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Functional Synergy between CD40 Ligand and HIV-1 Tat Contributes to Inflammation: Implications in HIV Type 1 Dementia

Ziye Sui,* Lynn F. Sniderhan,† Giovanni Schifitto,¶ Richard P. Phipps,‡‡ Harris A. Gelbard,** Stephen Dewhurst,*# and Sanjay B. Maggirwar—*†‡§

HIV type 1 (HIV-1)-associated dementia (HAD) is believed to occur due to aberrant activation of monocyte-derived macrophages and brain-resident microglial cells by viral proteins as well as by the proinflammatory mediators released by infected cells. To investigate the inflammatory aspects of the disease, we examined the levels of soluble CD40L (sCD40L) in paired samples of plasma and cerebrospinal fluid obtained from 25 HIV-infected individuals. A significantly higher level of sCD40L was detected in both cerebrospinal fluid from HIV-infected patients with cognitive impairment, compared with their nonimpaired counterparts. The contribution of sCD40L to the pathogenesis of HAD was then examined by in vitro experiments. rCD40L synergized with HIV-1 Tat to increase TNF-α release from primary human monocytes and microglia, in an NF-κB-dependent manner. The mechanistic basis for this synergism was attributed to a Tat-mediated up-regulation of CD40 in monocytes and microglia. Finally, the CD40L-mediated increase in TNF-α production by monocytes was shown to be biologically important; immunodepletion experiments revealed that TNF-α was essential for the neurotoxic effects of conditioned medium recovered from Tat/CD40L-treated monocytes. Taken together, our results show that CD40 signaling in microglia and monocytes can synergize with the effects of Tat, further amplifying inflammatory processes within the CNS and influencing neuronal survival.

HAART (11). Thus, HIV-1-associated neurologic complication remains an important and growing problem in the post-HAART era.

Untreated HIV-1 infection is associated with increases in markers of vascular activation, including the release of soluble markers of both endothelial and platelet activation (12–15). These include the candidate HIV-1 neurotoxin, platelet-activating factor (PAF) (16), as well as soluble VCAM and von Willebrand factor (12, 13). HAART has been shown to reduce markers of endothelial activation (soluble VCAM, von Willebrand factor) in HIV-1-infected individuals (17, 18), but to have no effect on soluble platelet activation markers such as PAF acetylhydrolase (19) and soluble CD40L (sCD40L, also known as CD154) (15); indeed, in at least one study, HAART has been associated with an increase in serum sCD40L levels (14).

CD40L is a 33-kDa type II membrane glycoprotein that is predominantly expressed by activated T cells, B cells, myeloid cells, and platelets (20). Truncated, soluble derivatives of this molecule are secreted/shed from activated cells, and retain the ability to bind and activate CD40, which is a 45- to 50-kDa type I integral membrane glycoprotein and a member of the TNFR superfamily (21, 22). Cells known to express CD40 include B cells, dendritic cells, endothelial cells, and macrophages, as well as astrocytes and microglia (23–26).

In this report, we show that sCD40L is present at an elevated level in the plasma and cerebrospinal fluid (CSF) of HIV-1-infected individuals with cognitive impairment, when compared with HIV-1-positive subjects with normal cognitive function, and with untreated HIV-1-positive subjects with normal cognitive function. In vitro experiments were conducted to determine whether this association between elevated sCD40L levels and neurologic disease might reflect a direct involvement of CD40L in virally induced neurotoxicity. Initial studies showed that rCD40L synergized with HIV-1 Tat to increase the production of TNF-α by human monocytes and microglia. This appeared to be a result of Tat’s ability to up-regulate CD40 expression on these cells, thereby sensitizing them to CD40L. Finally, experiments were conducted to evaluate the neurotoxicity of conditioned medium (CM) from Tat/CD40L-exposed monocytes. These studies showed that CM from Tat/CD40L-exposed monocytes elicited a higher level of neurotoxicity than CM from monocytes exposed to Tat or CD40L alone, and that immunity of depletion of TNF-α from CM resulted in a near-complete loss of neurotoxicity. Taken together, these findings reveal a novel interaction between HIV-1 Tat and CD40L signaling and suggest that such interactions can influence neuronal survival.

Materials and Methods

Reagents

HIV-1 Tat (1-101) was obtained from ImmunoDiagnostics; HIV-1 (BAL, IIIB, and MN strain) gp120 and anti-Tat were obtained from the AIDS Research and Reference Reagent Program, HIV-1 Tat (1-72) and CD40L-neutralizing Abs (MK13) were gifts from Drs. A. Nath (Johns Hopkins University, Baltimore, MD) and M. R. Kehry (Boehringer Ingelheim). BAY 11-7082, helenalin, and carbamyl-PAF (cPAF) were purchased from Biomol, whereas thrombin was obtained from Sigma-Aldrich. rCD40L was purified from SF9 insect cells infected with baculovirus vectors expressing human CD40L as described (27). Species-appropriate CD40L was used in experimental assays (i.e., murine CD40L was used in studies with murine BV-2 cells and human CD40L was used with human cells). For control studies, the same purification method was used in conjunction with uninfected SF9 cells; this preparation was then used as a negative control. Analogous to our previous reports (28, 29) and that of others (27), the concentrations of CD40L used in experimental analyses are described in terms of the relative dilution of the CD40L-containing stock prepared.

Patient material

Twenty-five HIV-seropositive patients were clinically evaluated at the University of Rochester Medical Center (Rochester, NY). The patients underwent a standard neurological and neuropsychological evaluation as previously described (30). Cognitive impairment was defined as either a moderate abnormality in at least two neuropsychological tests (one SD below normative data) or a deficit in at least a single test (two SD below normative data; see Dana Consortium on Therapy for HIV Dementia and Related Cognitive Disorders (31)). All subjects with cognitive impairment had an AIDS dementia complex stage ≥0.5. These patients were also characterized for their CD4+ T cell counts in blood by flow cytometry and for viral load in plasma and CSF by using the NucliSens QT assay (BioMerieux) (limit of detection 50 copies/ml). Plasma and CSF samples were tested for the presence or absence of gamma globulin using standard methods, aliquoted, frozen, and stored at −80°C until use. All patients gave written consent for all procedures, which were approved by the University of Rochester Research Subjects Review Board.

Cell cultures

Human platelets. Platelet-rich plasma was obtained by centrifugation of blood (from anonymous donors obtained via the American Red Cross) at 1800 X g for 8 min, followed by removal into a transfer bag (Charter Medical) at room temperature as described (32). A Pall Biomedical Pure-cell LRF high-efficiency leukoreduction filter was used to remove leukocytes, microaggregates, and anaphylatoxins. Platelets were then washed with 0.9% saline using a COBE 2991 Blood Cell Processor and used in experiments. The maximum numbers of contaminant nonplatelet cells were 0.0001% (for white blood cells) and 0.1818% (for RBC).

Human monocytes. Human peripheral monocytes were isolated from soft-spin buffy coats derived from HIV-1 and hepatitis B virus-seronegative donors, using immunomagnetic isolation methods as described (33). Briefly, PBMC were isolated from buffy coats after centrifugation on a lymphoprep gradient (AXIS-Shield). Monocytes were then isolated by positive selection with anti-CD14 MACS beads (Miltenyi Biotec), and cultured in RPMI 1640 medium supplemented with 10% FBS for 18–24 h before use. The monocyte cultures used in our experiments were ≥95% pure as determined by flow cytometric analyses using FITC-conjugated anti-CD14 Abs.

Human microglial cells and astrocytes. Human microglial cells and astrocytes that were isolated from fetal human brain were supplied by Clonexpress and SciencCell Research Laboratories, and maintained in DMEM containing 10% FBS, 2 mM glutamine, and antibiotics.

Murine microglial cell-line (BV-2). The BV-2 cell line (gift from Dr. R. Donato, University of Perugia, Perugia, Italy) was maintained in DMEM containing 10% FBS, 2 mM glutamine, and antibiotics.

Cerebellar granule neurons (CGNs). Seven-day-old Sprague-Dawley rats were euthanized following carbon dioxide inhalation and cerebellar brain tissue was harvested in accordance with the Animal Welfare Act and National Institutes of Health guidelines, as described (33). In brief, cerebellum was collected, washed, and separated into a single-cell suspension using gentle trypsinization, trituration with a polished glass pipette, and filtration through nylon mesh. Following Percoll density gradient centrifugation to remove glia, the neurons were collected and washed twice in sterile medium without serum, then resuspended in DMEM/F12 medium with 10% horse serum. Cells were plated on poly-l-lysine (70–150 K molecular mass; Sigma-Aldrich)-coated 48-well plates at a density of 2 × 10^5 cells/well. One day later, 5-fluorodeoxyuridine (20 mg/ml) and uridine (50 mg/ml) were added to eliminate proliferative cells (astrocytes); the purity of the neuronal population was verified by immunocytochemical staining for microtubule-associated protein-2. Under these conditions, the cultures were ≥95% homogeneous for neurons. Neurons were cultured for ≤7 days at 37°C in a humidified atmosphere containing 5% CO2 and suspended in serum-free DMEM/F12, for 24 h before use.

ELISA

sCD40L was measured in plasma and CSF samples derived from HIV-1-infected individuals using a human CD40L ELISA kit (R&D Systems) and methods outlined previously (32, 34). This assay has a minimum sensitivity of 2–10 pg/ml. The same kit was also used to measure sCD40L levels in platelet culture supernatants.

TNF-α levels were measured in culture supernatant (precollected by brief centrifugation) by using a human or mouse TNF-α ELISA kits (as appropriate; BioSource International) according to the manufacturer’s instructions. These kits have a minimum sensitivity threshold of 2 (human) or 3 pg/ml (mouse). Briefly, 100 µl of cell culture supernatant was incubated in a 96-well plate precoated with a TNF-α-specific mAb for 1 h. After extensive washing, binding of TNF-α was detected by incubation with biotinylated Abs, followed by streptavidin-peroxidase; colorimetric enzyme assays were performed to detect bound POD (33). Levels of IL-6 and IL-8 were also measured in culture supernatant by using ELISA kits specific to...
human IL-6 (sensitivity: 2 pg/ml) and to human IL-8 (sensitivity: 5 pg/ml). Both the kits were obtained from BioSource International.

Real-time RT-PCR
Total RNA was extracted using the high pure RNA isolation kit (Roche Diagnostics) and the protocol provided by the manufacturer. cDNA synthesis was performed using 2 μg of total RNA, oligo-dT primers, and the Superscript III first-strand synthesis system (Invitrogen Life Technologies). Specific pairs of primers and TaqMan probes were generated using Beacon Designer 2.06 software (Premier Biosoft International). Primers and probe sequences are as follows: 1) TNF-α primers, forward 5’-ACCTCCAGA ACATCTTGGAATGAC-3’; reverse 5’-GGGATCACTGTTTTCTGT GC-3’; TNF-α probe, 6-FAM-5’-AAC CAA GCA GCC CAG CCA GTT-3’-TAMRA; 2) β-actin primers, forward 5’-ACCTGACAGACT CTTAGGA-3’; reverse 5’-AGACAACTAGATGCATTTGTCC-3’; β-actin probe, 6-FAM-5’-TCT TGA CCG ACG GTG ACT ACA GC-3’-TAMRA. Each PCR contained 12.5 μl of 2 × TaqMan Universal PCR Master mix (Applied Biosystems), 1 μl of template cDNA, 900 nM forward primer, 900 nM reverse primer, and 200 nM probe in a total volume of 25 μl. After denaturation for 10 min at 95°C, the PCR was subjected to 40 cycles of 95°C for 15 s and 60°C for 60 s using the iCycler instrument (Bio-Rad). Agarose (1.7%) gel electrophoresis of the PCR was used to identify the PCR products. cDNA constructs expressing mouse TNF-α (NM_012693) and β-actin (NM_007393) were generated by inserting corresponding full-length PCR products into pCR2.1 vector using the TA Cloning kit (Invitrogen Life Technologies). Serial dilutions of these plasmids were then used to generate a standard curve. β-actin served as an internal control in these experiments.

Flow cytometry
BV-2 cells (1 × 10⁶) were grown in 24-well tissue culture plates and prepared for flow cytometry by washing the cell suspension in PBS containing 2% FBS and 0.1% sodium azide. For cell surface CD40 staining, cells were fixed with 2% paraformaldehyde and incubated directly with anti-mouse CD40-allophycocyanin Abs (BD Pharmingen). Incubation of cells was then fractionated by SDS-PAGE and electrophoretically transferred to Hybond ECL nitrocellulose membrane (Amersham). The membranes were analyzed for immunoreactivity with primary Abs raised against human CD40 or α-tubulin (Santa Cruz Biotechnology) at a dilution of 1:500. Bound Abs were detected with HRP-linked sheep secondary Abs (Amersham), followed by the addition of ECL reagent (Pierce Biotechnology) and subsequent exposure to x-ray film. Equal loading and uniformity of protein transfer to the nitrocellulose membrane were verified by confirming that nonspecific bands detected following the enhanced ECL reaction were present at equivalent levels in all lanes, and/or by stripping and reprobing the nitrocellulose membrane using primary Abs specific for α-tubulin.

TUNEL assays
Apopotic cells were visualized by using in situ TUNEL assay, according to the manufacturer’s instructions (Intergen) (33). Briefly, CGN cells (1.5 × 10⁶; DIV 7) were treated with conditioned medium (at dilution 1/6) derived from experimentally treated human monocytes, for 24 h. Cells were then washed with cold PBS, fixed with 4% paraformaldehyde, placed in equilibration buffer and incubated in a reaction buffer containing TdT and dUTP for 60 min at 37°C. After rinsing, the cells were incubated with peroxidase-conjugated anti-digoxigenin (as well as Triton X-100 and a blocking agent). The cells were then reacted with 0.5 mg/ml diamino-benzidine and 0.05% hydrogen peroxide to generate a brown reaction product. The percentage of TUNEL-positive cells (brown) was assessed by analysis of digitized images from nine or more microscopic fields of TUNEL-stained cells; image capture and digitization was performed using Adobe Photoshop software (version 7.0).

Statistical analysis
Mean data values and the SEM were computed for each variable. Mean data values for the two groups of patients were compared by using the Student t test for unpaired data. All other data (which involved the analysis of multiple sample groups) were analyzed by one-way ANOVA followed by Bonferroni’s test for multiple comparisons. A value of p < 0.05 was designated as statistically significant.

Results
sCD40L levels are increased in the plasma and CeSF of HIV-1-infected individuals with cognitive impairment, when compared with matched HIV-1-positive subjects without cognitive impairment
We tested whether levels of sCD40L in the plasma and CeSF of HIV-1-infected patients were altered following the onset of symptomatic neurologic disease. To do this, we collected paired samples of plasma and CeSF from 25 HIV-positive individuals most of whom were receiving antiretroviral therapy. Sixteen of these individuals were clinically diagnosed with cognitive impairment (AIDS dementia complex stage ≥0.5) while the other nine were judged to be cognitively normal; the other characteristics of these
two patient groups, such as duration of infection, viral load, and CD4⁺ T cell count, were very similar (Table I). The history of drug abuse among these patients was not available. Cognitive impairment was defined as having abnormalities in at least two neuropsychological tests one SD below normative data or one test two SD below normative data (see, Refs. 30 and 31). sCD40L levels in plasma and CeSF samples obtained from HIV-positive patients, both before and after antiretroviral therapy (38, 39), and 3) sCD40L in plasma is believed to arise almost solely (95%) from activated platelets (40). In these assays, we exposed human platelets either to thrombin, a powerful platelet activator, to the HIV-1 gene products Tat and gp120, or to PAF, a proinflammatory mediator that has been implicated in the pathogenesis of HAD (16). High levels of sCD40L release were detected in thrombin- or PAF-treated cultures, but not in cultures treated with Tat or gp120 (Fig. 1B). This suggests that proinflammatory mediators induced by HIV-1 infection, such as PAF, may contribute to platelet activation in vivo and lead to the observed elevation in sCD40L levels. It is noteworthy that the degree of neurologic dysfunction in HIV-1-infected patients has been shown to be associated with increased levels of PAF in CeSF (16).

![FIGURE 1](image1)

**FIGURE 1.** Levels of sCD40L are elevated in plasma and CeSF of HIV-1-infected individuals with cognitive impairment, compared with matched HIV-1-positive subjects without cognitive impairment. A, sCD40L levels in plasma and CeSF samples obtained from HIV-positive patients (without cognitive impairment, n = 9) and with cognitive impairment (n = 16) were analyzed by ELISA and shown as mean ± SEM. Statistical significance was determined by unpaired t test; * and #, p < 0.001, as compared with respective samples from HIV-positive subjects without cognitive impairment. B, Purified human platelets (6 × 10⁹) were exposed to thrombin (0.8 U/ml), cPAF (20 nM), HIV-1 Tat (1-101; 100 nM), HIV-1 gp120 (Bal, 100 nM), or vehicle alone (NT) for 1 h. Supernatants were then collected and analyzed for sCD40L levels by ELISA. Data represent mean values derived from three experiments; the bars represent the SEM. Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *, p < 0.01, as compared with NT.
Hyperactivation of primary human monocytes by CD40L and Tat

The pathogenesis of HAD is believed to involve activation of peripheral monocytes, and subsequent recruitment of these cells into the CNS, where they undergo cellular differentiation into brain-resident microglial phenotype and serve as a sanctuary for HIV-1 replication and production of secretory neurotoxins (41, 42). We therefore examined whether CD40L could activate human monocytes. To do this, human peripheral blood-derived monocytes were treated with rCD40L, alone or together with Tat, and release of TNF-α, IL-6, and IL-8 was measured (as markers of monocyte activation) in cell culture supernatants using ELISA. CD40L alone failed to stimulate synthesis of TNF-α, IL-6, and IL-8 (Fig. 2, A–C). As expected (43), treatment of monocytes with Tat rapidly up-regulated release of these cytokines; this was further elevated by simultaneous exposure to CD40L. This effect of CD40L on TNF-α synthesis was specific, because coadministration of CD40L-neutralizing Abs (MK-13) abolished the synergy between CD40L and Tat (data not shown). Moreover, CD40L failed to synergize with gp120 (Fig. 2D) or PAF (data not shown) to stimulate release of TNF-α, indicating that the synergistic effects of CD40L are limited to Tat. Together, these results show that CD40L can synergize with Tat to hyperactivate monocytes and thereby increase production of potentially neurotoxic proinflammatory cytokines.

Synergistic activation of primary human microglia by CD40L and Tat

We also examined whether CD40L might have a similar effect on brain-resident microglial cells. Primary human microglia were treated with CD40L (D), or HIV-1 gp120 (E); in D and E, Tat was used at a standard concentration (100 nM). NT represents vehicle-treated control cells. TNF-α release was measured by ELISA and presented as picograms per milliliter (A), or as picograms per milligram of total cellular proteins in the culture well (B–E; to account for cell division in BV-2 cell cultures). Results are shown as mean ± SEM of values derived from three replicates from a single representative experiment; the experiment was performed twice. In B, *, **, and # indicate statistical significance (p < 0.01), as compared with the corresponding time points for cells exposed to Tat alone. In C, *, # indicate statistical significance (p < 0.01), as compared with vehicle-treated cells (NT); * or # indicates statistical significance (p < 0.01) compared with cells exposed to Tat alone (see C for TNF-α release by cells exposed to 100 nM Tat only). Exposure of cells to Tat alone or Tat plus gp120 gave statistically indistinguishable data values for TNF-α release (E).
origin (monocytes and microglia) and not primary human astrocytes (cells of neuroectodermal origin), produced higher levels of TNF-α in response to CD40L and Tat (data not shown), suggesting that the synergistic effect of CD40L and Tat is limited to certain cell types.

Kinetics of TNF-α synthesis in microglial cells by CD40L and Tat

Next, we investigated whether CD40L and Tat induce TNF-α release in microglial cells in a time-, and dose-, dependent, fashion. For this purpose, we used a murine microglial cell line (BV-2) that is highly sensitive to inflammatory stimuli such as Tat (45). Treatment of BV-2 cells with Tat alone led to a significant secretion of TNF-α that peaked at 12 h and was diminished by 24 h (Fig. 3B), whereas, time-dependent increase in TNF-α synthesis was not observed in CD40L-treated cells (data not shown). BV-2 cells exposed simultaneously to Tat and CD40L produced higher levels of TNF-α at all time points post 4 h, and TNF-α levels remained elevated until 24 h after addition of Tat plus CD40L. These data demonstrate that the synergistic effect of CD40L and Tat on TNF-α production is not transient. Similarly, increasing doses of Tat (Fig. 3C) and CD40L (Fig. 3D) were able to produce escalating amounts of TNF-α in these cells. These effects of Tat or CD40L were specific, because addition of Tat- or CD40L-neutralizing antisera completely inhibited synergy (data not shown).

To test whether the observed synergy between Tat and CD40L was unique, we examined the effect of HIV-1 gp120 on Tat-induced TNF-α release (46). To do this, BV-2 cells were treated with increasing amounts of recombinant gp120 alone, or in the presence of a fixed amount of Tat; TNF-α levels were then measured in the culture supernatant. As shown in Fig. 3E, similar levels of TNF-α were released by BV-2 cells following exposure to Tat alone or Tat plus HIV-1 gp120 (only data for gp120 Bal is shown, but similar results were obtained for gp120 derived from both the MN and IIIB strains of HIV-1). These data demonstrate that the ability to enhance Tat-induced hyperactivation of monocytes and microglia is limited to certain proinflammatory molecules (such as CD40L).

Mechanism of CD40L and Tat-mediated TNF-α synthesis in microglial cells

To understand better how CD40L might synergize with Tat to produce higher amounts of TNF-α, BV-2 cells were treated with Tat and CD40L, alone, or in combination, and the abundance of TNF-α transcripts was measured by real-time RT-PCR. These experiments revealed that coadministration of CD40L and Tat led to a marked accumulation of TNF-α mRNA when compared with cells that were treated with Tat alone (Fig. 4A). This suggested that the synergy between CD40L and Tat, in regulating TNF-α synthesis, might be occurring at the transcriptional level.

Because previous studies have implicated Tat-mediated activation of the transcription factor NF-κB as being critical for Tat-induced up-regulation of TNF-α in human monocytes (47), we examined activation of NF-κB by Tat and CD40L in BV-2 cells. The cells were transiently transfected with a luciferase reporter plasmid driven by the κB enhancer (κB-TATA-luciferase). After 24 h, the cells were either left untreated or treated with Tat, CD40L, or Tat plus CD40L for 8 h and harvested for luciferase assays. As expected, κB-directed luciferase gene expression was potently induced in the cells that were treated with Tat (Fig. 4B), indicating activation of NF-κB in these cells. Modest increase in NF-κB activation was observed in CD40L-treated cells, however, this effect was statistically not different from nontreated cells. Moreover, NF-κB-dependent transcription of luciferase was even higher in Tat plus CD40L-treated BV-2 cells, showing that NF-κB activation was further increased following Tat and CD40L treatment.

To test the role of NF-κB in regulating Tat/CD40L-stimulated production of TNF-α, BV-2 cells were treated with Tat or Tat plus
CD40L, in the absence or presence of the NF-κB inhibitor Bay 11-7082, and TNF-α levels in the culture supernatant were measured. As shown in Fig. 4C, addition of Bay 11-7082 blunted TNF-α synthesis triggered by both Tat alone and by the combination of Tat and CD40L. Although the action of Bay 11-7082 involves inhibition of the NF-κB regulatory kinase, IκB kinase (48), it remained possible that the inhibition of TNF-α synthesis by Bay 11-7082 might be due to nonspecific effects of this drug on other pathways. To exclude this possibility, we transfected BV-2 cells with a vector encoding a highly selective molecular inhibitor of NF-κB (a degradation-resistant IκBα mutant, plκBα S32/36A); the cells were then treated with Tat and CD40L. Expression of this IκBα mutant potently inhibited the synthesis of TNF-α in Tat and CD40L-treated cells (Fig. 4D), underscoring the importance of NF-κB activation in Tat and CD40L-mediated TNF-α production.

**Tat-mediated induction of CD40 expression in primary cultures of human monocytes and microglia.**

To understand the molecular basis for the observed synergy between Tat and CD40L, we conducted experiments to determine whether Tat might induce expression of CD40, and thereby promote CD40L-mediated effects on cytokine production by monocytes and microglia. As determined by immunoblot analyses, low levels of CD40 were detected in primary human monocytes (Fig. 5A). Within 8 h, these levels were profoundly elevated by Tat treatment. We also performed indirect immunofluorescence assays to examine cell surface expression of CD40 in primary human microglia and astrocytes. As shown in Fig. 5B, CD40 was undetectable in vehicle-treated microglia, but could be readily visualized in cells that had been exposed to Tat. Tat failed to induce CD40 expression in primary human astrocytes (data not shown).

To quantitatively assess the effect of Tat on cell surface expression of CD40, we conducted flow cytometric analyses. BV-2 cells were exposed to either vehicle or Tat for 8 h; some cells were treated with other HIV toxins such as gp120 or PAF. The cells were then fixed, stained and prepared for flow cytometry using an anti-CD40-specific Ab. Approximately 12% of vehicle-treated BV-2 cells were positive for cell surface CD40 expression (Fig. 5C); following exposure to Tat, the percentage of CD40-positive cells increased by 5-fold. In contrast, exposure of cells to either gp120 or PAF had no effect on CD40 expression (Fig. 5C), indicating that the effect of Tat on CD40 expression is selective.

In light of these results (Figs. 3 and 5), we investigated whether Tat might directly induce expression and release of CD40L in microglial cells and whether this might contribute to Tat-induced release of TNF-α. To do this, we treated BV-2 cells with Tat in the absence or presence of CD40L-neutralizing Abs and then measured TNF-α release by ELISA. We found no change in Tat-mediated production of TNF-α following addition of CD40L-neutralizing Abs (or irrelevant control IgG; data not shown). These results are also consistent with the fact that Tat failed to stimulate CD40L release from human platelets (Fig. 1B).
Mechanism of CD40 induction in Tat-treated microglial cells

CD40 gene expression is known to be regulated by NF-κB (49), suggesting a possible pathway by which Tat might up-regulate expression of this cell surface receptor. We therefore tested whether Tat-mediated activation of NF-κB might be important for the regulation of CD40 expression in microglial cells. To do this, BV-2 cells were treated with Tat in the absence or presence of NF-κB inhibitors, Bay 11-7082 and helenalin, and CD40-positive cells were quantitated by flow cytometry. Cultures exposed to Tat alone contained high numbers of CD40-positive cells when compared with untreated cultures (Fig. 6A). This Tat-mediated up-regulation of CD40 expression was completely abrogated in cultures that were cotreated with either of the two NF-κB inhibitors. Overexpression of the degradation-resistant IκBα mutant (pIκBα S32/36A) in BV-2 cells also blunted Tat’s effect on CD40 expression (data not shown). These data demonstrate that Tat-mediated up-regulation of CD40 in microglial cells is dependent on Tat-induced activation of NF-κB.

The most likely mechanism by which NF-κB might contribute to Tat-mediated up-regulation of cell surface CD40 is through direct effect on CD40 gene expression. To evaluate this, BV-2 cells were transiently transfected with plasmids containing the luciferase reporter gene under the transcriptional control of either a minimal murine CD40 promoter element (–195 to +22, designated as WT) or an otherwise identical promoter that lacks the NF-κB-binding site at residues −128 to −119 (designated as ΔNF-κB). Some cells were transfected with 5 μg of an OCT-1-driven luciferase reporter plasmid (designated as OCT-1 Luc). Twenty-four hours later, the cells were either left untreated or treated with Tat (1–101, 100 nM) for 8 h and luciferase activity was measured. Results are expressed as mean values (±SEM) of relative light units derived from three replicates. *, p < 0.01 as compared with untreated cells (NT); **, p < 0.01 as compared with cells exposed to Tat alone. B, BV-2 cells (1.5 ⋅ 10⁵) were transiently transfected with 5 μg of plasmid vectors containing a luciferase reporter construct, OCT-1 Luc, the NF-κB-binding site present in the full-length, native promoter (designated as OCT-1 Luc). Twenty-four hours after transfection, the cells were treated with either vehicle or Tat for 8 h, and luciferase activity was measured. As shown in Fig. 6B, Tat induced CD40-driven reporter gene expression in a NF-κB-dependent manner. The BV-2 cells were also transfected with an OCT-1-driven luciferase reporter, whose activity was not altered by Tat treatment, indicating that Tat’s effect on NF-κB is specific. The relatively modest effect of Tat on CD40-driven luciferase reporter gene expression in this experiment (a 2-fold enhancement) may reflect the fact that we used a minimal CD40 promoter element which lacks an additional upstream NF-κB-binding site present in the full-length, native promoter (−562 to −553) (49).

Enhanced synthesis of TNF-α in CD40L and Tat exposed human monocytes contributes to neuronal apoptosis

The association between elevated levels of sCD40L in CSF and plasma, and cognitive impairment of HIV-1-infected individuals (Fig. 1A), suggested to us that CD40L might potentiate the release
of soluble neurotoxins. To address this hypothesis, and to test whether TNF-α might directly contribute to the neurotoxicity of secretions produced by Tat/CD40L-treated monocytes, we performed in vitro experiments using CM collected from Tat- and Tat/CD40L-treated monocytes. The monocyte CM was then added to primary cultures of rat CGNs and neuronal apoptosis was quantitated by TUNEL assay. It should be noted that the rat CGNs represent a well-characterized model system for measuring toxic effects of HIV-1 Tat (33, 50). In some experimental groups, the neurons were also cotreated with neutralizing Abs to human TNF-α (anti-TNF), TNF-α receptor 1-blocking Abs (anti-TR1), or isotype-matched irrelevant Abs (IgG). As shown in Fig. 7, a high level of apoptosis was detected in CGN cultures that were treated with CM collected from Tat-treated monocytes (CM-Tat), but not in neuronal cultures exposed to CM from untreated monocytes (CM-NT). CM from monocytes treated with CD40L plus Tat elicited an even higher level of apoptosis (CM-Tat plus CD40L). Immunodepletion of TNF-α (using a TNF-neutralizing Ab; anti-TNF) abrogated the neurotoxicity of the conditioned medium from both Tat- and Tat/CD40L-treated monocytes. Blockade of TNFR1 also abrogated the neurotoxicity of the monocyte conditioned medium (anti-TR1), but addition of an irrelevant control Ab had no effect on neurotoxicity (IgG). These findings show that CD40L potentiates the ability of HIV-1 Tat to elicit neurotoxic production by human monocytes, and that TNF-α represents the major neurotoxic effector molecule in conditioned medium from Tat/CD40L-treated monocytes.

Discussion

Onset and progression of HAD is known to be associated with recruitment of myeloid cells to the brain and elevated levels of proinflammatory mediators within the CNS. Previous studies have also shown a marker of platelet activation, PAF, is present at an elevated level in the CSF of HIV-1-infected subjects with symptomatic CNS disease (16). Taken together, these findings suggest that platelet activation in HIV-1 infected individuals might potentiate the activation of mononuclear phagocytes and thereby lead to increased production of neurotoxic inflammatory mediators within the CNS.

The experiments reported here provide strong support for this hypothesis. We now show that PAF triggers the release of soluble CD40L from human platelets and that circulating levels of sCD40L are elevated in cognitively impaired HIV-1-positive subjects, when compared with matched HIV-1-infected individuals without cognitive impairment. This raises the intriguing possibility that plasma and CSF levels of sCD40L might serve as a useful biomarker either for onset or progression of HAD. Further studies, including careful longitudinal analysis of samples from HIV-1-infected subjects who either did or did not progress to HAD may shed light on this question. It will also be of interest to determine how changes in the level of platelet activation markers may be related to the platelet decline that occurs in some HIV-infected individuals and which has been linked to the onset of CNS disease in SIV-infected macaques (51).

Elevated circulating levels of sCD40L have been observed in a variety of diseases in which sCD40L is thought to initiate or potentiate inflammation; these include atherosclerosis (52), acute coronary syndromes (53), and type 1 diabetes (54). Consistent with this notion, in the present study, we show that CD40L potentiates the ability of HIV-1 Tat to hyperactivate monocytes and microglial cells, leading to the secretion of neurotoxic inflammatory mediators (notably, TNF-α). This synergistic interaction depends upon a two-step activation process. First, Tat stimulates the NF-κB-mediated up-regulation of CD40. The ability of Tat to up-regulate CD40 may explain the previously described increase in CD40 expression in microglial cells within brain tissue from HIV-1-infected subjects (55) and is probably mediated by a calcium-dependent signaling pathway. However, it remains controversial as to whether Tat triggers release of calcium from intracellular stores (56) or whether it enhances uptake of extracellular calcium (57, 58).

The second step in the interplay between Tat and CD40L involves the action of sCD40L on newly up-regulated CD40 receptors, which results in CD40-mediated signal transduction and enhanced stimulation of NF-κB in monocytes and microglia. Hyperactivated monocytes and microglia then produce high level of neurotoxic inflammatory mediators, including TNF-α, which contribute to the onset and progression of HAD. An analogous two-step activation model for microglial cells, in which the activation stimuli are delivered by encephalitogenic T cells, has recently been proposed to be important for disease progression in experimental autoimmune encephalomyelitis (59).

The concentration of recombinant Tat (100 nM) used in the present studies is consistent with those used in our previous in vitro studies (33) and with similar studies from other laboratories (57, 60, 61). These concentrations are considerably higher than the estimated physiological concentration of Tat in the tissues or body fluids from HIV-1-infected individuals (62). However, as described by Nath et al. (63), the technical factors such as the susceptibility of Tat to freeze/thaw cycles, its oxidation sensitivity, and its affinity to glass and plastic, have led to a massive underestimation of the neurotoxic potential of Tat in previous in vitro experiments. To be consistent with our previous studies, we have continued to use Tat under the same conditions described in our published work. However, it is important to note that the actual concentration of free, biologically active Tat is much lower than this concentration.

The results shown here implicate TNF-α as a major contributor to the neurotoxicity of conditioned medium from Tat/CD40L-treated monocytes and microglia. Our earlier results have shown that TNF-α-induced neurotoxicity occurs via the generation of ceramide in neurons, and is dependent on activation of neuronal PAF receptors (64). Furthermore, PAF catabolism reduces TNF-α-mediated neurotoxicity (64). Collectively, these observations suggest that HAD may be characterized by a positive feedback loop between monocyte/microglial stimulation and platelet activation. Specifically, virotoxins such as HIV-1 Tat may trigger monocyte and microglial activation, leading to release of neurotoxic inflammatory mediators that include TNF-α and PAF. PAF then contributes to the ongoing activation of platelets and the generation of sCD40L, which in turn potentiates Tat-mediated stimulation of myeloid cells. This model predicts that drug regimens which simultaneously target activation of both monocytes/microglia and platelets may of therapeutic value in the treatment of HAD. In addition, pharmacologic agents that inhibit the generation of sCD40L, such as statins and glitazones (65), may also merit exploration as neuroprotective agents in HAD.

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Disclosures

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