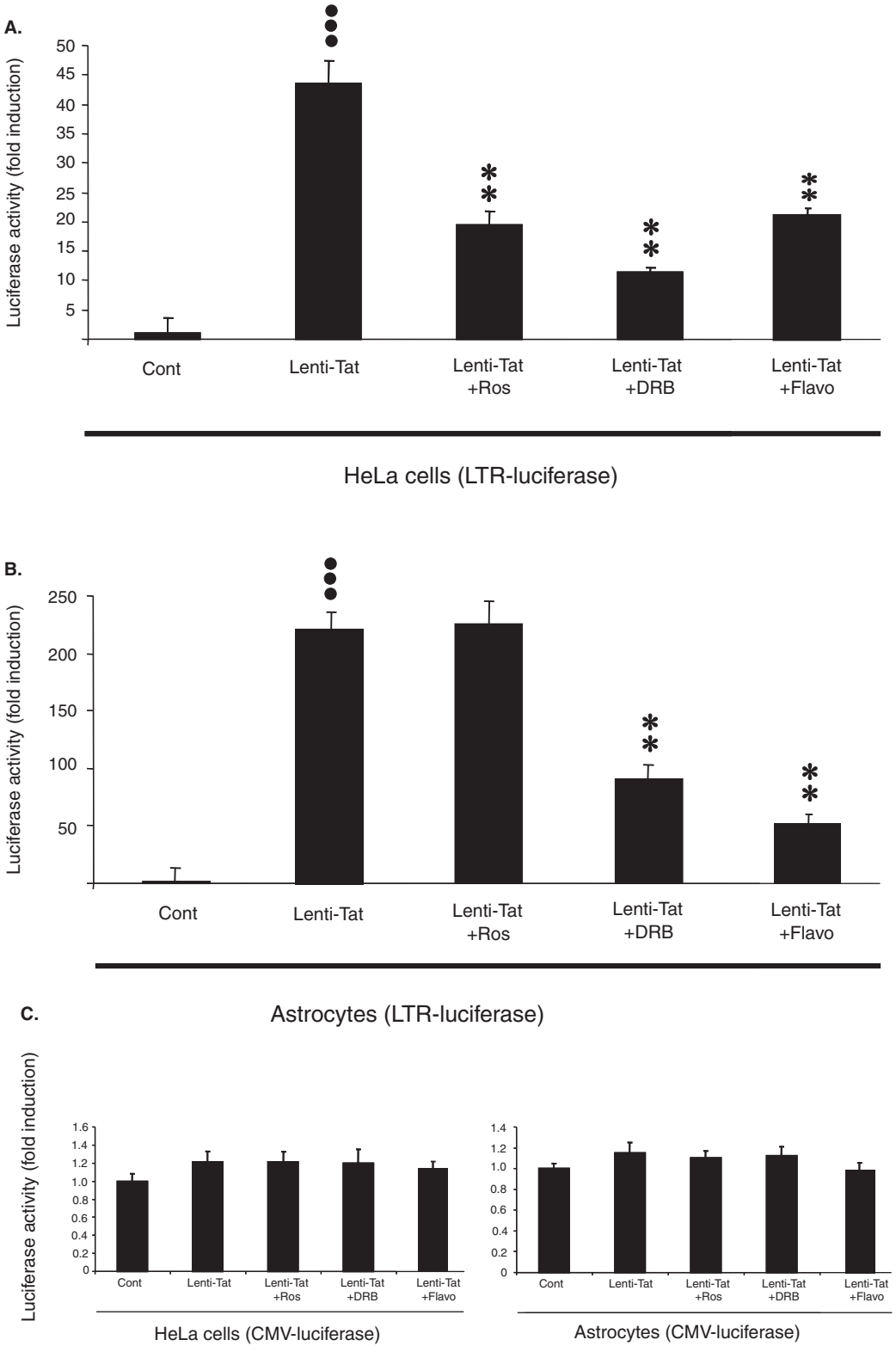

Figure 5 Inhibition of HIV transcription by cdk inhibitors. HeLa cells (**A**) and astrocytes (**B**) were infected with lentivirus carrying the HIV-1 LTR luciferase reporter gene or coinfected with the lentivirus encoding Tat protein. As a control, the cells were infected with lentivirus expressing the CMV luciferase or coinfected with lentivirus-Tat (**C**). We added 10 μM roscovitine, 25 μM DRB, or 100 nM flavopiridol at the time of infection. After treatment, luciferase activity and protein concentration were determined in cell extracts, for the calculation of luciferase activity/mg protein. Each data set was normalized, with 1 equal to the activity of cells infected with lentivirus expressing the luciferase reporter gene alone. Data are mean ± SEM of $n = 4$ independent determinations performed in duplicate. $^{***}P < .001$ versus basal values; $^{**}P < .01$ versus Tat-induced values.

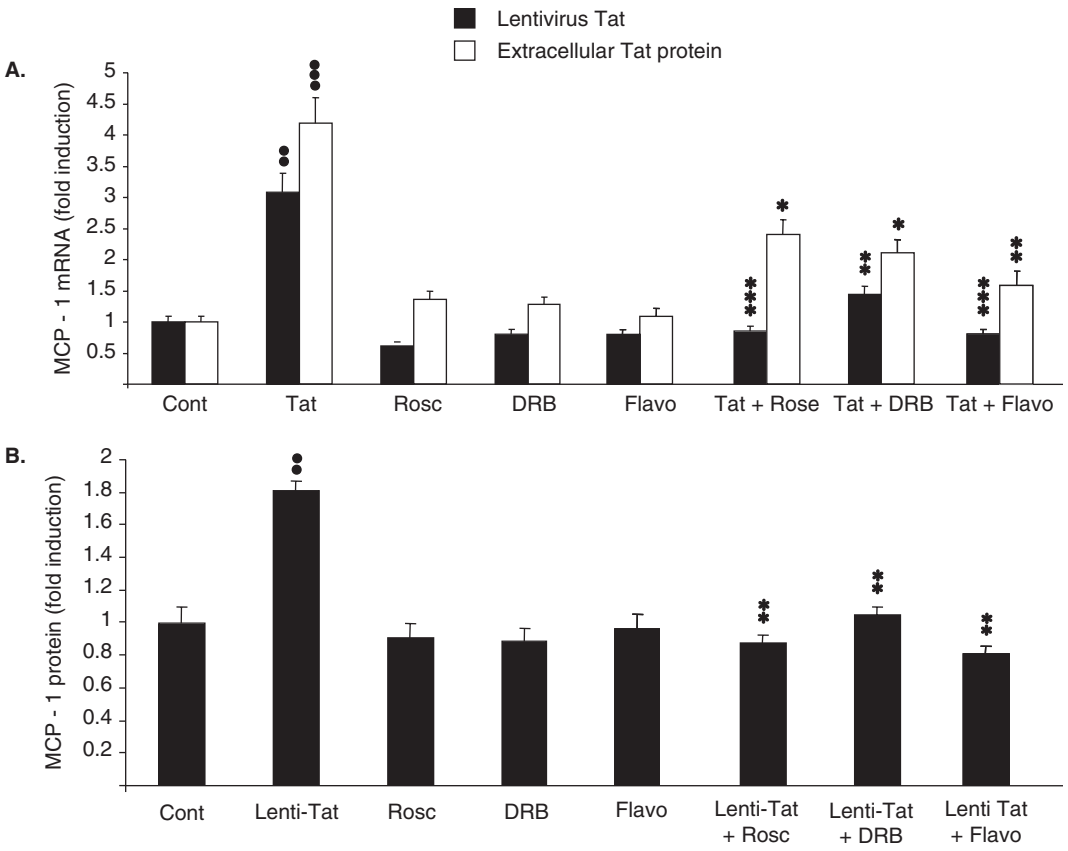

Figure 6 Effects of roscovitine, DRB, and flavopiridol on Tat-induced MCP-1/CCL2 gene expression. Effects of cdk inhibitors on Tat-induced MCP-1/CCL2 (**A**) and MCP-1/CCL2 secretion (**B**). Cultured astrocytes were infected with lentivirus-Tat 48 h before treatment or were incubated with extracellular Tat (5 μg/ml) for 6 h at the time of treatment. These cells were treated for 6 h with roscovitine (10 μM), with DRB (25 μM) or with flavopiridol (100 nM). MCP-1/CCL2 mRNA levels were determined by rRT-PCR analysis. Results were normalized with respect to 18 S rRNA levels and are expressed as fold induction with respect to basal conditions, set at 1. Each point represents the mean ± SEM of $n = 3$ independent determinations performed in duplicate. Negative results were obtained with ddH_2O controls, in all runs. MCP-1/CCL2 concentrations in culture supernatants were analyzed by ELISA and are expressed as fold induction with respect to basal conditions, set at 1. Data are means ± SEM of $n = 3$ independent determinations performed in duplicate. $^{••}P < .01$ and $^{•••}P < .001$ versus basal values; $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ versus Tat-induced values.

dominant-negative mutant and siRNA. The dn-cdk9 mutant has been used to inhibit Tat transactivation and HIV-1 replication both in cell lines (Foskett *et al*, 2001) and in peripheral blood lymphocytes (Salerno *et al*, 2007). Similarly, an siRNA targeting cdk9 has been shown to be effective at inhibiting HIV-1 replication in HeLa cells without affecting cell viability (Chiu *et al*, 2004). We found that MCP-1/CCL2 induction by Tat was inhibited by both siRNA and dn-cdk9. In addition, in dn-cdk9–transfected cells without Tat stimulation, MCP-1/CCL2 mRNA levels were significantly lower than those in control astrocytes, suggesting that cdk9 is also involved in the basal expression of these genes. It was suggested that dn-cdk9 would be more effective to block cdk9 activity than siRNA (Garriga *et al*, 2009). Interestingly, in a previous study, the targeted expression of the human cyclin T1 gene in $HIV_{JR\text{-}CSF}$ transgenic mice has been shown to increase MCP-1/CCL2 production in monocytes/macrophages (Sun *et al*, 2006), suggesting that MCP-1/CCL2 gene expression is stimulated by Tat and P-TEFb.

We also studied the possible involvement of the transcription factor NF-κB, which plays an important role in the up-regulation of many cytokines (Blaecke *et al*, 2002), including MCP-1/CCL2, which is encoded by a gene containing two closely spaced NF-κB sites (Mori *et al*, 2000). The treatment of human astrocytes with an IKK2 inhibitor had no effect on the induction of MCP-1/CCL2 by Tat. We also observed neither IκBα phosphorylation nor IκBα degradation after treatment with Tat or expression of the gene encoding Tat in astrocytes. These results contrast with previous findings suggesting that Tat protein activates NF-κB in human astrocytes cultured in the presence of 10% fetal calf serum (FCS), with activation potentially related to these culture conditions (Conant *et al*, 1998). Indeed, when we cultivated astrocytes in the presence of 10% FCS, the phosphorylation of IκBα is also

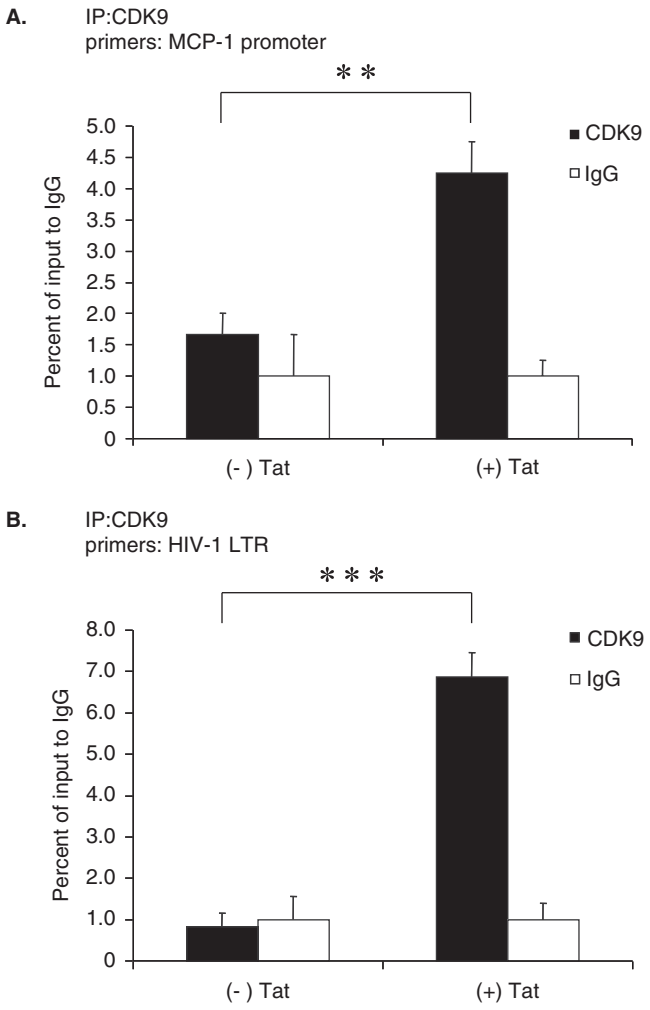

Figure 7 Cdk9 recruitment to the MCP-1/CCL2 and HIV-1 LTR promoters in astrocytes in the presence of Tat. ChIP assays were performed in lentivirus-Tat–infected astrocytes and in control astrocytes infected with lentivirus LTR-luciferase alone. (**A**) The data indicated that the cdk9 transcription factor bound to the MCP-1/CCL2 promoter in astrocytes expressing Tat but not in control astrocytes. (**B**) Cdk9 transcription factor bound to the HIV-1 LTR promoter in astrocytes expressing Tat but not in control astrocytes. CDK9, immunoprecipitation (IP) with anti-cdk9; IgG, IgG immunoprecipitation with an isotype control. Values were expressed as percent of amplified chromatin obtained after immunoprecipitation with the cdk9-specific antibody (anti-cdk9; dark column) to input chromatin, normalized to amplified chromatin immunoprecipitated with IgG (white column), as determined by quantitative PCR. Data are means ± SEM of $n = 3$ independent determinations performed in duplicate. $^{**}P < .01$ and $^{***}P < .001$ versus Tat-induced values.

observed (data not shown). It has been shown that Tat does not activate NF-κB in neural cells that differentiated or have been cultured in serum-free medium (New *et al*, 1998; Ramirez *et al*, 2001). Prolonged NF-κB inactivation has also been reported in serum-deprived neural cells, due to a sustained increase in the levels of IκBα and IκBβ (Kovacs *et al*, 2004). In humans, NF-κB activation in neurons and astrocytes in the brains of children with HIV or in patients with HAD is similar to that in controls (Dollard *et al*, 1995; Rostasy *et al*, 2000). These data suggest that, in the absence of serum, NF-κB is not stimulated by Tat in neural cells.

As P-TEFb regulates the productive elongation phase of RNA polymerase II activity, which is sensitive to roscovitine, DRB, and flavopiridol through cdk9 inhibition (Biglione *et al*, 2007; Pumfery *et al*, 2006), we decided to study the effects of these inhibitors on HIV-1 LTR and MCP-1/CCL2 levels. We used roscovitine at a concentration of 10 μM, DRB at a concentration of 25 μM, and flavopiridol at a concentration of 100 nM, these concentrations being the lowest causing nearly 100% decrease in Tat-induced MCP-1/CCL2 levels and known to inhibit cdk without toxicity in normal human astrocytes (Di Giovanni *et al*, 2005; Kim *et al*, 2004) and neural cells (Morris and Geller, 1996). We first evaluated the effect of these inhibitors on Tat-induced HIV-1 LTR activity. Using lentivirus infection, we showed that Tat induced basal HIV-1 LTR activity by a factor of 225 ± 15 times in human astrocytes and 43 ± 5 times in HeLa cells. These results confirm those of other studies, demonstrating that HIV-1 LTR transcription begins immediately after the infection

of the cell by the virus and increases after Tat synthesis (Chipitsyna *et al*, 2006). Interestingly, if DRB or flavopiridol were applied at the time of infection, HIV-1 LTR activity was inhibited in both astrocytes and HeLa cells. By contrast, roscovitine treatment was effective only in HeLa cells. Treatment with DRB or roscovitine 48 h after infection did not affect the Tat-induced expression of HIV-1 LTR in astrocytes, whereas significant inhibition was observed in HeLa cells. In addition, our results show that the induction of HIV-1 LTR promoter by Tat was stronger in astrocytes than in HeLa cells. Interestingly, we found that the two isoforms of cdk9 (42 and 55 kDa) were equally abundant in primary astrocytes, whereas the 55-kDa form represents only 10% to 20% in HeLa cells (Liu and Herrmann, 2005; Shore *et al*, 2005). This difference may account for the higher HIV transcription in astrocytes than in HeLa cells. This may also be explained by a possible differential involvement of transcription factors other than P-TEFb in HIV-1 LTR transcription.

Further experiments are required to account for the difference in reactivity between astrocytes and HeLa cells in response to pharmacological inhibitors of cdk.

Roscovitine, DRB, and flavopiridol decreased the Tat-induced production of MCP-1/CCL2 in astrocytes infected with lentivirus-Tat or treated with extracellular Tat. These results are consistent with the effect of specific cdk9 inhibitors. Roscovitine is considered to be a selective inhibitor of cdks, primarily inhibiting cdk2, cdk5, cdk7, and cdk9 (McClue *et al*, 2002). DRB, a classical transcription inhibitor, principally inhibits cdk9 (Marshall and Price, 1995; Zhu *et al*, 1997). However, it also inhibits other protein kinases involved in cellular metabolism, such as casein kinase type II (Zandomeni *et al*, 1986). Flavopiridol is known to be the most effective and specific P-TEFb inhibitor currently identified (Biglione *et al*, 2007).

To ascertain the direct implication of cdk9 in Tat-induced MCP-1/CCL2 expression, it was necessary to study the binding of cdk9 to the MCP-1/CCL2 promoter region. To demonstrate the presence of cdk9 in the proximity of the MCP-1/CCL2 promoter, we performed ChIP assay. We found that cdk9 binding to the MCP-1/CCL2 promoter was significantly enhanced in astrocytes expressing Tat. This result shows that cdk9 is directly involved in Tat-mediated MCP-1/CCL2 induction. In our study, we used HIV-1 LTR as a control. Interestingly, it was already shown that Tat and cdk9 stimulate HIV-1 promoter in human astrocytes (Kaniowska *et al*, 2006). As already shown in Jurkat T cells (Kim *et al*, 2006), we found that cdk9 bind to HIV-1 LTR promoter only in the presence of Tat in human astrocytes.

Previous work has shown that the specific inhibition of cdk9 with a dominant-negative mutant of the enzyme does not inhibit T-cell activation, had little effects on RNA polymerase II transcription, RNA synthesis, proliferation, and viability of peripheral blood lymphocytes, but does selectively inhibit HIV-1 replication, suggesting that specific pharmacological inhibitors of cdk9 might be effective drugs against HIV-1 infection (Salerno *et al*, 2007). Interestingly, it was suggested that small pharmacological compounds targeting cdk9 with enhanced selectivity could be developed (Canduri *et al*, 2008). Our results suggest that, in astrocytes, such a strategy may not only decrease HIV-1 LTR expression but also decrease the induction of cytokines induced by HIV-1 Tat proteins, making this approach potentially even more interesting.

Materials and methods

Human astrocyte cultures and cell lines

Authorization for experimentation with fetal tissue was obtained from the local ethics committee, in accordance with French guidelines. Brain tissue was removed from embryos aborted at 7 to 10 weeks of gestation. Adherent blood vessels and meninges were removed. The brain tissue was then mechanically dissociated and plated at a density of 5×10^5 cells/ml on 10 cm diameter Petri dishes coated with DL-polyornithine (40 kDa, 5 μg/ml), as previously described (Deshayes *et al*, 2004). The medium used was Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% heat-inactivated FCS, and antibiotics (penicillin at a concentration of 100 IU/ml, streptomycin at 100 μg/ml). Cells were cultured for 28 to 35 days at 37°C in a humidified atmosphere containing 5% CO_2, 95% air, and the medium was changed every 3 days. The cells were subcultured when they reached confluence, every 2 to 3 weeks, by dilution in fresh medium (1:2 or 1:3), using trypsin-EDTA to detach the cells. A cell population enriched in astrocytes was generated by subculturing cells at a 1:10 dilution. Astrocyte cultures were stained for GFAP, using a mouse monoclonal antibody (1:200; DAKO A/S, Glostrup, Denmark). Under these culture conditions, GFAP was detected in 90% to 99% according to batch of cells. HEK293T and HeLa cells were cultured in DMEM supplemented with 10% FCS and antibiotics. P4C5 is a cell line derived from HeLa cells in which Tat transactivation induces expression of the *Escherichia coli lacZ* gene under the control of HIV-1 LTR promoter (Pleskoff *et al*, 1997). This cell line was maintained in complete DMEM supplemented with G418 (Geneticin; 1 mg/ml; Gibco) and hygromycin (300 μg/ml).

Compounds

DRB and flavopiridol was obtained from Sigma Aldrich (St Quentin-Fallavier, France) and resuspended in ethanol and in dimethyl sulfoxide (DMSO) to generate a 10 mM and a 2 mM stock

solution, respectively. Roscovitine, synthesized and resuspended in DMSO to generate 10 mM stock solutions, was generously provided by Dr. Laurent Meijer (CNRS, Roscoff, France). SC514 was obtained at a concentration of 25 mM in DMSO from Calbiochem (VWR International S.A.S, Fontenay sous Bois, France). The full-length Tat protein of the HIV-1 Lai strain (^{1}MEPVDPRLEPWKHPGSQPKTACTTCYCKKCCFHCQVCFTTKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQPTSQPRGDPTGPKE^{86}) was chemically synthesized and purified, as previously described (Chaloin *et al*, 2005). Tat samples were stored lyophilized, at −80°C, to prevent oxidation.

Lentiviral plasmids
The pEV731 plasmid, bearing an LTR-Tat-IRES-GFP construct—in which the open reading frames of both the *tat* and *gfp* genes are transcribed by the HIV promoter to generate a single transcript—was obtained from Dr Eric Verdin (University of San Francisco) (Jordan *et al*, 2001). For translation of the downstream *gfp* open reading frame, an internal ribosome entry site was inserted between the *tat* and *gfp* open reading frames. The 101-amino acid Tat protein is encoded by a gene with two exons. The first exon encodes the first 72 amino acids of Tat, the remaining amino acids being encoded by the second exon. The pWPXL-GFP plasmid, in which *gfp* gene expression is directed by the EF-1alpha promoter, was obtained from Prof. Didier Trono (EPFL, Switzerland) (Wiznerowicz and Trono, 2003). This vector allowing expression of *gfp* gene was used as a control relative to LTR-Tat-IRES-GFP vector. pHR'P-Luc, containing a luciferase reporter gene under the control of the HIV-1 LTR promoter, and pHR'P-SIN-18-Luc, a SIN lentiviral vector carrying a luciferase reporter gene under control of an internal human CMV immediate-early promoter, were obtained from Dr Jonathan Karn (Case Western Reserve University, Cleveland, USA), (Tyagi and Karn, 2007). All plasmids were purified with the EndoFree plasmid maxi kit (Qiagen S.A., Courtaboeuf, France).

Production of HIV-based vectors and cell infections
HIV-based vectors were generated by transfecting HEK293T cells with plasmids. Viral particles containing the HIV-based vector LTR-Tat-IRES-GFP construct or the HIV-based vector-GFP construct were obtained by transfecting 4×10^6 HEK293T cells with the lentiviral plasmids (50 μg), pCMV-R8.91 (50 μg) and a VSV-G expression plasmid (pCMV-VSVG, 10 μg) in a 150-cm^2 flask. The medium was replaced after 16 h, and supernatants containing viral particles were harvested 24 and 48 h later. Supernatants were passed through filters with 0.45-μm pores, immediately frozen and stored at −80°C. The number of HIV-based vector LTR-Tat-IRES-GFP construct-containing infective particles per ml was established by infecting 1×10^5 P4C5 cells with various amounts of viral suspension. The number of HIV-based vector-GFP construct expressing infective particles per ml was determined by infecting 1×10^5 HeLa cells and analyzing GFP levels 2 days later, by fluorescence microscopy. In infection experiments, 1 × 105 P4C5 or HeLa cells were seeded in DMEM/10% FCS in 35 mm diameter Petri dishes, 24 h before infection. The cells were then overlaid with 1 ml of viral supernatant, at dilutions of 1:10 to 1:10,000 in 1 ml of medium, and incubated overnight at 37°C. The medium was then removed and the cells were incubated with fresh medium supplemented with 10% FCS for 24 to 48 h. Infected cells were identified based on their β-galactosidase activity, by 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) staining, as previously described (Solly *et al*, 2005). Viral titers are expressed in CFU/ml. GFP virus titer was determined from the number of GFP-positive colonies.

Envelope-minus ('delta env') VSV-G–pseudotyped HIV-1 virus, produced by the transient transfection of HEK293T cells, was generously provided by Dr. Pierre Charneau, Institut Pasteur, France (Arhel *et al*, 2006).

Human astrocyte infection and treatments
In infection experiments, astrocytes were seeded in 6-well plates coated with DL-polyornithine, at a density of 5×10^5 cells/well. The following day, cells were transduced overnight with HIV-based vectors at the same final concentration (10^6 CFU/ml; MOI = 2 CFU/cell) in 1 ml of medium. The medium was replaced the next day. In a first series of experiments, we incubated astrocytes in culture medium containing either 10% or 0.5% FCS and measured MCP-1/CCL2 levels in cell supernatants by ELISA. The levels of MCP-1/CCL2 induced in lentivirus-Tat–infected astrocytes were higher in the presence of 0.5% FCS than in the presence of 10% FCS (data not shown). This stronger induction probably results from basal levels of MCP-1/CCL2 being much lower in serum-starved astrocytes than in the presence of 10% FCS. Therefore, for all experiments, the cells were cultured with 10% FCS for 36 h, and then with 0.5% FCS for 12 h. The next day, these cells were treated with roscovitine, DRB, or flavopiridol for various periods of time, in the presence of 0.5% FCS. Cell-free supernatants were harvested for MCP-1/CCL2 ELISA, and cells were used to prepare total RNA. Controls were performed by infecting astrocytes with lentivirus expressing GFP. For experiments with synthetic Tat protein, the cells were cultured with 0.5% FCS for 12 h before replacing the medium with Tat protein in 0.5% FCS/DMEM.

MCP-1/CCL2 chemokine measurements
We quantified MCP-1/CCL2 with a human MCP-1/CCL2 ELISA development kit (PeproTech France, Neuilly-Sur-Seine, France) and a sandwich ELISA

method, used according to the manufacturer's instructions. Briefly, 96-well microtiter plates (Nunc MaxiSorp; VWR International, Fontenay-sous-Bois, France) were coated with rabbit anti-hMCP-1/CCL2 antibody by overnight incubation at room temperature. The plates were washed with phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (PBS-T) and blocked by incubation for 1 h at room temperature in 1% BSA in PBS. A standard curve was constructed with human recombinant MCP-1/CCL2. After 2 h of incubation with samples at 37°C, the plates were washed with PBS-T. A biotinylated polyclonal antibody against MCP-1/CCL2 was then added and the plates were incubated for 2 h at room temperature. The plates were washed, avidin–horseradish peroxidase conjugates were added and the plates were incubated for a further 30 min at room temperature. The plates were washed again; 100 µl of azinoethylbenzthiazolinesulfonic acid substrate solution (ABTS; Sigma Aldrich) was added, and the plates were incubated for 20 min. Absorbance was then measured at 405 nm, with wavelength correction set at 650 nm. Results are from at least three independent experiments and are expressed in pg/ml. The ELISA had a sensitivity of 100 pg/ml.

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from cultured cells with Trizol reagent (Invitrogen, Cergy Pontoise, France), according to the manufacturer's recommendations. RNA concentration was determined by measuring absorbance at 260 nm and 280 nm, ensuring that the ratio of these absorbance values was at least 1.8. Total RNA (1 µg) was digested by incubation for 15 min at 37°C with 1 U of DNase I (amplification grade; Invitrogen). The DNase was then inactivated and the RNA reverse transcribed with 200 units of reverse transcriptase (Superscript kit; Invitrogen), using random hexamer primers (Promega, Charbonnières, France), as previously described (Boukari *et al*, 2007). PCR was performed with 200 ng cDNA in the presence of qPCRTM Mastermix Plus for SybrTM Green I (Eurogentec, Seraing, Belgium), with 300 nM specific primers. The sense and antisense primers were as follows: MCP-1/CCL2, forward primer: 5′-TCCCAAAGAAGCTGTGATCTTCA, reverse primer: 5′-TGCTTGTCCAGGTGGTCCAT; 18S ribosomal RNA, forward primer: 5′-GTGCATGGCCGTTCTTAGTTG, reverse primer: 5′-CATGCCAGAGTCTCGTTCGTT; LTR, forward primer: 5′- GGGAACCCACTGCTTAAGCCT, reverse primer:5′-CTGCTAGAGATTTTCCACACTGAC;GFAP, forward primer: 5′-CTGGAGGTTGAGAGGGACAATC, reverse primer: 5′- CAGGGTGGCTTCATCTGCTT; CDK9, forward primer: 5′-GCTGCTTAACGGCCTCTACTACA, reverse primer: 5′-CAGCTTCAGGACCCCATCAC. The reaction mixture was heated at 95°C for 10 min, and subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. Controls without reverse transcriptase and without template were included to check that fluorescence was not overestimated due to the amplification of residual genomic DNA or the formation of primer dimers. RT-PCR products were also analyzed by plotting a postamplification fusion curve to ensure that a single amplicon was obtained. Real-time PCR was carried out on an ABI 7000 Sequence Detector (Applied Biosystems, Courtaboeuf, France). The PCR fragment corresponding to the 18S RNA, which was used as an internal standard in quantitative assays, was first cloned into pGEMT-easy, kindly provided by Dr. Marc Lombès, INSERM U693, Bicêtre, France (Boukari *et al*, 2007). For the MCP-1/CCL2 standard, we used the pCDNA3-7ND plasmid, containing the human MCP-1/CCL2 cDNA sequences to be amplified, which was kindly provided by Prof. Kensuke Egashira, Kyushu University, Japan (Egashira *et al*, 2000). For the LTR standard, we used pEV731. Standard curves were generated using serial dilutions of linearized standard plasmids. We amplified the ribosomal 18S RNA as an internal control for data normalization. The relative expression of a given gene is expressed as the ratio: attomoles of mRNA/femtomole of 18S.

Western blot analysis

Cell proteins were extracted and analyzed by Western blotting. Briefly, 7×10^5 cells were cultured in 6-well plates. The medium was replaced with 0.5% FCS in DMEM 12 h before treatment. This medium was removed and the cells were washed with PBS at 4°C. For analysis of transduction of Tat proteins into astrocytes, cells were extensively washed with trypsin and harvested for the preparation of cell extracts. Cells were incubated in lysis buffer (50 mM Tris-HCl pH 8, 10% glycerol, 0.1 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol [DTT]) containing protease inhibitors (Roche Diagnostics, Meylan, France) for 1 h at 4°C. Lysates were clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. The supernatants were isolated, and their protein concentrations were determined by the Bradford method (Bio-Rad, Marne la Coquette, France). Equal amounts of total cellular protein (50 µg) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels. The proteins were transferred to Hybond-P membranes (GE Healthcare–Amersham Biosciences, Les Ulis, France) by electroblotting. These membranes were probed with antibodies (Abs) recognizing Tat (1:500; Santa Cruz Biotechnology, Tebu, France), phospho-IκBα (Ser32/36) (1:1000; Cell Signaling, Ozyme, France), IκBα (1:1000; Cell Signaling), and cdk9 (C12F7, 1:1000; Cell Signaling) and β-tubulin (1:5000; Sigma Aldrich), the binding of which was detected by incubation with horseradish peroxidase–conjugated secondary Abs. The blot was stripped and reprobed for β-tubulin, used as a loading control. The labeled bands, detected by chemiluminescence (ECL+ reagent; GE Healthcare–Amersham Biosciences),

were visualized by autoradiography, using Kodak Biomax autoradiographic film (Sigma Aldrich). The files were scanned to generate TIFF files, which were recorded and analyzed with Image J software. The amounts of Tat, phospho-IκBα, IκBα, and cdk9 were determined and standardized with respect to β-tubulin protein levels.

Nucleofection with Amaxa-Nucleofector

Approximately 2×10^6 astrocyte cells were resuspended in Primary Mammalian Neurons Nucleofector solution (Amaxa, Cologne, Germany). DNA or siRNA and cells were mixed in the Amaxa cuvette and placed directly in the Nucleofector device. For DNA, we transfected cells with 5 μg pMaxGFP (Amaxa) or pFlag-CMV2-hCdk9-D167N, a plasmid encoding a dominant negative mutant form of human cdk9 (dn-cdk9) generously provided by Prof. Andrew P. Rice (Baylor College of Medicine, Houston, TX) (Gold *et al*, 1998). For siRNA, we used a pool of siRNAs against cdk9 (ON-TARGETplus smart pool; Dharmacon [Perbio Science, Brebières, France]) or a control ON-TARGETplus nontargeting siRNA. Primary human astrocytes were treated with program T-020. This program was optimized as recommended by the manufacturer. The cell suspension was removed immediately from the cuvette, by adding prewarmed medium with the Amaxa pipette. The cell suspension was added to a 6-well plate containing prewarmed medium and incubated at 37°C, under a humidified atmosphere containing 5% CO_2. The cells were harvested 48 h after transfection, and GFP levels were evaluated by FACS analysis. All transfection experiments were carried out five times.

FACS analysis

Infected cells were washed twice with PBS and dissociated into single cells by incubation with 0.125% trypsin/0.01% EDTA. The cells were centrifuged briefly, resuspended in 2% paraformaldehyde, and stood on ice for 5 min. They were then washed with PBS and analyzed using a FACS flow cytometer (Beckman Coulter, Villepinte, France) equipped with an argon laser with emission at 488 nm. Untransfected cells were used as a negative control. The percentage positive cells and staining intensity were evaluated. Gating criteria for the analysis of GFP-expressing cells were set according to the level of autofluorescence of a nontransfected control.

Luciferase assay

Luciferase activity was determined as previously described (Zennaro *et al*, 1996). Transfected cells were washed twice with ice-cold PBS and lyzed at 4°C in 250 μl lysis buffer. Supernatant (100 μl) was mixed with 100 μl luciferin reagent (0.07 mg/ml; Sigma Aldrich), and luciferase activity was determined using the Perkin-Elmer Victor 3V luminometer plate reader model 1420. Background luminescence readings were obtained and subtracted from the luminescence data. Protein concentrations were determined with the Bradford protein assay. The luminescence of each protein lysate was calculated in arbitrary units of light per microgram of protein.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed according to the manufacturer's protocol (Upstate, Millipore, St Quentin-en-Yvelines, France). Briefly, 5×10^5 astrocytes/well were cultivated and infected with HIV-based vectors in 6-well plates, as described above. Cells were then fixed with 1% formaldehyde for 10 min at 37°C, washed with ice-cold PBS containing a protease inhibitor mixture (Complete Mini; Roche Diagnostics), lysed, and sheared by sonication (5 sets of 30-s pulses and 60-s off on wet ice using a sonicator [Bioruptor; Diagenode, Liège, Belgium] set to its minimum power gave the appropriate length [200- to 500-bp] DNA fragments). Immunoprecipitation was performed overnight at 4°C using 0.5 μg/ml rabbit anti-cdk9 clone C12F7 antibody or rabbit IgG controls (Cell Signaling) followed by precipitation using protein A coupled to agarose beads. After washing, treatment with high salt and proteinase K (Fermentas, St Rémylès-Chevreuse, France), immunoprecipitated DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation. Quantitative PCR was performed on immunoprecipitated DNA fragments using specific sets of primers to amplify MCP-1/CCL2 and HIV-1 LTR sequences. The sense and antisense primers were as follows: MCP-1/CCL2 promoter, forward primer: −112 to −92; 5′- CCTCTGCCCGCTTTCAATAA, reverse primer: −38 to −15; 5′-AGTGCGAGCTTCAGTTTGAGAAT; HIV-1 LTR, forward primer: −192 to −172; 5′-GTTTGACAGCCGCCTAGCAT, reverse primer: −86 to −69; 5′-CCACGCCTCCCTGGAAA. Values were expressed as percent of amplified chromatin obtained after immunoprecipitation with the cdk9-specific antibody to input chromatin, normalized to amplified chromatin immunoprecipitated with IgG.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA). Values of $P < .05$ were considered statistically significant.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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