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Activation of p38 MAPK Is Required in Monocytic and Neuronal Cells for HIV Glycoprotein 120-Induced Neurotoxicity

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HIV-1 envelope protein gp120 has been implicated in neurotoxin production by monocytic cells (i.e., macrophages and microglia), as well as in the pathogenesis of HIV-1–associated neurocognitive disorders. We previously showed in cerebrocortical cell cultures from rodents containing microglia, astrocytes, and neurons that overall inhibition of p38 MAPK signaling abrogated the neurotoxic effect of HIV-1 gp120. However, the time course of p38 MAPK activation and the contribution of this kinase in the various cell types remained unknown. In this study, we found that active p38 MAPK is required in monocytic lineage cells (i.e., macrophages and microglia) and neuronal cells for HIV gp120-induced neurotoxicity to occur. In cerebrocortical cell cultures, HIV-1 gp120 stimulated a time-dependent overall increase in active p38 MAPK, and the activated kinase was primarily detected in microglia and neurons. Interestingly, increased activation of p38 MAPK and neuronal death in response to gp120 were prevented by prior depletion of microglia or the presence of CCR5 ligand CCL4 or p38 MAPK inhibitors. In human monocytic THP-1 cells and primary monocyte-derived macrophages, HIV gp120-stimulated production of neurotoxins was abrogated by prior introduction into the cells of a dominant-negative p38 MAPK mutant or p38 MAPK small interfering RNA. In addition, the neurotoxic effects of cell-free supernatants from gp120-stimulated monocytic THP-1 cells were prevented in microglia-depleted cerebrocortical cells pretreated with a pharmacological inhibitor of p38 MAPK. Thus, p38 MAPK signaling was critical, upon exposure to HIV gp120, for the neurotoxic phenotype of monocytic cells and subsequent toxin-initiated neuronal apoptosis. The Journal of Immunology, 2010, 185: 4883–4895.

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Abbreviations used in this paper: AdV, adenoviral expression vector; AdV-DNp38, adenovirus expressing dominant-negative p38 MAPK; AdV-GFP, adenovirus expressing GFP, ATF, activating transcription factor; CM, conditioned media; G, glia only; gp120Δp38, envelope glycoprotein of HIV-1 strain SF2; gp120Δp38Δ, envelope glycoprotein of HIV-1 strain SF162; HAND, HIV-1–associated neurocognitive disorder; LME, leucine methyl ester; MAP, microtubule-associated protein; MDM, monocye-derived macrophage; MOL, multiplicity of infection; N+G, neurons plus glia; NMDA, N-methyl-D-aspartic acid; NT, nontargeting; pJNK, phospho–c-Jun; pp38, phospho-p38 MAPK; RPMI-ABS, RPMI 1640 containing 2 mM glutamine, antibiotics (penicillin/streptomycin), and 10% human AB serum; SAPK, stress-activated protein kinase; siRNA, small interfering RNA.

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support of the latter, depletion or inactivation of microglia in mixed neuronal-glial cell cultures completely abrogates HIV-1 gp120-induced neuronal death (15, 21). Furthermore, we previously showed that CCR5 and CXCR4 can mediate the neurotoxic effect of gp120, depending on the coreceptor usage of the virus strain from which the envelope protein originated (16).

In addition, β-chemokines and natural CCR5 ligands CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) were reported to be major suppressors of HIV-1 infection and disease progression (22). These reports suggested that suppression occurs through steric hindrance, receptor internalization, and/or via CCR5-mediated protective signaling against HIV. In support of this, our group and other investigators found that the CCR5 ligands CCL4 and CCL5 block neuronal death caused by HIV-1 gp120 in vitro (14, 15).

Macrophages or microglia are principal targets for β-chemokines and gp120 variants, but the mechanism by which gp120-induced chemokine receptor signaling in macrophages/microglia results in a neurotoxic phenotype is not well defined. However, previous studies showed that possible mechanisms of HIV-1 neuropathogenesis involve the perturbation of intracellular signaling by HIV-1 gp120 and the subsequent release of neurotoxic factors from activated macrophages and microglia (3, 12, 15, 19). Previous studies showed that possible mechanisms of HIV-1 neuropathogenesis involve the perturbation of intracellular signaling by HIV-1 gp120 and the subsequent release of neurotoxic factors from activated macrophages and microglia (3, 12, 15, 19). Intracellular signaling pathways, such as those of Src family kinase Lyn (20), PI3K (20), Akt (16), the focal adhesion factors from activated macrophages and microglia (3, 12, 15, 19), and gp120-induced chemokine receptor signaling in macrophages/microglia results in a neurotoxic phenotype is not well defined. However, previous studies showed that possible mechanisms of HIV-1 neuropathogenesis involve the perturbation of intracellular signaling by HIV-1 gp120 and the subsequent release of neurotoxic factors from activated macrophages and microglia (3, 12, 15, 19, 23–25). Intracellular signaling pathways, such as those of Src family kinase Lyn (20), PI3K (20), Akt (16), the focal adhesion-related proline-rich tyrosine kinase Pyk2 (20, 25), phosphatidylinositol 3-kinase (C26), proteins of the MAPK family (15, 16, 25, 27), and the transcription factor p53 (21, 27), have been implicated as potential pathways responsible for gp120-induced macrophage activation and neurotoxicity. Of these, MAPKs are involved in several diverse biological activities, including differentiation, proliferation, apoptosis, and inflammation (28). The stress-activated p38 MAPK was shown to be responsible for neuronal death, microglial/macrophage activation, and proinflammatory cytokine production in several CNS disorders (15, 16, 29–32). In fact, we previously showed that inactivating p38 MAPK with p38-specific pharmacological inhibitor SB203580 or dominant negative p38α (p38αAF) in mixed neuronal-glial cultures prevented neuronal death triggered by HIV-1 gp120, whereas other investigators reported that blocking p38 MAPK activity also ameliorated glutamate toxicity in neuron-rich cell cultures (15, 16, 29, 32). However, the molecular mechanism of p38 MAPK activation and its cell-specific responses downstream of chemokine receptor signaling in gp120-mediated neurotoxicity are still unknown.

For this purpose, we investigated the neurotoxic effects of HIV-1 gp120 on cell type-specific p38 MAPK signaling pathways using primary mixed neuronal-glial cerebrocortical cultures from rodents and human mononuclear/monocytic THP-1 cells or primary human monocyte-derived macrophages (MDMs) as models for macrophages and microglia. We found that HIV-1 gp120 activated p38 MAPK primarily in neurons and microglia but not in astrocytes. We also found that depletion of microglia from mixed cultures and pretreatment with neuroprotective CCL4 reduced the activation of neuronal p38 MAPK after gp120 exposure. In addition, monocytic/macroage-like THP-1 cells and MDMs required p38 MAPK for gp120-induced neurotoxicity production. However, inhibiting p38 MAPK activation in neurons prevented death induced by neurotoxic media derived from gp120-stimulated THP-1 cells, whereas the absence of HIV coreceptors in cerebrocortical neurons and astrocytes failed to protect the cell cultures from gp120-induced neurotoxicity of the monocytic cells. Our results suggested that p38 MAPK signaling is responsible for the neurotoxic phenotype of macrophages/microglia, as well as the initiation of neuronal apoptosis upon exposure to HIV gp120.

Materials and Methods

Reagents

Recombinant gp120 from different HIV-1 strains, SF2 (gp120SF2), dual-tropic or SF162 (gp120SF162; CCR5 preferring), were obtained from National Institutes of Health AIDS Research and Reference Reagent Program. Recombinant human chemokines were purchased from R&D Systems (Minneapolis, MN). A specific p38 MAPK inhibitor, SB203580, was obtained from Calbiochem (San Diego, CA). Chemokines and HIV gp120 were reconstituted in 0.1% BSA at 100× the final concentration, and controls received BSA vehicle alone (0.001% final concentration). Kinase inhibitor was dissolved in DMSO at 1000× the final concentration (0.1–10 µM) and was added to the cultures for 15 min prior to treatment with 200 pM or 1 nM HIV-1 gp120 strains.

Preparation of cerebrocortical, mixed neuronal-glial cell cultures

Rat mixed neuronal/glial cerebrocortical cultures were prepared from embryos of Sprague-Dawley rats at day 15–17 of gestation, as previously described by our group (15, 16). In brief, cells were cultured in 35-mm dishes with poly-L-lysine–coated glass coverslips (1.87 × 10⁵ cells per dish) or poly-L-lysine–coated clear-bottom 96-well plates for imaging (0.087 × 10⁵ cells per well; BD Falcon, BD Biosciences, San Jose, CA) and D10C medium containing 80% DMEM with high glucose (Invitrogen, Carlslbad, CA), 10% FBS (Hyclone Laboratories, Logan, UT), 10% F12 (Hyclone), 3% 1 M HEPES (Omega), 200 mM l-glutamine (Invitrogen), and 100 U/ml penicillin with 100 mg/ml streptomycin (Sigma). Cell populations consisted of ~30% neurons, ~70% astrocytes, and ~0.1–1% microglia (16) and, generally, were used after 17 d in vitro when the majority of neurons were considered fully differentiated and susceptible to N-methyl-D-aspartic acid (NMDA) toxicity. For most experiments, rat cultures were transferred into prewarmed Earle’s balanced salt solution containing 1.8 mM Ca²⁺ and 5 mM glycine without Mg²⁺. For some experiments in which the contribution of microglia to neuropathogenesis was investigated, microglia were depleted by pretreatment of the cultures overnight with 7.5 mM l-leucine methyl ester (15). Importantly, rodents express CXCR4 and CCR5 homologs, which are capable of interacting with HIV-1 via gp120 binding (15, 16). Murine cerebrocortical cell cultures from CCR5/CXCR4 double-knockout embryos were prepared, as previously published by our group (16); they are similar to their above-described counterpart derived from rat embryos, except that they contain ~4–5% microglia. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the Sanford-Burnham Medical Research Institute.

Isolation and preparation of MDMs

The preparation of human primary MDMs, using a Ficoll gradient, was performed as previously described (33, 34). In brief, whole blood from consenting healthy donors was obtained through the Normal Blood Donor Service of The Scripps Research Institute at Scripps Health Green Hospital (La Jolla, CA). Following an initial centrifugation step of the heparinized blood at 200 × g for 20 min,uffy coat cells were isolated using density gradients of Ficoll-paque (1.073; GE Health Life Sciences, Piscataway, NJ) and transferred into 75-cm² cell-culture flasks (35). After allowing adherence in RPMI 1640 containing 2 mM glutamine, antibiotics (penicillin/streptomycin), and 10% human AB serum (RPMI-ABS) at 37°C, 5% CO₂, in humidified atmosphere, nonadherent cells were removed by rigorous washing with warm RPMI 1640 (37°C) without supplements. Adherent monocytes were then cultured for 7 d in the above serum-containing medium to allow for differentiation into MDMs (35). For harvest and further experimentation, the cells were detached by treatment with PBS containing EDTA (0.2 g/l; Sigma, St. Louis, MO) for 5–10 min at 37°C and scraping with a rubber policeman. After washing three times in PBS, cells were resuspended in 0.25 × 10⁶ cells/0.5 ml medium per well in 24-well plates. Cell viability was monitored with trypan blue exclusion and usually exceeded 95%.

Cell culture of human mononuclear THP-1 cells

Human mononuclear THP-1 cells, a model for monocyte lineage cells, including microglia and macrophages (12, 36), were maintained in medium containing 90% RPMI 1640 (Life Technologies, Carlsbad, CA), 10% FBS (Hyclone), 2 mM l-glutamine (Sigma), and a combination of 100 U/ml penicillin with 100 µg/ml streptomycin (Sigma) at 37°C with 5% CO₂; they usually were split 1:3–1:4 once or twice a week after reaching ~1 × 10⁶ cells/ml. For small interfering RNA (siRNA) experiments, THP-1 cells were plated at 0.2 × 10⁵ cells/ml after reaching a density of 0.3–0.4 × 10⁶ cells/ml. Upon reaching a density of 0.6–0.7 × 10⁵ cells/ml, cells were
collected for nucleofection of siRNA duplexes using the Amaxa Nucleofector system (Lonza Walkersville, Walkersville, MD) and the supplier’s protocol.

Assessment of neuronal death

Neuronal apoptotic death and loss were analyzed as previously described (16). In brief, the number of neurons was quantified by immunostaining for the neuron-specific marker microtubule-associated protein 2 (MAP-2)-2, and apoptotic nuclei were identified morphologically after staining nuclei with the DNA dye Hoechst 33342. Alternatively, apoptosis was quantified by TUNEL (Apoptosis Detection System/Fluorescein; Promega, Madison, WI) to detect fragmented nuclear DNA in apoptotic nuclei, as seen in Fig. 9. Neuronal survival was calculated from the percentage of neurons remaining after subtraction of those that had undergone apoptosis. Three to eight independent experiments were performed for each treatment.

Mononuclear cell-mediated neurotoxicity

To obtain conditioned cell-culture supernatants, human mononuclear THP-1 cells or primary MDMs were incubated for 24 h or 4 d in the presence or absence of recombinant viral envelope gp120 of HIV strains SF2 and SF162 in the same medium used to culture rat cerebrocortical cells (THP-1 cells) or RPMI-ABS (MDMs). The controls without gp120 received 0.001% BSA (vehicle) only or CCL4/MIP-1β or CCL5/RANTES (both at 20 nM). For some experiments, mononuclear cells were transduced using adenoviral expression vectors (AdVs) encoding a dominant negatively interfering mutant of p38 MAPK or GFP as control 2 d before exposure to HIV-1 gp120 or BSA control in THP-1 culture media or RPMI-ABS in the case of MDMs (16). All AdVs were kindly provided by Dr. J. Han (The Scripps Research Institute) (37) and amplified and titrated using standard procedures. Following a 1- or 4-d stimulation period, cell-free supernatants were transfected at a final 10% concentration to the mixed rat neuronal-glial cultures depleted of microglia maintained in D10C medium as readout for neurotoxicity (12, 36). Unless stated otherwise, neuronal survival was assessed after 72 h of incubation, as described above. In addition, cell lysates were collected as described below for immunoblotting.

siRNA nucleofection

MAPK14 (p38α MAPK) siRNA or nontargeting (NT) siRNA duplexes were obtained from Dharmacon, Thermo Fisher Scientific (Lafayette, CO) and Ambion (Austin, TX)/Applied Biosystems (Carlsbad, CA). Silencer MAPPK14 siRNA (ID# 1217 and 1312) duplexes from Ambion are referred to as si3p38α-1 and si3p38α-2, respectively, in this article. ON-TARGETplus MAPPK14 siRNA (J-003512-21) from Dharmacon is referred to as si3p38α-3. The siRNA duplexes were transfected into THP-1 cells at a density of 1 × 10^5 cells/ml using Amaxa Nucleofector kits, according to the manufacturer’s instructions. Briefly, 48 h after nucleofection with 2 μg siRNA, THP-1 cells were treated for 24 h with 200 μM HIV-1 gp120 (or gp120(Fc)2). Supernatants or conditioned media (CM) from these treatments were collected and used at 10% final concentration on microglia-depleted rat cerebrocortical cultures, as previously described for neurotoxicity analysis. The mononuclear THP-1 cells were collected and analyzed for p38 MAPK activation via Western blot.

Immunofluorescence staining and deconvolution microscopy

After treatment and washing with PBS, rat cerebrocortical cultures were fixed for 25 min with 4% PFA at 4°C and subsequently permeabilized with 0.2% Triton X-100 for 5 min at room temperature (for staining of MAP-2 or TUNEL alone) or 100% methanol for 10 min at −20°C (for staining of phosphorylated p38 with or without MAP-2). Primary Abs included mouse anti–MAP-2 (1:500, M4403; Sigma-Aldrich), chicken anti–MAP-2 (1:5000, ab5932; Abcam, Cambridge, MA), rabbit anti–ACTIVEp38 (1:1000, V1211; Promega), and mouse anti–CD11b (1:150, MCA257; Serotec, abdersen; Raleigh, NC). Secondary Abs included goat anti-rabbit, anti-mouse, or anti-chicken IgG Alexa 594/488/647 (1:2000; Invitrogen) and horse anti-mouse IgG Texas Red (1:150; Vector Laboratories, Burlingame, CA). Controls were included in which primary Abs were omitted or replaced with irrelevant IgG of the same subclass. Nuclear DNA was stained with Hoechst H33342. Deconvolution microscopy was performed, as described earlier, with filters for DAPI, FITC, CY3, and CY5, with the only modification being that a constraint iterative algorithm was used for deconvolution (Slidebook software; Intelligent Imaging Innovations, Denver, CO) (16).

Cell lysates

After washing with PBS, cerebrocortical or mononuclear THP-1 cells were harvested by adding 1 × Cell Lysis Buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na3-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin; Cell Signaling Technology, Beverly, MA) supplemented with 5 mM NaF (serine/threonine protein phosphatase inhibitor) and Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) on ice for 10 min. The lysed samples were transferred to microcentrifuge tubes, sonicated four times for 5 s, and then cleared by centrifugation (13,200 rpm, 10 min) at 4°C. Lysate total-protein concentrations were determined using the BCA protein assay kit (Pierce). Immunoblotting results were analyzed using immunoblotting (1:1000; Cell Signaling Technology).

Immunocomplex kinase assay for activity of p38 MAPK or stress-activated protein kinase/JNK

The kinase assay for phosphorylated p38 MAPK and JNK was performed using kits from Cell Signaling Technology, following the supplier’s instructions. In brief, immobilized anti–phospho-p38 MAPK mAb was used to immunoprecipitate active p38 MAPK from cell lysate (200–400 μg total protein), followed by an in vitro kinase assay using activated transcription factor (ATF)-2 as a substrate. ATF-2 phosphorylation was detected by Western blotting using phospho–ATF-2 Ab (1:1000; Cell Signaling Technology). For stress-activated protein kinase (SAPK)/JNK kinase activity, c-Jun fusion protein linked to agarose beads was used to pull down JNK enzyme from cell extracts (200–400 μg total protein); and phospho–c-Jun Ab was measured via Western blotting (1:1000; Cell Signaling Technology). All protein kinase assays were standardized for total cellular protein amount and lysate volume used in the initial immunoprecipitation step.

Immunoblotting

For Western blotting analysis of whole-cell lysates or substrates of kinase assays, 4X lithium dodecyl sulfate sample buffer and 10X reducing agent (Invitrogen) were added to samples containing equal amounts of total protein (10, 30, or 50 μg for lysates; 2 μg kinase substrate) and heated for 5 min at 100°C. Alternatively, cell lysates in SDS-containing gel loading buffer were made 41 mM in DTT and heated. Samples were electrophoretically separated on SDS-PAGE gels (NUPAGE; Invitrogen) under reducing conditions and subsequently electro-transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking, membranes were incubated with gentle agitation overnight at 4°C with specific primary Abs against total and phospho forms of the proteins of interest (ATF-2 as a substrate). ATF-2 phosphorylation was detected using peroxidase-conjugated secondary Abs (1:10,000; Cell Biosciences). The membranes were then incubated with secondary Abs conjugated with horseradish peroxidase for 1 h and exposed to SuperSignal Pico chemiluminescent detection kit (Pierce). Immunoblotting results were analyzed using Adobe Photoshop (Adobe Systems, San Jose, CA), and densitometric measurements were normalized against β-actin expression levels.

Statistical analysis

The data are expressed as mean values ± SEM for three to nine independent experiments. Statistical analysis was performed using one-way ANOVA, followed by the Fisher protected least significant difference post hoc test or the Student t test, with the StatView software package (version 5.0.1; SAS Institute, Cary, NC). The significance level was set at p < 0.05. The immunoblotting and immunofluorescence images are representative of the at least three independent experiments performed.

Results

Kinetics of p38 MAPK activity in cerebrocortical cell cultures upon exposure to HIV gp120

We previously showed that envelope protein gp120 derived from CCR5-prefering, CXCR4-prefering, and dual-tropic HIV-1 strains can induce neurotoxicity and that this toxicity is mediated equally by the respective HIV coreceptors (16). To extend the analysis of the HIV-1 coreceptor signaling pathways responsible for mediating neuronal death, we investigated the kinetics and involvement of different MAPK pathways in induction of neuronal death by gp120. We used in vitro nonradioactive immunocomplex kinase assays for the stress-activated protein kinase (SAPK) p38 MAPK and,

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for comparison, SAPK/JNK (JNK) with phospho–ATF-2 and phospho–c-Jun as substrate readouts, respectively. Cerebrocortical cell cultures were incubated for various time periods with HIV gp120 or vehicle as control, as described for neurotoxicity assays (15, 16). Subsequently, the cells were transferred onto ice, quickly washed once with ice-cold PBS, and lysed. The active kinases were precipitated from the cell lysate and exposed to assay substrates in the presence of ATP. Phosphorylated substrates were visualized by Western blotting and quantified by densitometry. Baseline and HIV-1 gp120-induced activities of the protein kinases were assessed for each time point. In response to HIV-1 gp120SF2 exposure, activation of p38 MAPK occurred early, in a short-lived peak at 5 min, followed by a temporary decrease below baseline at 1 h. After the decreased kinase activity at 1 h, p38 kinase activity showed a sustained increase that was most pronounced above baseline at 3–24 h (Fig. 1A). Thus, despite some variability between different batches of primary cerebrocortical cell cultures, which is to be expected, we observed a consistent pattern at early and late time points of initial, temporary up- and downregulation, with an overall increase in p38 MAPK activity after exposure to gp120 (Fig. 1A). Altogether, the data indicated an overall trend toward increased kinase activity for p38 MAPK after gp120 exposure over a 24-h period (Fig. 1B, trend line). For comparison, the activity of JNK, which was monitored in the same way, showed a less pronounced deviation from the baseline over 24 h, except for at the 5-min time point. Fig. 1C shows representative Western blot data for p38 MAPK (phospho–ATF-2) and JNK (phospho–c-Jun) kinase assays at the significant time points of 5 min and 1, 3, and 24 h. In addition, direct immunoblotting for phospho-p38 MAPK was used to detect the active kinase within rat cerebrocortical cultures (Fig. 1D, 1E). Increased phosphorylation of p38 MAPK occurred in response to gp120 from CXCR4-, CCR5-, and CCR5/CXCR4-prefering, dual-tropic HIV-1 strains (IIIB, SF162, and SF2, respectively; Fig. 1D). Representative Western blot data showing increased phospho-p38 MAPK after treatment with 200 pM gp120SF2 for 24 h are shown in Fig. 1E. Based on the finding that the activity of p38 MAPK deviated from baseline upon exposure to HIV gp120 most consistently at 5 min and 1, 3, and 24 h, we focused primarily on p38 MAPK activity at those time periods in subsequent experiments.

Active p38 MAPK is required for HIV-1 gp120-induced neuronal death in mixed neuronal-glial cerebrocortical cell cultures in a dose-dependent fashion

After observing that activation of p38 MAPK to less than twice the baseline activity mediated neuronal death, we assessed the dose dependence of the neuroprotective effect that pharmacological inhibition of p38 MAPK showed in earlier studies. Therefore, we exposed primary rat cerebrocortical cultures to increasing doses of the p38 MAPK-specific pharmacological inhibitor SB203580 prior to HIV-1 gp120 exposure. SB203580 acts as a competitive ATP-binding inhibitor that interrupts the kinase activity of p38 MAPK (38, 39). We treated the cultures with the compound at concentrations of 0.1, 1.0, and 10 μM 15 min prior to treatment with 200 pM dual-tropic viral envelope protein gp120 from HIV-1 SF2 strain for 24 h. Controls received the vehicles for SB203580 (DMSO) and gp120 (PBS/BSA, 0.001% final concentration) (15, 16). Subsequently, we assessed neuronal survival as readout for neurotoxicity by fluorescence microscopy using a combination of immunolabeling of neurons for MAP-2 with nuclear DNA staining by H33342, as described in Materials and Methods. As expected, HIV gp120 reduced neuronal survival by \( \sim 23 \pm 3.3\% \) in the

![FIGURE 1](http://www.jimmunol.org) Kinetics of p38 MAPK and JNK activation in mixed neuronal-glial cerebrocortical cell cultures upon exposure to neurotoxic HIV envelope protein gp120. HIV-1 gp120SF2 causes significant deviation of p38 MAPK activity from baseline control condition at 5 min and 1, 3, and 24 h, with an overall increase (A and B for trend line for p38 MAPK activity). A and C. In contrast, activity of JNK is only significantly elevated at 5 min. Cerebrocortical cell cultures were incubated with dual-tropic gp120SF2 (200 pM) for the indicated time periods prior to immunoprecipitation and kinase assay analysis. The kinase activity of p38 MAPK and JNK using ATF-2 and c-Jun as substrates, respectively, was detected by immunoblotting following the supplier’s protocol, as mentioned in Materials and Methods. A. The kinase activity in untreated controls was measured for each time point and defined as the 100% baseline value. A and B, Error bars represent the SEM of four to nine independent experiments. C. Representative Western blots of phospho–ATF-2 (p-p38) and phospho–c-Jun (p-JNK) kinase assay results are shown after exposure to gp120 for 5 min and 1, 3, and 24 h. D, In addition, gp120 of CXCR4-prefering (IIIB), dual-tropic (SF2), and CCR5-prefering (SF162) HIV-1 activate p38 MAPK in cerebrocortical cells at 24 h. E, Examples of active phospho-p38 (pp38) MAPK, phospho MAPK, and actin Western blots used for densitometry analysis 24 h after gp120SF2 treatment. D and E, Equal amounts of cellular protein (10 μg/lane) were analyzed by SDS-PAGE and Western blotting. E, The two bands detected as total p38 MAPK represent the α isoform (lower) and β isoform (upper) of the kinase, whereas the phospho-p38 MAPK is mostly of the α isoform. D, Error bars represent the SEM of three independent experiments. *p < 0.05, compared with control.
Although all cells within the rat cerebrocortical cultures express p38 MAPK protein (Fig. 4), the spatial distribution of p38 MAPK activity induced by exposure to HIV-1 gp120 had not been defined. To localize activated p38 MAPK, we performed immunostaining on cultures treated for 24 h with 200 pM HIV-1 gp120 or vehicle control. These cultures were costained for active p38 MAPK, the neuronal marker (MAP-2), microglia marker (CD11b), and/or nuclear DNA staining by H33342. Error bars represent the SEM of four independent experiments. *p < 0.05, compared with control.

Presence of the neuroprotective chemokine CCL4 reduces HIV-1 gp120-induced neuronal injury and death.

Neuroprotective β-chemokine CCL4 reduces HIV-1 gp120-induced p38 MAPK activation

Previously, we found that the presence of CCL4, the natural ligand for CCR5, abrogated the neurotoxicity of gp120 in cerebrocortical cells (16, 32). Using the same experimental approach as in the previously reported neurotoxicity studies and the above-discussed kinase assay, we found that treatment with CCL4 during exposure to the viral envelope protein suppressed the increase in p38 MAPK activity at 3 and 24 h but not at 5 min (Fig. 3). This finding suggested that an increased activity of p38 MAPK at 3 and 24 h, but not at 5 min, reflected a neurotoxic process. Although under protective conditions, such as the presence of the CCR5 ligand, p38 kinase activity remained close to background levels except for the early 5-min time point. Compared with the 1-, 3-, and 24-h time points, the activity of p38 MAPK at 7 and 15 h was more variable and, on average, closer to baseline than what was to be expected for this time period from the experiments shown in Fig. 1A. Neuroprotective CCL4 (20 nM) was added immediately prior to HIV-1 gp120 (200 pM) incubation (15, 16). p38 MAPK activity was detected by immunoblotting and evaluated using densitometry of phospho–ATF-2, as described in Materials and Methods. The kinase activity in BSA vehicle-treated controls was measured for each time point and defined as the 100% baseline value. As a separate control, activity of p38 MAPK for treatment with CCL4 alone was assessed at 5 min and 1, 3, and 24 h only. Error bars represent the SEM of three to six independent experiments. *p < 0.01, compared with control.

Inhibition of p38 MAPK prevents gp120-induced neuronal apoptosis in a dose-dependent fashion.

Rat cerebrocortical cultures were incubated for 24 h with or without gp120 (200 pM) in the presence or absence of 0.1, 1, or 10 μM of p38 MAPK inhibitor SB203580. Neuronal survival was assessed using fluorescence microscopy after fixation, permeabilization, and immunostaining of neurons for MAP-2 in combination with nuclear DNA staining by H33342. Error bars represent the SEM of three to six independent experiments. *p < 0.001, compared with control; †p < 0.02, compared with gp120; ‡p < 0.01, compared with control and gp120. ANOVA with the Fisher protected least significant difference post hoc test.

Presence of the neuroprotective chemokine CCL4 reduces the activation of p38 MAPK in the presence of neurotoxic gp120 in rat cerebrocortical cultures at 3 and 24 h. Note that the activity of p38 MAPK at 7 and 15 h was more variable and, on average, closer to baseline than was to be expected for this time period from the experiments shown in Fig. 1A. Neuroprotective CCL4 (20 nM) was added immediately prior to HIV-1 gp120 (200 pM) incubation (15, 16). p38 MAPK activity was detected by immunoblotting and evaluated using densitometry of phospho–ATF-2, as described in Materials and Methods. The kinase activity in BSA vehicle-treated controls was measured for each time point and defined as the 100% baseline value. As a separate control, activity of p38 MAPK for treatment with CCL4 alone was assessed at 5 min and 1, 3, and 24 h only. Error bars represent the SEM of four independent experiments. *p < 0.05, compared with control.

To localize activated p38 MAPK, we performed immunostaining on cultures treated for 24 h with 200 pM HIV-1 gp120 or vehicle control. These cultures were costained for active p38 MAPK, the neuronal marker (MAP-2), microglia marker (CD11b), and/or nuclear DNA dye Hoechst H33342. Phosphorylated or activated p38 MAPK was detected by immunostaining experiments indicated that removal of neurons reduced the basal levels of activated p38 MAPK by 77.8 ± 4.5% compared with control cerebrocortical culture (neurons and glia) (Fig. 4D, 4E). In contrast, depletion of microglia with leucine
methyl ester (LME) did not detectably change the amount of active or total p38 MAPK at basal levels (Fig. 4D, 4E).

**Microglial depletion reduces HIV-1 gp120-induced p38 MAPK activation in cerebrocortical neurons**

In previous studies, depletion of microglia from cerebrocortical cultures with LME abrogated the neurotoxicity of gp120 (15, 16). Using the same experimental approach as in the previously reported neurotoxicity studies, we found that depletion of microglia before exposure to the viral envelope protein also suppressed the increase in p38 MAPK activity at 3 and 24 h but not at 5 min (Fig. 5). Hence, the increased activity of p38 MAPK in response to HIV-1 gp120 from 3 h onward is dependent on the presence of microglia. Our observation also suggested that the early peak of p38 MAPK activity at 5 min occurred independently of microglia and, thus, through a direct interaction of gp120 with astrocytes and/or neurons. Moreover, and similar to the findings in the experiments with CCL4, this peak at 5 min was apparently not associated with neurotoxicity.

**Mononuclear cells require p38 MAPK activation for gp120-induced neurotoxin production**

In an alternative approach to assess the role of mononuclear lineage cells and HIV-1 coreceptors in the neurotoxicity of gp120, we used human mononuclear THP-1 cells and primary MDMs as models for macrophages and microglia. Like primary human MDMs, monocyctic THP-1 cells express CXCR4 and CCR5, in addition to CD4; are permissive to HIV infection; and produce neurotoxins in response to HIV-1 virus and gp120 (12, 36, 40). In the current study, THP-1 cells and MDMs were incubated with 200 pM or 1 nM HIV-1 gp120, and then the cell-free supernatants from GM120SF162 or 1 nM HIV-1 gp120 (4 d) and then the cell-free supernatants were transferred into microglia-depleted rat cerebrocortical cultures (at 10% cerebrocortical culture media volume) for 3 d. HIV-1 gp120 induced neurotoxin production in THP-1 cells (Fig. 6) and MDMs (Fig. 7). THP-1 plus gp120 CM reduced neuronal survival in an envelope dose-dependent fashion by ~40–60% of rat cerebrocortical cultures exposed to control CM or not treated with CM (Fig. 6A). Control CM did not significantly affect neuronal survival. In contrast, control supernatants from BSA/vehicle-exposed THP-1 cells or control THP-1 CM spiked with HIV gp120 did not significantly increase neuronal death in microglia-depleted cerebrocortical cell cultures.

Interestingly, THP-1 cells exposed to gp120SF162 also showed increased levels of phosphorylated p38 MAPK in a dose-dependent manner after 4 d, suggesting that activation of the kinase in mononuclear cells correlated with neurotoxin production (Fig. 6B, upper panel). Furthermore, we found that cell-free neurotoxic THP-1 media, generated by incubation of the cells with 200 pM gp120, caused an increase in active p38 MAPK in microglia-depleted cerebrocortical neuronal cells, compared with control CM, after 1 and 3 d of exposure (Fig. 6B, lower panels).
Because the media conditioned by THP-1 cells and MDMs in the presence of gp120 must be expected to carry over viral envelope protein onto microglia-free cerebrocortical cells, it might be possible that gp120 acted directly on neurons and astrocytes, contributing to neurotoxicity in synergy with a factor secreted by gp120-stimulated, but not control-treated, mononuclear cells. To assess a potential role for HIV coreceptors in such a hypothetical scenario, we introduced 15,000 monocytic THP-1 cells into 300,000 murine cerebrocortical cells deficient in both HIV coreceptors (CCR5<sup>−/−</sup>/CXCR4<sup>−/−</sup>) that were pretreated with LME to deplete endogenous microglia (5% final concentration of THP-1 cells as replacement for microglia). For introduction into murine microglia-free cerebrocortical cells, THP-1 cells were first suspended in CM of the same mouse cell culture at 1.5 × 10<sup>6</sup> cells/ml. This cell suspension was then distributed at 10 μl/well into the cerebrocortical cells that were previously treated with LME and washed to remove potentially remaining microglial cell debris and excess LME. However, THP-1 cells did not adhere to cerebrocortical cultures, even in the presence of HIV/gp120 (data not shown). Of note, cerebrocortical cells comprising neurons, astrocytes, and microglia but lacking both major HIV coreceptors are resistant to induction of gp120 neurotoxicity (16). The chemokine receptor-deficient cerebrocortical cultures with and without THP-1 cells were then exposed to gp120 or BSA (as a control) for 24 h. After fixation, permeabilization, and immunolabeling of neurons with MAP-2, in combination with nuclear staining using DAPI, neuronal survival was determined by fluorescence microscopy (Fig. 6C). Exposure of cerebrocortical cultures to gp120 in the absence of THP-1 cells or to THP-1 cells alone (after pretreatment with LME) did not significantly change neuronal survival. However, addition of gp120 in the presence of THP-1 cells caused a significant demise of neurons. This finding suggested that the presence of mononuclear cells expressing CXCR4 and CCR5 (in addition to CD4) was able to restore susceptibility to gp120 toxicity in combined CCR5-CXCR4-deficient cerebrocortical cells and further supported a critical role for monocyte-lineage cells and their HIV coreceptors in gp120-induced neurotoxicity. In contrast, the findings also showed that an interaction with neuronal or astrocytic HIV coreceptors of any potentially carried-over gp120 in the CM of mononuclear cells was dispensable for neurotoxicity to occur. Finally, this experimental approach revealed that a 24-h incubation of THP-1 cells with HIV gp120 produced significant neurotoxicity.

Therefore, we next analyzed whether gp120 triggered activation of p38 MAPK in THP-1 cells during a 24-h exposure. The monocytic cells were exposed for 5, 15, and 30 min and 24 h to gp120 or BSA vehicle as control, and p38 MAPK activity was subsequently assessed in cell lysates using an immunocomplex kinase assay, as described above for rat cerebrocortical cells. The kinase assay showed that exposure of monocytic cells to gp120 resulted in a significant increase in active p38 MAPK at all four time points (Fig. 6D).

Similar to THP-1 cells, MDM plus gp120 CM reduced neuronal survival to 70–80% of rat cerebrocortical cultures treated with control MDM CM (Fig. 7A). Of note, MDM plus gp120 CM retrieved after 4 d was not significantly more toxic than CM of the same sample collected earlier, after only 24 h of incubation. Control MDM CM collected at 24 h and 4 d, or spiked with gp120 before addition to microglia-depleted cerebrocortical cells, and CM from MDMs incubated with CCL4 or CCL5 lacked neurotoxicity, thus confirming the specificity of the toxic effect triggered by HIV gp120.

Comparable to THP-1 cells, although less pronounced, MDMs exposed to 200 pM gp120 of HIV-1 SF2 and SF162 showed increased levels of phosphorylated p38 MAPK after 4 d, again suggesting that activity of the kinase in mononuclear cells correlated with neurotoxin production (Fig. 7B).

Next, we assessed the importance of p38 MAPK signaling in monocyte-lineage cells after gp120 treatment. To analyze whether p38 MAPK in mononuclear cells contributes to gp120-induced neurotoxicity, we successfully infected THP-1 cells and MDMs, each at a multiplicity of infection (MOI) of 10, with adenoviruses expressing GFP (AdV-GFP) or dominant-negative p38α AF MAPK (AdV-DNp38α) (37) 2 d prior to HIV-1 gp120 exposure. AdV-DNp38α AF, but not AdV-GFP, strongly reduced the release of neurotoxins by THP-1 cells and MDMs as the result of HIV-1 gp120<sub>SF2</sub> treatment increasing neuronal survival (Figs. 6E, 7C, respectively). Reduction of neurotoxin release was due to the presence of the dominant-negative p38α kinase mutant and not the result of decreased mononuclear cell viability, because the viability of the control (AdV-GFP) and AdV-DNp38α–infected cells, in THP-1 cells and MDMs, respectively, were equivalent (data not shown).

Altogether, the experiments showed that HIV gp120 triggered neurotoxin production equally in THP-1 cells and MDMs, which, in turn, could be prevented by inhibiting active p38 MAPK and, thus, supported the suitability of THP-1 cells as a model for macrophage-mediated neurotoxicity of the viral envelope protein. Hence, to further investigate the role of endogenous p38 MAPK in gp120-induced neurotoxicity, we used siRNA technology to knock down expression of p38α MAPK in THP-1 cells prior to incubation with HIV-1 gp120<sub>SF162</sub>. We were able to efficiently reduce expression of endogenous p38 MAPK protein by 50–75% compared with NT siRNA (p < 0.001) using three different and
separate p38α MAPK siRNA duplexes: sip38α-1, -2, and -3, with or without gp120SF162 treatment (Fig. 8A, 8B). CM collected from THP-1 cells nucleofected with p38α MAPK siRNA for 48 h (efficient knockdown between 48 and 72 h; data not shown) and treated with gp120SF162 for an additional 24 h (p38α siRNA: THP-1 gp120SF162 CM) were significantly less neurotoxic than CM from THP-1 cells nucleofected with NT siRNA and treated with gp120SF162 (NT siRNA:THP-1 gp120SF162 CM; Fig. 8C). The incubation with gp120 was limited to 24 h to stay within the time frame of maximal reduction of p38 MAPK protein expression in the presence of the specific siRNAs. Importantly, these experiments also confirmed that incubation with gp120 for 24 h was sufficient to obtain significantly neurotoxic CM. Direct neuronal interaction with gp120 did not seem to cause significant amounts of neuronal death in the absence of microglia, as observed after addition of gp120 to microglia-depleted cultures. In contrast, as a control, a 20-min exposure to 300 μM NMDA prior to a 24-h incubation in control CM was still able to induce excitotoxic neuronal death (Fig. 8C).

Inhibition of p38 MAPK activation in cerebrocortical neurons prevents neuronal death stimulated by gp120-induced neurotoxic media from mononuclear THP-1 cells

We next tested whether inhibition of p38 MAPK activity in neurons would prevent neuronal death upon exposure to neurotoxic stimuli from THP-1 cells treated with HIV-1 gp120. Therefore, prior to treatment of microglia-depleted rat cerebrocortical cultures with gp120-induced neurotoxic THP-1 CM, we treated the cerebrocortical cultures with 10 μM SB203580 or DMSO as vehicle control for 15 min (15, 16). As before, THP-1 cells were incubated separately with HIV-1 gp120SF2 or gp120SF162 or vehicle for 4 d to obtain neurotoxic and nontoxic control supernatants. The THP-1 CM were transferred onto SB203580-pretreated microglia-depleted rat cerebrocortical cultures (at 10% cerebrocortical culture media volume) for 3 d. Interestingly, inhibition of p38 MAPK by 10 μM SB203580 in microglia-depleted rat cerebrocortical cultures prior to the addition of gp120-induced neurotoxic THP-1 CM prevented neuronal apoptotic death (Fig. 9), indicating that p38 MAPK is a crucial mediator in toxin-producing macrophages/microglia, as well as in toxin-sensitive neurons. Again, spiking nontoxic THP-1 CM with additional 200 μM gp120SF2 or gp120SF162 before application to microglia-depleted rat cerebrocortical cultures failed to produce neurotoxicity, confirming the requirement of microglia for gp120-induced neuronal death (Fig. 9A, THP-1 CM control plus SF2 or SF162).

Discussion

The interaction of HIV gp120 with CD4 and the viral coreceptors, CCR5 and/or CXCR4, was shown to be vital to productive infection and to stimulation of intracellular signaling cascades presumably responsible for most pathological effects of viral–host interactions (3, 10, 14–17, 19, 20, 24–26, 32, 41, 42). Previously, we and other investigators showed that the stress-related p38 MAPK can apparently mediate gp120-induced neuronal death (10, 15, 16). Furthermore, activation of p38 MAPK was observed following gp120 treatment in macrophages and, thus, was implicated in HIV pathogenesis and the development of AIDS (25, 43). However, the current study supplies evidence that p38 MAPK and its activity are essential to the reduction of neuronal survival by HIV-1 gp120, as well as the neurotoxic phenotype of gp120-exposed microglia and macrophages. In addition, both consequences of exposure to HIV-1 gp120, the overall increased activation of p38 MAPK in neurons and neuronal death, are dependent on the presence of microglia. Therefore, to our knowledge, this is the first study to...
show that p38 MAPK in two different cell types may be culpable and necessary for gp120-induced neurotoxicity in a mixed glial-neuronal environment.

Activation of p38 MAPK above baseline by HIV envelope protein gp120 was reported for striatal neurons for a time frame of up to 4 h of exposure (10). However, our results presented in this article showed that p38 MAPK activity fluctuates at earlier time points after a short-lived initial increase; after a decrease at 1 h, it displays an overall increasing trend over a 24-h time course. This difference may be due to the fact that Singh et al. (10) used striatal neuron-rich cultures as their experimental model and treated them with more than twice the amount of gp120 (500 pM) than applied in our study (200 pM) (15, 16, 32). In contrast, the early increase in p38 MAPK activation after 5 min of gp120 exposure was seen in both studies. This early peak in p38 MAPK activity does not correlate with neuronal death in our study, because it was not abolished by pretreatment with CCL4 or by microglial depletions from mixed cultures, which was shown in our studies to inhibit gp120-induced reduction in neuronal survival (15, 16). The initial peak in p38 MAPK activity at 5 min due to CCL4 alone, gp120 alone, or CCL4 plus gp120 is most likely due to neuronal activation, because it is not abolished after microglial depletion, but it is absent in neuron-deficient cultures exposed to gp120. The reason why the temporary increase in p38 MAPK activity at 5 min is apparently not associated with neurotoxicity and the underlying question of how the increase in kinase activity at 5 min is different from the increase between 1 and 3 h and up to 24 h remain unknown and are the subjects of ongoing studies. One explanation for the gradual increase and decrease in p38 MAPK activity in surviving neurons may be a yet-to-be-defined mechanism leading to a disturbance of the regulatory balance between phosphorylation and dephosphorylation by phosphatases. However, CCL4 was previously shown to immediately activate p38 MAPK in C6 glioblastoma cell lines (44), and this was also the case in our...
neuronal cultures (Fig. 3). We were also able to show that CCL4-stimulated p38 MAPK activity diminished to basal levels within 1 h of exposure and stayed similar to control for up to 24 h. Thus, to our knowledge, our study is the first to show CCL4-induced p38 MAPK activity in neuronal cultures over a 24-h time period and how it differs from gp120-induced p38 MAPK activity. The question of why CCL4 was unable to block gp120-induced p38 MAPK at 5 min, when incubated together, also remains to be investigated, although we previously showed that CCL4 can prevent neuronal death caused by the viral envelope (16). That finding could be explained by direct competition at the receptor level and, in the case of CXCR4-preferring gp120, via heterologous desensitization, which we demonstrated in the same study. Both mechanisms may occur at the level of macrophage chemokine receptors, which is further supported by our present study.

Other related questions for future investigations are why CCL4, in contrast to gp120, fails to produce a lasting increase in active p38 MAPK and how a CCR5-binding viral envelope, but not the β-chemokine, can cause neurotoxicity. Although our present study cannot provide the answers to those two questions, CCL4 and gp120 represent different ligands for the same receptor; as such, they may elicit different or even opposing effects. Importantly, gp120 binds with greater affinity to chemokine receptors if CD4 is also present (as in macrophages/microglia and lymphocytes); previous studies by other groups showed that even different natural ligands for the same receptor, such as CCR5 and its ligands CCL3, -4, -5, -8 and -14, can have different activities and trigger diverse signaling within the same cell population (45, 46).

Although p38 MAPK activation is known to be important for gp120-induced neuronal death (10, 15, 16), the distribution of phosphorylated p38 MAPK had not been investigated. Our report shows that phosphorylated p38 MAPK is localized in neurons and microglia in mixed cerebrocortical cell cultures. Importantly, based on detection by immunofluorescence, active p38 MAPK in microglia occurs at early time points upon treatment with gp120 and may be sustained over 24 h, whereas neuronal p38 MAPK activation seems to occur downstream of microglial kinase activation after longer exposure to gp120 or possible neurotoxins from activated microglia. The number of neurons positive for phosphorylated p38 MAPK staining did not seem to be different from control at 5 min and 1 and 3 h, but it was significantly reduced at 24 h, in line with reduction in neuronal survival at 24 h. However, in addition to counting cells positive for phosphorylated p38 MAPK staining did not seem to be different from control at 5 min and 1 and 3 h, but it was significantly reduced at 24 h, in line with reduction in neuronal survival at 24 h. However, in addition to counting cells positive for phosphorylated p38 MAPK, a quantitative readout for p38 MAPK activity between control and gp120 treatments was provided by our kinase assay results. Baseline activity of p38 MAPK seemed to mostly reside within neurons, as shown by the low remaining activity in glial cells after deletion of the neuronal population, which made up ~30% of the total cell population in the rat cerebrocortical cultures. Furthermore, our results suggest that the majority of the

![FIGURE 9. Inhibition of p38 MAPK in microglia-depleted rat cerebrocortical cultures rescues neurons from gp120-activated THP-1 neurotoxicity. A, Cell-free CM from THP-1 was collected after treatment for 4 d with gp120SF2- or gp120SF162-, or 0.001% BSA. Microglia-depleted rat cerebrocortical cultures were treated with 10 μM SB203580 for 15 min prior to treatment with control or neurotoxic THP-1 CM for 3 d and analyzed for neuronal survival. Error bars represent the SEM of three independent experiments. Direct treatment of microglia-depleted cerebrocortical cultures with gp120 did not produce significant neuronal death (THP-1 Control CM + SF2 or SF162), t < 0.05; ANOVA with the Fisher post hoc test. B, Neuronal survival was assessed using quantification of apoptotic, TUNEL+ nuclei (green), neuronal MAP-2 immunostaining (red), and nuclear DNA (blue); representative examples of images used for quantification are shown (original magnification ×40).]
gp120-induced p38 MAPK activity originates in the neuronal cell population. In addition, the combination of immunofluorescence detection and immunocomplex kinase assay indicated that the increase or decrease of p38 MAPK activity during the first 3 h was not based on a significant change in the number of cells positive for active kinase; rather, it reflected a change in p38 MAPK activity within a given number of cells. Therefore, neurons surviving 24 h after gp120 exposure seem to represent a smaller cell population that is still in the process of dying or tolerates greater p38 MAPK activity than that occurring under the respective control conditions. Given the fact that inhibition of p38 MAPK activity was protective, the variable measurements of kinase activity between 3 and 24 h presumably reflect a temporary deactivation of the active kinase by phosphatases, as well as the death and consequent drop-out of neurons that previously contributed to an overall increasing kinase activity in the cell culture as a whole. An additional and not mutually exclusive explanation is that primary, mixed neuronal-glial cell cultures do not grow in the synchronized fashion that, for example, cell lines do; therefore, different neurons in a given culture may respond to neurotoxins and eventually enter into a cell death pathway (apoptosis) at different times. Thus, the fluctuations of p38 MAPK activity may result from the dynamic interplay of activation and inactivation of the kinase, as well as the gradual demise of neurons.

We also performed Western blotting experiments to address the question of active p38 MAPK in cerebrocortical neurons exposed to CM from THP-1 cells (Fig. 6). We detected increased p38 MAPK activity in the microglia-depleted cerebrocortical cultures at 24 h and 3 d after treatment with neurotoxic THP-1 CM. Of note, the results shown in Figs. 6 and 8 indicated that, at 24 h, the demise of a significant proportion of neurons had already occurred. Thus, our findings suggested that the remaining, surviving neurons (and astrocytes) may indeed have adapted, at least for the duration of the experiment, to an increased p38 MAPK activity. The possibility of such an adaptation in surviving neurons is a hypothesis that warrants future investigations.

Other groups showed that gp120 triggers p38 MAPK activation in macrophages and monocytes, which can enter the brain soon after HIV infection and presumably contribute to the development of HAND (25, 43). In concurrence with these previous studies, gp120 elicited p38 MAPK activation in rat microglia and in our models for microglia, human mononuclear THP-1 cells, and primary MDMs. These mononuclear cells produced significant neurotoxicity upon treatment with gp120 for 24 h–4 d. Activation of p38 MAPK by HIV-1 gp120 in these phagocytic cells must occur before activation of the same kinase increases in neurons, as evidenced by the kinetic of p38 MAPK in gp120-treated monocytic THP-1 cells, as well as the reduction in neuronal death (15, 16) and decrease in kinase activity signal in cerebrocortical cells after microglial depletion. In addition, suppressing p38 MAPK activity in monocytic THP-1 cells and primary MDMs via the dominant-negative, inactive p38 MAPK mutant caused a significant reduction in neurotoxicity. Thus, the dominant-negative inactive p38 MAPK mutant suggests a critical role for p38 MAPK signaling in macrophages and microglia for gp120-induced neurotoxicity. Furthermore, this finding was consistent with our observation of p38 MAPK-dependent gp120 neurotoxicity in the mixed rat cerebrocortical cultures that were obtained using the same adenooviral constructs (15, 16) and various dosages of the pharmacological inhibitor SB203580 (Fig. 2).

In addition, the similarity of the results obtained in this study with primary human MDMs and THP-1 cells strongly supports the notion that the latter cell type is a very suitable model of microglia and macrophages. Therefore, additional experiments were performed using monocytic THP-1 cells. In particular, knockdown of endogenous p38 MAPK via three separate siRNA duplexes all derived from exon 2, similar to studies using a macrophage-specific deletion of p38 MAPK in vivo (47), significantly and specifically reduced expression of the protein by 50–75% in mononuclear THP-1 cells. The α isoform of p38 MAPK is thought to be the most important isoform involved in inflammatory regulation (47, 48) and a possible therapeutic target for CNS disorders beyond HAND and HIV-associated dementia, such as Alzheimer’s disease (49). Importantly, in our study, knockdown of p38α MAPK by 50–75% abrogated gp120-induced neurotoxicity similar to the significant reduction seen with overexpression of the dominant-negative mutant of the kinase. This supports the theory that p38 MAPK is vital for macrophage-mediated neurotoxicity of HIV gp120, but it does not necessarily involve all p38 MAPK molecules available in the cell. This interpretation is in line with our observation that, in addition to partial depletion of p38 MAPK protein, lower concentrations of the p38 MAPK pharmacological inhibitor SB203580 than what we previously assessed (15, 16, 32) can sufficiently and specifically suppress HIV-1 gp120-induced neurotoxicity.

In our studies using mixed neuronal-glial cell cultures, direct neuronal interaction with gp120 did not seem to cause significant amounts of neuronal death in the absence of microglia, as observed when gp120 was added to microglia-deficient cultures directly or via spiking of control THP-1 supernatants. However, it might be possible that gp120 acted directly on neuron and astrocytes, contributing to neurotoxicity in synergy with a factor secreted by gp120-stimulated, but not control-treated, mononuclear cells. Therefore, we performed neurotoxicity assays in mouse cerebrocortical cultures deficient in both HIV coreceptors (double CCR5/CXCR4 knockouts) that are, even in the presence of endogenous microglia themselves, resistant to gp120-induced neurotoxicity (16). Therefore, we replaced endogenous microglia with monocytic THP-1 cells to selectively provide monocytes that can subsequently activate monocytes. The results showed that without HIV gp120 coreceptors present in neurons and astrocytes, gp120-treated THP-1 cells (and their supernatants) remained neurotoxic. Hence, the presence of mononuclear cells expressing CXCR4 and CCR5 (in addition to CD4) restored susceptibility to gp120 toxicity in combined CCR5-CXCR4–deficient cerebrocortical cells, and a direct interaction of gp120 with neurons or astrocytes, at least via chemokine receptors, was dispensable for induction of neuronal death.

In contrast, inhibition of p38 MAPK in rat cerebrocortical neurons and astrocytes, prior to the addition of gp120-induced mononuclear cell-derived neurotoxins, prevented neuronal apoptosis, as shown in Fig. 9. These data suggested that inhibition of stress-related signaling via p38 MAPK, at the level of neurons (and possibly astrocytes), also succeeded in protecting against gp120-induced macrophage/microglia neurotoxicity and, thus, revealed a potential mechanism to rescue mature neurons from HIV gp120-induced injury and death, even if neurotoxins are present. However, our findings also emphasize the potential of limiting or abrogating the upstream involvement of macrophages/microglia in HIV-1 gp120 neurotoxicity. Therefore, further investigation into the mechanism of macrophage/microglia-mediated gp120 neurotoxicity is warranted.

Importantly, our results revealed that p38 MAPK provides a causal link in the neurotoxic mechanism triggered by HIV-1 gp120 in two disparate cell types, similar to the behavior of the transcription factor p53 (21). This comparable requirement for p38 MAPK and p53 warrants further investigation, because it was reported that direct phosphorylation of p53 by p38 MAPK is a critical step in the induction of apoptosis in HIV-1/gp120–induced syncytia (27).

Although p38 MAPK activity in both cell types is important for the overall neurotoxic effect of HIV-1 gp120, and β-chemokines
and pharmacological inhibitors of p38 MAPK affect neuronal and glial cells, the blockade of p38 MAPK activity in microglia or neurons ameliorates the toxicity of gp120. Thus, our finding that blockade of the kinase in macrophages and microglia, the primary productively HIV-infected cell type in the brain, prevents the indirect neurotoxicity of the viral envelope protein may offer new cell-type-specific therapeutic strategies for protection of the CNS against HIV-associated injury and functional impairment.

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Disclosures

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