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PDGF Synergistically Enhances IFN-γ-Induced Expression of CXCL10 in Blood-Derived Macrophages: Implications for HIV Dementia[1]

Navneet Kaur Dhillon,* Fuwang Peng,* Richard M. Ransohoff,† and Shilpa Buch[2]*

There is increasing cumulative evidence that activated mononuclear phagocytes (macrophages/microglia) releasing inflammatory mediators in the CNS are a better correlate of HIV-associated dementia (HAD) than the actual viral load in the brain. Earlier studies on simian HIV/rhesus macaque model of NeuroAIDS confirmed that pathological changes in brains of macaques with encephalitis were associated with up-regulation of platelet-derived growth factor (PDGF) and the chemokine, CXCL10. Because the complex interplay of inflammatory mediators released by macrophages often leads to the induction of neurotoxins in HAD, we hypothesized that PDGF could interact with IFN-γ to modulate the expression of CXCL10 in these primary virus target cells. Although PDGF alone had no effect on the induction of CXCL10 in human macrophages, in conjunction with IFN-γ, it significantly augmented the expression of CXCL10 RNA & protein through transcriptional and posttranscriptional mechanisms. Signaling molecules, such as JAK and STATs, PI3K, MAPK, and NF-κB were found to play a role in the synergistic induction of CXCL10. Furthermore, PDGF via its activation of p38 MAPK was able to increase the stability of IFN-γ-induced CXCL10 mRNA. Understanding the mechanisms involved in the synergistic up-regulation of CXCL10 could aid in the development of therapeutic modalities for HAD. *The Journal of Immunology,* **2007, 179: 2722–2730.**

Chemokines are small chemotactic cytokines of ~10 kDa, which orchestrate the inflammatory response by attracting leukocytes to the sites of inflammation. Cerebral expression of various chemokines and their receptors has been shown to play a crucial role in HIV-associated dementia (HAD).[3] For example, the IFN-γ-inducible peptide, IP-10 (in the systematic nomenclature, CXCL10), has been detected in the CSF of individuals with HIV-1 infection (1) and in the astrocytes in brains of individuals with HAD (2–4). CXCL10 is a secreted polypeptide of 10 kDa that was first identified as an early response gene induced after IFN-γ treatment in a variety of cells (5, 6) and is a ligand for the receptor, CXCR3. CXCL10 has been shown to stimulate HIV-1 replication in monocyte derived macrophages (MDMs) and peripheral blood lymphocytes, and blocking studies involving the neutralization of endogenous CXCL10 or CXCR3 antagonists has led to a reduction of HIV-1 replication in macrophages and lymphocytes (7). Expression of CXCL10 and CXCR3 was also shown to be enhanced in SIV-encephalitis (8, 9). Additionally, CXCL10, a chemokine with neurotoxic properties (10, 11), is up-regulated (~20-fold increase) in the brains of simian HIV (SHIV)-infected macaques with lentiviral lesions. More recently, occupation of the CXCL10 receptor CXCR3 by the proteolytically cleaved chemokine SDF-1α has recently been shown to be apoptotic for neurons, thereby underscoring the role of CXCL10 and its homologues in neurodegeneration (12).

In the brain, CXCL10 can be induced by a variety of factors, including viral gp120, Tat, and Nef, and cellular host factors such as IFN-γ (13–15). Additionally, interactions of soluble host factors (IFN-γ and TNF-α) can also synergistically induce the expression of CXCL10 (16–19).

In our earlier studies aimed at exploring factors contributing to encephalitis caused by SHIV in the rhesus macaque model, we also observed the overexpression of the inflammatory mediator platelet-derived growth factor (PDGF)-B chain in the brains of diseased animals (20, 21). PDGF is a known inducer of the potent chemokine CCL2 (22–24), which has been implicated in the progression of HAD (3, 25) and various other pathological inflammatory diseases (26–31). Additionally, the enhanced expression of IFN-γ has been observed in the brains of HIV-1-infected patients and SIV-infected macaques (32–34).

HAD is often characterized by complex interactions of host/viral factors released by activated cells in the CNS, eventually leading to amplified neurotoxic responses. We therefore hypothesized that PDGF (21) and IFN-γ, both of which are known to be up-regulated in SIV/SHIV-E, could synergize to induce the expression of CXCL10. In the present study, we evaluated the mechanism by which PDGF synergizes with IFN-γ to up-regulate CXCL10 expression in macrophages, the major target cell for the virus in the CNS. We demonstrate that the combination of PDGF and IFN-γ can regulate CXCL10 expression by transcriptional and posttranscriptional mechanisms involving the JAK/STAT cascades, PI3K, MAPK, and NF-κB activation.

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[3] Abbreviations used in this paper: HAD, HIV-associated dementia; MDM, monocyte derived macrophages; SHIV, simian HIV; PDGF, platelet-derived growth factor; ISRE, IFN-stimulated response element; Act D, actinomycin D.

Materials and Methods

Macrophage cultures and treatments

PBMCs from human blood were obtained by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation and isolated and differentiated into macrophages as described earlier (21). PBMCs were seeded in 96-well tissue culture dishes (Costar) or on cover slips in 24-well plates at a concentration of 8×10^5 cells/200 μl per well. All experiments involving the treatment of cells with exogenous PDGF-BB (R&D Systems) and IFN-γ recombinant protein (R&D Systems), were conducted under serum-free conditions, because serum induces PDGF. Following overnight serum starvation, cells were treated with PDGF (100 ng/ml) and/or IFN-γ (1000 U/ml) for 3 or 6 h for RNA or protein analyses, respectively. For inhibitor studies, cells were preincubated with various kinase inhibitors for 30 min at 37°C before cytokine and/or growth factor treatment. The following inhibitors were used at the final concentration specified: JAK inhibitor 1 (1 μM, 10 μM, 20 μM; Calbiochem), LY294002 (20 μM; Calbiochem), U73122 (20 μM; Calbiochem), U0126 (20 μM; Calbiochem), SB203580 (20 μM; Calbiochem), and SP600125 (20 μM; Calbiochem). Studies were performed in macrophage cultures derived from at least three separate donors, and each experiment was performed in triplicate.

CXCL10 mRNA analysis

Total RNA was extracted from MDMs that were either untreated or treated with PDGF and/or IFN-γ for 3 h using TRIzol reagent (Invitrogen Life Technologies). Quantitative analysis of the RNA was then conducted using the Quantikine mRNA quantitation kit from R&D Systems according to the manufacturer's instructions. The minimum detectable dose of the kit was 7 amol/ml.

Quantitative analysis of CXCL10 mRNA in cells treated with IFN-γ and/or PDGF was also done by real-time RT-PCR using the SYBR Green detection method. RT2 PCR primer pair set for CXCL10 was obtained from SuperArray Bioscience and amplification of CXCL10 from first-strand cDNA was performed as described earlier (35) using ABI Prism 7700 sequence detector. Data were normalized using Ct values for the house-keeping gene hypoxanthine-guanine phosphoribosyl transferase in each sample. To calculate relative amounts of CXCL10, the average Ct value of the hypoxanthine-guanine phosphoribosyl transferase was subtracted from that for each target gene to provide changes in Ct value. The fold change in gene expression (differences in changes in Ct value) was then determined as $\log_2$ relative units.

CXCL10 protein analysis by ELISA

Supernatant fluids collected from MDMs treated with or without PDGF and/or IFN-γ were examined for CXCL10 protein accumulation using a CXCL10 ELISA kit (R&D Systems). Samples were analyzed for CXCL10 expression in three independent experiments in three to four replicates.

Chemokine protein analysis by Luminex

Expression of CXCL10 protein in supernatant fluids collected from treated and untreated MDMs was also confirmed using the Beadlyte human multicytokine Beadmaster kit from Millipore as per the manufacturer's instructions. In brief, the multicytokine standards 4 and 5 were resuspended in assay buffer and then serially diluted from 5000 to 6.9 pg/ml. Fifty microliters of standard or sample was added to each well of a 96-well plate with 25 μl of the Beadlyte anti-cytokine bead solution and was incubated overnight at 4°C. The Beadlyte reporter solution was added to each well and incubated at room temperature for 1.5 h. Beadlyte streptavidin-PE was diluted 1/25 in assay buffer and was added to each well and incubated at room temperature for 30 min before the addition of the Beadlyte stop solution. The plate was then analyzed on the Luminex xMAP 100 system (Luminex) and analyzed using Beadview multiplex data analysis software (Millipore). Supernatants were analyzed twice, in duplicate.

Luciferase assay

To detect the effect of PDGF and/or IFN-γ on transcriptional activation of CXCL10, differentiated MDMs were trypsinized and transfected using a Nucleofector kit (Amaxa Biosystems) with indicated luciferase reporter plasmids, TGL-CXCL10, p-IFN-stimulated response element (ISRE) mut and pκB mut. For normalization of transfection efficiencies, cells were also cotransfected with pRL-TK plasmid DNA that contains the *Renilla* luciferase gene. Four hours following transfection, cells were treated with PDGF and/or IFN-γ, and 4 h thereafter, the cells were lysed and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data represent results obtained from three independent experiments.

Phosphoprotein analysis by Beadlyte 8-plex MultiPathway Signaling kit

The Beadlyte Cell Signaling assay is a simple and sensitive fluorescence bead-based Ab sandwich immunoassay. Following overnight serum starvation, MDMs were treated with PDGF and/or IFN-γ and lysed at various time intervals using the Beadlyte Cell Signaling Universal Lysis Buffer containing 1 mM sodium orthovanadate, a phosphatase inhibitor. Additionally, complete protease inhibitors (Roche) were also added to this buffer before use. The protein concentration of each sample was determined using the microBCA method (Pierce). Cell lysate was diluted 1/1 in Beadlyte Cell Signaling Universal Assay Buffer, and 5 μg protein from each sample was taken for phosphoprotein analysis using the Beadlyte 8-plex MultiPathway Signaling kit (Upstate Biotechnology). The 8-plex signaling kit includes anti-phophoprotein beads against MAPK as follows: Erk/MAPK 1/2 (Thr185/Tyr187); JNK (Thr183/Tyr185); p38 (Thr180/Tyr182); p70 S6 kinase (Thr412); transcription factors STAT3 (Ser727), STAT5A/B (Tyr694/699), CREB (Ser133), and NF-κB inhibitor; and IκB-α (Ser32). The cell lysates were incubated overnight with anti-phosphoprotein beads followed by biotinylated reporter Ab and strepavidin-PE incubations and quantification using the Luminex xMAP 100 system as described above. Beadlyte Hela Cell Lysate, Multistim, that is provided in the kit was used as a lysate standard to create standard curves for relative quantification of different phosphoprotein analytes. The results were represented as lysate U/mg protein.

Western blot analysis

Cells treated with or without PDGF and IFN-γ treatment for different time intervals were lysed in Beadlyte Cell Signaling Universal Lysis Buffer containing phosphatase and protease inhibitors as described above. Equal amounts of protein samples were run on a 12% SDS-polyacrylamide gel in reducing conditions followed by transfer onto a polyvinylidene difluoride membrane. The blots were blocked with 5% nonfat dry milk in PBS. Western blots were then probed with Abs recognizing phosphorylated forms of STAT1 (Tyr701) (Cell Signaling Technology; 1/1000), STAT1 (Ser727) (Santa Cruz Biotechnologies; 1/200), STAT3 (Cell Signaling Technology; 1/200), Akt (Cell Signaling Technology; 1/500), PDK1 (Cell Signaling Technology; 1/200), p38 (Santa Cruz Biotechnologies;1/500), JNK (Santa Cruz Biotechnologies; 1/200), Erk1/2 (Cell Signaling Technology; 1/200), p70S6 (Cell Signaling Technology; 1/1000) MAPK, IκB-α (Cell Signaling Technology; 1/200), and β-actin (Sigma-Aldrich; 1/4000). The secondary Abs used were HRP-conjugated anti-mouse or anti-rabbit (Pierce; 1/5000) and detection was performed using the ECL system (Pierce).

Immunocytochemistry

Immunocytochemical analysis for NF-κB activation was performed on PDGF and/or IFN-γ-treated MDMs grown on cover slips. Following treatment, cells were fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization with 0.5% Triton X-100 in PBS. Cells were then incubated with a blocking buffer containing 5% BSA in PBS for 1 h at room temperature. Following blocking, anti-human NF-κB p65 rabbit polyclonal Ab (1/500; Abcam) was added to each coverslip and incubated for 2 h at room temperature. Finally, the secondary Ab, AlexaFluor 488 goat anti-rabbit IgG, was used at a 1/1000 dilution for 2 h to view NF-κB activation in cells. 4′,6′-diamidino-2-phenylindole was used to stain the cell nuclei. Fluorescent digital images were obtained using a Zeiss LSM510 confocal microscope equipped with an Argon/2 laser (25 mW) for the excitation (488 nm) and detection (band pass 505–530 nm filter; BP505–530) of the AlexaFluor 488. Images were acquired in multitrack channel mode (sequential excitation/emmision) with LSM510 (version 3.2) software and a Plan-Apochromat objective with a zoom factor of 1 or 2 and frame size of 1024×1024 pixels. Detector gain was set initially to cover the full range of all of the samples and background corrected by setting the amplifier gain, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. Control coverslips were comprised of 1) cells without any secondary Ab treatment and, 2) cells treated with secondary Ab only.

mRNA stability assays

For mRNA stability assays, 10 μg/ml actinomycin D (Act D) was added to MDMs 90 min following stimulation with PDGF and/or IFN-γ. At selected times after Act D treatment, total RNA was harvested and CXCL10 RNA molecules were amplified by real-time RT-PCR. The fold change in gene expression determined from RT-PCR assay was then used to calculate the percentage of mRNA remaining following Act D treatment.

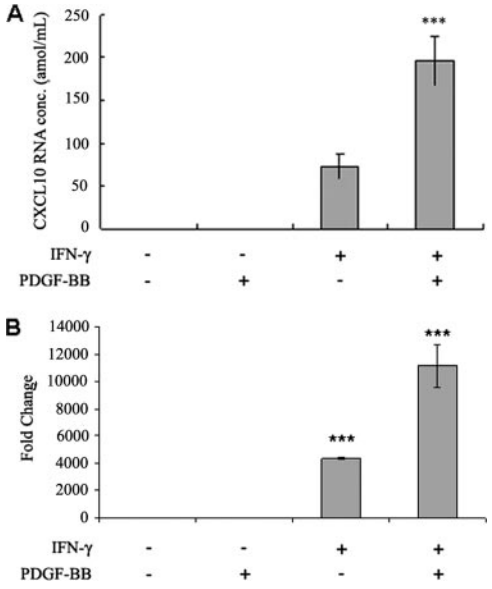

FIGURE 1. PDGF enhances IFN-γ-induced CXCL10 RNA expression in human macrophages. Overnight serum-starved MDMs were treated with recombinant PDGF-BB (100 ng/ml) and IFN-γ (1000 U/ml) either alone or in combination for 3 h followed by total RNA extraction and analyses of CXCL10 RNA by Colorimetric Quantikine mRNA Quantitation method (*A*). The figure shown is representative of three independent experiments. *B*, Real-time RT-PCR analysis of CXCL10 RNA in MDMs stimulated with PDGF-BB and/or IFN-γ. The average Ct value of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase was subtracted from that of the CXCL10 gene to give changes in Ct (dCt). The fold-change in gene expression (differences in dCt, or ddCt) was then determined as $\log_2$ relative units. The data represents the mean ± SD from three independent experiments (***, $p \leq 0.001$).

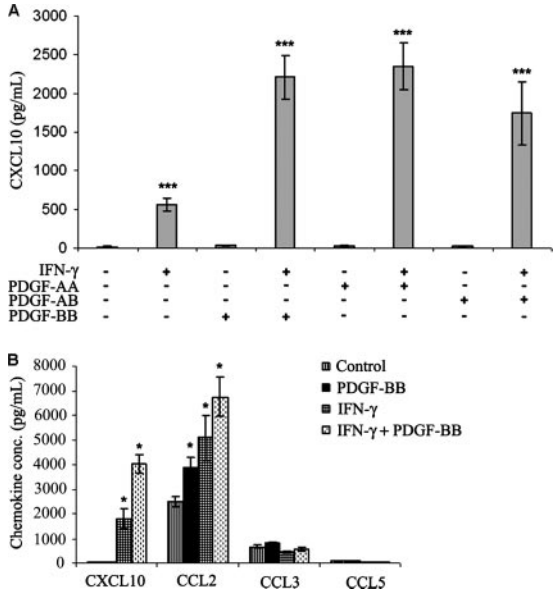

FIGURE 2. Synergistic induction of CXCL10 protein by IFN-γ and PDGF in human MDMs. Supernatants from cells treated with different isoforms of PDGF (100 ng/ml) in the presence or absence of IFN-γ (1000 U/ml) were collected at 6 h posttreatment followed by analysis of CXCL10 protein levels by ELISA (*A*) and by using fluorescence-based immunobead assay (*B*). Levels of CCL2, CCL3, and CCL5 were also analyzed by Luminex in the same supernatants. Statistical significance from three independent experiments was calculated (***, $p \leq 0.001$; *, $p \leq 0.05$).

Statistical analysis

All statistical analyses were performed by using a one-tail, independent, *t* test. Results were judged as statistically significant at values of $p \leq 0.05$.

Results

Synergistic induction of CXCL10 RNA by IFN-γ and PDGF-BB in MDMs

To determine whether PDGF could synergize with IFN-γ to induce the expression of CXCL10, human MDM cultures were either untreated or treated with recombinant PDGF-BB (100 ng/ml), IFN-γ (1000 U/ml), or PDGF-BB in combination with IFN-γ, and total RNA was then extracted 3-h posttreatment. As shown by the Quantikine colorimetric mRNA quantitation method in Fig. 1*A*, although PDGF alone was not able to induce CXCL10 mRNA expression, treatment of cells with IFN-γ alone resulted in the induction of CXCL10, a finding that is consistent with previous in vitro studies (18, 36). Treatment of cells with a combination of both IFN-γ and PDGF, however, resulted in significant enhancement of CXCL10 expression (almost 2.7-fold) compared with treatment with IFN-γ alone. Corroboration of these findings using the real-time RT-PCR assay (Fig. 1*B*) also showed a threefold increase in CXCL10 expression in the presence of both the inducers compared with its induction with IFN-γ alone.

PDGF treatment alone does not up-regulate CXCL10 expression, but synergistically it enhances IFN-γ-induced expression of CXCL10 in MDMs

Having determined the changes in the expression of CXCL10 at the RNA level, it was of interest to assess changes at the protein level following treatment with the inducers. Culture supernatant fluids from cells treated or untreated with PDGF and/or IFN-γ at 6 h were analyzed for accumulation of CXCL10 protein by ELISA. As shown in Fig. 2*A*, treatment of human MDMs with PDGF-BB alone did not induce expression of CXCL10 protein, whereas IFN-γ alone, as expected, was able to significantly induce the protein. Exposure of MDMs to a combination of PDGF-BB and IFN-γ, however, resulted in a robust induction of CXCL10 expression in the culture supernatant fluids. PDGF-BB at any of the tested concentrations (50–500 ng/ml) was not able induce CXCL10 protein expression in the absence of IFN-γ (data not shown). All of the PDGF isoforms tested, PDGF-BB, -AB, and -AA, were able to equally synergize with IFN-γ to induce the CXCL10 protein expression (Fig. 2*A*). Next, we wanted to explore whether the synergistic action of PDGF with IFN-γ in the induction of CXCL10 also extended to the over-expression of other unrelated chemokines. As shown in Fig. 2*B*, PDGF alone was able to induce the expression of CCL2, as expected (22–24), and a similar effect was observed in cells treated with IFN-γ. Although the combination of both PDGF and IFN-γ led to a further enhancement of CCL2 expression, this effect was neither synergistic nor additive. This was in contrast to CXCL10 protein induction, which was not induced with PDGF-BB alone, but in the presence of IFN-γ, PDGF synergistically up-regulated the expression of the chemokine. Chemokines, CCL3 and CCL5, in contrast, were not induced in the presence of either PDGF and/or IFN-γ (Fig. 2*B*).

Transcriptional activation of CXCL10 with PDGF and IFN-γ

To evaluate whether the cooperative effects of IFN-γ and PDGF on CXCL10 expression involved increased transcriptional activation of CXCL10, we cotransfected human MDMs with a TGL-luc CXCL10 construct, a promoter-reporter containing 435 bp of hCXCL10 sequence upstream of the transcriptional start site and a *Renilla* luciferase-expressing plasmid. Following transfection,

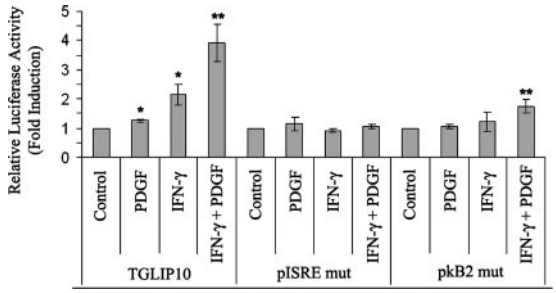

FIGURE 3. Effect of IFN-γ and/or PDGF on CXCL10 promoter activity in primary macrophages. MDMS were transiently transfected by a nucleofector kit with either a control plasmid containing the luciferase gene (pGL3-Luc) or plasmids containing the luciferase gene under the control of the human CXCL10 promoter (pTGLCXCL10-luc) or mutant promoters (pκB2-mut and pISRE3-mut) as indicated. After 4 h of recovery period, the cells were treated with or without IFN-γ (1000 U/ml) and/or PDGF-BB (100 ng/ml) for 4 h and lysed followed by determination of relative luciferase activities with a luminometer. The values shown are the mean ± SD of three independent experiments (∗, $p \leq 0.05$; ∗∗, $p < 0.01$).

cells were treated with PDGF and/or IFN-γ and the promoter activity was assessed by the relative luciferase activities of the cell lysates using the luminometer. As shown in Fig. 3, treatment with either PDGF-BB or IFN-γ resulted in significant activation of the TGL-CXCL10 luciferase construct, and this effect was further enhanced with combination treatments of IFN-γ and PDGF. These findings thus suggested that the synergistic effect of IFN-γ/PDGF on CXCL10 expression in MDMs is manifested at both the transcriptional and translational levels.

Having observed the PDGF-mediated synergy with IFN-γ for induction of CXCL10 at the transcriptional level, we addressed whether this process involved the cooperation of ISRE and NF-κB binding sites on the CXCL10 promoter. Mutant promoter reporter constructs blocking the IRF9 recognition site (pISRE mut) or one of two NF-κB recognition sites (pκB mut) were used in transient transfection assays as described above. The mutation in the ISRE site completely abrogated the CXCL10 promoter induction by IFN-γ alone or in combination with PDGF-BB (Fig. 3). Mutation in the NF-κB2 site resulted in the inhibition of robust CXCL10 induction by both IFN-γ and PDGF. Residual CXCL10 transcription in the presence of mutated NF-κB2 site construct could be due to compensatory effect of intact NF-κB1 site. This indicated that both NF-κB sites are required for robust synergistic induction of CXCL10 by IFN-γ and PDGF-BB.

Signaling pathways involved in the PDGF-BB and IFN-γ-mediated synergistic induction of CXCL10
Role of the JAK-STAT pathway

The primary pathway by which IFN-γ acts as an immunomodulator is through the regulation of gene expression via the JAK-STAT tyrosine kinase-dependent cascade (37, 38). To elucidate the mechanism(s) involved in the synergistic induction of CXCL10 in presence of IFN-γ and PDGF, we investigated the phosphorylation status of signaling proteins STAT1 and STAT 3. Human MDMs were stimulated with PDGF and IFN-γ for 15, 30, and 60 min, followed by cell lysis and analyses of various transcription factors. As shown by Western blot analysis (Fig. 4*A*), treatment of MDMs with both IFN-γ and PDGF resulted in the dramatic phosphorylation of STAT1 at Tyr701 as early as 5 min following treatment. Phosphorylation of STAT1 at Ser 727 increased with time following treatment with peak phosphorylation at 30 min. A low level of STAT3 activation was observed following treatment of MDMs with both PDGF and IFN-γ (Fig. 4*A*). Further confirmation of STAT3 activation with both PDGF and IFN-γ, either alone or in combination, was achieved by immunobead signaling assay, with a peak activation achieved at 30 min postinduction (data not shown).

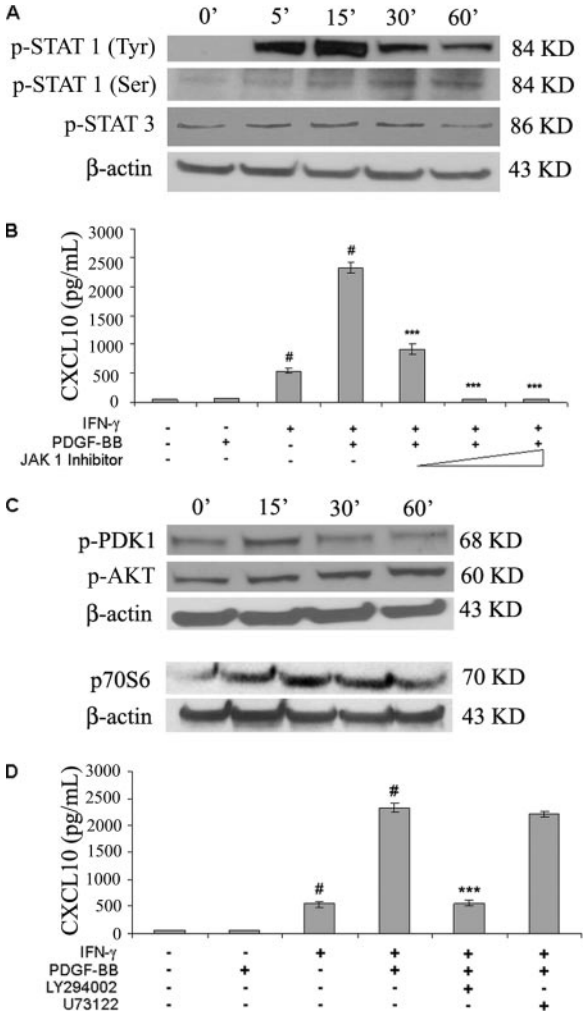

FIGURE 4. Analyses of JAK/STAT and PI3K pathways in IFN-γ and PDGF-BB stimulated macrophages. *A*, Western blot analyses was conducted sequentially using phosphorylated Abs against Tyr-701-STAT1, Ser-727-STAT1, and STAT3 on the total cell lysate from MDM cultures untreated or treated with IFN-γ (1000 U/ml) and/or PDGF (100 ng/ml) for indicated times. Blots were finally reprobed with β-actin Ab for normalization. *B*, Complete inhibition of the synergistic effect of PDGF on the IFN-γ-induced CXCL10 expression by JAK1 inhibitor. Serum-starved MDMs were preincubated with medium alone or with JAK1 inhibitor at various concentrations for 30 min, followed by incubation with IFN-γ (1000 U/ml) and PDGF (100 ng/ml) for 6 h. CXCL10 expression was then analyzed by ELISA in the collected supernatants. Values are mean ± SD from three independent experiments. #, $p < 0.001$ vs values with control, and ∗, $p < 0.001$ vs values with IFN-γ plus PDGF. *C*, Western blot analyses of the total cell lysate from MDM cultures untreated or treated with IFN-γ (1000 U/ml) and/or PDGF (100 ng/ml) were conducted sequentially using phosphorylated Abs against downstream targets of PI3K; PDK1, Akt, and p70S6. *D*, CXCL10 levels in the supernatant fluids were monitored by ELISA from MDMs pretreated with or without 20 μM PI3K inhibitor (LY294002) followed by stimulation with IFN-γ (1000 U/ml) and PDGF (100 ng/ml) for 6 h. Pretreatment of MDMs with the PI3K inhibitor led to the abrogation of PDGF-mediated synergistic induction of CXCL10 expression (mean ± SD; #, $p < 0.001$ vs values with control; and ∗, $p < 0.001$ vs values with IFN-γ plus PDGF) whereas the phospholipase C inhibitor U73122, in contrast, had no effect on the suppression of CXCL10.

STATs remain in an inactive state until they are phosphorylated by receptor-activated JAKs. To address the involvement of the JAK-STAT signaling pathway in the synergistic enhancement of the CXCL10 expression by PDGF, MDMs were pretreated with a pharmacological inhibitor of JAK (JAK inhibitor 1) before treating the cells with PDGF-BB and IFN-γ. Inhibition of JAK, which is an overlapping signaling molecule for both PDGF and the IFN-γ signaling pathway, completely abrogated CXCL10 induction by the two inducers (Fig. 4*B*). JAK inhibitor 1 at a 1 μM concentration reduced CXCL10 induction to 50% in the presence of PDGF and IFN-γ; however, at concentrations of 10 μM and 20 μM, levels of CXCL10 dropped to almost negligible levels similar to that observed in untreated cells. Cell viability remained unaffected at these concentrations of inhibitor (data not shown).

Role of PI3K pathway

The PI3K pathway plays a critical role in PDGF signaling upstream of PDK1/2 and Akt/PKB kinases (39). To dissect the role of this pathway in the IFN-γ and PDGF-mediated synergy of CXCL10 expression, cell lysates were examined for the phosphorylation of PDK1 and Akt. PDK1 was found to be activated at 15 min following IFN-γ and PDGF treatment, whereas Akt, downstream of PDK1, showed increased phosphorylation with time (Fig. 4*C*). Kinetics of activation of another MAPK, p70S6 kinase, which also lies downstream of PI3K, showed activation as early as 5 min with maximum phosphorylation signal at 30 min in cells treated with both the inducers (Fig. 4*C*). Analysis of phosphorylation of p70S6 kinase by immunobead assay also showed an earlier activation in cells treated with both the inducers whereas treatment with either inducer alone delayed the activation of p70S6 kinase (data not shown).

To address the functional role of PI3K in the synergistic induction of CXCL10, cells were pretreated with the PI3K inhibitor, LY 294002, followed by treatment of the cells with PDGF and IFN-γ and monitoring the cells for CXCL10 expression. As shown in Fig. 4*D*, pretreatment of MDMs with the PI3K inhibitor led to abrogation of PDGF-mediated synergistic induction of CXCL10 expression. Phospholipase C inhibitor, U73122, in contrast did not suppress CXCL10 induction.

Role of MAPK

Because MAPK are activated by a wide variety of extracellular signals, including growth factors, cytokines, and hormones (40), the activation of these kinases by PDGF and IFN-γ was evaluated. MDMs stimulated with PDGF and/or IFN-γ treatment for varying times followed by cell lysis were analyzed for changes in the relevant phosphorylated MAPK to evaluate activation of these pathways.

As shown in Fig. 5*A* (*top left*), maximal activation of Erk1/2 MAPK was observed in human MDMs treated with PDGF and IFN-γ for 30 min. By contrast, treatment of MDMs with PDGF or IFN-γ alone transiently activated Erk1/2 MAPK at 15 min, followed by return to basal levels. During these incubation periods, p38 MAPK was not activated by PDGF or IFN-γ, but simultaneous treatment with both of these stimuli activated the p38 MAPK with maximum activation at 30 min incubation (Fig. 5*A*, *top right*). JNK was also activated with IFN-γ and/or PDGF treatments, with maximum activation achieved at 30 min of incubation (Fig. 5*A*, *bottom*). PDGF alone or in combination with IFN-γ resulted in increased activation (2.5-fold) of JNK protein at 30 and 60 min compared with cells treated with IFN-γ alone. Corroboration of these immunobead findings was done by Western blot analyses of the cell lysate from cells treated with both IFN-γ and PDGF-BB, using Abs specific for the phosphorylated forms of Erk1/2, p38, and JNK (Fig. 5*B*).

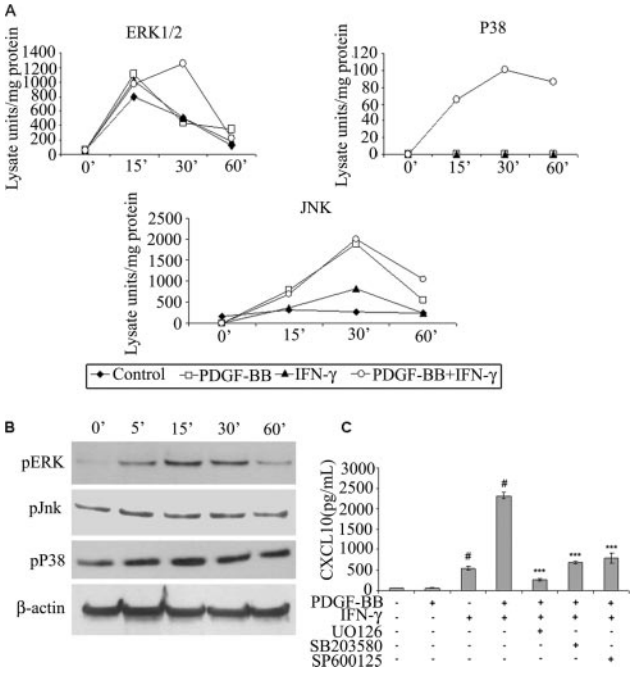

FIGURE 5. *A*, Multiplex analysis of MAPK in human MDMs treated with IFN-γ and/or PDGF-BB. Serum-starved MDMs were stimulated with IFN-γ (1000 U/ml) and/or PDGF (100 ng/ml) for 15, 30, and 60 min. Cells were then lysed in lysis buffer containing protease inhibitors. Twenty-five microliters of 125 μg/ml of each lysate was used for analysis of Erk1/2 (*top left*), p38 (*top right*), and JNK (*bottom*) MAPK. The median fluorescence intensity was measured with the fluroresecence-based immunobead assay. Standard curves obtained from Beadlyte Hela Cell Lysate standard were used to convert median fluorescence intensity of each analyte into lysate units and data was plotted as lysate U/mg protein. *B*, Western blot analysis of MAPK in macrophages treated with IFN-γ and PDGF-BB. Total cell lysates were obtained from MDM cultures untreated or treated with IFN-γ (1000 U/ml) and/or PDGF (100 ng/ml) for indicated times and sequentially immunoblotted with Abs specifically directed to the phosphorylated forms of Erk1/2, p38, and JNK MAPK and finally reprobed with β-actin Ab for normalization. *C*, Inhibition of MAPK(s) activity results in down modulation of PDGF and IFN-γ-mediated induction of CXCL10. Serum-starved MDMs were preincubated in the absence or presence of various MAPK inhibitors (U0126:MEK1 and 2 inhibitor; SB203580:p38MAPK inhibitor and SP600125: stress-activated protein kinase/JNK inhibitor) at a concentration of 20 μM for 30 min, followed by incubation with IFN-γ (1000 U/ml) and PDGF (100 ng/ml) for 6 h. Supernatant fluids collected were then analyzed for CXCL10 levels by ELISA. Data represents mean ± SD from three independent experiments (#, $p < 0.001$ vs values with control, and ∗, $p < 0.001$ vs values with IFN-γ plus PDGF).

The functional roles of these MAPK in the synergistic induction of CXCL10 were addressed by pretreating the cells with semiselective pharmacological inhibitors before induction with the cytokines. As shown in Fig. 5*C*, pretreatment of cells with the MEK1/2 inhibitor, U0126, led to inhibition of PDGF and IFN-γ-mediated induction of CXCL10 expression to levels less than that observed in the presence of IFN-γ alone. Both p38 MAPK/stress-activated protein kinase 2 inhibitor (SB203580) and JNK inhibitor II (SP600125) also inhibited the synergistic induction of CXCL10 by PDGF and IFN-γ. Taken together, these findings confirmed the involvement of specific MAPK in the synergistic induction of CXCL10 by PDGF and IFN-γ.

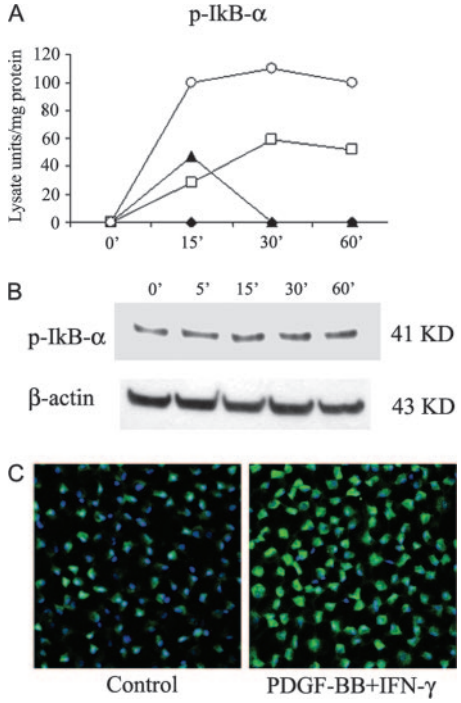

FIGURE 6. Involvement of NFκB in synergistic induction of CXCL10 by IFN-γ and PDGF-BB. *A*, Serum-starved MDMs were either untreated (♦) or treated with PDGF (□), IFN-γ (▲), or both PDGF and IFN-γ (○) and cell lysates analyzed for phospho IκB-α by fluoresecence based immunobead assay. *B*, Western blot analyses of the cell lysates from MDMs treated with both stimulants simultaneously. *C*, Human MDMs grown on cover-slips were either untreated (*left*) or treated with PDGF and IFN-γ for 30 min (*right*), and stained with anti-NF-κB p65 polyclonal Ab followed by treatment with Alexa Fluor 488-conjugated secondary Ab. Macrophage-specific FITC-tagged mouse anti-CD68 Ab was then used as second primary Ab. After the final washing, the slides were mounted in Slow Fade antifade reagent (with DAPI, blue stain) and images were captured by confocal microscopy (magnification ×250).

NF-κB signaling

The transcription factor NF-κB plays a pivotal role in inflammatory and immune responses. This family of transcription factors is present in the cytosol in an inactive state complexed with the inhibitory IκB proteins. Activation occurs via the phosphorylation of IκB-α, resulting in the release of active NF-κB. Using the immunobead assay, increased levels of phosphorylated IκB-α were detected in cells treated with both PDGF and IFN-γ (Fig. 6*A*). Cell lysates from IFN-γ-treated cells had increased phosphorylation of IκB-α as early as 15 min after IFN-γ treatment followed by a decrease to undetectable levels similar to that observed in untreated cells. Western blot analyses of the cell lysates from PDGF and IFN-γ-treated macrophages were performed to confirm these findings (Fig. 6*B*).

The nuclear translocation of NFκB p65 was demonstrated by immunocytochemistry in PDGF and IFN-γ-treated cells. Treatment with both the inducers for 30 min induced the nuclear translocation of active NFκBp65 in ~90% of infected cells; a representative example is shown in Fig. 6*C* (*right*). In contrast, in the nuclei of untreated cells there was no detectable NFκBp65 (Fig. 6*C*, *left*). The induction of NFκBp65 translocation following simultaneous treatment with PDGF and IFN-γ correlated well with the rapid phosphorylation of ERK1/2, JNK, and IκB-α as demonstrated in Figs. 5*A* and 6*A*, respectively, showing multiple simultaneous convergent signaling pathways.

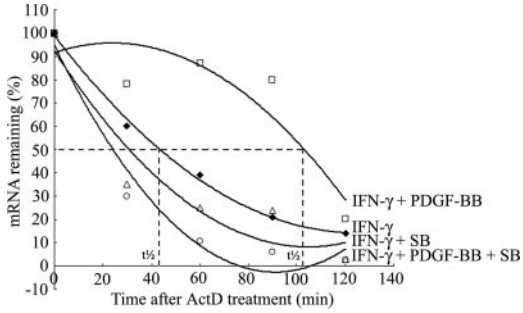

FIGURE 7. PDGF-BB treatment in combination with IFN-γ enhances the stability of CXCL10 mRNA. Human MDMs were stimulated with IFN-γ and/or PDGF-BB for 90 min before incubation with 10 μg/ml Act D in presence or absence of SB203580 (SB), and RNA was harvested at the indicated time points. The levels of CXCL10 mRNA were then determined using real-time RT-PCR and expressed as a percentage of mRNA remaining vs time after Act D treatment. Stability assays were performed in two sets.

Increased CXCL10 mRNA stability in IFN-γ- and PDGF-treated MDMs compared with cells treated with IFN-γ alone

Because CXCL10 mRNA and protein accumulation in MDMs following PDGF and IFN-γ treatment increased 4-fold compared with induction by IFN-γ alone, whereas the transcriptional activation increased only 2-fold, we hypothesized that the synergistic induction of CXCL10 by IFN-γ plus PDGF might be mediated in part by an increase in CXCL10 mRNA stability. Human MDMs were treated with PDGF and IFN-γ for 90 min to induce CXCL10, followed by incubation with Act D at varying times to block de novo mRNA synthesis. CXCL10 transcript levels were quantified by quantitative RT-PCR at the time of Act D addition and at subsequent time points. IFN-γ and PDGF-BB treatment resulted in an increased half life of CXCL10 mRNA (T1/2 = 103 min) compared with a mRNA half life of 43 min with IFN-γ alone (Fig. 7). CXCL10 transcript stabilization is known to be mediated via p38MAPK (41, 42). We therefore examined whether the increased stability of CXCL10 mRNA in the presence IFN-γ and PDGF could be blocked by an inhibitor of p38 MAPK. The p38 MAPK inhibitor, SB203580, significantly reduced CXCL10 mRNA half life in both IFN-γ and in PDGF and IFN-γ-treated cells, underscoring the importance of p38 MAPK in CXCL10 mRNA stabilization.

Discussion

HIV-1 infection often leads to dementia and related neurologic disorders in children and in 20% of adults with AIDS, and is associated with the infiltration of the brain by activated T lymphocytes and macrophages (43–45). CXCL10, a chemokine that specifically acts upon activated T cells and macrophages has been found to be closely associated with the progression of HIV-1-related CNS infection and neuropyschiatric impairment (3, 8, 9). Macrophages or microglia, but not neurons, are the predominant CNS reservoirs for the virus (43, 46, 47). Furthermore, the number of activated macrophages/microglia in the CNS correlate with HAD (48, 49, 50). Therefore, the interplay of soluble host factors mediating the induction of CXCL10, may represent additional neuropathogenic mechanism(s) underlying the progression of HAD.

Our earlier findings on microarray analysis of brains of macaques with $SHIV_{89.6P}$ encephalitis revealed an enhancement of CXCL10 and PDGF-B chain RNA in encephalitic macaques compared with animals without encephalitis (20). Apart from the induction of CXCL10, by viral factors, host factors, such as IFN-γ, IFN-αβ, and TNF-α, either alone or in combination, can also induce

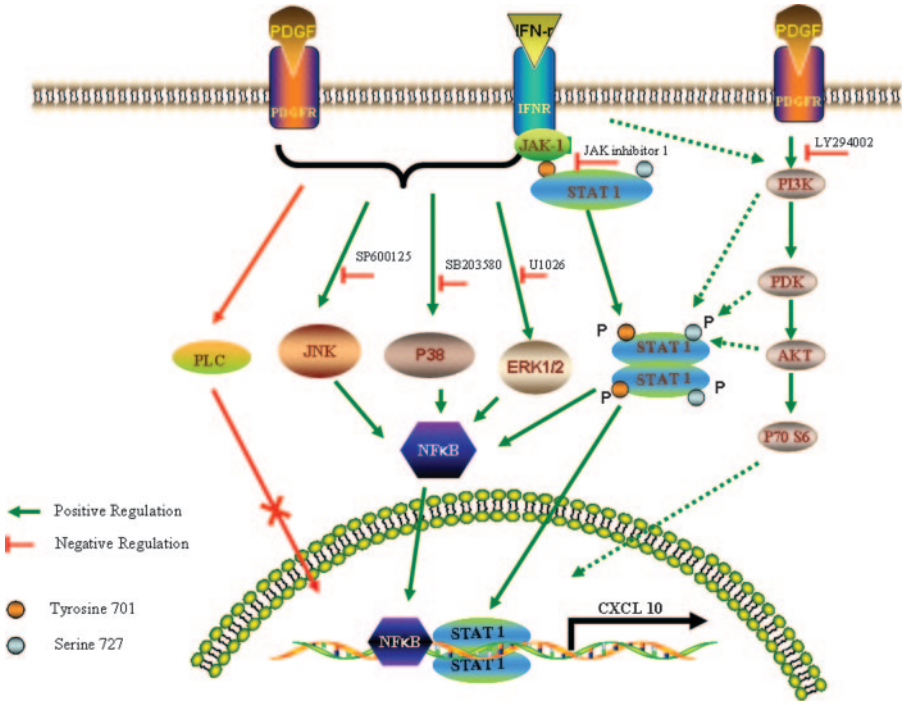

FIGURE 8. Schematic of signaling pathways involved in the synergistic induction of CXCL10 by IFN-γ and PDGF-BB in macrophages. The major signaling pathways activated by IFN-γ and PDGF-BB engagement in the induction of CXCL10 involve the JAK/STATs, MAPK, and PI3K. The latter two signals converge in the activation of NF-κB, resulting in transcription of the CXCL10 gene.

the chemokine in various cell types (16–19). Tannenbaum et al. (51) have previously reported the induction of CXCL10 mRNA expression in PDGF-stimulated BALB/c 3T3 fibroblasts. Based on the stimulation of CXCL10 with diverse stimuli (16–19) and the studies demonstrating increased IFN-γ and IFN-inducible genes in the brains of humans/macaques with HIV/SIV-related encephalopathies (1, 8, 9, 32–34), we evaluated whether the inflammatory factor, PDGF, could synergize with IFN-γ to induce CXCL10 expression in macrophages, the target cells for HIV in the brain. Furthermore, we also examined the intracellular molecular mechanisms by which the two cytokines could cooperatively regulate CXCL10 gene expression. It was interesting to note that, contrary to findings by Tannenbaum et al. (51), we did not observe the induction of CXCL10 RNA in MDMs treated with PDGF alone. A novel finding of this study was that while PDGF alone failed to induce CXCL10 protein expression in MDMs, it synergistically enhanced the induction of CXCL10 by IFN-γ in the same cells.

PDGF activates a variety of intracellular signaling pathways in many cell types via two tyrosine kinase receptors, PDGF α and β (52, 53). PDGF receptors can phosphorylate and activate STAT proteins directly or indirectly via the JAK activation pathway (54). Activated PDGF receptors also generate two lipid second signals, diacylglycerol and phosphatidylinositol trisphosphate (55–57) and drive the Ras/Raf/MAPK cascade (58). We evaluated several of these pathways for their involvement in the PDGF-mediated synergistic increase of CXCL10 in the presence of IFN-γ. The data presented here clearly define a role for JAK-STAT, PI3K, and MAPK pathways in the cooperative interaction of PDGF and IFN-γ in the induction of CXCL10. Fig. 8 is a schematic demonstrating the signaling pathways involved in the synergistic induction of CXCL10 by IFN-γ and PDGF-BB in macrophages.

IFN-γ activates the JAK/STAT pathway through its receptor, α- and β-subunits (37, 59). In almost all cells, IFN-γ binds to the α- and β-subunits of its receptor, leading to activation of JAK1 and JAK2 kinases followed by tyrosine phosphorylation of STAT1 (37, 60, 61). Complete activation of STAT 1 by IFN-γ requires phosphorylation of the following: 1) Tyrosine 701 and, 2) Serine 727 (62, 63). The phosphorylation of the former is a receptor-mediated event, while that of the latter has been shown to be regulated by activated PI3K and its effector kinase Akt following IFN-γ receptor occupancy (64). Additionally, PDGF treatment also leads to phosphorylation of STAT1 on serine 727 (65). A significant increase in the phosphorylation of STAT1 at tyrosine 701 was evident following treatment of MDMs with both the inducers. PDGF synergy also involved activation of the following: 1) STAT1 at serine 727, albeit at lower levels than that observed at tyrosine 701 and, 2) PI3 kinase pathway. These findings lead us to speculate that PDGF-mediated activation of PI3 kinase cascade may play a role in fully activating STAT 1 (also at serine 727). Simultaneous treatment of human MDMs with both the inducers also resulted in activation of STAT 3, which, in rare instances, has been shown to be activated by IFN-γ (66, 67, 68). As expected, treatment of macrophages with the JAK inhibitor 1 completely abrogated the PDGF-mediated synergistic induction of CXCL10 by IFN-γ, thus underpinning the role of JAK-STAT pathway in this synergy.

In addition to the role of protein factors, STAT-1 and p65 (NFκB subunit), we also found involvement of ISRE and NFκB binding sites on the CXCL10 promoter to be critical in the transcriptional activation of CXCL10 by IFN-γ and PDGF-BB. This is in keeping with previous findings demonstrating the coordinated action of p48 ISRE recognition factor, STAT-1α and p65 subunit of NFκB for sustained, synergistic transcription of CXCL10 induction (16).

JAK/STAT signaling is essential but not sufficient for full responses to the IFNs, most cytokines, and some growth factors (69, 70, 71). Activation of Erk and JNK kinases were also involved in the synergistic induction of IFN-γ inducible CXCL10 by PDGF as demonstrated by pharmacological inhibitors and phosphoprotein studies.

IFN-γ is known to induce two parallel but coordinated pathways regulating the expression of genes encoding proinflammatory molecules. Transcript synthesis is regulated by the JAK-STAT pathway, whereas transcript stabilization is regulated by the p38 MAPK pathway (41, 42). Although PDGF is also known to activate p38 MAPK (58) in some cells, we were not able to detect

activation of this kinase with either PDGF or IFN-γ alone. Interestingly, when cells were treated with PDGF and IFN-γ together, there was a significant activation of p38MAPK at all the time points tested, thus leading us to suggest that p38MAPK in the presence of both PDGF and IFN-γ may be involved in increasing the stability of CXCL10 mRNA induced by IFN-γ. Using Act D chase experiments, we observed enhanced stability of the CXCL10 transcript in the presence of both PDGF and IFN-γ.

The importance of synergistic interactions between soluble host factors is becoming increasingly well recognized, specially, in the progression of HAD (72). Our studies establish that a PDGF-triggered signal transduction mechanism(s) can cooperate with common pathways activated by IFN-γ to induce CXCL10 and thus define a major proinflammatory mechanism by which PDGF may play a role in the pathogenesis of HAD. These findings have important implications in the pathogenesis of AIDS-associated dementia, because CXCL10 is a known potent neurotoxin (10–12, 14). Enhanced expression of this chemokine mediated by interaction of over-expressed host factors, such as PDGF and IFN-γ, can not only lead to increased influx of inflammatory cells in the CNS, but can also result in increased neuronal dysfunction and death, thereby amplifying the toxic pathological responses that are the hallmark of end-stage HAD. Such complex interactions between antiviral and inflammatory factors can thus tip the balance from protection to disease progression.

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Disclosures

The authors have no financial conflict of interest.

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