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Mechanisms for HIV Tat upregulation of IL-10 and other cytokine expression: Kinase signaling and PKR-mediated immune response

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Abstract HIV Tat has been known to have multiple regulatory roles including replication of HIV and modulation of cellular kinases. We investigated whether signaling kinase PKR plays a critical role in mediating Tat-induced cytokine dysregulation. We showed Tat induction of IL-10 dysregulation is associated with PKR activation. To examine the mechanism involved, inhibition of PKR activity abrogated the Tat-induced cytokine induction. We next identified that the MAP kinases including ERK-1/2 and p38 are downstream of PKR in these Tat-induced pathways. Thus, PKR may play a critical role in mediating the subversive effects of HIV Tat resulting in IL-10 induction.

Keywords: HIV-1; HIV Tat protein; Protein kinase PKR; Cytokines; Transcription factors

1. Introduction

The genome of HIV encodes a number of essential structural proteins as well as regulatory proteins to enhance its replication and survival in the host. Tat, one of the regulatory proteins, acts as a potent transcriptional activator by binding to the transactivation response (TAR) element of the HIV long terminal repeat (LTR) to mediate viral gene expression [1]. Additionally, HIV utilizes Tat to perturb intracellular functions and evade immune responses of the host. To achieve this, it has been shown that Tat is secreted from infected cells for subsequent uptake by other cells to exert its biological effects including cytokine expression [2]. Tat also induces the expression of specific chemokine receptors such as CCR5 and CXCR4, which are important for HIV infection [3].

Following virus infection, the intercellular coordination of host response is regulated, at least in part, by interferon (IFN) and proinflammatory cytokines. Binding of IFN to its cognate receptor induces PKR, a double-stranded RNA-activated protein kinase. This results in phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α), leading to inhibition of translation [4]. Additionally, other stress inducers including LPS can induce PKR by activation of cell surface or transmembrane receptors including CD14 or Toll-like receptors [5]. This leads to induction of cytokines including interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α to fight the invading pathogen. Other biological functions of PKR including its role in mediating TNF-α- or virus-induced apoptosis have been previously reported [6,7]. Among these, we have shown tumor suppressor gene p53 acts downstream of PKR in TNF-regulated apoptosis.

Several reports have indicated PKR plays an important role in resistance to HIV infection due to its role in mediating IFN's effects on the inhibition of HIV-1 translation [8,9]. Moreover, the regulation of HIV-LTR transactivation is mediated by PKR through the transcription factor NF-κB [10]. NF-κB is known to regulate the expression of cytokines including IL-6, IL-8 and TNF-α. Similarly, the expression of these proinflammatory cytokines is regulated by nuclear factor NF-IL6, implicating its potential role in HIV pathogenesis.

In light of the critical role of PKR in immune defense, we examined the signaling pathways involved in HIV Tat interactions with PKR, and its consequent effects in the activation of transcription factors and induction of cytokines. We further delineated that the cytokine induction is mediated by mitogen-activated protein (MAP) kinases including p38 and p44 and p42 MAP kinases (ERK-1/2), which act as downstream effectors of PKR in cellular signaling transduction.

2. Materials and methods

2.1. Cell culture and primary blood monocyte isolation

Primary blood monocytes (PBM) were isolated from buffy coats of healthy blood donors (Source: Hong Kong Red Cross Blood Transfusion Service) by Ficoll-Paque (Amersham Pharmacia Biotech.) density gradient centrifugation. PBM were resuspended in RPMI 1640 medium supplemented with 5% autologous plasma and cultured for 1 h. Following removal of non-adherent cells, adherent cells have been shown to be >90% positive using anti-CD14 antibodies staining by flow cytometer. Additionally, we used derivatives of U937 promonocytic cells (U9K-M) that are deficient in PKR activity to confirm the role of PKR in the Tat-induced cytokine expression[11]. The activity of PKR in U9K-M (results not shown) was confirmed by different experiments including phosphorylation of eIF2α and PKR-mediated apoptosis assays as previously reported [6].

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Abbreviations: 2-AP, 2-aminopurine; ERK-1/2, p44 and p42 MAP kinases; IL, interleukin; MAP, mitogen-activated protein; PBM, human primary blood monocytes; RT, reverse transcription; TNF, tumor necrosis factor

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2.2. Recombinant HIV-1 Tat protein

The recombinant HIV Tat protein was produced by using GST-Tat 1 86R plasmid (NIH AIDS Research and Reference Reagent Program). Cleaving GST-Tat protein with thrombin produced the recombinant Tat protein. The mock control was prepared by using pGEX-2T plasmid without Tat cDNA insert. The purity and the quantity of the fusion protein were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. The levels of endotoxin were measured by Pyrotell assay kit (Associates of Cape Cod) and were shown to be less than 0.5 EU/ml in these samples. The biological activity of the Tat protein was confirmed by HIV-LTR luciferase activity (results not shown).

2.3. RNA extraction and RT-PCR

Total cellular RNA was extracted by TRIzol and reverse transcribed into cDNA by SuperScript II system (Invitrogen). The reverse transcriptase (RT)-PCR primer sets for cytokines and GAPDH were as follows. IL-6 (upstream:5′-ATGAACTCCTTCTCCACAAGCGC-3′; downstream:5′-GAAGAGCCCTCAGGCTGGACTG-3′), IL-10 (upstream: 5′-ATGCCCCAAGCTGAGAACCAAG-3′; downstream: 5′-TCTCAAGGGGCTGGGTCAGCTA-3′), TNF-α (upstream: 5′-GGCTCCAGGCGGTGCT TGTCC-3′; downstream: 5′-AGACGGCGATGCGGCTGATG-3′), and GAPDH (upstream: 5′-ACCACAGTCCATGCCATCAC-3′; downstream: 5′-TCCACCACCCTGTTGCTGTA-3′). The thermal cycling condition for primer annealing was at 60 °C, followed by 25–35 cycles of amplification depended on the different genes.

2.4. Real-time RT-PCR

Real-time PCR was performed by using SYBR® Green PCR Master Mix (Applied Biosystems). The reactions were cycled 40 times under the following parameters: 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s. The mRNA level of Tat-induced cytokines was normalized to that of β-actin and compared with the untreated control. Samples were prepared from at least 3 separate experiments. All the reagents used for TaqMan Real time RT-PCR were purchased from Applied Biosystems. Two hundred nanograms of RNA were reverse transcribed using GeneAmp RNA PCR kit to synthesize cDNA. The IL-6, IL-10 and TNF-α TaqMan probes were purchased from the Applied Biosystems and 18S RNA was used as an internal control.

2.5. ELISA

The culture supernatants of Tat- and other kinase inhibitors-treated PBM were collected at different time intervals and stored at −70 °C. The level of TNF-α was measured by a TNF-α specific ELISA kits (R&D system).

2.6. Role of PKR and MAP kinases in signal transduction

The PBM were incubated with inhibitors for individual kinases including 2-aminopurine (2-AP) (Sigma–Aldrich) for PKR, PD98059 for ERK-1/2 kinase, or SB203580 for p38 MAP kinase (Calbiochem), respectively, for 1 h prior to Tat protein treatment. Protein and RNA were extracted from the treated cells at different time points for further analysis.

2.7. Electrophoretic mobility shift assay (EMSA)

The DNA-protein binding reaction was performed by incubating 2 μg of nuclear extracts of Tat-treated cells with 12 fmole of ^{32}P-labeled double-stranded NF-IL6 oligdeoxyonucleotide probe (wild-type: 5′-GATCGGACGTCACATTGCACAATCTTAATAAT-3′; mutant: 5′-GGACGTCACACTACAAACTCTTAATAA-3), 1× binding buffer (25 mM HEPES pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% NP-40, 12% glycerol, and 50 mM NaCl), and 0.5 μg of poly (dI:dC) at room temperature for 30 min. The reaction product was resolved in 5% non-denaturing PAGE in Tris–glycine buffer, dried and detected by autoradiography.

2.8. Western blot analysis

The procedure for collection of protein extracts has been described previously [12]. Thirty micrograms of cytoplasmic proteins or two micrograms of nuclear proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane for probing overnight with primary antibodies. The antibodies against eIF2α, phosphorylated eIF2α, p38 MAP kinase, phosphorylated p38 MAP kinase, ERK-1/2 and phosphorylated ERK-1/2 were purchased from Cell Signaling Technology. The anti-PKR, anti-C/EBP-β, anti-p65 (Rel A), anti-actin and anti-IκB-α antibodies were purchased from Santa Cruz Biotechnology. The antibody against phosphorylated PKR was purchased from Biosource International. Following washings, the membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (BD Transduction Lab). The signal was visualized by enhanced chemiluminescence kit (Amersham Pharmacia Biotech.).

2.9. Statistical analysis

Statistical analysis of the comparison between groups is calculated by using Student's t test. Data were presented as the mean ± standard deviation (S.D.). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Tat induces the expression of cytokines in a dose- and time-dependent manner

To examine the signaling pathways of Tat-induced intracellular events, we initially demonstrated that Tat induces the expression of cytokines in PBM. Real-time RT-PCR results showed that Tat induced the transcription of IL-6, IL-10 and TNF-α in a dose-dependent manner (Fig. 1A). When followed over a period of 12 h, the induction of IL-6, IL-10 and TNF-α mRNA in PBM treated with Tat (500 ng) peaked at 3 h (Fig. 1B and C, lane 3). For IL-6, the elevated mRNA level was maintained up to 3 h and started to decline between 3 and 6 h after Tat treatment. In contrast, the mRNA levels of IL-10 and TNF-α returned to the basal level at 6 h (Fig. 1B and C, lane 4). We further demonstrated that the levels of TNF-α protein started to increase at 3 h and peak at 10 h following Tat treatment (Fig. 1D). Additionally, we have shown that extracellular Tat protein, immobilized by coating on culture wells, is capable of inducing cytokine expression in the PBM (results not shown).

3.2. Inhibition of PKR results in abrogation of Tat-induced cytokine expression

Since PKR regulates IFN induction in virus infections [7], we investigated whether Tat-induced cellular effects are dependent on PKR activity. Following incubation with different concentrations of 2-AP, a PKR inhibitor, for 1 h, PBM were treated with Tat protein for another 3 h. The induction of IL-10 and TNF-α were potently inhibited by 2 mM of 2-AP (Fig. 2A, lanes 2 and 3). For IL-6, a higher concentration (5 mM) of 2-AP was needed to inhibit the induction (Fig. 2A, lane 4). At these concentrations of 2-AP (up to 10 mM), the cells did not show any signs of cytotoxicity and remained viable in subsequent cell assays (results not shown)[13]. Another set of experiments using PKR-deficient U9K-M cells was performed to confirm the 2-AP effects. After 6 h of Tat protein treatment, the induction of IL-10 and TNF-α mRNA was observed in the control U9K-C cells (transfected with the control plasmid without PKR insert [11]) but not in the U9K-M cells (Fig. 2B, lanes 3 versus 6, $P < 0.05$). We next measured the TNF-α production in PBM pre-treated with or without 2-AP for 1 h before the addition of Tat protein for 10 h. As shown in Fig. 2C, the production of TNF-α by Tat was abrogated by 2-AP treatment (lanes 3–5). In the presence of 2-AP, we have demonstrated that there was inhibition of Tat-induced phosphorylation of the PKR protein (Fig. 2E).

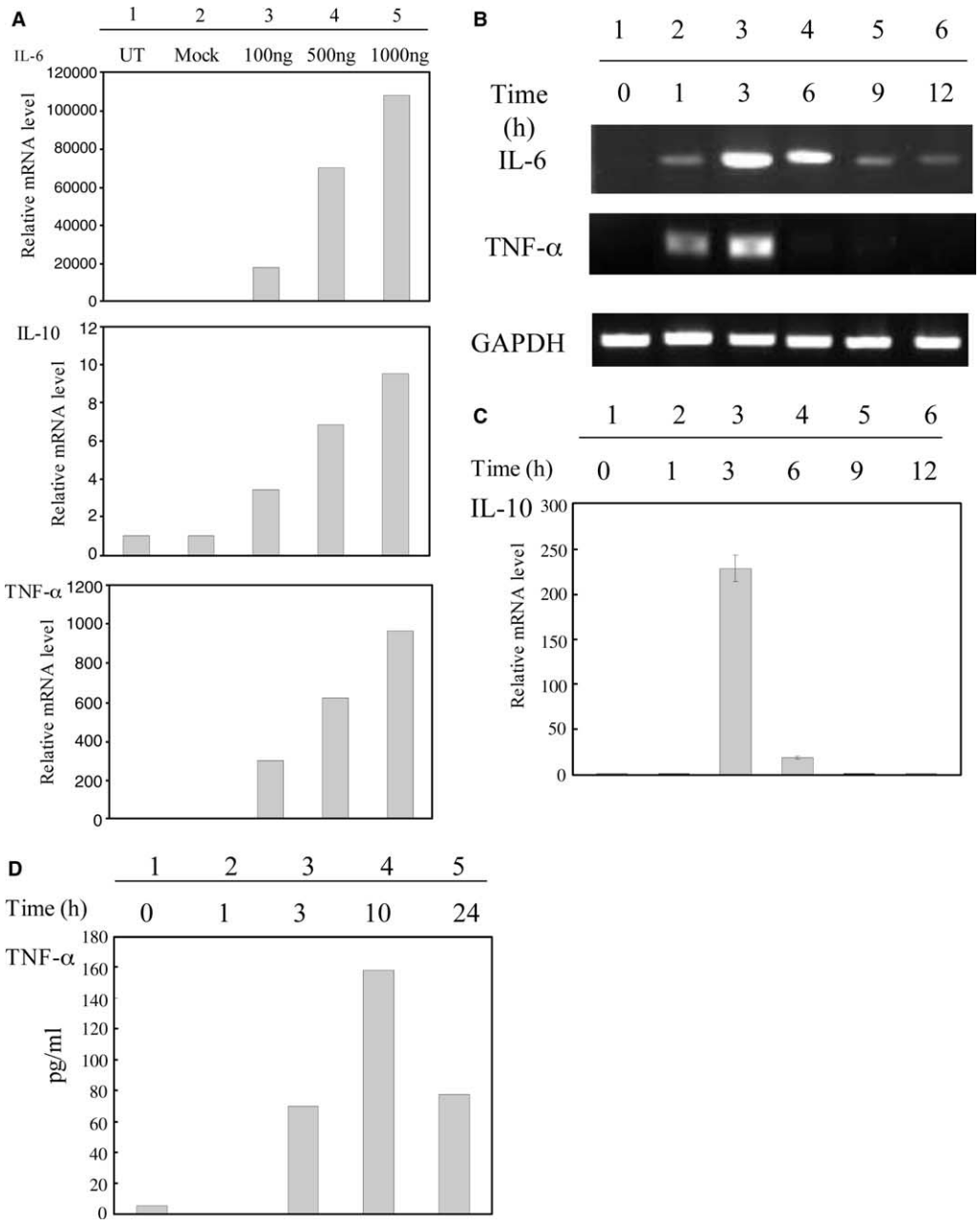

Fig. 1. Dose effects and kinetics of Tat-induced cytokine expression in primary blood monocytes (PBM). (A) Real time RT-PCR of IL-6, IL-10 and TNF-α was performed on PBM treated with Tat for 3 h. The untreated cells and mock reagent described in Section 2 was used as a control (lanes 1 and 2). The results have been normalized with the measurement of 18S mRNA levels. The experiment shown is representative of three independent experiments. (B) RT-PCR analysis of IL-6, TNF-α and GAPDH of PBM treated with Tat (500 ng). The results shown are representative of at least three independent experiments. (C) The cDNA from (B) were used to perform real-time quantitative PCR for IL-10. The results (mean ± S.D.) shown have been normalized with the measurement of β-actin mRNA levels, and they are representative of three experiments performed. (D) PBM were incubated with 500 ng of Tat protein over a period of 24 h. The culture supernatants were assayed for TNF-α by ELISA assays ($n = 3$).

3.3. Tat induces PKR kinase activity

As the above results showed that inhibition of PKR activities abrogated the Tat-induced cytokine expression, we next examined whether Tat can regulate the kinase activity of PKR. We measured the levels of phosphorylated PKR in Tat-treated cells at different time intervals by immunoblot assays. The activity of PKR increased gradually from 30 min to 2 h (Fig. 2D), indicating the activation of PKR.

3.4. PKR mediates Tat-induced NF-IL6 activation but not NF-κB

To determine whether PKR regulates Tat-induced activation of transcription factors, we investigated the nuclear translocation of NF-κB and NF-IL6 by Western analysis. Our results showed that Tat could induce the degradation of NF-κB inhibitor, IκB-α, (Fig. 3A, lane 3). This degradation persisted in the Tat-treated cells that were pretreated with 2-AP suggesting

that PKR is not involved (Fig. 3A, lane 4). Similarly, the nuclear translocation of the NF-κB subunit, p65/RelA protein, was unaffected by the treatment of 2-AP (Fig. 3B, lanes 3 and 4).

Nuclear translocation of NF-IL6 was detected in Tat-treated PBM (Fig. 3C, lane 3). Such translocation of NF-IL6 could be abrogated by inhibiting PKR (Fig. 3C, lane 4). In addition, the EMSA results showed that the Tat-induced NF-IL6 DNA-binding activity was inhibited by 2-AP (Fig. 3D, lanes 3 and 4). We have performed densitometric analysis of the results obtained in Fig. 3D. The difference between the 2-AP treatment (Fig. 3D, lane 5) and untreated control (Fig. 3D, lane 1) was insignificant. The slightly increased binding in the 2-AP-treated cells may be due to non-specific activities (results not shown). The use of ^{32}P-labeled double-stranded NF-IL6 mutant oligo was to demonstrate the specificity of the NF-IL6 oligo. The ^{32}P-labeled mutant oligo consists of modified NF-IL6 enhancer sequence and thus would not allow for binding of the NF-IL6 protein. Consequently, the binding of wild-type NF-IL6 was observed in lane 3, while there was no binding to the ^{32}P-labeled mutated NF-IL6 oligo in lane 7 (Fig. 3D). These results illustrated that the correct enhancer binding sites are critical to the binding in the EMSA for NF-IL6. Taken together, the results indicated that PKR mediates the activation of NF-IL6 but not NF-κB following induction by Tat in monocytic cells.

3.5. p38 and ERK-1/2 MAP kinases mediate Tat-induced cytokine induction

Previous studies showed that MAP kinases play key roles in cytokine induction mediated by PKR in cells treated with LPS [14]. We investigated whether these kinases are also involved in

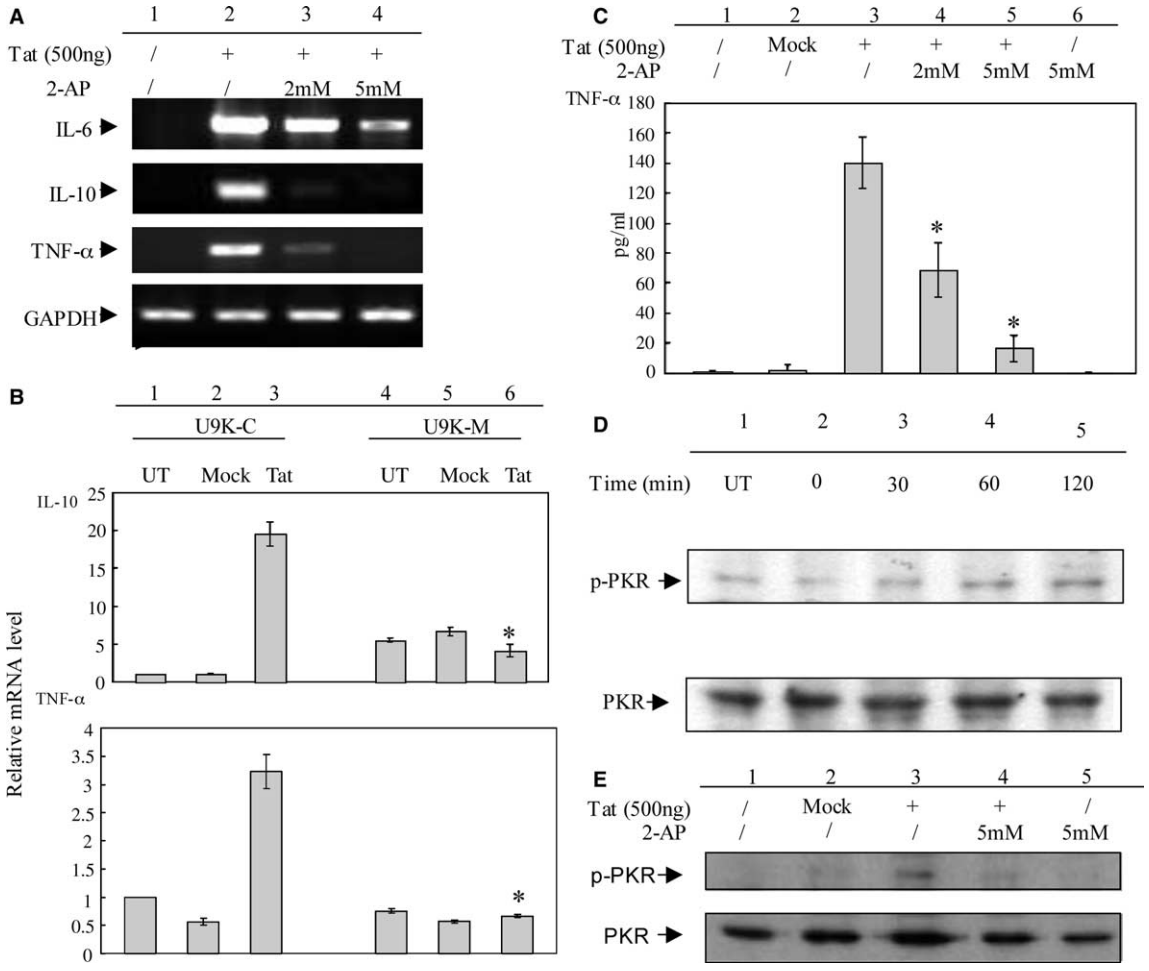

Fig. 2. PKR mediates Tat-induced cytokine expression. (A) PBM were pre-treated with different concentrations of 2-AP for 1 h and followed by incubation with Tat protein for 3 h (lanes 2–4). PBM without treatment (lane 1) were used as controls. (B) Effects of Tat on cytokine expression in PKR-deficient U9K-M cell. Total RNA extracted from U9K-C and U9K-M cells treated with Tat for 6 h were used to perform real-time PCR analysis. The untreated PBM (lanes 1 and 4) and those treated with mock reagent (Mock, lanes 2 and 5) were used as controls. The results (mean ± S.D.) have been normalized with the measurement of β-actin mRNA levels ($n = 3$). The statistical significance of the fold of induction compared with untreated/mock is indicated as follows: *, $P < 0.05$. (C) Effects of PKR inhibition on TNF-α production in PBM after 10 h of Tat treatment: lane 1, PBM without treatment; lane 2, PBM treated with mock reagent; lane 3, PBM treated with Tat; lanes 4 and 5, PBM treated Tat plus 2-AP; and lane 6, 2-AP treatment alone. Mean values and standard deviation of the mean from triplicate experiments are as shown. (D) PBM were treated with 500 ng of Tat protein from 0 to 2 h (lanes 2–5). Cytoplasmic protein (30 g) was transferred to nitrocellulose membrane and then immunoblotted using antibodies specific for phosphorylated PKR. The same membrane was stripped and incubated with antibodies specific for PKR protein to show the equal loading of protein. (E) PBM were treated with 500 ng Tat protein (lanes 3 and 4) with or without the presence of 2-AP. The experiment shown is representative of three independent experiments performed on PBM obtained from different donors.

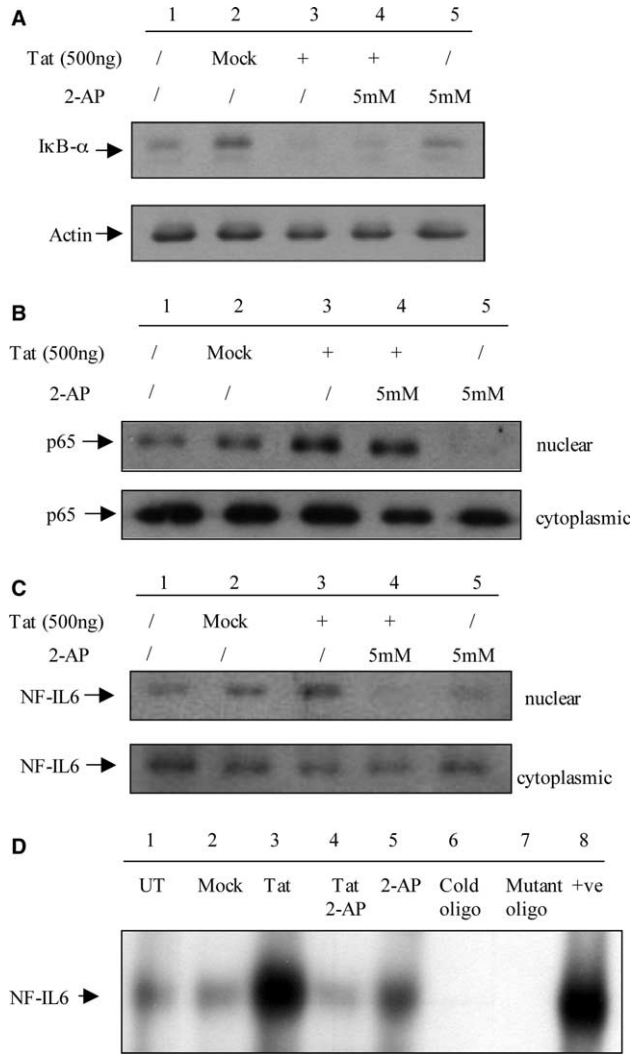

Fig. 3. Effects of 2-AP on Tat-mediated activation of NF-IL6 and NF-κB. (A) Cytoplasmic proteins (30 μg) of PBM treated with Tat for 1 h in the absence or presence of 5 mM 2-AP (lanes 3 and 4) were assayed for IκB-α. The same membrane was stripped and incubated with anti-actin antibodies to measure the total amounts of protein loaded. The experiment shown is representative of three independent experiments. Nuclear translocations of NF-κB (B) and NF-IL6 (C) in PBM were as shown. Nuclear (2 μg, upper panel) and cytoplasmic (30 μg, lower panel) proteins extracted from PBM that were treated with 500 ng of Tat protein for 1 h with or without 2-AP treatment (lanes 3 and 4) were analyzed by Western blot with antibodies against p65/Rel A protein (B) or C/EBP-β (C). Cells without treatment (lane 1) or treated with mock reagent (lane 2) were used as controls. (D) Effects of 2-AP on Tat-induced activation of NF-IL6 nuclear DNA binding activity. EMSA was performed on nuclear proteins (2 μg) from PBM treated with Tat protein (lane 3) or pre-treated with 2-AP followed by Tat protein treatment for 1 h (lane 4). The 50× unlabelled oligos (lane 6) and radiolabeled mutated NF-IL6 oligos (lane 7) were used as controls to test for specificity of the binding. Cells without treatment (lane 1), treated with mock reagents (lane 2), or 2-AP (lane 5) were used as controls. Additionally, U937 cells treated with PMA for 48 h were used as positive controls for NF-IL6 activation (lane 8). The experiment shown is representative of three independent experiments from three different donors.

these Tat-induced and PKR-mediated cellular events. The results showed that pretreatment with ERK-1/2 inhibitor (PD98059, 1 μM) blocked the induced expression of IL-6 and TNF-α mRNA by 83% ($P < 0.05$) and 52% ($P < 0.05$), respectively (Fig. 4A, lane 6). For the p38 MAP kinase inhibitor (SB203580, 1 μM), it could inhibit both IL-6 and IL-10 by 90% ($P < 0.05$) and 66% ($P < 0.05$), respectively (Fig. 4A, lane 4). These results showed that the Tat-induced IL-6 mRNA transcription is mediated by both p38 and ERK-1/2 MAP kinases, whereas the expression of IL-10 and TNF-α is individually regulated by p38 and ERK-1/2 MAP kinases, respectively. To examine the effects of p38 and ERK-1/2 on TNF-α protein levels, PBM were pre-treated with the respective inhibitors before Tat incubation. As shown in Fig. 4B, the production of TNF-α was abrogated by SB203580 (62.6%, $P < 0.05$) or PD98059 (74.6%, $P < 0.05$), thus confirming the role of these kinases in cytokine regulation by Tat.

3.6. p38 and ERK-1/2 MAP kinases act downstream of PKR in Tat-induced PBM

We next investigated the relationship of p38 and ERK-1/2 MAP kinases with PKR in cytokine regulation. Since eIF2α is an endogenous substrate of PKR, measuring the phosphorylation status of eIF2α is indicative of PKR activity. As shown, both inhibitors failed to inhibit the phosphorylation of eIF2α (Fig. 4C, lanes 3–5; and lanes 3, 7 and 8). In other words, both of the p38 and ERK-1/2 MAP kinases could not regulate the activity of PKR. In order to confirm that PKR acts upstream of both MAP kinases, the Tat-induced PBM were pre-treated with 2-AP. This resulted in reduced activation of p38 and ERK-1/2 MAP kinases as reflected by significant decreases in the phosphorylated form of the proteins (Fig. 4D, lanes 2–4). Thus, the findings indicated that p38 and ERK-1/2 MAP kinases may act as downstream targets of PKR in regulating cytokine induction by Tat.

4. Discussion

Previous studies on the interaction of Tat and PKR have yielded intriguing results. For instance, two reports demonstrated the ability of Tat binding to PKR, resulting in inhibiting its kinase activity in an in vitro model of protein–protein interaction [15,16]. Furthermore, Tat can compete with PKR for binding to eIF2α, a cellular substrate for kinase activity of PKR [17]. These results suggest that Tat may act as an inhibitor of PKR. Additionally, another report showed that PKR mediates the activation of NF-κB by Tat [10]. In investigating the mechanisms involved, our results revealed that Tat induces the activity of PKR (Fig. 2D). These discrepancies in results could be due to different experimental settings. Most of the previous studies have focused on the protein–protein interaction of Tat and PKR in vitro using purified recombinant proteins [15,16]. In our study, exogenous addition of the recombinant Tat protein may interact with cell surface receptors or was directly transported into the cell to trigger a cascade of signal transduction.

Here, we demonstrated the relationship between exogenous Tat and PKR in cytokine induction in human blood monocytes. With the inhibition of PKR, the Tat-induced cytokine expression was abrogated (Fig. 2). Thus, our results showed that PKR may play a critical role in this intracellular signal transduction process by Tat.

The host immune system utilizes multiple signaling pathways and different transcription factors to mediate immune

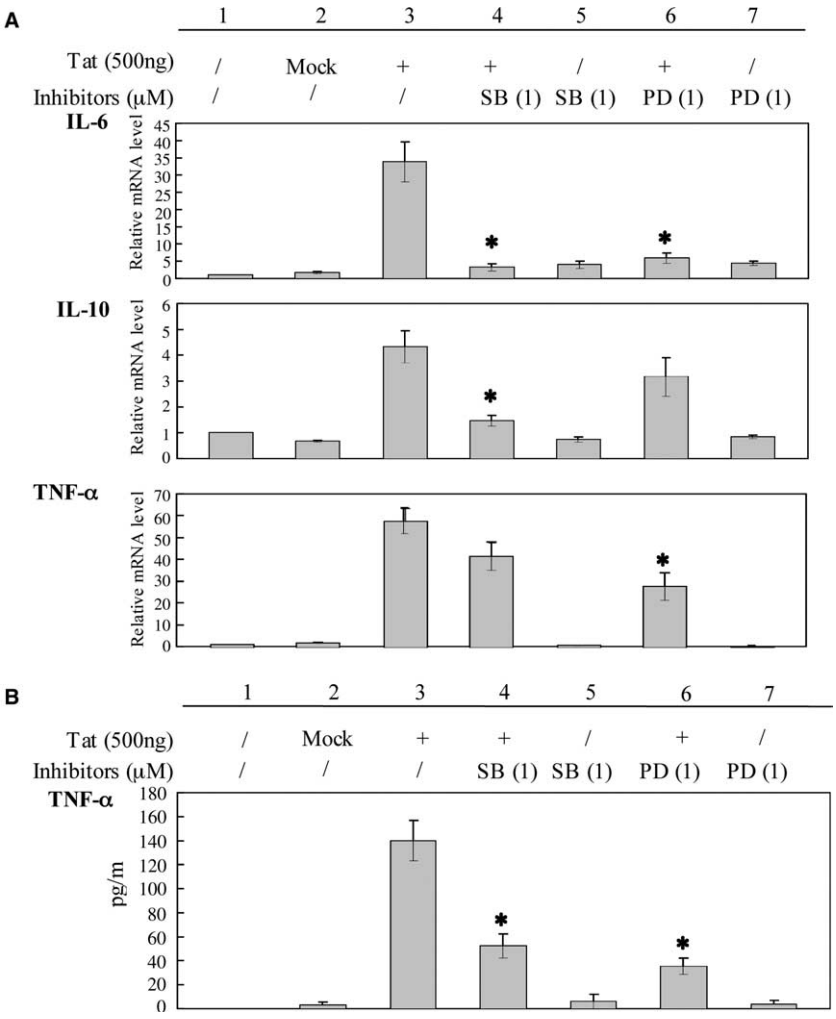

Fig. 4. The role of p38 and ERK-1/2 MAP kinases in Tat-induced cytokine induction. (A) Effects of inhibitors SB203580 (p38 kinase) and PD98059 (ERK1/2 MAP kinase) on Tat-induced expression of cytokine genes. Real-time PCR was performed on total RNA of PBM treated as follows: lane 1, no treatment; lane 2, mock treatment; lane 3, Tat alone; lane 4, SB203580 pretreatment for 1 h and Tat for 3 h; lane 5, SB203580 pretreatment; lane 6, PD98059 pretreatment and Tat for 3 h; lane 7, PD98059 treatment alone. (B) Effects of MAP kinase inhibitors on Tat-induced production of TNF-α protein. PBM were incubated with Tat protein for 10 h as follows: lane 1, no treatment; lane 2, mock treatment; lane 3, Tat treatment; lane 4, Tat and SB203580 treatment; lane 5, SB203580 alone; lane 6, Tat and PD 98059 treatment; lane 7, PD98059 alone. Mean values and standard deviation of the mean from triplicate experiments are shown. (C) Effects of kinase inhibitors on Tat-induced phosphorylation of eIF2α. Anti-phosphorylated eIF2α antibodies were used to perform Western blots on PBM treated as follows: lane 1, no treatment; lane 2, mock treatment; lane 3, Tat treatment; lanes 4 and 5, Tat and SB203580 treatment; lane 6, SB203580 alone; lanes 7 and 8, Tat and PD98059 treatment; and lane 9, PD98059 alone. The same membrane was stripped and incubated with eIF2α antibodies to detect the total amounts of eIF2α. The experiment shown is representative of three independent experiments. (D) Effects of 2-AP on Tat-induced phosphorylation of p38 MAP and ERK-1/2 kinases. Cytoplasmic proteins (30 μg) were extracted from PBM treated as follows: lane 1, no treatment; lane 2, Tat treatment; lanes 3 and 4, Tat with 2-AP treatment; lane 5, 2-AP alone. The cytoplasmic proteins were immunoblotted with a panel of antibodies specific for phosphorylated-p38, p38, phosphorylated-ERK-1/2 or ERK-1/2 as shown. The experiment shown is representative of three independent experiments.

responses. For example, NF-κB has been shown to mediate cytokine expression including IL-6, IL-10 and TNF-α [18–20], and its activation is regulated by multiple kinase pathways [21]. For further controls, transcription factors including C/EBP, NF-AT and NF-IL6 are involved in cytokine expression [22,23]. In light of critical roles of NF-B and NF-IL6, we investigated whether these factors play a role in the Tat-activated and PKR-mediated pathways. Our results demonstrated that Tat induced the degradation of IκB-α and subsequent nuclear translocation of NF-κB subunit (Fig. 3A and B). The nuclear translocation and binding activities of NF-IL6 were also enhanced by Tat treatment (Fig. 3C and D). The activities of NF-IL6 but not NF-κB can be abrogated by the inhibition of PKR with the use of 2-AP. Thus, we identified that Tat can activate both NF-κB and NF-IL6 but the activation of NF-κB is PKR-independent.

Transcription factor NF-IL6, which was initially identified in IL-6 induction, is characterized by a basic DNA-binding domain linked to a basic leucine zipper dimerization motif. Subsequent reports showed that IL-8, TNF-α, and CCR5 also have the functional NF-IL6 binding sites in their respective promoters [22,24,25]. NF-IL6 also has been shown to mediate the transcriptional activation of HIV-1 LTR [26]. Interactions of NF-IL6 with other transcription factors have resulted in

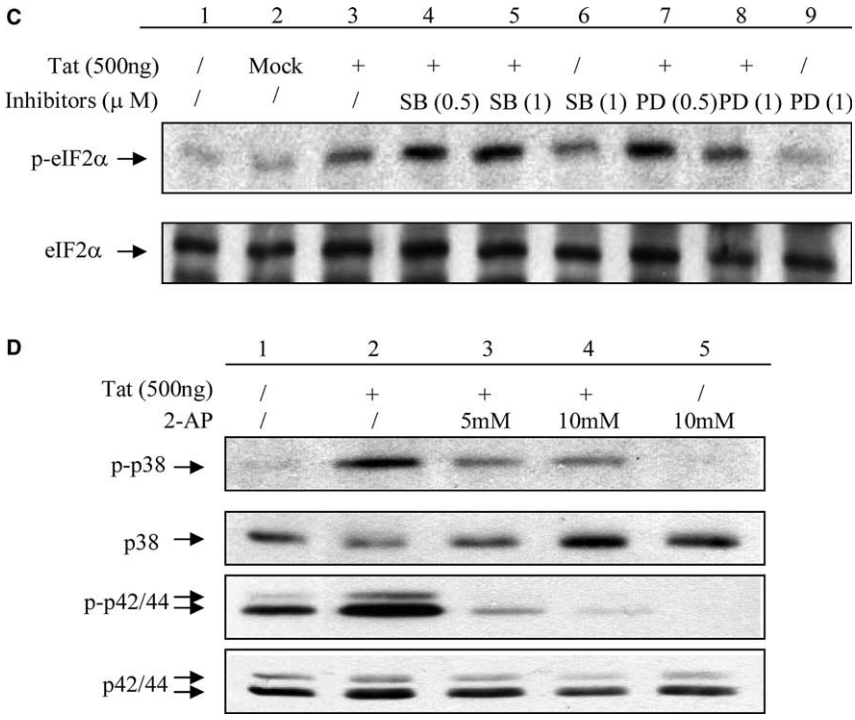

Fig. 4 (*continued*)

enhanced synthesis of cytokines [22,23]. These reports indicate that NF-IL6 serves a supportive role in multiple pathways for mediating the effects of extracellular inducers.

We also have identified downstream signaling kinase pathways regulated by PKR that may mediate Tat-induced cytokine expression. The key players examined were p38 and ERK-1/2 MAP kinases that are known to be critical in regulating cytokine expression [27]. Our results showed that ERK-1/2 and p38 MAP kinases have distinct roles in Tat-induced and PKR-mediated cytokine production. Both ERK-1/2 and p38 MAP kinases played a role in IL-6 transcription (Fig. 4A, upper panel), whereas only p38 kinase activity was involved in IL-10 regulation (Fig. 4B, middle panel). The mechanism of PKR regulation of p38 kinase is through the phosphorylation of MKK3/6, an upstream effector of p38 kinase [14]. The Tat-induced TNF-α expression can be regulated at both transcriptional and post-transcriptional levels. For TNF-α transcription, ERK-1/2 plays a significant role in the Tat-induced cellular process (Fig. 4A, lower panel). For TNF-α protein synthesis, both ERK-1/2 and p38 MAP kinases could play a role as reflected by the use of the respective inhibitors (Fig. 4B). Taken together, these results suggest that the regulation of IL-10 and TNF-α mRNA synthesis are mediated by multiple pathways including the involvement of MAP kinases.

By its nature, cytokines have pleiotropic properties and their regulated pathways have overlapping effects in signal transduction. We showed that ERK-1/2 and p38 kinase are downstream effectors of PKR, since inhibition of PKR abrogate the phosphorylation of MAP kinases (Fig. 4D) but not vice versa (Fig. 4C). The results in Fig. 4C showed that there were increases in p-eIF2α levels when cells were treated with both Tat and MAPK inhibitors, compared to either alone. In this case, it remains possible that MAPK may act upstream to inhibit PKR activity and reduced eIF2α levels. Taken together, these findings suggest that Tat-induced PKR acts upstream of MAP kinases in regulating its phosphorylation and activation. In previous finding, PKR-mediated activations of transcription factors are via the activation of p38 MAP kinase with LPS treatment [14]. Additionally, both the ERK-1/2 and p38 MAP kinases have been shown to regulate the activation of NF-IL6 [28]. Taken together, it appears that Tat-induced and PKR-regulated cytokine induction is mediated by p38 and ERK-1/2, leading to activation of transcription factors.

Increased expression of cytokines in AIDS patients could be due to the presence of opportunistic pathogens or due to direct HIV infection of the immune cells [29,30]. It is also possible that the elevated cytokine levels are due to Tat protein secreted by the HIV-infected cells. In turn, Tat activates monocytes and related immune cells through the activation of PKR and MAP kinases to produce the cytokines. Consequently, these proinflammatory cytokines activate NF-κB and NF-IL6 to further enhance the replication of HIV via binding to their respective enhancer sites in the HIV-LTR.

In summary, we have further delineated the mechanisms of HIV Tat-induced PKR-mediated events in the host cell. The consequences of such induction are most likely cell type specific. In response to the virus infection, immune cells activate antiviral pathways including PKR and related IFN-stimulated genes. In its counter-attack, HIV interacts with antiviral kinases including PKR to activate the immune system and exploits nuclear factors including NF-IL6 and NF-κB to enhance its own replication. Thus, HIV evades the immune system by exploiting the antiviral proteins and cytokines to enhance its own life cycle.

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