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Cocaine-mediated enhancement of virus replication in macrophages: Implications for human immunodeficiency virus–associated dementia

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Injection drug use has been recognized as a major risk factor for acquired immunodeficiency syndrome (AIDS) from the outset of the epidemic. Cocaine, one of the most widely abused drugs in the United States, can both impair the functions of macrophages and $CD4^+$ lymphocytes and also activate human immunodeficiency virus (HIV)-1 expression in these cells. Because the brain is the target organ for both cocaine and HIV, the objective of the present study was to explore the effects of cocaine on virus replication in macrophages, the target cells for the virus in the central nervous system (CNS). Cocaine markedly enhanced virus production in simian human immunodeficiency virus (SHIV)–infected monocyte-derived macrophages (MDMs) and in U1 cells, a chronically infected promonocytic cell line as monitored by enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry. Cocaine treatment also resulted in the activation of nuclear factor (NF)-κB and transcriptional activation of the HIV-LTR (long terminal repeat) gag-GFP (green fluorescent protein). Analyses of chemokines in cocaine-treated macrophages by real-time reverse transcriptase–polymerase chain reaction (RT–PCR) and Luminex assays suggested increased expression of interleukin (IL)-10, a cytokine that is known to promote HIV replication in MDMs. In addition to enhancing IL-10 expression, cocaine also caused an up-regulation of the macrophage activation marker, human leukocyte antigen (HLA)-DR, in MDMs. The synergistic effect of cocaine on virus replication and its enhancement of host activation markers suggest that cocaine functions at multiple pathways to accelerate HIV-associated dementia (HAD). *Journal of NeuroVirology* (2007) **13,** 483–495.

Keywords: cocaine; HIV-1; IL-10

Introduction

Intravenous drug use (IVDU) and humanimmunodeficiency virus (HIV) infections are two linked global health crises because needle sharing is a well-recognized mode of HIV transmission. Whereas HIV infection is the leading cause of death among Americans 25 to 44 years old, injection drug use now accounts for about one third of all new U.S. acquired immunodeficiency syndrome (AIDS) cases reported each year. Epidemiological studies on abused drug users and AIDS link abuse of cocaine (by different routes), even more than other drugs, to increased incidence of HIV seroprevalence and progression to AIDS. (Anthony *et al*, 1991; Baldwin *et al*, 1998; Chaisson *et al*, 1989, 1991; Doherty *et al*, 2000). Cocaine targets the central nervous system (CNS) and is associated with brain dysfunction. Cellular mechanisms involved in HIV-1 neuropathogenesis within

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the context of substance abuse have been extensively reviewed (Nath *et al*, 2000, 2002; Tyor and Middaugh, 1999; Hauser *et al*, 2007; Shapshak *et al*, 1996).

HIV-encephalitis (HIV-E), one of the major complications of HIV-1 infection, results from a cascade of virus-host interactions that lead to cytokine and chemokine imbalance, monocytic infiltration, formation of microglial nodules, highly productive infection in the brain macrophage/microglia, and neuronal dysfunction and death (Nath, 1999; Kaul *et al*, 2001). The pathogenesis of HIV encephalopathy revolves around two processes characterized first, by productive replication of the virus in macrophages in the brain, a process that leads to encephalitis, and second, neuronal degeneration that results from by-products of the infected macrophages, leading to dementia.

Use of cocaine either by snorting, smoking, or by intravenous injection has been known to promote HIV-1 infection and disease progression, including acquisition of secondary opportunistic infections in HIV-1–infected individuals (Bell *et al*, 1998; Fiala *et al*, 1998; Goodkin *et al*, 1998; Nath *et al*, 2001, 2002; Reyes *et al*, 1991;Tyor and Middaugh, 1999). Cocaine has multiple immunomodulatory effects, including the ability to influence cytokine release in immunoeffector cells. It is known to impair the functions of macrophages and CD4+T cells (Klein *et al*, 1993; Mao *et al*, 1996; Baldwin *et al*, 1997; Eisenstein and Hilburger, 1998; Friedman *et al*, 2003) and activate HIV-1 expression in these cells (Peterson *et al*, 1990; Bagasra and Pomerantz, 1993; Nair *et al*, 2000; Roth *et al*, 2002; Steele *et al*, 2003), thus leading to progression of HIV-1 infection. The interaction between cocaine and HIV-1 has also been evaluated *in vivo* under more physiologic conditions using a hybrid-mouse model (huPBL SCID mouse) infected with HIV-1 in the presence and absence of cocaine. In this model, systemic cocaine administration led to accelerated HIV-1 infection of human peripheral blood leukocytes (PBLs), a decrease in $CD4^+$ cells, and a dramatic rise in circulating virus load (Roth *et al*, 2002). In a series of studies by Peterson *et al*, cocaine was reported to increase the production of p24 antigen in HIV-infected, mitogen- or cytomegalovirus (CMV)-activated human peripheral blood mononuclear cells (PBMCs) by a mechanism involving transforming growth factor (TGF)-β (Peterson *et al*, 1991, 1992, 1993). Cocaine effects on enhancement of viral invasion and cerebrovascular complications have been associated with cocaine-mediated cascade of augmented expression of inflammatory cytokines and endothelial adhesion molecules (Fiala *et al*, 1998). Cocaine is also known to modulate the expression of interleukin (IL)-10 (Stanulis *et al*, 1997; Gardner *et al*, 2004), a cytokine that has been shown to promote HIV-1 replication (Finnegan *et al*, 1996). The use of cocaine therefore exacerbates factors that promote HIV-1 replication in the brain.

Because macrophages are the target cells for HIV-1 replication and for the release of toxic mediators in the CNS, it is critical to explore the combinatorial effects of cocaine and HIV-1 in this class of cells. In the present study we therefore sought to examine the effect of cocaine on (a) the synergistic enhancement of virus replication in the target cells, the macrophages; and (b) the expression of host factors associated with HIV-associated dementia (HAD).

Results

Cocaine up-regulates HIV_{Bal}/SHIV replication in human/macaque MDMs

We first examined the replication of HIV_{Bal} in human macrophages (MDMs) exogenously treated with increasing amounts of cocaine-HCl (10 nM to 100 μM). Cell supernatants at various days post infection were collected, and assayed for release of virus p24 protein using the enzyme-linked immunosorbent assay (ELISA) kit. The results shown in Figure 1A reveal that at day 4 of infection, 1 μM cocaine was the optimal concentration for enhancing virus replication, almost twofold compared to the virus replication in infected cells not treated with cocaine. Macaque MDMs also responded with increased virus replication in the presence of 1 to 10 μM cocaine (Figure 1B). Unlike the human cells, macaque MDMs demonstrated delayed kinetics of virus replication. Subsequent studies were done with human MDMs treated with 1 μM cocaine.

Further confirmation of the increased virus replication in MDMs cultured in the presence of 1 μM cocaine was assessed using the immunocytochemical analysis. MDMs cultured on glass coverslips were infected with HIV-1, washed, and subsequently either left untreated or treated with 1 μM cocaine followed by immunostaining with viral anti-p24 antibody. As shown in Figure 1C (*right panel*), increased numbers of virus-positive, multinucleated giant cells with syncytia were present in HIV-infected MDMs exposed to cocaine as compared to cells cultured in the absence of cocaine (Figure 1C, *left panel*). Syncitium formation, the fusion of cells into multinucleated giant cells, is commonly observed in cultures infected with retroviruses as a consequence of cytopathic effect of the infection.

Cocaine potentiates HIV-1 activation in latently infected promonocytic U1 cells

Promonocytic U1 cells are chronically infected cells that harbor the HIV-1 provirus in a latent state and upon stimulation with the appropriate cytokine result in activation of the HIV-1 genome (Emiliani *et al*, 1996). Because cocaine enhanced virus replication in macrophages, the next question we asked was whether cocaine treatment could lead to the release of HIV genome from its latency state in the U1 cells.

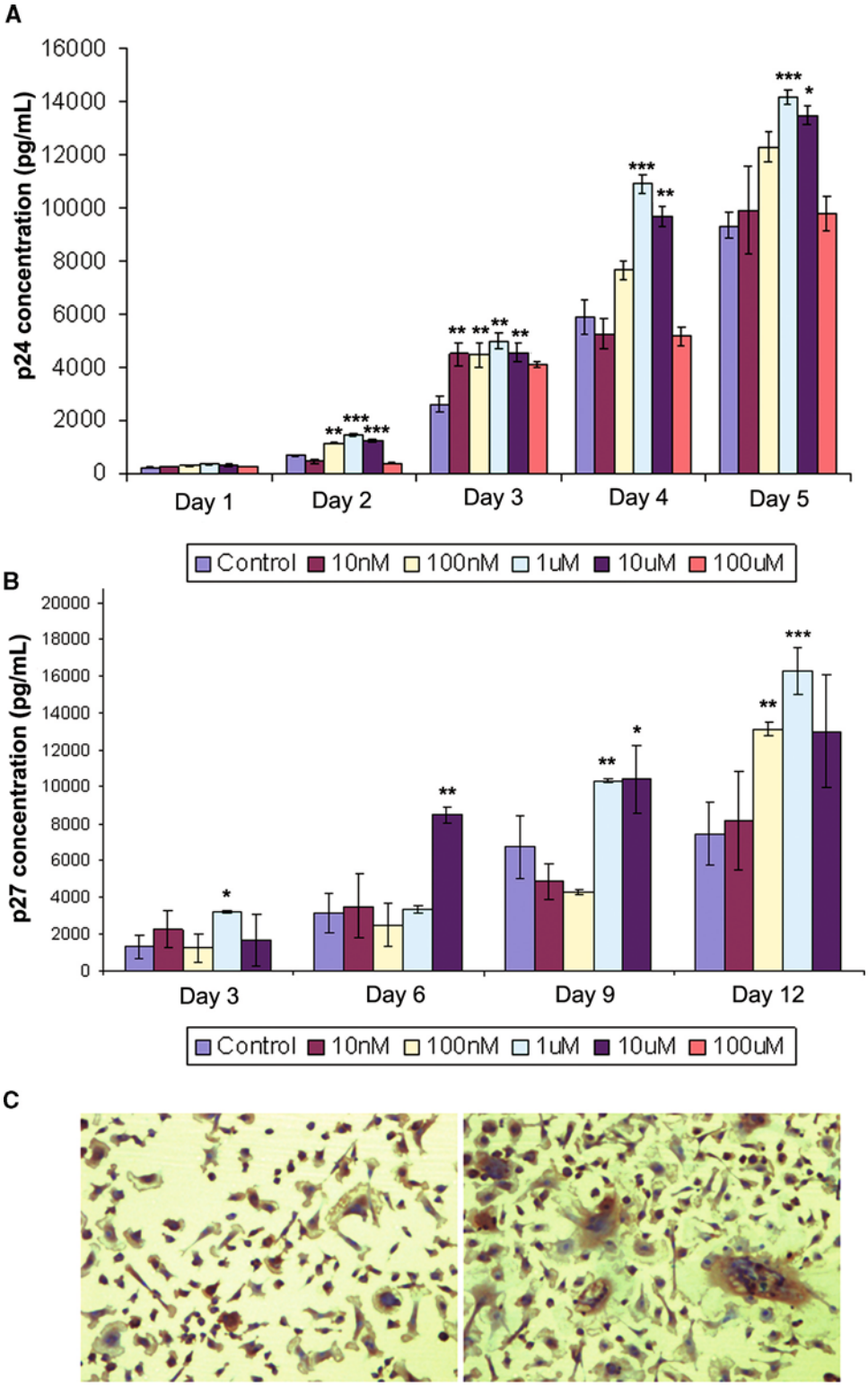

Figure 1 Cocaine potentiates HIV_{Bal}/SHIV replication in human/macaque MDMs. (**A**) HIV-1_{Bal}–infected human MDMs were treated with varying doses of cocaine (10 nM to 100 μM) and supernatants collected every day for analyses of viral p24 protein by ELISA. Data are presented as mean ± SD from three separate experiments. Statistical significance (cocaine treated versus control: $^{*}P < .05$; $^{**}P \leq .01$; $^{***}P \leq 0.001$) was determined by one-way analysis of variance (ANOVA). (**B**) $SHIV_{89.6}$P-infected macaque MDMs were treated with varying doses of cocaine (10 nM to 100 μM) and supernatants collected every third day for analyses of viral p27 protein by ELISA. Data are presented as ± SD from three separate experiments. Statistical significance (cocaine treated versus control: $^{*}P < .05$; $^{**}P \leq .01$) was determined by one-way ANOVA. (**C**) Immunocytochemical staining of viral p24 protein in HIV-1_{Bal}–infected MDMs cultured for 48 h in the absence (*left panel*) or presence of 1 μM cocaine-HCl (*right panel*).

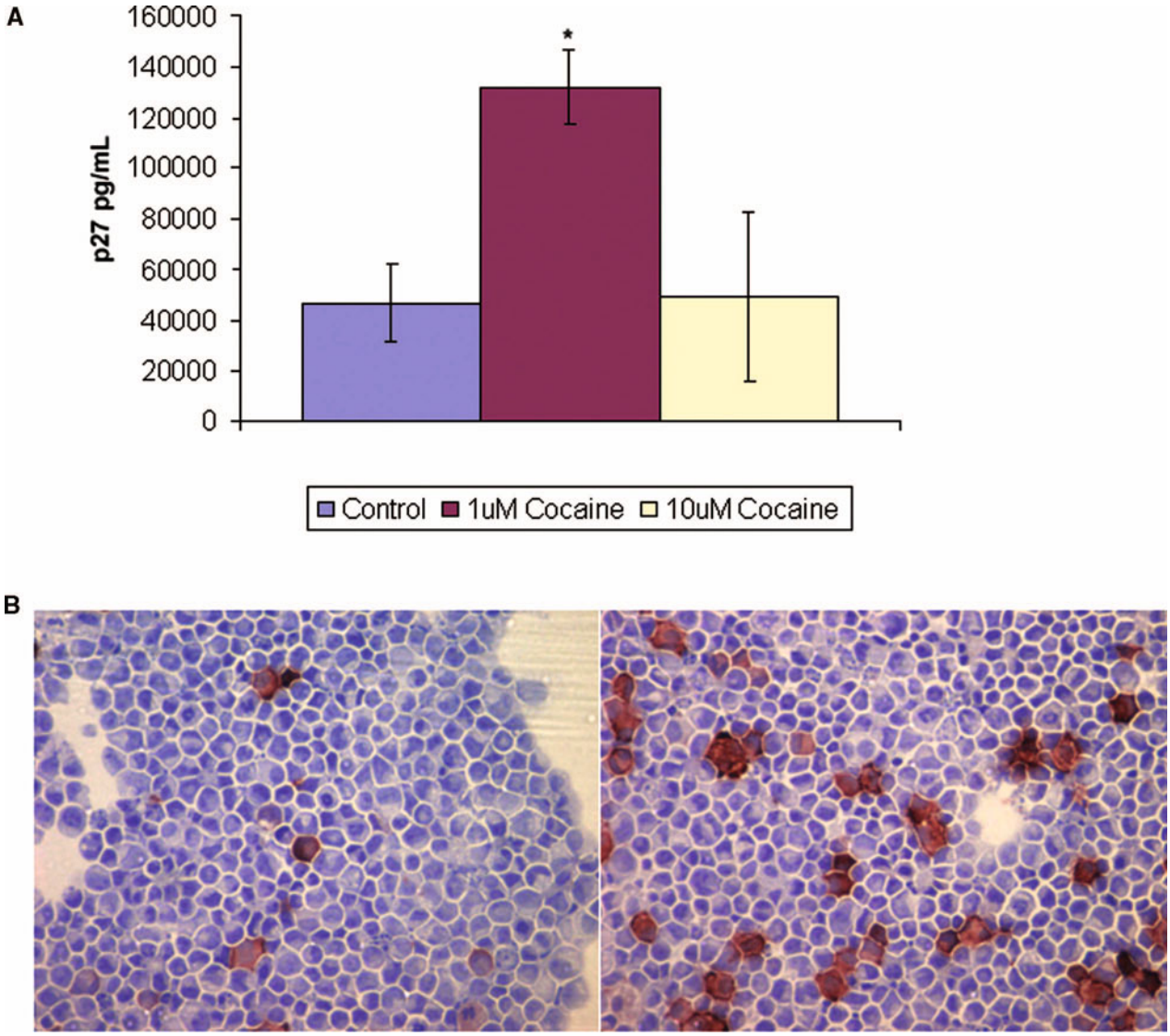

Figure 2 Cocaine potentiates HIV-1 replication in latently infected U1 cells. (**A**) Promonocytic U1 cells were either untreated or treated with 1 or 10 μM cocaine and supernatants were collected 24 h later and assessed for HIV-1 p24 antigen by ELISA. The data represent mean ± SD of three independent experiments ($^{*}P < .01$). (**B**) Immunocytochemistry of HIV-1 p24 protein in U1 cells treated with (*right panel*) or without (*left panel)* cocaine for 24 h.

We tested the ability of cocaine to activate HIV-1 proviral expression in the U1 cells at 1 and 10 μM cocaine concentrations. Following 24 h of treatment with cocaine, supernatant fluids were collected and examined for release of viral p24 protein by ELISA. As shown in Figure 2A, treatment of U1 cells with 1 μM cocaine resulted in a significant increase in p24 levels in the cell supernatant compared to untreated cells. Treatment of cells with 10 μM cocaine, however, did not demonstrate any enhancement of virus replication. Immunostaining of fixed U1 cells cultured in the presence or absence of 1 μM cocaine for 24 h with anti-p24 antibody also showed increased virus-positive cells in the presence of cocaine (Figure 2B, *right panel*) as compared to untreated cells (Figure 2A, *left panel*), which exhibited very few virus-positive cells.

Activation of HIV-1 LTR–directed transcription and NF-κB by cocaine

Because cocaine modulated virus replication in both MDMs and U1 cells, it was of interest to examine whether this effect was mediated by transcriptional activation of the HIV-LTR (long terminal repeat). Adherent MDMs cultured in 6-well plates were gently trypsinized and transfected with the HIV-LTR-GFP (green fluorescent protein) construct using the Nucleofector kit (Amaxa Biosystems, MD). Cells were also transfected with the empty vector to serve as untreated controls. Following transfections, cells were

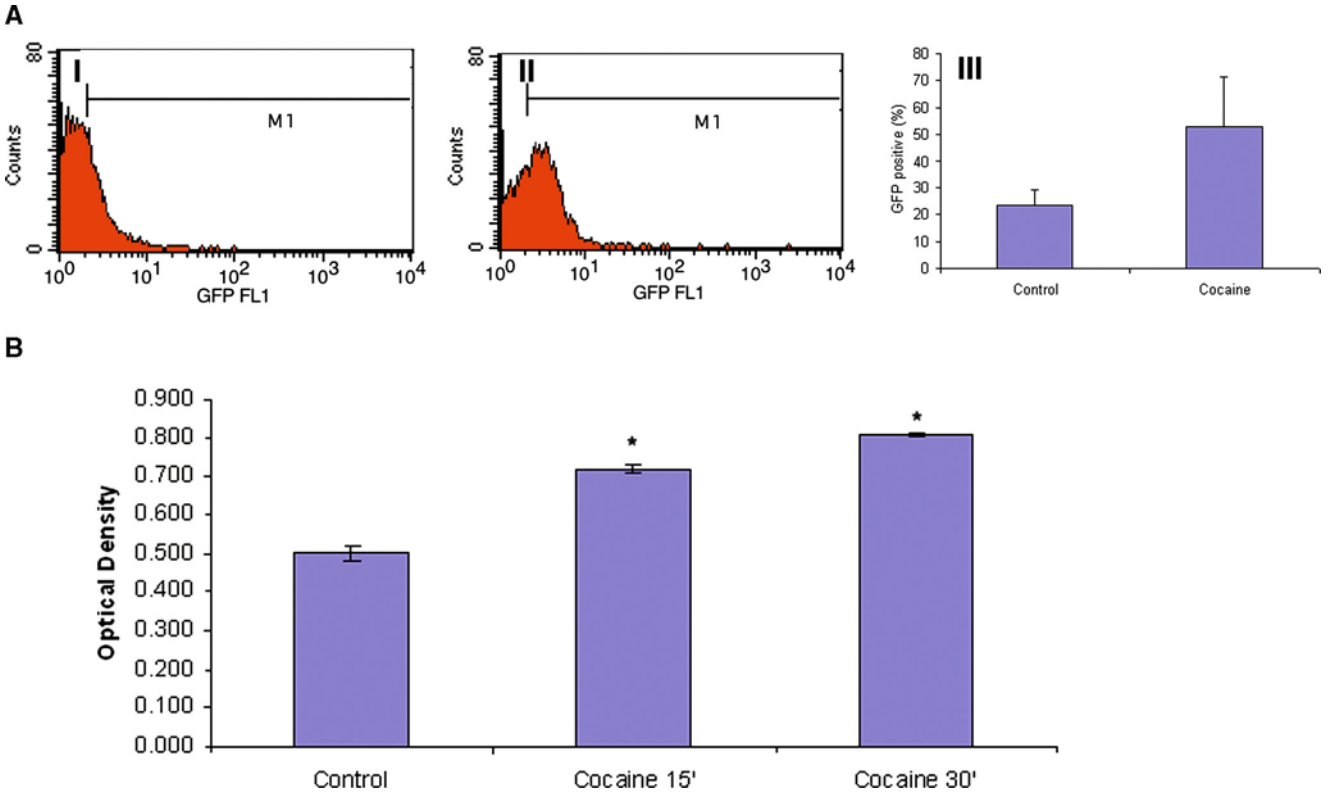

Figure 3 (**A**) Transcription regulation of HIV-1 LTR by cocaine. MDMs were transfected with the HIV-LTR-GFP construct using the Amaxa Nucleofection method. Twenty-four hors later cells were treated with (II) or without (I) 1 μM cocaine for another 24 h and analyzed for percentage of GFP-positive cells by flow cytometry. The histogram (panels I and II) is a representative of three independent experiments. Quantified GFP-positive cells with and without cocaine treatment are shown as a bar diagram in panel III and represent the mean $\pm$ SD of three independent experiments. (**B**) Activation of NF-κB transcription factor by cocaine in human MDMs. Human MDMs were treated with or without 1 μM cocaine for 15 or 30 min. Nuclear extracts from these treated cells were then analyzed for NF-κB activity by Transbinding NF-κB p50 Assay Kit. The average absorbance obtained by transbinding assay from two independent experiments is shown (treatment versus control: $^{*}P = .01$).

treated with 1 μM cocaine and 24 h later, the cells were harvested and processed for flow cytometry to monitor percentage of GFP-positive cells. As shown in Figure 3A, treatment with cocaine resulted in 30% increase in GFP-positive cells compared to cells not treated with cocaine.

Stimulation of HIV-1 transcription is known to be mediated via nuclear transcription factors capable of transactivating the viral LTR. To determine if cocaine-mediated activation of HIV-LTR the involved sequence-specific DNA binding protein nuclear factor (NF)-κB, human MDMs were treated with 1 μM cocaine for 15 or 30 min, followed by extraction of nuclear proteins. The nuclear extracts were assessed for their DNA-binding activity using the Transbinding NF-κ B p50 Assay Kit. Treatment of MDMs with cocaine at both 15 and 30 min resulted in marked up-regulation of the DNA binding activity of NF-κB to its consensus sequence, as compared to binding of nuclear extracts from cells not treated with cocaine (Figure 3B). These findings suggested that cocaine mediates the activation of NF-κB, a transcription factor that is critical for HIV transcription (Osborn *et al*, 1989).

Induction of HIV-1 replication by cocaine is mediated via IL-10 expression in human macrophages

IL-10 is known to synergize with multiple cytokines in enhancing HIV-1 production in MDMs (Finnegan *et al*, 1996) and in latently infected promonocytic U1 cells (Weissman *et al*, 1995). We thus sought to examine the effect of cocaine in modulating IL-10 expression in human MDMs that were either uninfected or infected with HIV_{Bal}. Virus-infected and uninfected MDMs that were treated with or without 1 μM cocaine were assessed for IL-10 expression by semiquantitative reverase transcriptase–polymerase chain reaction (RT–PCR) using the IL-10 primers. As shown in Figure 4A, which is a representative of three individual experiments, MDMs, which were either uninfected or infected with HIV-1, expressed low levels of IL-10 RNA. Cocaine treatment, on the other hand, dramatically enhanced IL-10 expression in these cells and this effect of cocaine was more pronounced in the presence of virus. This effect of cocaine on IL-10 expression was subsequently confirmed at the protein level using the fluorescence bead array assay (Figure 4B).

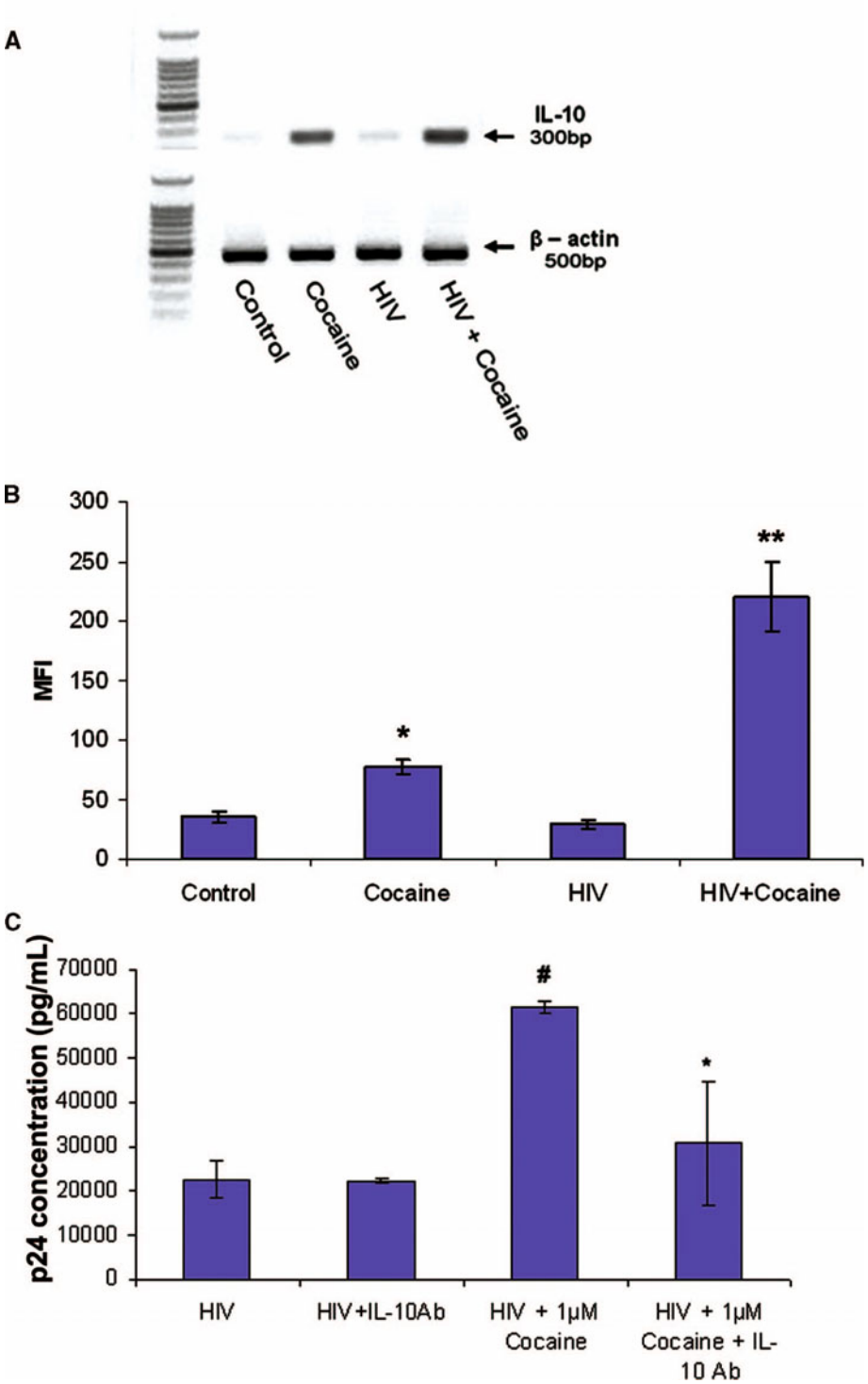

Figure 4 Cocaine induces IL-10 expression in the cells of macrophage lineage. (**A**) RT–PCR Analysis of IL-10 expression in MDMs in the presence of HIV_{Bal} and/or cocaine. (**B**) Luminex analysis of IL-10 protein in the supernatant fluids of HIV-infected or uninfected human MDMs treated with (1 μM) or without cocaine at day 6 post infection. The data represent mean ± SD of two independent experiments ($^{*}P \leq .05$, $^{**}P \leq 0.01$, treated versus control). (**C**) P24 ELISA in the day 6 supernatant of HIV-infected MDMs that were pretreated with neutralizing IL-10 antibody followed by treatment of cells with or without cocaine. Supernatant fluids from HIV-infected MDMs exposed to cocaine or untreated cells were also included as controls. The data represent two independent experiments. (HIV versus HIV + cocaine: $^{\#}P < .01$; HIV + cocaine versus HIV + cocaine + IL-10Ab: $^{*}P < .05$.) (**D**) Luminex analysis of IL-10 protein in supernatant fluids of U1 cells treated with 1 μM cocaine at day 1 post treatment. Supernatants were collected from two independent experiments done in triplicates and the data are a mean of the two experiments ($^{*}P \leq .001$, cocaine treated versus control). (**E**) Real-Time RT–PCR for *gag* RNA in U1 cells treated with cocaine and/or IL-10 antibody. RNA isolated from U1 cells preincubated with antibody specific for rat IgG was used as control. The data are presented as mean ± SD from two independent experiments. (*continued*)

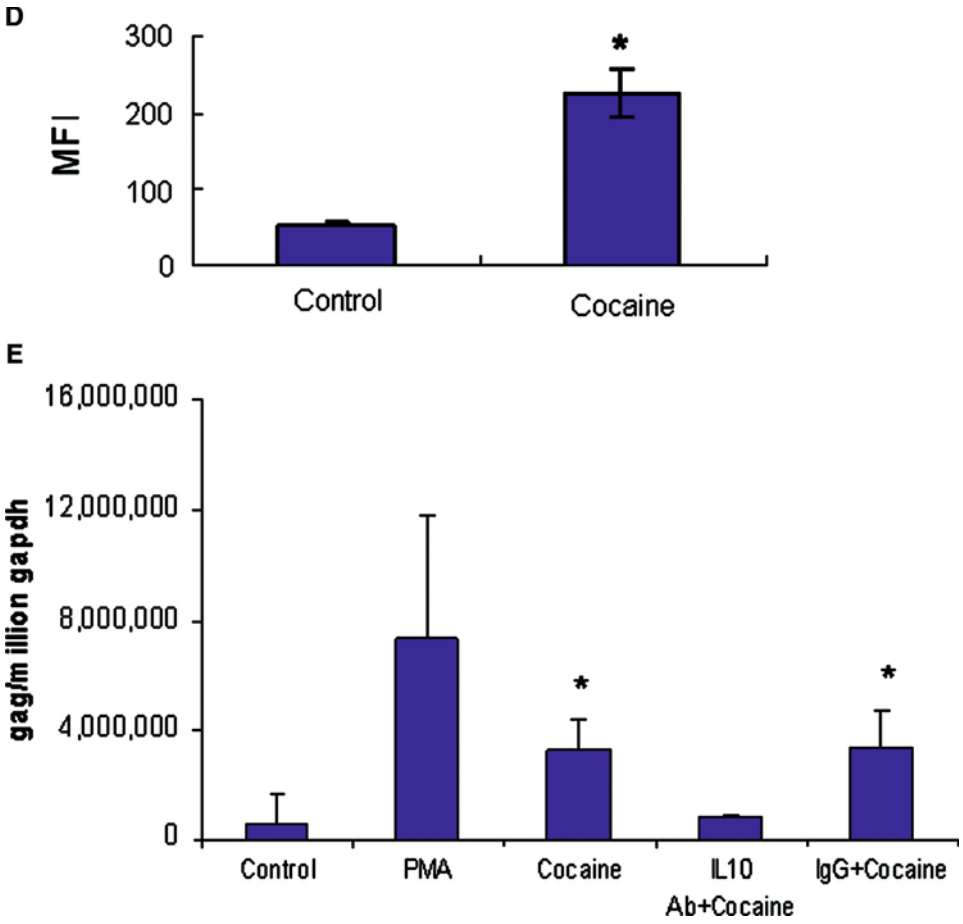

Figure 4 (Continued)

Because cocaine enhanced virus replication in MDMs and also increased IL-10 expression in these cells, we next enquired whether cocaine-meditated up-regulation of virus replication involved IL-10 expression. Virus-infected MDMs were pretreated with neutralizing antibody specific for IL-10 prior to stimulation with or without cocaine. As shown in Figure 4C, cocaine treatment enhanced virus replication in MDMs as expected and pretreatment of MDMs with anti-IL-10 antibody prior to cocaine stimulation resulted in inhibition of cocaine-mediated enhancement of virus replication. Treatment of virus-infected cells with IL-10 antibody alone had no effect on virus replication as compared to virus-infected cells.

We also monitored the expression of IL-10 in supernatants from U1 cells exposed to cocaine using the Luminex bead array. As shown in Figure 4D, treatment with 1 μM cocaine resulted in increased expression of IL-10 protein. Higher concentration of cocaine (10 μM) did not result in enhancement of IL-10 expression and this effect could be attributed to increased cell toxicity (data not shown). To examine whether the cocaine-meditated release of viral genome from its proviral state was functioning through IL-10 in U1 cells, cells were pretreated with IL-10 neutralizing antibody prior to cocaine exposure and RNA extracted from the treated cells was then assessed for viral transcript by real-time RT-PCR analysis. As shown in Figure 4E, treatment of U1 cells with IL-10 antibody reduced the expression of HIV-1 *gag* as compared to cocaine-exposed cells not treated with the antibody. These data thus suggest that the action of cocaine-mediated activation of HIV-1 genome from its latency involves IL-10 expression.

Macrophage activation in the presence of cocaine

Increasing evidence now suggests that microglial/macrophage activation and not the virus load within the CNS is the key determinant of the underlying neuropathology of HIV-E (Glass *et al*, 1995; Gray *et al*, 2000). We therefore investigated whether treatment of macrophages with cocaine alone could activate these cells. Macrophages not only express chemokines in response to the changed environment but also become activated to express diverse surface markers to amplify inflammation, including major histocompatibility complex (MHC) class II molecules. To investigate the effect of cocaine on MHC-II antigen processing and presentation, U1 cells were treated with or without cocaine and expression

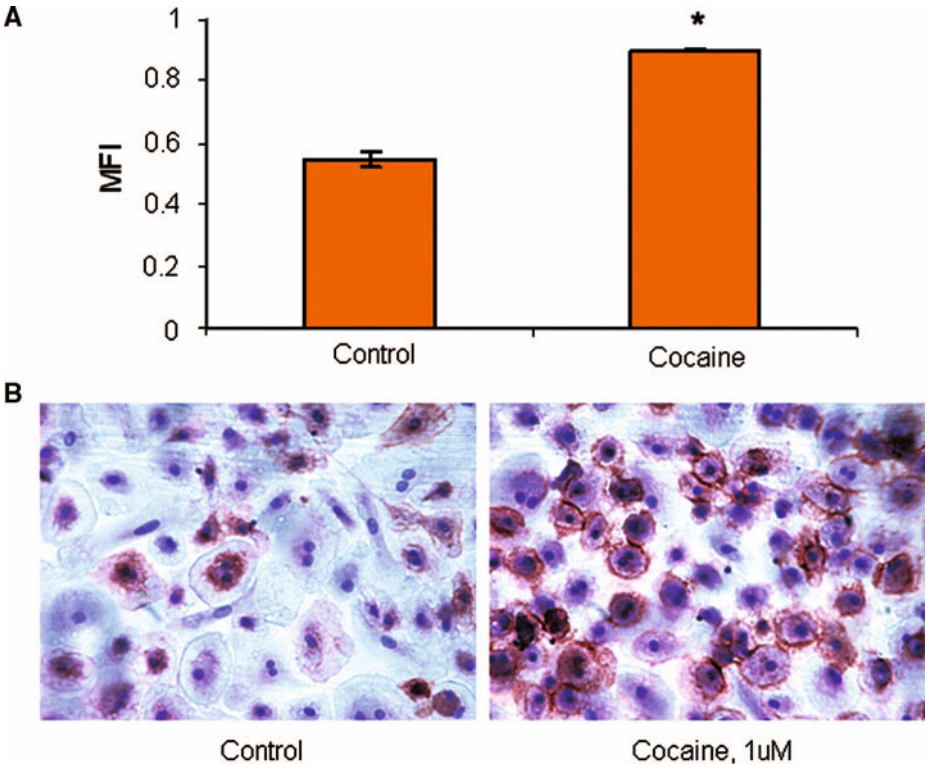

Figure 5 Cocaine mediates up-regulation of activation marker HLA-DR in U1 cells. (**A**) U1 cells were treated with 1 μM cocaine for 24 h, followed by analysis of HLA-DR expression by flow cytometry. The graph shows the mean fluorescence intensity from two different experiments (mean $\pm$ SD; $^{*}P \leq .001$). (**B**) Immunocytochemical localization of the activation marker HLA-DR in human MDMs treated with (*right panel*) or without (*left panel*) 1μM cocaine.

of human leukocyte antigen (HLA)-DR was measured by flow cytometry. As shown in Figure 5A, addition of 1 μM cocaine to U1 cells resulted in an almost twofold increase in the mean fluorescence intensity for HLA-DR compared to cells not treated with cocaine. Similar findings were obtained for human MDMs cultured in the absence or presence of cocaine (data not shown). Confirmation of these findings was also carried out by immunostaining MDMs treated with or without cocaine with monoclonal antibody specific for HLA-DR. As shown in Figure 5B, there is a clear translocation of MHC-II molecules from the cytosol to the cell membrane in the presence of 1 μM cocaine (right panel) as compared to cells not treated with cocaine (*left panel*).

Discussion

Injection drug use has been recognized as a major risk factor for AIDS from the outset of the epidemic (Selwyn *et al*, 1992). Cocaine has been one of the most widely abused drugs in the United States. Based on extensive literature demonstrating that substances of abuse, such as cocaine, can both impair the functions of macrophages and $CD4^{+}$ lymphocytes (Klein *et al*, 1993; Mao *et al*, 1996; Baldwin *et al*, 1997; Eisenstein and Hilburger, 1998; Friedman *et al*, 2003) and activate HIV-1 expression in these cells (Roth *et al*, 2002; Peterson *et al*, 1991, 1992, 1993; Bagasra and Pomerantz, 1993), it has been postulated that cocaine may could serve as a cofactor in susceptibility and progression of HIV-1 infections (Fiala *et al*, 1998; Larrat and Zierler, 1993). Mounting evidence suggests that HIV-infected drug abusers have accelerated and more severe neurocognitive dysfunction compared with non–drug-abusing HIV-infected populations (Nath *et al*, 2001, 2002; Goodkin *et al*, 1998; Avants *et al*, 1997; Durvasula *et al*, 2000). Because most drugs of abuse are CNS stimulants and because the brain is the target organ for both cocaine and HIV-1, it stands to reason that these drugs may synergize with soluble factors released during the course of HIV-1 infection and thus amplify the end-stage toxic response.

The pathogenesis of HIV-E revolves around two processes, characterized first by productive replication of the virus in macrophages in the brain, a process that leads to encephalitis, and second, neuronal degeneration that results from byproducts of the infected macrophages, leading to dementia. In the present study, we have analyzed the impact of cocaine on HIV-1 infection of macrophages. We also explored the potential mechanism(s) by which cocaine enhances HIV-1 infection in these target cells.

Our study demonstrated that cocaine potentiates the expression of HIV-1 in human macrophages and

also in latently infected promonocytic U1 cells, consistent with the previous reports that cocaine fosters replication of HIV-1 in peripheral blood mononuclear cells (Peterson *et al*, 1991, 1992; Bagasra and Pomerantz, 1993). We demonstrated that increased HIV-1 replication in human macrophages exposed to cocaine was associated with (1) activation of NF-κB and egr-1, with a modest increase in proviral LTR activity that was independent of Tat; (2) increased levels of IL-10 production; and (3) activation of macrophages.

Cocaine-mediated increase in viral gag expression in macrophages and U1 cells was also dependent on the soluble factor, IL-10. In HIV-1 infection, an increased level of IL-10 production in serum correlates with dramatic decrease in $CD4^+$ T cell counts and progression to AIDS (Srikanth *et al*, 2000; Stylianou *et al*, 1999). IL-10 levels are elevated in HIV-1–infected individuals (Denis and Ghadirian, 1994) and are also induced during HIV-1 infection of monocyte/macrophages (Denis and Ghadirian, 1994; Borghi *et al*, 1995). Secretion of IL-10 from stimulated PBMCs of HIV-infected patients was increased in advancing disease (Clerici *et al*, 1993) and increased constitutive expression of IL-10 mRNA was observed in lymphoid tissues of HIV-infected individuals (Pantaleo *et al*, 1993). In cocaine-abusing HIV individuals, cocaine may thus indirectly increase the viral load via IL-10 up-regulation, thereby contributing to a further increase in the IL-10 pool and a concomitant enhancement of disease progression.

One of the mechanisms by which cocaine could assist the activation of HIV-LTR, independent of tat, could be by targeting the NF-κB activation pathway directly or by activating protein(s) such as IL-10 that further activate this pathway. IL-10 is known to enhance HIV-1 production by synergizing with multiple cytokines, including tumor necrosis factor (TNF)-α in MDMs (Weissman *et al*, 1995) and in latently infected promonocytic U1 cells via the action of NF-κB (Finnegan *et al*, 1996).

Although macrophages and microglia are the main reservoir of infectious virus in the brain, it is now becoming increasingly well recognized that both HIV-E and HAD correlate better with the degree of monocyte infiltration and microglial activation rather than the CNS viral load per se (Glass *et al*, 1993, 1995); Tyor *et al*, 1995; Gray *et al*, 2000). This implies that, although microglia do become infected (Budka, 1991; Wiley *et al*, 1999), the key determining factor of neurological impairment is not the virus within the CNS, but rather the activation status of microglia. Additionally, chronic activation of these cells is also thought to fuel an inflammatory process that leads to neurodegeneration (Glass *et al*, 1995; Tyor *et al*, 1995; Gray *et al*, 2000). Inflammatory mediators released by the activated cells initiate a cascade of events that ultimately culminate into amplification of toxic responses in the CNS, a hallmark feature of end-stage HAD (Asensio and Campbell, 1999). The studies reported here suggest that cocaine by itself can also activate macrophages as evidenced by the up-regulation of the cell surface marker, MHC class II receptor. Resting macrophages express low or undetectable levels of most surface receptors. However, when macrophages sense changes in their environment, they respond by up-regulating MHC class II, CD40, and also secrete mediators that are known to be neurotoxic. Increased expression of the activation marker MHC class II observed in macrophages exposed to cocaine suggest that substance abuse may predispose the cells in the CNS to release soluble mediators, which in turn can aid in amplifying the toxic cascade associated with end-stage disease.

The findings presented in this report thus suggest that cocaine, in addition to its role as a virus enhancement factor in the macrophages and a factor that activates the virus from its latency, also has the potential to activate the virus target cells, the macrophages. These studies have clinical implications for cocaine-abusing HIV-infected individuals because cocaine, which has the potential to act as a catalyst in multiple pathways in the virus life cycle, can augment the severity and progression of HAD.

Materials and methods

Monocyte-derived MDMs

MDMs derived from both peripheral blood of uninfected humans or rhesus macaques were used for virus replication studies. Briefly, mononuclear cells were purified from heparinized blood by centrifugation through Ficoll-hypaque gradients (Buch *et al*, 2001). The cells were then cultivated in RPMI supplemented with 20% fetal bovine serum (FBS), amacrophage colony-stimulating factor (M-CSF) (5 U/ml), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 U/ml) in tissue culture flasks. Monocytes in the cell preparations differentiated to become adherent MDMs in culture vessels. Approximately 7 days after differentiation of monocytes, MDMs were used for specific experiments described below.

Cocaine treatment and assessment of viral expression

Cocaine hydrochloride was obtained from Sigma Aldrich (St. Louis, MO) and diluted in saline prior to use. Latently infected promonocytic U1 cells were either untreated or treated with varying concentrations of cocaine. Human MDMs were pretreated with cocaine at indicated concentrations, followed by inoculation with HIV_{Bal} (National Institute of Health AIDS Research and Reference Reagent Program) at a multiplicity of 0.01. After 4 h of absorption with HIV/SHIV (simian human immunodeficiency virus) at 37°C, cells were washed three times with RPMI and supplemented with culture medium containing cocaine.

Supernatant fluids were collected sequentially at various days in triplicates and examined for virus content using an ELISA for viral Gag p27 (Coulter Laboratories, FL).

Viral RNA concentration in U1 cells was assessed using real-time RT-PCR assay as previously described (Smith *et al*, 2002). Briefly, total RNA isolated from frozen tissues was treated with DNase and subjected to real-time RT–PCR (ABI, Foster City, CA) using *gag* primers (HIV gag A [forward primer]: 5′-CCAGAAGTAATACCCATGTTTKCA [K = G+T]; HIV gag B [reverse primer] 5′-CCACTGTGTTTAGCATGGTGTT) and a Taqman probe (5′-TCAGAAGGAGCCACCCCACAAGA) with thermal cycler conditions as described. Levels of hypoxanthine phosphoribosyl transferse (HPRT) mRNA, a housekeeping gene were also measured by real-time RT–PCR to normalize the viral load. The amplification efficiencies of the *gag* and HPRT targets can be considered essentially equal as the difference in their slopes (ΔS) of the standard curves was within 0.2.

Semiquantitative RT–PCR analysis

Total RNA was extracted from U1 cells using Trizol reagent (Life Technologies, Grand Island, NY). Semiquantitative RT–PCR analysis for IL-10 expression was performed on the RNA samples from cocaine-treated and untreated cells *using* the Access RT–PCR kit (Promega, Madison, WI) in a Perkin-Elmer (Emeryville, CA) DNA Thermal Cycler 480 as described earlier (Dhillon *et al*, 2005). The primer set used for IL-10 was 5′-TGA AGG GAT CAG CTG GAC AAC-3′ and 5′-TCG TTC ACA GAG AAG CTC AG-3′.

Real-time RT–PCR

Quantitative analyses of CCL2, CCR2, and CXCL10 mRNAs in macrophages was done by real-time RT–PCR using the SYBR Green detection method (Ferreira *et al*, 2006). We used RT^2 PCR primer pair set from SuperArray Bioscience (Frederick, MD, USA). Total RNA was isolated from cells treated with and without cocaine by lysis in Trizol. It was then converted into first-strand cDNA, the template for the PCR using the Reaction Ready First Strand cDNA Synthesis Kit (SuperArray). Amplification was performed with the following PCR cycle sequence: 15 min at 95°C (stage 1), 30 s at 95°C, 30 s at 55°C, followed by 30 s at 72°C (stage 2) (repeated for 40 cycles), and then finally at 72°C for 5 min (stage 3). Detection was performed with an ABI Prism 7700 sequence detector (ABI, Foster City, CA, USA). Data were normalized using Ct (defined as the threshold cycle of PCR at which amplified product is first detected) values for GAPDH in each sample (ABI). The replication efficiencies of platelet-derived growth factor (PDGF) B chain or the two receptors and the housekeeping gene GAPDH were found to be similar. So to calculate relative amounts of target gene, the average Ct value of the GAPDH was subtracted from that for each target gene to provide changes in Ct (ΔCt) value. The fold-change in gene expression (differences in ΔCt, or ΔΔCt) was then determined as $\log_2$ relative units.

Immunocytochemistry

Immunocytochemical analysis was performed on zinc formalin–fixed macrophage cultures grown on coverslips in 6-well plates or U1 cells cytospun on glass slides. Slides were treated with murine monoclonal antibody to p24, the gag protein of HIV-1 (Advanced Biotechnologies, Columbia, MD), or with mouse monoclonal antibody to HLA-DR (Abcam, Boston, MA), followed by treatment with biotinylated goat anti-mouse immunoglobulin G (IgG) (DAKO, Carpenteria, CA), peroxidase-conjugated streptavidin (DAKO), and NovaRed substrate (Vector Laboratories, Burlingame, CA), which yields a reddish reaction product (Hicks *et al*, 2002).

For immunofluorescence staining, cells were first treated with the primary antibody, egr-1 (Santa Cruz Biotechnologies, Santa Cruz, CA), followed by treatment with Alexa Fluor 488–conjugated secondary antibody. After the final washing, the slides were mounted in SlowFade anti-fade reagent with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR), and images were captured using a Zeiss LSM510 confocal microscope. Control slides included (1) cells without secondary antibody treatment for autofluorescence, and (2) green secondary antibody only for non-specific binding.

NF- κB activation assay

We used the ELISA-based TransBinding NF-κB Assay Kit to detect and quantify NF-κB activation, specifically NF-κB p50. Nuclear extracts from macrophages treated with or without cocaine were plated on a NF-κB binding site–specific oligonucleotide-coated plate. Complex bound to the oligonucleotide was detected spectrophotometrically at 450 nm by antibody directed against the p50 subunit and horseradish peroxidase (HRP)-conjugated secondary antibody.

Transfections

Human MDMs were transfected with HIV-LTR GFP using Nucleofector Kit V (Amaxa Biosystems) according to the manufacturer's protocol. Briefly, 7×10^5 cells were resuspended in nucleofection solution containing 5 μg DNA and transfection was achieved in the Nucelofactor I apparatus. Immediately after transfection, cells were recovered in prewarmed serum-free RPMI medium and incubated for 3 h at 37°C/5% CO_2, followed by treatment with 1 μM cocaine.

Flow cytometry

Flow cytometry was used to assess expression of GFP in MDMs transfected with HIV-LTR-GFP and of HLA-DR in latently infected U1 cells. After 24 h of treatment of U1 cells with and without cocaine

(1 μM), cells were labeled with flourescein isothiocyanate (FITC)–conjugated mouse antihuman HLA-DR (BD Pharmingen, San Jose, CA) on ice in the dark for 1 h, washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), followed by fixation with 500 μl of 2% formalin, and analyzed by flow cytometry. The results were recorded as mean fluorescence intensity (MFI) and as percentage of positive staining population. Negative-control cells were stained with isotype control primary conjugated antibodies. Samples were prepared and analyzed in duplicate, and a minimum of 10,000 cells was counted for each sample.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance. Results were judged statistically significant if $P < .05$ by analysis of variance.

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