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CCAAT/enhancer binding protein β expression is increased in the brain during HIV-1-infection and contributes to regulation of astrocyte tissue inhibitor of metalloproteinase-1

Jerel Fields,* Jessica Gardner-Mercer,† Kathleen Borgmann,* Ian Clark‡ and Anuja Ghorpade*

*Department of Cell Biology and Anatomy, University of North Texas Health Science Center, Fort Worth, TX, USA

†University of Nebraska Medical Center, Omaha, NE, USA

‡School of Biological Sciences, University of East Anglia, Norwich, UK

Abstract

Human immunodeficiency virus (HIV)-1-associated neurocognitive disorders (HAND) associated with infection and activation of mononuclear phagocytes (MP) in the brain, occur late in disease. Infected/activated MP initiate neuroinflammation activating glial cells and ultimately disrupting neuronal function. Astrocytes secrete tissue inhibitor of metalloproteinase (TIMP)-1 in response to neural injury. Altered TIMP-1 levels are implicated in several CNS diseases. CCAAT enhancer-binding protein β (C/EBPβ), a transcription factor, is expressed in rodent brains in response to neuroinflammation, implicating it in Alzheimer's, Parkinson's, and HAND. Here, we report that C/EBPβ mRNA levels are elevated and its isoforms differentially expressed in total brain tissue lysates of HIV-1-infected and HIV-1 encephalitis patients. *In vitro*, HAND-relevant stimuli additively induce C/EBPβ nuclear expression in human astrocytes through 7 days of treatment. Over-expression of C/EBPβ increases TIMP-1 promoter activity, mRNA, and protein levels in human astrocytes activated with interleukin-1β. Knockdown of C/EBPβ with siRNA decreases TIMP-1 mRNA and protein levels. These data suggest that C/EBPβ isoforms are involved in complex regulation of astrocyte TIMP-1 production during HIV-1 infection; however, further studies are required to completely understand their role during disease progression.

Keywords: astrocyte and neuroinflammation, CCAAT enhancer-binding protein β, human immunodeficiency virus-1-associated dementia, tissue inhibitor of metalloproteinase-1.

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Human immunodeficiency virus (HIV)-1 infects approximately 33 million people worldwide and 40–70% of these have associated complications in the CNS. The most severe form of HIV-1-associated neurocognitive disorders (HAND) is HIV-1-associated dementia (HAD); dementia associated with reactive astrogliosis and neuronal dysfunction/death (Lindl *et al.* 2010). While the advent of antiretroviral therapy has transformed HIV-1 infection into a manageable, chronic condition and lowered the incidence of HAD, the prevalence of HAD has increased because of the extended life span of HIV-1 infected individuals (Lindl *et al.* 2010). Here, we investigate the expression of CCAAT enhancer-binding protein (C/EBP) β during HIV-1 infection and its regulation of astrocyte tissue inhibitor of metalloproteinase (TIMP)-1 production.

Astrocytes, important homeostatic regulators of the brain, are activated during HAD and display an altered gene expression profile during neuroinflammation (Faulkner *et al.* 2004; Heales *et al.* 2004; Sofroniew 2005; Laird *et al.* 2008; Yadav and Collman 2009; Sofroniew and Vinters 2010). Although the cellular expression of TIMP-1 in the CNS varies with disease state (La Fleur *et al.* 1996; Rivera *et al.*

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Address correspondence and reprint requests to Anuja Ghorpade, PhD, Department of Cell Biology and Anatomy, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107, USA. E-mail: anuja.ghorpade@unthsc.edu

Abbreviations used: AP, activator protein; C/EBPβ, CCAAT enhancer-binding protein β; CP, control plasmid; GFAP, glial fibrillary acidic protein; HAD, human immunodeficiency virus-1-associated dementia; HAND, human immunodeficiency virus-1-associated neurocognitive disorders; HIV+, human immunodeficiency virus-1-infected; HIVE, human immunodeficiency virus-1 encephalitis; IL-1β, interleukin-1β; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pTIMP-1-luc, TIMP-1 promoter-driven firefly luciferase; RT^2PCR, real time PCR; siC/EBPβ, C/EBPβ specific short interfering RNA; siCON, non-specific control short interfering RNA; TIMP, tissue inhibitor of metalloproteinase; WT, wild-type C/EBPβ-expressing plasmid.

1997; Bugno *et al.* 1999; Jaworski 2000), astrocytes are major producers of this (Pagenstecher *et al.* 1998; Jaworski 2000; Suryadevara *et al.* 2003) physiological antagonist of matrix metalloproteinases (MMPs) that stimulates cellular proliferation and inhibits apoptosis (Hornebeck 2003; Jourquin *et al.* 2005; Ould-yahoui *et al.* 2009). TIMP-1 is a multifunctional molecule that may affect pathology in multiple ways besides MMP inhibition, and this places an impetus on understanding how astrocyte TIMP-1 expression contributes to overall TIMP-1 levels during HAD. TIMP-1 expression levels are dysregulated in HAD and other CNS pathologies (Gardner and Ghorpade 2003). Reduced TIMP-1 expression in the CSF and brain tissues of HAD patients was previously reported (Suryadevara *et al.* 2003), but the mechanism causing this deficit is unknown. *In vitro*, astrocyte activation with the HIV-relevant stimulus, interleukin (IL)-1β, was shown to initially increase TIMP-1 expression, which decreased with long-term stimulation (Suryadevara *et al.* 2003). Although, the TIMP-1 : MMP balance is implicated in several CNS pathologies (Yong *et al.* 1998) and the TIMP-1 knockout mice showed impaired learning and memory, the mechanism for this is unknown and is likely multifaceted (Lorenzl *et al.* 2002, 2003, 2008; Hornebeck 2003; Chaillan *et al.* 2006). TIMP-1 expression is well studied in several model systems, but the distinct mechanisms controlling short-term versus long-term regulation in astrocytes are incompletely understood. The 1.7 kb sequence upstream of exon 1 and part of intron 1 contains numerous regulatory elements including five CCAAT boxes (Clark *et al.* 1997; Phillips *et al.* 1999).

Several transcriptional regulators are known to be up-regulated in astrocytes during neuroinflammation (Panenka *et al.* 2001; Brambilla *et al.* 2005, 2009; Abraham *et al.* 2006; Gris *et al.* 2007; Chen *et al.* 2008; Herrmann *et al.* 2008; Sofroniew and Vinters 2010). Recently, it was reported that the transcription factor C/EBPβ is expressed in rodent astrocytes in response to inflammatory stimuli (Albertini *et al.* 1998; Ejarque-Ortiz *et al.* 2007) and is involved in many cellular processes of the CNS (Alberini *et al.* 1994; Sterneck and Johnson 1998; Yukawa *et al.* 1998; Cardinaux *et al.* 2000; Menard *et al.* 2002; Cortes-Canteli *et al.* 2004; Nadeau *et al.* 2005; Ejarque-Ortiz *et al.* 2007; Sandhir and Berman 2010). Although, C/EBPβ is a prolific transcription factor that is expressed in microglia and neurons (Sterneck and Johnson 1998; Ejarque-Ortiz *et al.* 2007), it is unclear what proportion each of these cell types contributes to C/EBPβ expression in the brain. It is likely that C/EBPβ plays a distinct role in regulating the response of each cell type during neuroinflammation. However, astrocytes are well-accepted major producers of brain TIMP-1, thus, in this study, we focus on the role of C/EBPβ in astrocytes (Pagenstecher *et al.* 1998; Crocker *et al.* 2006). Alternative start site initiation results in a single C/EBPβ mRNA being translated into three isoforms: 42, 40, and 20 kDa. The two larger isoforms have transcriptional activation properties, whereas the 20 kDa isoform is a transcriptional silencer (Sears and Sealy 1994). C/EBPβ functions by dimerizing with other factors to regulate transcription (Sears and Sealy 1994). In this study, we hypothesize that C/EBPβ is expressed in astrocytes during HAND and contributes to the initial increase in TIMP-1 production by astrocytes, and possibly to the following dysregulation of TIMP-1 that we reported from *in vitro* studies and clinical cases (Suryadevara *et al.* 2003).

Here, we show that C/EBPβ is differentially expressed in the brains of HIV-1-infected (HIV+) and HIV encephalitis (HIVE) brain specimens. We report that primary human astrocytes express C/EBPβ in response to HAND-relevant stimuli and the transcription factor contributes to the complex regulation of astrocyte TIMP-1. Overall, this work identifies C/EBPβ as a transcription factor that contributes to astrocyte TIMP-1 regulation and is expressed in the human brain during HIV infection. These findings may have broader implications in many other neuroinflammatory CNS pathologies.

Experimental procedures

Preparation of human brain lysates

Brain lysates were prepared from specimens obtained from the NNTC, Center for Neurovirology and Neurodegenerative Disorders brain bank and Rapid Autopsy Program at the University of Nebraska Medical Center as previously described by Suryadevara *et al.* (2003). Protein concentration was determined by bicinconic acid method as suggested by the manufacturer (Pierce, Rockford, IL, USA).

Isolation, cultivation, and activation of human astrocytes

Human astrocytes were isolated from first- and early second-trimester aborted specimens obtained from the Laboratory of Developmental Biology, University of Washington, Seattle; in full compliance with the ethical guidelines of the NIH, University of Washington and University of North Texas Health Science Center. Astrocytes were isolated from specimens as described by Gardner *et al.* (2006). Astrocyte activation was achieved by stimulating with IL-1β, tumor necrosis factor (TNF)-α, and/or HIV-1_{JR-FL} for various time periods. All treatment conditions were derived empirically through testing a range of concentrations for maximal activation of astrocytes. Astrocytes were treated with HIV_{JR-FL} at 6000 counts reverse transcriptase activity/mL/min and IL-1β was used at 20 ng/mL. The concentrations used were in the range described in the current literature (Liu *et al.* 1996; Suryadevara *et al.* 2003; Dhar *et al.* 2006; Gardner *et al.* 2006). Furthermore, an *in vivo* correlate, mouse models utilizing adenovirus-driven IL-1β over-expression in the brain, achieved expression levels around 10 ng/mg total protein 7 days post-injection (Ferrari *et al.* 2006) or a mean of 41 ng in the whole striatum 8 days post-injection (Ferrari *et al.* 2004), respectively. During prolonged activation, cells received a medium exchange every 4 days with the original treatment concentrations. *De novo* TIMP-1 synthesis was measured in activated astrocytes by

blocking translation with 10 μg/mL cycloheximide (Sigma Chemicals, St. Louis, MO, USA) for 2, 8, and 24 h. Data presented are representative of a minimum of three independent experiments with two or more independent donors.

RNA isolation and real time PCR (RT^2PCR)

RNA from activated astrocytes was extracted (Qiagen, Alameda, CA, USA) and reverse transcribed into cDNA as per the manufacturer's instructions (PE Applied Biosystems, Inc., Foster City, CA, USA). TaqMan 5′ nuclease RT^2 PCR assays were performed using an ABI Prism 7500 sequence-detection system (PE Applied Biosystems, Inc.). The following TaqMan Gene Expression Assay primers were used: TIMP-1 (C/N: Hs99999139_m1), C/EBPβ (C/N: Hs00270923_s1), and glyceraldehyde-phosphate dehydrogenase (C/N: 4310859). The reactions were carried out at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were analyzed in triplicate. All experiments and analyses meet the minimum standard guidelines for fluorescence-based RT^2PCR experiment. One-way analysis of variance (ANOVA) was used to analyze RT^2PCR data.

Western blot

Astrocytes were cultured as adherent monolayers in 75 cm^2 flasks at a density of 8×10^6 cells per flask. The following day, cells were treated with IL-1β (20 ng/mL). Cells were lysed, and nuclear extracts were isolated at 72 and 168 h post-IL-1β treatment using nuclear extraction reagent (Fisher, Waltham, MA, USA). Equal amounts of protein (30 μg/lane from astrocytes and 160 μg/lane from brain lysates) were resolved by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane using i-Blot (Invitrogen, Carlsbad, CA, USA). The membrane was incubated with anti-C/EBPβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA; C/N : C-19 at a dilution of 1 : 200 for astrocyte lysates and 1 : 40 for brain lysates), and then in secondary antibody at a concentration of 1 : 5000. β-actin was used as a loading control.

Measurement of TIMP-1 protein and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) levels

TIMP-1 levels in astrocyte supernatants were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). The MTT assay was performed at appropriate time points according to the method originally described by Manthrope *et al.* (1986). The ELISA determinations yielded quantities of protein in units of ng/mL, and were normalized to MTT values. Results were analyzed with GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) using one-way ANOVA with Newman–Keul's post-test for multiple comparisons.

TIMP-1 promoter constructs and C/EBPβ-expressing plasmids

A TIMP-1 promoter-driven firefly luciferase (pTIMP-1-luc) construct was used to measure TIMP-1 promoter activity in transfected astrocytes. The −1718/+988 portion of the TIMP-1 sequence was cloned into the pGL3-Basic reporter vector (Promega, Madison, WI, USA) and kindly provided by Dr Ian Clark at the University of East Anglia, UK (Clark *et al.* 1997). The wild-type (WT) C/EBPβ-expressing plasmid expresses the 42, 40, and 20 kDa isoforms, whereas the mutated plasmid has a mutation in the 3′-translation start sites and predominantly expresses the 42 kDa isoform. Dr Calkhoven kindly provided the C/EBPβ-expressing plasmids from Leibniz Institute for Age Research, Fritz Lipman Institute in Germany.

Transfection of primary human astrocytes with luciferase reporter constructs

Astrocytes were cultured as adherent monolayers in a 48-well plate at a density of 0.15×10^6 cells/well. The following day, cells were transfected with 1.5 μg of total DNA. Total DNA consisted of a mixture of the pTIMP-1-luc and the simian vacuolating virus promoter-driven *Renilla* luciferase (Promega) plasmids with or without C/EBPβ-expressing plasmids, or a control plasmid (CP). We used lipofectamine reagent to transfect the plasmid as per the manufacturer's instructions (Invitrogen). Untreated and mock-transfected controls were maintained for comparison. Mock controls were transfected without plasmid. At 2 days post-transfection promoter activity was measured as luciferase activity in cell extracts using the Dual-Glo Luciferase Assay System as per the manufacturer's instructions (Promega). Luciferase activity was determined as a ratio of firefly to *Renilla* luciferase using the Tecan Infinite Pro 200 luminometer (Tecan, Mannedorf, Switzerland). *Renilla* luciferase activity was thus used as an internal control. All data were analyzed with GraphPad Prism 4.0 in triplicate using one-way ANOVA with Newman–Keul's post-test for multiple comparisons unless otherwise specified. Data presented are representative of a minimum of three independent experiments with two or more independent donors.

Transfection of primary human astrocytes with siRNA

Astrocytes were resuspended in nucleofector transfection reagent (Lonza, Walkersville, MD, USA) at a concentration of 80×10^6 cells/mL and transfected with short interfering (si) C/EBPβ (GGCCCUGAGUAAUCGCUUA – 100 nM) or non-specific control short interfering RNA (siCON; Dharmacon, Lafayette, CO, USA) as per the manufacturer's instructions. Cells were then cultured in 25 cm^2 flasks and allowed to recover for 24 h prior to treatment with IL-1β (20 ng/mL).

Statistical analyses

Statistical analyses were carried out using GraphPad Prism 4.0 software, with one-way ANOVA and Newman–Keuls post-test for multiple comparisons. Significance was set at $p < 0.05$ and data represent mean values ± SEM. Data presented are representative of a minimum of three independent experiments with two or more independent donors.

Results

C/EBPβ and TIMP-1 expression in HIV-1-infected individuals

C/EBPβ is a prolific transcription factor that regulates gene expression during inflammation (Alberini *et al.* 1994; Sterneck and Johnson 1998; Yukawa *et al.* 1998; Cardinaux *et al.* 2000; Menard *et al.* 2002; Cortes-Canteli *et al.* 2004; Nadeau *et al.* 2005; Ejarque-Ortiz *et al.* 2007; Sandhir and Berman 2010). To determine C/EBPβ expression patterns in

the brain of HIV-1-infected patients, we performed RT^{2}PCR (n = 14) and western blotting (n = 8) using total brain tissue lysates from the frontal cortex of control, HIV+, and HIVE patients (Table S1). The control samples used for RT^{2}PCR were made up of four females and two males (age: 37–52); of the HIV-infected patients, seven were male and one was female (25–55), and all had progressed to AIDS. Of the AIDS patients, four showed signs of encephalitis and the remaining four showed no signs of CNS pathology. There were no detectable signs of opportunistic infection in the CNS of any patients studied. C/EBPβ mRNA levels were significantly higher in HIV+ patients compared with HIVE and control patients (Fig. 1a). Low levels C/EBPβ mRNA and protein were detected in the brain tissues from the control patients. All HIV-1-infected patients showed expression of at least one isoform of C/EBPβ. C/EBPβ was detected in the brain lysates of three HIV+ patients, in which we detected a 42 kDa isoform and an unreported band around 25 kDa (Fig. 1b). The 20 kDa isoform was detected in two brain lysates from HIVE patients. Overall, this work shows that C/EBPβ expression is markedly increased in the brains of these HIV-1-infected individuals compared with uninfected controls, C/EBPβ mRNA, and protein levels are lower in the brain tissues of HIVE compared with HIV+ patients. Furthermore, the expression pattern of C/EBPβ isoforms varies between HIV+ and HIVE patients. A 42 kDa isoform and an unreported band at 25 kDa were detected in HIV+ tissues, whereas a 20 kDa isoform was detected in HIVE patients.

HIV-relevant stimuli induce C/EBPβ expression in human astrocytes

Cultured human astrocytes were used to determine if HIV-relevant stimuli induce C/EBPβ expression. We treated astrocytes with IL-1β (20 ng/mL) for days 1, 3, and 7, and then collected total RNA and protein (Fig. 2a and b). C/EBPβ mRNA was significantly ($p < 0.001$) increased in IL-1β-treated astrocytes compared with untreated controls at all time points. C/EBPβ mRNA was increased 1.4-fold in IL-1β-treated astrocytes at day 3 ($p < 0.01$) and increased 2.5-fold further at day 7 (***$p < 0.001$), (Fig. 2a). Normalized levels of C/EBPβ mRNA in control astrocytes were not significantly different at any time-point. We performed immunoblotting for C/EBPβ in days 3 and 7 nuclear extracts from IL-1β-treated astrocytes as described in the 'Experimental procedures' (Fig. 2b). C/EBPβ levels were increased in IL-1β-treated astrocytes compared with untreated controls at days 3 and 7. To determine if HAND-relevant stimuli, other than IL-1β, are capable of inducing C/EBPβ expression, we treated astrocytes with HIV_{JR-FL} (6000 counts RT activity/mL/min), IL-1β (20 ng/mL), and TNF-α (20 ng/mL), alone, or in combination for 1 and 3 days (Fig. 2c). IL-1β and TNF-α both induced increases in C/EBPβ mRNA compared with control, and led to a further increase through day 3 of stimulation. Treatment with HIV_{JR-FL} enhanced the IL-1β- and TNF-α-mediated increase in C/EBPβ mRNA. Treatment with TNF-α or HIV_{JR-FL} alone had no significant effect on C/EBPβ mRNA levels at day 1, but resulted in a twofold ($p < 0.01$) increase in at day 3 compared with untreated controls. HIV_{JR-FL} combined with TNF-α further enhanced the induction of C/EBPβ mRNA to threefold, compared with untreated controls at day 3. Treatment with IL-1β significantly ($p < 0.001$) increased C/EBPβ mRNA levels at days 1 and 3 compared with untreated controls. We isolated protein from HIV-relevant stimuli-treated astrocytes 2 days post-treatment, and performed western blot for C/EBPβ (Fig. 2d). Protein levels were markedly increased in IL-1β-, TNF-α-, IL-1β + TNF-α-, and HIV_{JR-FL} + IL-1β + TNF-α-treated astrocytes compared with untreated controls. Treatment with TNF-α alone had less effect than

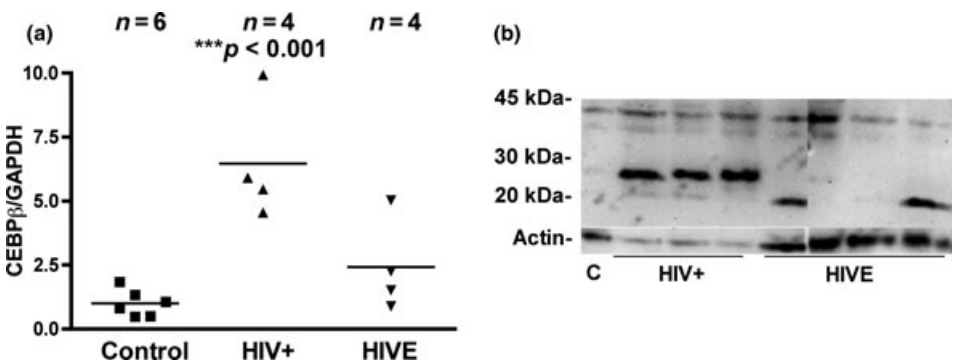

Fig. 1 CCAAT enhancer-binding protein β (C/EBPβ) mRNA levels are elevated in the brain tissues from HIV+ patients. C/EBPβ 42 kDa and 20 kDa isoforms are detectable in brain lysates of HIV+ and HIVE patients. Total RNA isolated from brains of control, HIV+, and HIVE patients was reverse transcribed to cDNA and subjected to RT^{2}PCR for C/EBPβ and TIMP-1. (a) C/EBPβ mRNA levels were significantly (***$p < 0.001$) higher in samples from HIV+ patients compared with control or HIVE patients. (b) Low levels of the 42 kDa isoform were detected in lysates from control patients whereas increased 42 kDa, a light band at 20 kDa, and a strong band at 25 kDa was detected in all samples from HIV+ patients.

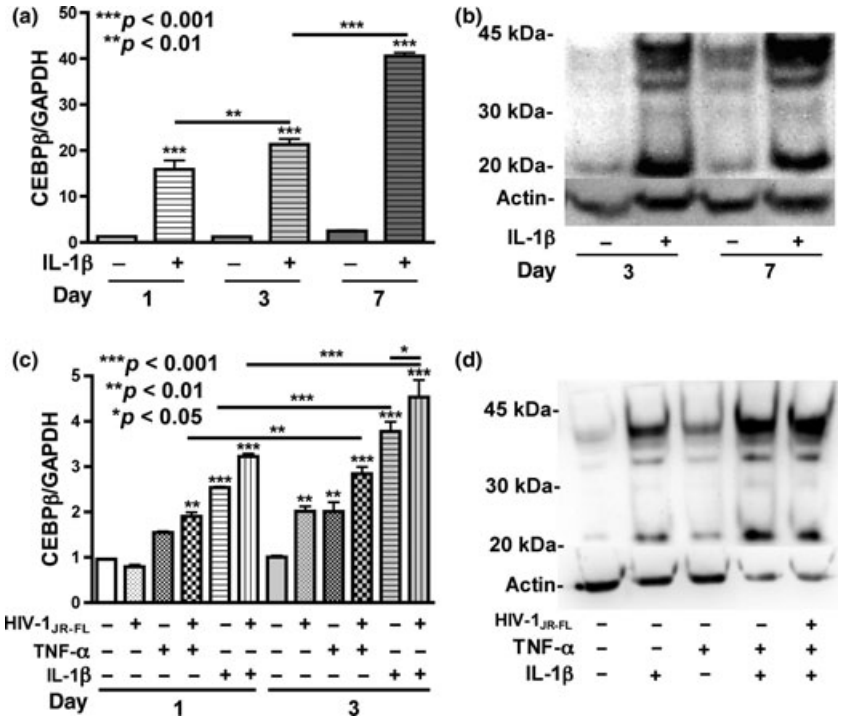

Fig. 2 HIV-1-associated neurocognitive disorders (HAND)-relevant stimuli induce CCAAT enhancer-binding protein β (C/EBPβ) expression induced in human astrocytes. (a) C/EBPβ transcripts were measured in mRNA isolated from primary human astrocytes treated with IL-1β (20 ng/mL) for 1, 3, or 7 days. C/EBPβ mRNA expression was significantly increased compared with untreated at 1, 3, and 7 days (***$p < 0.001$). C/EBPβ mRNA continued to increase, as levels at 3 days were significantly higher than at 1 (**$p < 0.01$) and levels at 7 days were significantly higher than at 3 (***$p < 0.001$). (b) C/EBPβ protein levels were assayed by immunoblotting. C/EBPβ protein levels were increased compared with untreated controls at 3 and 7 days. (c) C/EBPβ transcripts were measured in total RNA isolated from primary human astrocytes treated with IL-1β (20 ng/mL), tumor necrosis factor (TNF)-α (20 ng/mL), and/or HIV_{JR-FL} (6000 counts RT activity/mL/min) for 1 or 3 days. C/EBPβ mRNA expression was significantly increased compared with untreated control at 1 and 3 days in IL-1β- and TNF-α-treated astrocytes (***$p < 0.001$). (d) Primary human astrocytes were treated with IL-1β (20 ng/mL), TNF-α (20 ng/mL), and/or HIV_{JR-FL} (6000 counts RT activity/mL/min) for 2 days and then cell lysate was immunoblotted for C/EBPβ. C/EBPβ protein levels were increased compared with untreated controls in all samples. Data represent mean values ± SEM in at least three independent experiments in at least two independent donors.

IL-1β alone, and combinations IL-1β + TNF-α and HIV_{JR-FL} + IL-1β + TNF-α resulted in the most robust increase in C/EBPβ protein expression. Taken together, both C/EBPβ mRNA and protein levels are robustly increased in response to HIV-relevant stimuli.

We treated astrocytes with IL-1β for 48 h, fixed and co-localized glial fibrillary acidic protein (GFAP) as an astrocyte-specific marker (red) with C/EBPβ (green) in activated human astrocytes (Fig. 3b). In control cells, GFAP is present throughout the cell body of the astrocytes. Control astrocytes have a larger cell body, (Fig. 3a), whereas activated astrocytes have extensive processes protruding from their soma and more dense staining of GFAP (Fig. 3b and c). Low levels of C/EBPβ are present in the nuclei of control human astrocytes compared with activated astrocytes, where the green signal from the nuclei is markedly enhanced. Together with the mRNA and protein expression data represented earlier, this confirms that C/EBPβ expression is increased and localized to the nucleus of astrocytes in response to HAND-relevant stimuli.

TIMP-1 production *de novo*

Prior to verifying the specific effects of C/EBPβ on TIMP-1 promoter regulation, we first evaluated whether TIMP-1 is synthesized *de novo* or instead released from intracellular stores upon activation. Human astrocytes were treated with translation inhibitor, cycloheximide at 10 μg/mL in conjunction with 20 ng/mL IL-1β for 2, 8, or 24 h (Fig. 4). TIMP-1 levels normalized to MTT units demonstrated that cycloheximide significantly inhibited TIMP-1 production following 8 and 24 h of IL-1β activation (***$p < 0.001$). These data show that protein synthesis is necessary for TIMP-1 up-regulation in acutely activated astrocytes and that TIMP-1 is synthesized *de novo* in astrocytes. Hence, regulation of mRNA transcription may serve as a promising target to increase expression.

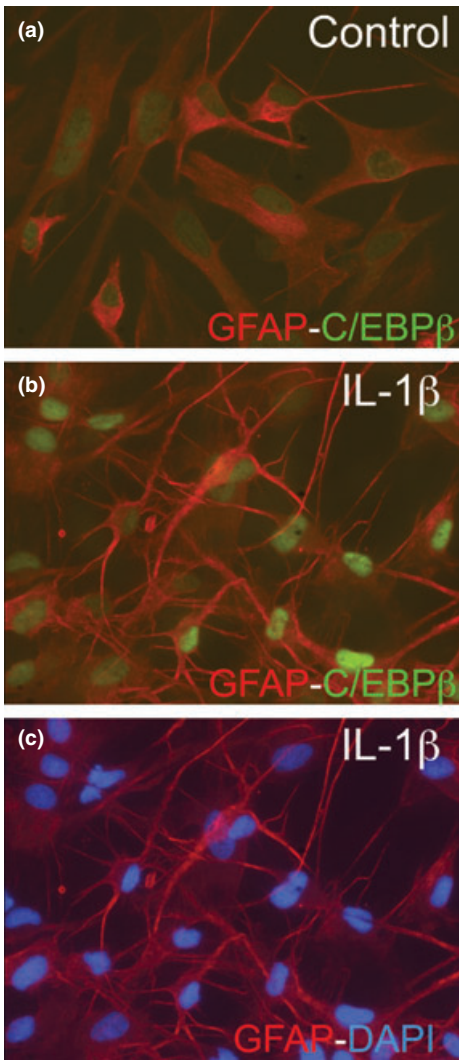

Fig. 3 CCAAT enhancer-binding protein β (C/EBPβ) localizes to the nucleus in response to IL-1β. (a) Untreated astrocytes were stained for glial fibrillary acidic protein (GFAP) (red) and C/EBPβ (green). Red signal is detected throughout the cytoplasm and green is localized to the nucleus. (b) In IL-1β-treated astrocytes C/EBPβ (green) is localized to the nucleus and demonstrates greater intensity as compared with control astrocytes. (c) 4′,6′-diamidino-2-phenylindole staining in IL-1β-treated astrocytes. All pictures were taken at 200× and data represent at least three independent experiments in at least two independent donors.

C/EBPβ over-expression increases TIMP-1 promoter activity, mRNA, and protein levels

We used a panel of plasmids that over-express C/EBPβ isoforms, in combination with luciferase-expressing plasmids driven by the TIMP-1 promoter (Fig. 5a). Two C/EBPβ over-expression plasmids were used. The WT with three translation start sites leading to the production of three isoforms of C/EBPβ (42, 40, and 20 kDa), and the mutant plasmid in which the two 3′-translation start sites are mutated to allow for predominant expression of the 42 kDa isoform. The −1718/+988 region of the TIMP-1 promoter was used to drive expression of the firefly luciferase gene. The −1718/+988 region contains five CCAAT sites possibly involved in promoter activity (Clark *et al.* 1997). We transfected human astrocytes, by nucleofection and isolated nuclear extracts 48 h post recovery. We resolved nuclear extracts from primary human astrocytes transfected with WT (lane 1) and mutant (lane 2) plasmids, and performed western blot for C/EBPβ (Fig. 5b). Bands for all three isoforms were detected in the astrocytes transfected with WT, whereas the mutant transfected astrocytes expressed predominantly the 42 kDa isoform. Next, we cotransfected astrocytes with pTIMP-1-luc (−1718/+988) and CP, WT, or mutant plasmids (Fig. 5c). Cotransfection with the WT or mutant plasmids significantly increased activity from the −1718/+988 region of the TIMP-1 promoter 2.6- and 3.0-fold, respectively, compared with cotransfection with CP (***$p < 0.001$). Overall, over-expression of C/EBPβ in primary human astrocytes significantly increases TIMP-1 promoter activity.

We transfected human astrocytes with C/EBPβ WT or CP by nucleofection, and activated with IL-1β for 24 h to determine if over-expressing C/EBPβ would increase the initial astrocyte TIMP-1 response. Maximum C/EBPβ over-expression was achieved between 24 and 48 h post-transfection in time course studies. We collected supernatant and RNA 1-day post-treatment (Fig. 6a and b). IL-1β treatment significantly increased (***$p < 0.001$) TIMP-1 mRNA compared with untreated cells. Over-expressing C/EBPβ significantly increased TIMP-1 mRNA 1.4-fold compared with the CP-transfected astrocytes with IL-1β treatment (***$p < 0.001$) (Fig. 6a). Over-expressing C/EBPβ increased (***$p < 0.001$) TIMP-1 secretion from activated astrocytes by 1.5-fold compared with CP-transfected-activated astrocytes. These data confirm that over-expressing C/EBPβ enhances TIMP-1 production in response to IL-1β (Fig. 6b).

C/EBPβ knockdown decreases TIMP-1 mRNA and protein expression

C/EBPβ-specific short interfering RNA (siC/EBPβ) was used to evaluate the effect of C/EBPβ knockdown on TIMP-1 mRNA and protein levels in activated astrocytes (Fig. 7). We achieved 65% knockdown (***$p < 0.001$) of C/EBPβ mRNA through 72 h in astrocytes transfected with siC/EBPβ, compared with mock-transfected cells (Fig. 7a). We isolated nuclear extracts from astrocytes transfected with siC/EBPβ, siCON, and mock 4 days post-transfection, and found undetectable C/EBPβ levels in siC/EBPβ-transfected astrocytes compared with siCON-transfected cells (Fig. 7b). Analysis of total RNA from siC/EBPβ-transfected astrocytes showed significant down-regulation in TIMP-1 mRNA (**$p < 0.01$) compared with siCON- or mock-transfected cells (Fig. 7c). TIMP-1 levels supernatants from siC/EBPβ-transfected astrocytes were significantly lower (*$p < 0.05$) than those from siCON- or mock-transfected cells (Fig. 7d).

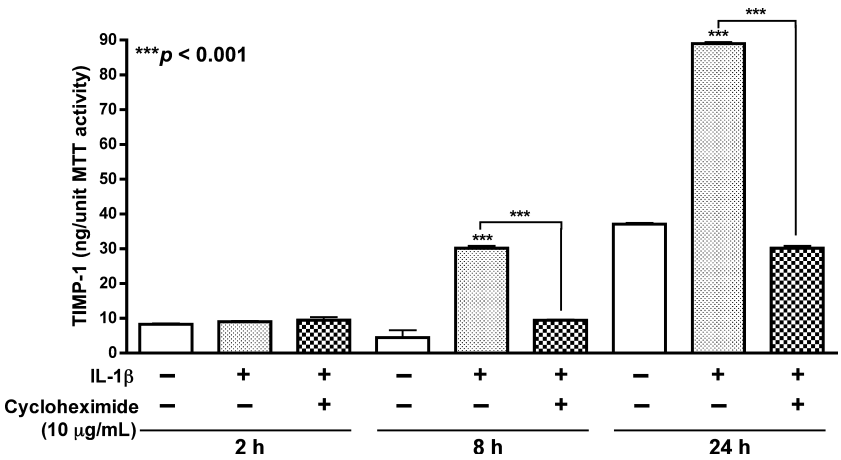

Fig. 4 *De novo* synthesis of astrocyte tissue inhibitor of metalloproteinase (TIMP)-1. Cycloheximide at 10 μg/mL was applied to astrocytes in conjunction with 20 ng/mL IL-1β for 2, 8, or 24 h. TIMP-1 levels in astrocyte supernatants were measured by ELISA and normalized to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity. Cycloheximide significantly inhibited TIMP-1 production following 8 and 24 h of IL-1β activation, (***$p < 0.001$) compared with untreated controls. Data represent mean values ± SEM of TIMP-1 protein levels determined in duplicate, in at least three independent experiments and two independent donors.

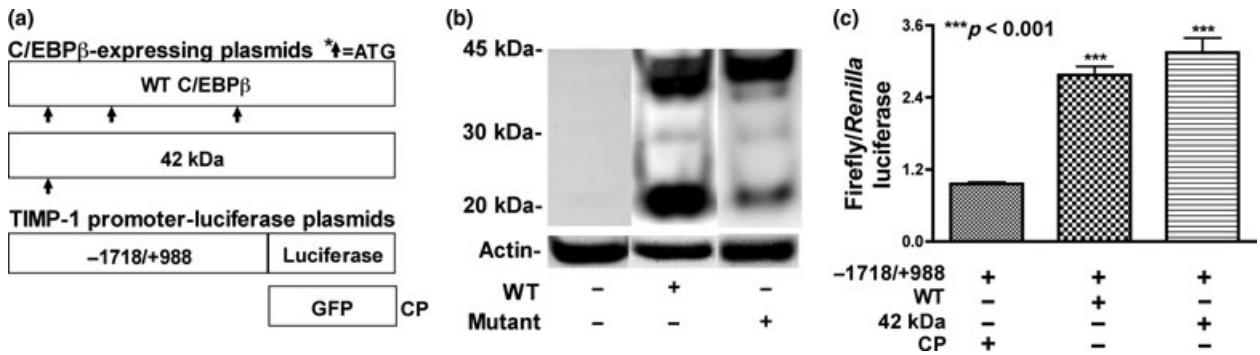

Fig. 5 Over-expression of CCAAT enhancer-binding protein β (C/EBPβ) wild-type (WT) and mutant C/EBPβ increases tissue inhibitor of metalloproteinase (TIMP)-1 promoter activity. (a) Constructs used. (b) Primary human astrocytes were transfected with WT or mutant by nucleofection or Lipofectamine. WT expresses the C/EBPβ mRNA that has three translation start sites; generating two large (42 and 40 kDa) isoforms from the first two start sites and one smaller 20 kDa isoform from the most 3′-start site. The mutant has a mutation in the two 3′-translation start sites, and predominately generates the 42 kDa isoform. (c) Cotransfection with WT or 42 kDa with pTIMP-1-luc (−1718/+988) significantly (**$p < 0.01$ and ***$p < 0.001$, respectively) increased activity. Data represent mean values ± SEM of luciferase activity in triplicate, in at least three independent experiments and at least two independent donors.

Human astrocytes transfected with siC/EBPβ expressed approximately 30% less TIMP-1 mRNA and protein compared with siCON-transfected astrocytes. These data illustrate that knockdown of C/EBPβ by siRNA results in down-regulation of astrocyte TIMP-1 at the mRNA and protein levels.

Discussion

Antiretroviral therapy has transformed HIV-1-infection into a chronic-manageable illness in the industrialized world; however, HAND represents a group of complications that are increasingly prevalent and require continued investigation (Yadav and Collman 2009). Focus is shifting from the dysfunctional neurons to glia as an important contributing factor to neuroinflammation and to pathologies of the CNS like HAND. Here, we focus on astrocytes, the most abundant cells in the CNS, and their production of TIMP-1 in response to injury. Previously, we reported that TIMP-1 production is dysregulated in HIV-infected brain specimens, and *in vitro* experiments showed astrocytes up-regulate TIMP-1 when acutely activated with IL-1β; however, long-term IL-1β exposure was shown to down-regulate TIMP-1 production (Suryadevara *et al.* 2003; Gardner *et al.* 2006). In these studies, we sought to delineate the mechanism of astrocyte TIMP-1 dysregulation in the HIV-infected brain. We report that total C/EBPβ mRNA and protein levels are higher in the brains of HIV+ patients compared with control

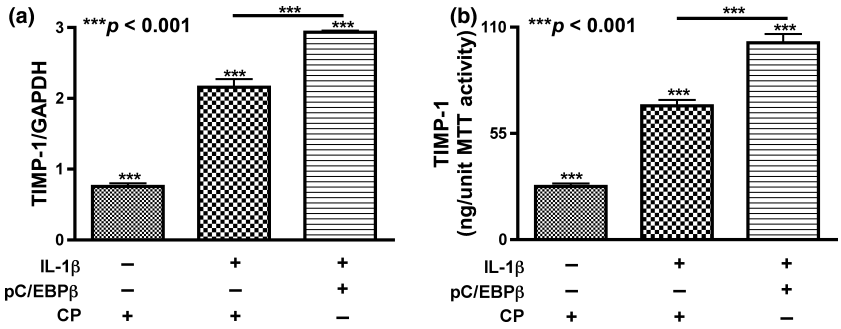

Fig. 6 Over-expression of wild-type (WT) CCAAT enhancer-binding protein β (C/EBPβ) increases tissue inhibitor of metalloproteinase (TIMP)-1 expression. Astrocytes were transfected with plasmids over-expressing WT or CP nucleofection, treated with IL-1β and total mRNA, and supernatant was collected 48 h post-transfection. (a) TIMP-1 mRNA levels were significantly (***$p < 0.001$) increased in astrocytes transfected with WT as compared with CP-transfected cells. Data represent mean values ± SEM of TIMP-1 mRNA levels determined in triplicate in at least three independent experiments and two independent donors. (b) TIMP-1 protein levels were significantly (***$p < 0.001$) increased in the supernatant of astrocytes transfected with WT as compared with CP-transfected cells. Data represent mean values ± SEM of TIMP-1 levels determined in duplicate, in at least three independent experiments and two independent donors.

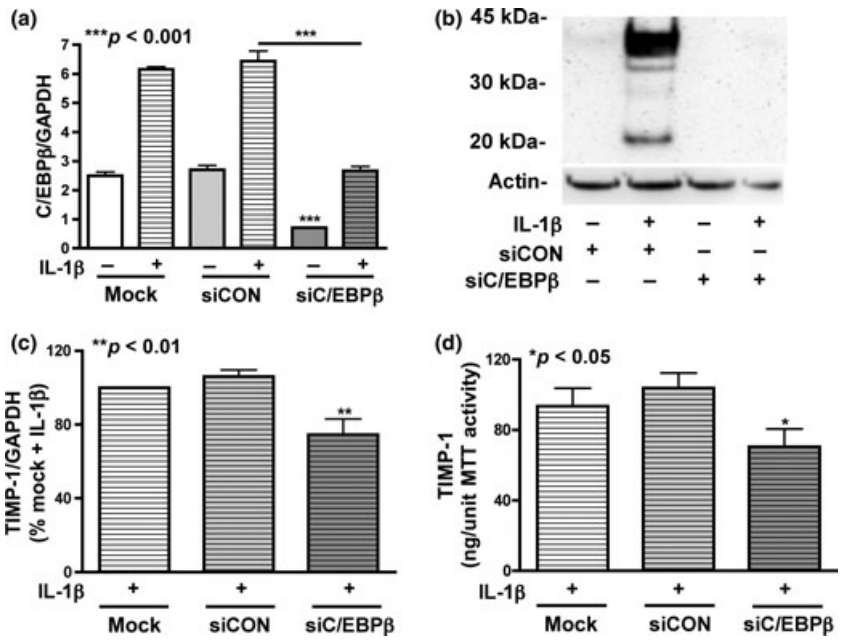

Fig. 7 Knockdown of CCAAT enhancer-binding protein β (C/EBPβ) with siRNA decreases tissue inhibitor of metalloproteinase (TIMP)-1 expression. C/EBPβ mRNA was measured in primary human astrocytes 3 days after transfection with siC/EBPβ or non-specific control short interfering RNA (siCON) by nucleofection. (a) C/EBPβ mRNA expression was significantly (***$p < 0.001$) decreased in untreated and IL-1β-treated astrocytes. (b) Four days after transfection, cell lysates were immunoblotted for C/EBPβ. C/EBPβ protein was detected in siCON-transfected astrocytes, but not in those transfected with siC/EBPβ. (c) TIMP-1 mRNA expression was significantly decreased (**$p < 0.01$) at 3 days in astrocytes transfected with siC/EBPβ compared with mock or siCON-transfected astrocytes. (d) TIMP-1 was measured using ELISA 3 days post-transfection. Supernatant from astrocytes transfected with siC/EBPβ had significantly less (*$p < 0.05$) TIMP-1 protein than controls. Data represent mean values ± SEM of TIMP-1 mRNA and protein levels determined in duplicate, in at least three independent experiments and two independent donors.

individuals. Confirming similar results from rodent models, HAND-relevant stimuli is shown to induce C/EBPβ nuclear expression in human astrocytes, and this response is enhanced by co-stimulation with HIV_{JR-FL}. Lastly, over-expression of C/EBPβ increases TIMP-1 production, whereas knockdown of C/EBPβ decreases TIMP-1 production in human astrocytes in response to the HAND-relevant stimulus, IL-1β.

Astrocytes react to neural injury by changing gene expression that in turn leads to changes in cellular function, morphology, and replication (Sofroniew and Vinters 2010). C/EBPβ regulates inflammatory responses in multiple cell

types (Akira *et al.* 1990) through promoter regulation; the TIMP-1 promoter harbors five sequences predicted to bind C/EBPβ. Therefore, we focused on characterizing C/EBPβ expression in the brain during HIV-1 infection and its involvement in injury-response and astrocyte TIMP-1 production. As C/EBPβ is highly expressed in glia during inflammation (Alberini *et al.* 1994; Sterneck and Johnson 1998; Yukawa *et al.* 1998; Cardinaux *et al.* 2000; Menard *et al.* 2002; Cortes-Canteli *et al.* 2004; Nadeau *et al.* 2005; Ejarque-Ortiz *et al.* 2007; Sandhir and Berman 2010), it was exciting to detect C/EBPβ up-regulation at the mRNA and protein level in HIV-1-infected brain specimens. Our finding that C/EBPβ mRNA and protein levels were highest in HIV+ brains and higher in HIVE than control brains suggests that C/EBPβ may be involved in the initiation of neuroinflammation following HIV-1 infection rather than a consequence of it. However, our data are derived from total brain tissue lysates, thus, the source of C/EBPβ in these brain tissue specimens likely represents expression by cells other than astrocytes as well. The presence of transcription-activating C/EBPβ isoforms in HIV patients versus the expression of the 20 kDa isoform detected in HIVE patients could represent a switch that contributes to TIMP-1 dysregulation during HAD. Low expression of the 42 kDa isoform of C/EBPβ was detected in control patients, and mRNA levels were minimal.

It is established that human astrocytes are morphologically and functionally more complex than their counterparts in rodents; therefore, we aimed to confirm that the C/EBPβ response is conserved in human astrocytes following activation with HAND-relevant stimuli (Oberheim *et al.* 2009). To our knowledge, this is the first report of C/EBPβ expression in primary human astrocytes in response to IL-1β. Interestingly, C/EBPβ expression showed a significantly augmented increase when co-stimulated with HIV-$1_{JR\text{-}FL}$, and other HAND-relevant stimuli. This phenomenon, however, requires further investigation. C/EBPβ expression continues to rise through 7 days of stimulation, which suggests a prolonged contribution to changes in astrocyte gene expression during inflammation. Along with other reports, this study supports the idea that C/EBPβ could be an integral part of the regulatory mechanism in a general glial response to neuroinflammation (Alberini *et al.* 1994; Sterneck and Johnson 1998; Yukawa *et al.* 1998; Cardinaux *et al.* 2000; Menard *et al.* 2002; Cortes-Canteli *et al.* 2004; Nadeau *et al.* 2005; Ejarque-Ortiz *et al.* 2007; Sandhir and Berman 2010). We focused on IL-1β stimulation of human astrocytes for the remainder of the studies, as it is a prototypical inflammatory cytokine associated with HAND.

In our previous studies, we identified a robust increase in astrocyte TIMP-1 mRNA and protein production response to HAND-relevant stimuli (Gardner *et al.* 2006). Here, we show that TIMP-1 is produced *de novo* upon stimulation with HAND-relevant stimuli rather than released from intracellular stores. This suggests that transcriptional regulation may be a promising therapeutic target to enhance astrocyte TIMP-1 production. Regulation of TIMP-1 through the promoter has been studied and is very complex (Clark *et al.* 1997; Dean and Clark 1999; Dean *et al.* 2000; Fassina *et al.* 2000). Among other elements, the TIMP-1 promoter harbors five C/EBPβ-binding sites (Clark *et al.* 1997). As we predicted, over-expression of WT C/EBPβ enhanced TIMP-1 promoter activity, and over-expression of the 42 kDa isoform alone showed further enhancement. This could be explained by the transcriptional silencing activity of the 20 kDa isoform. Over-expression of C/EBPβ increased TIMP-1 promoter activity, mRNA, and protein expression in human astrocytes treated with the HAND-relevant stimulus, IL-1β. Knockdown of C/EBPβ resulted in the opposite effect on TIMP-1 expression; decreasing mRNA and protein levels in response to IL-1β activation. Overall, these studies suggest that C/EBPβ contributes to regulating TIMP-1 production in activated astrocytes. Thus, identifying TIMP-1 as one of genes regulated by C/EBPβ may provide a target for increasing TIMP-1 expression during neuroinflammation. It is noteworthy that C/EBPβ knockdown did not completely ablate TIMP-1 production in response to IL-1β, however, over-expression of C/EBPβ did enhance TIMP-1 production. These data support the hypothesis that C/EBPβ binds to some or all of the five CCAAT sites in the TIMP-1 promoter to regulate transcription, but this is most likely not the sole factor influencing transcription. It is possible that C/EBPβ regulates the transcription of other factors that influence TIMP-1 expression. Additional studies are necessary to determine other contributors for TIMP-1 transcription, to identify C/EBPβ-binding partners and the precise binding site for C/EBPβ in the TIMP-1 promoter.

Neuroinflammation contributes to HAND, but the precise mechanism required to transition from activated and infected glia in the brain to dysfunctional neurons, is not fully understood (Borjabad *et al.* 2009; Yadav and Collman 2009). Here, we show that HIV-1-infected patients, not yet suffering from cognitive deficits or any CNS pathology, express C/EBPβ known to mediate inflammatory responses. C/EBPβ detected in control patients is very low. Patients with signs of cognitive deficits or CNS pathology also express C/EBPβ; although they show different pattern of isoform expression. This links observations about C/EBPβ to HIV infection of the CNS, and, most importantly, to pathology in humans. However, neurons and microglia also express C/EBPβ. Previously, we reported decreased TIMP-1 protein in the CSF and brain of HIV and HIVE compared with control patients (Suryadevara *et al.* 2003). Taken together with the C/EBPβ isoforms detected in the eight patient samples, a shift in C/EBPβ isoform expression may contribute to dysregulation of TIMP-1 during HIV-1 infection. Further studies, using a large and well-characterized cohort of patient samples, will be useful in determining if

the different C/EBPβ isoforms are expressed at different stages of HIV infection, the cell types in which they are expressed, if this contributes to dysregulation of TIMP-1 during disease, and if the unidentified 25 kDa isoform plays a role. There is no evidence, in the literature or our studies, that C/EBPβ or TIMP-1 expression is influenced by gender.

Exciting new findings are revealing alternative roles for secreted TIMP-1 as essential for maintaining homeostasis by altering extracellular matrix architecture and acting as a growth factor (Hornebeck 2003; Jourquin *et al.* 2005; Ouldyahoui *et al.* 2009). These new findings support a need for studies that culminate in the understanding of the mechanisms regulating this tightly controlled gene. TIMP-1 promoter regulation has been extensively studied, however, little is known about the regulation of astrocyte TIMP-1 (Clark *et al.* 1997). This study opens the door to discovering additional functions for C/EBPβ in the CNS as well as another mechanism potentially regulating astrocyte TIMP-1 production during neuroinflammation. Further studies are needed to delineate the roles of C/EBPβ in the CNS, and the regulation of TIMP-1 during neuroinflammation.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1. Patient information.

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