Published in final edited form as:
J Neuroimmune Pharmacol. 2009 March ; 4(1): 129–139. doi:10.1007/s11481-008-9128-0.

Methamphetamine Enhances HIV-1 Infectivity in Monocyte Derived Dendritic Cells

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Abstract

The US is currently experiencing an epidemic of methamphetamine (Meth) use as a recreational drug. Recent studies also show a high prevalence of HIV-1 infection among Meth users. We report that Meth enhances HIV-1 infectivity of dendritic cells as measured by multinuclear activation of a galactosidase indicator (MAGI) cell assay, p24 assay, and LTR-RU5 amplification. Meth induces increased HIV-1 infection in association with an increase in the HIV-1 coreceptors, CXCR4 and CCR5, and infection is mediated by downregulation of extracellular-regulated kinase (ERK2) and the upregulation of p38 mitogen-activated protein kinase (MAPK). A p38 inhibitor

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(SB203580) specifically reversed the Meth-induced upregulation of the CCR5 HIV-1 coreceptor. The dopamine D2 receptor antagonist RS ± sulpiride significantly reversed the Meth-induced upregulation of CCR5, demonstrating that the Meth-induced effect is mediated via the D2 receptor. These studies report for the first time that Meth fosters HIV-1 infection, potentially via upregulating coreceptor gene expression. Further, Meth mediates its regulatory effects via dopamine receptors and via downregulating ERK2 with a reciprocal upregulation of p38 MAPK. Elucidation of the role of Meth in HIV-1 disease susceptibility and the mechanism through which Meth mediates its effects on HIV-1 infection may help to devise novel therapeutic strategies against HIV-1 infection in high-risk Meth-using HIV-1-infected subjects.

Keywords

dendritic cells; HIV-1 infectivity; MAGI assay; dopamine D_2 receptor; p38 MAPK; chemokine receptors

Introduction

The risk for HIV-1 infection attributable to methamphetamine (Meth) use continues to increase and the US is currently experiencing a grave epidemic of Meth use as a recreational drug (National Drug Threat Assessment 2005, 2007; National Institute of Drug Abuse INFO FACTS 2005). Meth use has surpassed cocaine use as of July, 2005 (National Survey on Drug Use and Health 2003; http://www.jointtogether.org/news/headlines/inthenews/2006/global-meth-use-exceeds.html). Meth may be smoked, taken orally, or injected intravenously. In recent years, the use of Meth has spread from the West Coast to the East Coast. In 2006, a national survey results estimated 5.77% of the US population aged 12 or older used Meth at least once in their lifetime (Office of National Drug Control Policy 2007). Recent studies indicated higher use of crystal Meth in young adults in the US than previously reported (Iritani et al. 2007).

Dendritic cells (DC) are the most potent cells involved in the generation of primary and secondary immune responses (Macatonia et al. 1989). They are the first line of defense against any infection including HIV-1 infection. HIV-1 may use DCs to obtain access to its major cellular targets, CD4+ T lymphocytes (Langhoff et al. 1991; Pope et al. 1994; Weissman et al. 1995). The formation of a complex between C-type lectin receptors like dendritic-cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN; CD209) and HIV-1 gp120 may be the first contact between the virus and its new host (Cameron et al. 1996; Geijtenbeek et al. 2000). Previous studies have shown that blood monocyte-derived dendritic cells (MDCs) serve as an effective model to study HIV-1 infection in injection drug users (Blauvelt et al. 1997). Several research efforts are now focused on understanding DC-HIV-1 interactions, with the hope that this knowledge could lead to strategies aimed at blocking early events in transmission. We have attempted to model primary HIV-1 infection by exposing normal DC (derived from monocytes isolated from whole blood) to HIV-1 in vitro. Studies by Blauvelt et al. (1997) have shown that HIV-1 interacts with DC using two different pathways, both of which can occur simultaneously and independently of one another. The first pathway suggests that productive infection of DC is HIV-1 coreceptor dependent and requires proliferation of DC, while the second pathway suggests that the ability of DC to capture HIV-1 is independent of HIV-1 binding to CD4+ T cells, HIV-1 coreceptor usage, HIV-1 reverse transcription, and DC proliferation. If the mechanisms of DC-HIV-1 interactions could be defined more clearly, blocking the transmission of virus from DC to T cells may serve as a possible antiviral strategy for HIV-1-infected subjects. Although the role of Meth as a cofactor in HIV-1 pathogenesis is being increasingly recognized, no studies have examined the effect of Meth

on HIV-1 infectivity in vitro and the mechanistic role of Meth-mediated effect on HIV-1 disease. We hypothesize that Meth treatment exacerbates HIV-1 infection in monocyte-derived DC by increasing the expression of chemokine receptors CCR5 and CXCR4 and that the activation of the mitogen-activated protein kinase (MAPK) signal transduction pathways are involved in regulation of HIV-1 infectivity. The mechanism involved in Meth-enhanced HIV-1 infectivity could be via the release of dopamine leading to dopamine oxidation in the cytosol, and this Meth-induced dopamine release is predominantly regulated by D2 receptor (Larsen et al. 2002; Watanabe et al. 1989).

Materials and methods

Human subjects

Blood donors were apprised of this study and consents were obtained consistent with the policies of SUNY Buffalo and the National Institutes of Health. Peripheral blood samples from healthy individuals were drawn into a syringe containing heparin (20 units per milliliter).

Isolation and generation of MDC and IDC

DC were propagated from peripheral blood mononuclear cells (PBMC) using protocols described earlier (Dauer et al. 2003; Cao et al. 2000). Briefly, PBMC were separated on a density gradient and adhered to plastic culture plates in media containing serum. Nonadherent cells were removed after 1 h at 37°C and adherent cells were cultured for 6 days in media containing 100 U/ml of rhGM-CSF and 100 U/ml of IL-4 (R & D systems, Minneapolis, MN, USA). After 6 days of culture, immature dendritic cells (IDC) were removed by gently swirling the plate to resuspend them for use in the experiments. These IDC were allowed to progress to mature dendritic cells (MDCs) by incubating for five more days with media containing 1,000 U/ml of rhGM-CSF and 1,000 U/ml of IL-4 as above. Phenotypic characterization of IDC and MDC by flow cytometry was done. Both IDC and MDC were washed in FACS buffer (eBioscience, San Diego, CA, USA), incubated with nonspecific IgG (20 μg/ml) for 10 min at 4°C to block Fc receptors, stained with specific antibodies for DC surface markers, and analyzed by flow cytometry. Both MDC and IDC express CD80, CD86, CD40, HLA-DR, DQ, and CD11c at different levels. However, MDCs predominantly express CD83 and CCR7 as described (Caux et al. 1994; Zhou and Tedder 1996).

Cell culture

IDC and MDC were cultured at a concentration of 5×10^5 cells per milliliter with Meth (10 to 100 μM) in six-well plates. Meth used in the present experiments was obtained from Sigma Chemical Co (Cat# 2222) as a lyophilized form and dissolved in culture media. The purity of Meth is 60–90%, and it is predominantly *d*-methamphetamine which has greater CNS potency than the *l*-isomer or the racemic mixture. Commonly abused doses are 100–1,000 mg/day and up to 5,000 mg/day in chronic binge use. A single dose of 10 mg of Meth results in a maximum blood concentration of about 10 ng/ml at 24 h and a urine concentration of 2,400–33,300 ng/ml in abusers. Based on these numbers, we expect that an average Meth abuser would have a Meth blood concentration ranging from 200 to 4,000 ng/ml. The in vitro doses of Meth we used in our experiments ranged from 10- to 100-μM concentration of Meth which correspond to 185 to 1,850 ng/ml which are well within the dynamic range of a Meth abuser (Baselt 1982).

Treatment of dendritic cells with HIV-1 isolates

MDCs were infected with HIV-1 IIIB [X4] (NIH AIDS Research and Reference Reagent Program Cat# 398) at a concentration of $10^{3.0}$ $TCID_{50}$ per milliliter cells for 3 h as described (Nair et al. 2005). Cells were washed and cultured ±Meth (10 to 100 μM) once for 15 days and the culture supernatants were quantitated for p24 antigen. Meth concentrations selected are similar to levels found in the blood, urine, or tissue samples of Meth-using subjects (Takayasu et al. 1995; Schepers et al. 2003). Additionally, after a postinfection period of 24 h, RNA was isolated from a set of infected MDC, and quantitative real-time polymerase chain reaction (PCR) was used to amplify the LTR-R/U5 region which represents early stages of reverse transcription of HIV-1.

RNA extraction

Cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described (Chomczynski and Sacchi 1987). Cultured DC were centrifuged and resuspended in a 4-M solution of guanidinium-thiocyanate. Cells were lysed by repeated pipetting and then extracted with phenol–chloroform in the presence of sodium acetate. After centrifugation, RNA was precipitated from the aqueous layer by adding an equal volume of isopropanol and the mixture was kept at −20°C for 1 h and then centrifuged to sediment the RNA. The RNA pellet was washed with 75% ethanol to remove any traces of guanidinium. The final pellet was dried and resuspended in diethyl pyrocarbonate water and the amount of RNA was determined using a spectrophotometer at 260 nm. DNA contamination in the RNA preparation was removed by treating the RNA preparation with DNAse (1 IU/μg of RNA) for 2 h at 37°C, followed by proteinase K digestion at 37°C for 15 min and subsequent extraction with phenol–chloroform and NH_4OAc–ETOH precipitation. The DNA contamination in the RNA preparation was checked by including a control in which reverse transcriptase enzyme was not added in the PCR amplification procedure. RNA preparation, which is devoid of any DNA contamination, was used in the subsequent experiments in our semiquantitative PCR. The isolated RNA was stored at −70°C until used.

Real-time quantitative RT-PCR

Gene expression were quantitated using real-time PCR. Relative abundance of each mRNA species was assessed using the SYBR green master mix from Stratagene (La Jolla, CA, USA) to perform real-time semiquantitative PCR using the ABI Prism 5700 instrument that detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches the threshold (defined as the threshold cycle number or C_T). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube. Relative mRNA species expression was quantitated and expressed as transcript accumulation index ($TAI=2^{-\text{delta delta CT}}$), calculated using the comparative C_T method (Shively et al. 2003). All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin. In addition, results on RNA from treated samples were normalized to results obtained on RNA from the control untreated sample.

FACS analysis

Flow cytometry using FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) was used to identify and quantify signal transduction molecules p38 and extracellularly

responsive kinases (ERK) MAPKs expressed by MDC. Phycoerythrin-conjugated monoclonal antibodies against p38 MAPK, ERK2, and an appropriately matched isotype control were obtained from BD Biosciences-Pharmingen (San Diego, CA, USA). Stained cells were subjected to light scatter analysis and a fixed population of cells were gated after quadrant markers were set, based on the isotype control. Cells positive for specific marker were expressed as a percentage of the total cells gated.

Western blot

MDCs were cultured with Meth (10–100 μM) for 72 h and the lysates were analyzed by Western blot (Coligan et al. 1991) using antibodies specific for both total and phosphorylated forms (only 10 to 30 min) of ERK2 and p38 MAPKs. The specific monoclonal antibodies were used as recommended by the manufacturer.

MAGI assay to measure HIV-1 infectivity

To observe the effects of Meth on HIV-1 infectivity in MDC, we used the multinuclear activation of a galactosidase indicator (MAGI) assay. This assay was intended to quantitatively measure virus infectivity (Chackerian et al. 1997). The MAGI cells are HeLa-derived cells stably transfected with CD4 and a reporter construct consisting of the β-galactosidase gene (which is modified to localize to the nucleus) driven by a truncated HIV-1 LTR. Expression of the β-galactosidase gene is Tat dependent such that an incoming virus must produce active Tat protein to drive expression of the reporter. MAGI cells (4×10^4 cells per well) were plated in a 24-well plate and were treated in duplicate with a 100-μl cell suspension of MDCs that were treated with Meth (10–100 μM) and subsequently infected with HIV-1 III virus. A total of 200 μl of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, and 300 μg/ml glutamine containing DEAE-Dextran at a concentration of 15 mg/ml was added to the MAGI cells and infected cells were incubated for 3 days at 37°C, 5%CO_2. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and blue cells were counted as infected cells.

p24 measurements

A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix, Buffalo, NY, USA) was used to quantitate p24 in culture supernatants.

Statistics

Data were analyzed by one-way analysis of variance (ANOVA), followed by Bonferroni multiple range post hoc comparisons where appropriate. Comparisons between two groups were conducted using Student's *t* test. Results were considered significant when *P* was <0.05, with a two-tailed test. Data analysis was performed with the Statistical Program (Primer for Biostatistics; McGraw Hill, New York City, NY, USA).

Results and discussion

Effect of Meth on HIV-1 infection by DC

We examined if Meth treatment could affect HIV-1 infectivity in vitro, as measured by quantitating the expression of the LTR-R/U5 region which represents early stages of reverse transcription of HIV-1. Our results (Fig. 1a) demonstrate that Meth at 10-μM (TAI=1.18±0.04, 18% increase, p=0.05) and 100-μM (TAI=1.57±0.085, 57% increase, p=0.015) concentrations significantly upregulated viral replication in MDC, compared to HIV-1-treated control cultures. However, Meth at 10 μM (TAI=1.54±0.12, 54% increase, p=0.003) and 100 μM (TAI=2.48±0.08, 148% increase, p=0.001) showed significantly

increased HIV-LTR-R/U5 gene expression in HIV-1-treated control cultures in IDC, compared to MDC.

We also measured HIV-1 infectivity using the MAGI assay that allows detection of HIV-1 after a single viral replication cycle in supernatant of infected Meth-treated MDC cultures. Our results show an increase in number of HIV-1-infected cells as represented by the increased number of blue cells with increasing concentrations of Meth. Percentage of HIV-1-infected cells at 25-, 50-, and 100-μM concentrations of Meth were 8% (p=0.08 [NS]), 12% (p=0.03), and 21% (p=0.015), respectively, as compared to the 3% in the HIV-1-infected Meth-untreated control (Fig. 1b).

HIV-1 p24 antigen levels in the culture supernatants were quantitated using a p24 ELISA kit (ZeptoMetrix Corporation, Buffalo, NY, USA) on day 15 postinfection. The assay had a minimum detectability of 10 pg/ml, according to manufacturer's guidelines. The p24 antigen level in 50-μM Meth-treated HIV-1-infected MDC was 7,600±3,005 (p=0.01) compared to the untreated control (1,100±520 pg/ml; Fig. 1c).

Kinetics and dose response effect of Meth on CXCR4 and CCR5 gene expression in MDC

Since our data showed that Meth facilitates HIV-1 infection of DC as measured by MAGI assay, p24 antigen, and LTR amplification, our next goal was to examine whether Meth could modulate the expression of HIV-1 entry coreceptors.

We examined the kinetics and dose response effect of Meth on CXCR4 and CCR5 expression using a quantitative real-time PCR. Granelli-Piperno et al. (1996) have shown that, in purified dendritic cells, HIV-1 infectivity occurs via interaction with multiple chemokine coreceptors. Our results (Fig. 2a) show a dose and time kinetics in MDC treated with Meth (10 and 100 μM) at 12, 24, and 48 h. Relative mRNA species expression was quantitated and expressed as transcript accumulation index (TAI=$2^{-\text{delta delta CT}}$), calculated using the comparative C_T method. Our results show that, at 12 h after Meth treatment, the CXCR4 gene expression at 100 μM (TAI=1.74, 74% increase) was increased as compared to the untreated control (TAI=1.0). At 24 h after Meth treatment, the CXCR4 gene expression at 100 μM (TAI=3.04, 204% increase) was increased as compared to the untreated control (TAI=1.0). Further at 48 h, Meth treatment at 100 μM (TAI=3.19, 219% increase) significantly increased CXCR4 gene expression as compared to the untreated control (TAI=1.0; Fig. 2a). Kinetic experiments demonstrate that at 24 and 48 h the CXCR4 gene expression was higher compared to 12 h. Data in Fig. 2b show a dose-dependent effect of Meth on CCR5 gene expression. Our results show that MDC treated with Meth for 24 h showed a significant dose-dependent increase in the CCR5 gene expression at 10 μM (TAI=1.11, 11% increase), 25 μM (TAI=1.68, 68% increase), 50 μM (TAI=2.88, 188% increase), and 100 μM (TAI=3.03, 203% increase) compared to the untreated control (TAI=1.0).

Signal transduction mechanisms that mediate Meth-induced effects on HIV-1 entry coreceptor expression

Signal transduction via MAPKs plays a significant role in cellular immune responses. The best-characterized subfamilies of the MAPK superfamily are the ERKs and the p38 MAPKs. Several studies implicate ERK–MAPK as the virion-associated kinases which regulate HIV-1 infectivity (Yang and Gabuzda 1999; Jacque et al. 1998; Popik et al. 1998). Thus, we examined whether Meth-induced modulation of HIV-1 infectivity via coreceptor modulation is mediated via virion-associated MAPK by real-time quantitative PCR. Our results (Fig. 3a) show that Meth significantly decreased ERK2 gene expression at 10-μM (TAI=0.55, 45% decrease, p=0.02) and 100-μM (TAI=0.43, 57% decrease, p=0.01) concentrations. However,

Meth significantly increased p38 gene expression at both 10 μM (TAI=1.38, 38% increase, p=0.04) and 100 μM (TAI=1.74, 74% increase, p=0.005; Fig. 3a). Since our gene expression data showed significant modulation of ERK2 and p38 MAPK, we investigated protein expression (level) and phosphorylation status (activity) of the extracellular-regulated kinase ERK2 and p38 MAPK, respectively. Meth treatment significantly decreased ERK2 protein expression (Fig. 3b) while reciprocally upregulated p38 MAPK protein expression at 10-μM (24%, p<0.05) and 100-μM (62%, p<0.01) concentrations, respectively (Fig. 3c).

Using FACS analysis, we further examined the effect of Meth on the phosphorylated form of ERK2 and p38 MAPK on MDC (Fig. 3di–iv). Our results show that Meth significantly downregulated (15.16%, p<0.01) the phenotypic expression of ERK2-positive MDC (Fig. 3di–ii) at 10 min of Meth treatment compared to untreated control culture (20.4%). Meth also significantly upregulated the percentage of MDC expressing the phosphorylated form of p38 MAPK (Fig. 3diii–iv) at 15 min (23.4%; p<0.05) and 30 min (24.34% p<0.05) of treatment as compared to the untreated control (20.8%). Thus, our Western blot and FACS analysis confirm our gene expression data.

Further, to confirm the participation of p38 MAPK pathways in the modulation of Meth-induced effects, we used a p38 MAPK inhibitor SB203580 for its potential ability to reverse the Meth-induced upregulation of CCR5 coreceptor gene expression by MDC. In these experiments (Fig. 3e), treatment of MDC with SB203580 significantly reversed Meth-induced upregulation of CCR5 gene expression (55%, p<0.05) compared to Meth-alone-treated cultures. These results suggest that Meth-induced upregulation of CCR5 coreceptor may be mediated through the activation of p38 MAPK.

Role of dopamine receptors in Meth modulation Of HIV-1 infectivity

Meth is known to exert its effects through interaction with dopamine receptors on cells, inducing the release of neurotransmitters and by inhibiting their uptake resulting in the increase of extracellular dopamine concentrations (Granelli-Piperno et al. 1996). In order to examine whether Meth-induced effects are mediated through the D1 receptor, we used a D1-receptor-specific siRNA (AF498961) to knock down the D1 receptor. The knockout of D1 receptor was validated by investigating the effect of D1-receptor-specific siRNA on the D1 receptor gene expression in siRNA-transfected mature dendritic cells (Fig. 4a). The results obtained show that D1-specific siRNA transfection (60 pM) inhibited the gene expression of D1 receptor (lane 3 TAI=0.3, p<0.03) compared to untransfected control (lane 1 TAI=1.0) or scrambled siRNA-transfected cultures (lane 5 TAI=1.01). Further, D1 siRNA-transfected cultures treated with Meth (100 μM) produced significantly lower level of D1 receptor gene expression (lane 4 TAI=0.32, p<0.01) compared to untransfected DCs treated with Meth (100 μM; lane 2, TAI=1.65). Additionally, to evaluate whether Meth-induced upregulation of HIV-1 infectivity and coreceptor modulation are mediated through dopamine receptor D1 and D2, we investigated the effect of siRNA directed against D1R and the use of a D2R antagonist for their potential effect to reverse Meth-induced upregulation of CCR5 gene expression. The data presented in Fig. 4b show that MDC treated with Meth (100 μM) significantly upregulated CCR5 gene expression (lane 2; TAI=2.32, p<0.003) compared to the untransfected control culture (lane 1; TAI=1.0) or scrambled siRNA-transfected culture (lane 6; TAI=1.05). However, MDC transfected with D1R-specific siRNA and treated with Meth completely reversed Meth-induced CCR5 gene upregulation (lane 3; TAI=0.85; p<0.03) compared to nontransfected Meth-treated control (lane 2, TAI=2.32). Data presented in Fig. 4c show the effects of D2 receptor antagonist, RS ± sulpiride on Meth-induced upregulation of CCR5. Thus, studies on siRNA against D1R and antagonist against D2R suggest that both D1 and D2 receptors are involved in Meth-mediated effects on CCR5 gene expression. The reversal of the Meth-induced upregulation of the HIV-1 coreceptor CCR5 by both the D1 receptor antagonist (SCH23390) and the D2 receptor antagonist (RS ±

sulpiride) suggest the specificity of the Meth-induced effects. The dopamine agonist SKF-82958 has previously shown to produce similar effects in a rat model, suggesting dopaminergic involvement (Munzar and Goldberg 2000). In our study also, SKF-82958 also produced identical results as Meth (data not presented).

Our study is the first report that the drug of abuse Meth enhances HIV-1 infectivity in human monocyte-derived DC and the mechanisms of these effects may be by upregulating HIV-1 coreceptors. Gavrilin et al. (2002) have shown that Meth induced the upregulation of the CXCR4 receptor in feline immunodeficiency virus astrocytes. Further, we have shown the ERK2 and p38 MAPK pathways that are involved in these Meth-mediated effects. Additionally, our data suggest that both dopaminergic D1 and D2 are associated with Meth-induced modulation of HIV-1 infectivity and coreceptor modulation. These studies provide important information on fundamental aspects of Meth and HIV-1 transmission and pathogenesis and may help to develop novel therapeutic and preventative strategies using HIV-1 coreceptors or dopamine receptor antagonists, which will be beneficial in treating Meth-abusing HIV-1-infected population.

Acknowledgments

This work was supported in part by the National Institute on Drug Abuse Grants RO1-DA012366, RO1-DA014218, RO1-DA015628, RO1-DA021537, and RO1AA017405.

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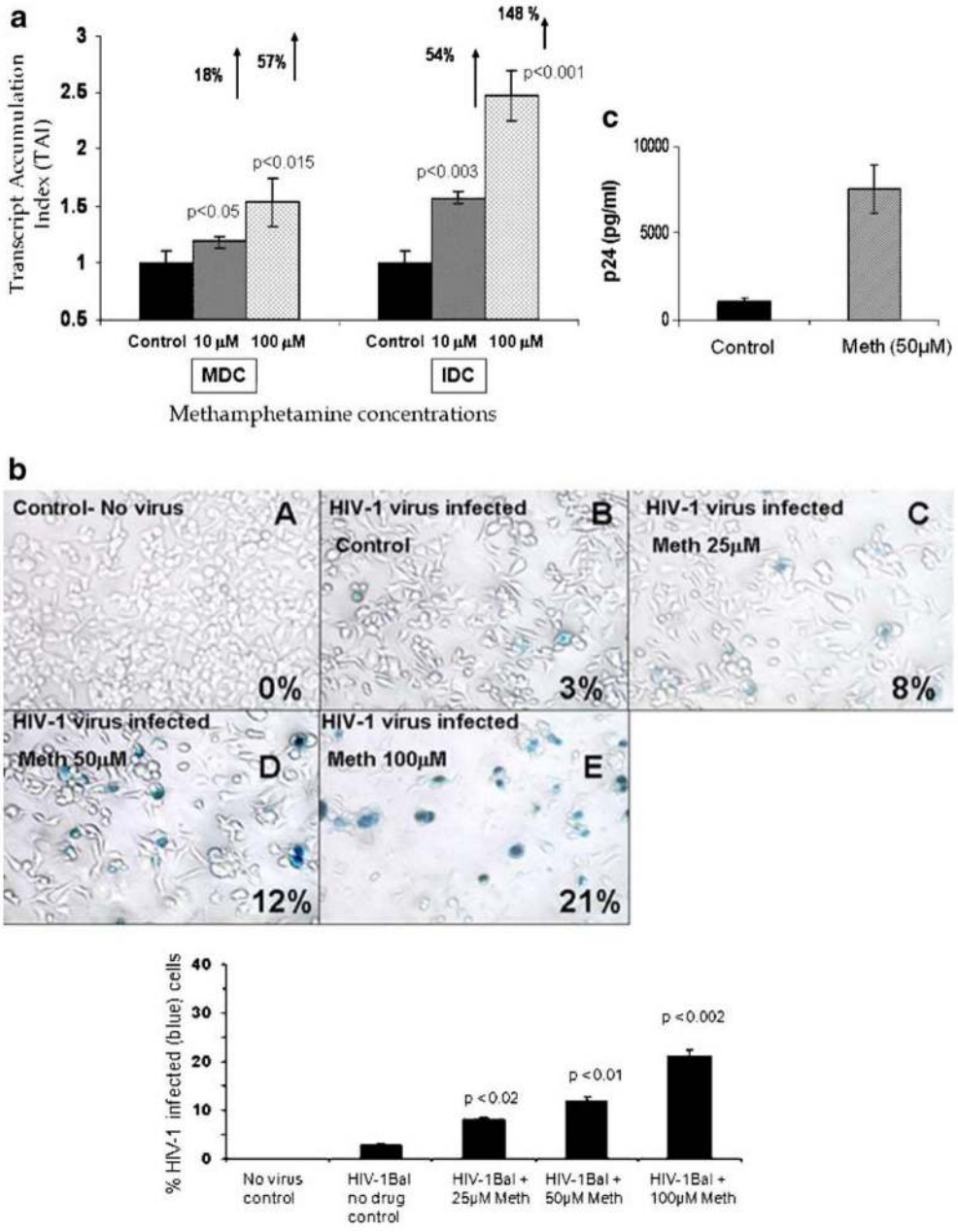


Fig. 1.

a *Meth enhances HIV-1 replication*. MDC and IDC (5×10^5 cells per milliliter) were infected with native HIV-1 IIIB [X4] (NIH AIDS Research and Reference Reagent Program Cat# 398) at a concentration of $10^{3.0}$ $TCID_{50}$ per milliliter cells overnight and washed three times with Hank's balanced salt solution (GIBCO-BRL, Grand Island, NY, USA) before being returned to culture with and without Meth (10 and 100 μM) for 24 h. The RNA was extracted, reverse-transcribed, and followed by quantitative real-time PCR against the LTR-RU5 and the housekeeping gene, β-actin, and the 18S RNA primers as internal controls. The data represent mean±SD of three independent experiments. Statistical analysis was done using Student's *t* test. **b** *Meth enhances HIV-1 infectivity as measured by MAGI assay*. The MAGI cells are HeLa-derived cells stably transfected with CD4 and a reporter construct consisting of the β-galactosidase gene (which is modified to localize to the nucleus) driven

by a truncated HIV-1 LTR. Expression of the β-galactosidase gene is Tat dependent such that an incoming virus must produce active Tat protein to drive expression of the reporter. HIV-1-infected cells stained blue with the X-Gal dye. MAGI cells (4×10^4 cells per well) were plated in a 24-well plate and were treated in duplicate with a 100-μl supernatants from MDCs that have been infected with HIV-1 IIIB virus and treated with or without Meth. A total of 200 μl of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, and 300 μg/ml glutamine containing DEAE-DEXTRAN at a concentration of 15 mg/ml was added to the MAGI cells and infected cells were incubated for 3 days at 37°C, 5%CO_2. Cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) and blue cells were counted as infected cells. The graph represents the percentage of infected cells in each treatment group as observed using a ×20 inverted Nikon microscope. **c** *Meth enhances p24 production*. MDCs (5×10^5 cells per milliliter) from normal subjects were infected with native HIV-1 IIIB (NIH AIDS Research and Reference Reagent Program Cat# 398) at a concentration of $10^{3.0}$ $TCID_{50}$ per milliliter cells overnight and washed three times with Hank's balanced salt solution (GIBCO-BRL, Grand Island, NY, USA) before being returned to culture with and without Meth (50 μM) for 14 days. The culture supernatants were quantitated for p24 antigen using a p24 ELISA kit (ZeptoMetrix Corporation, Buffalo, NY, USA). The data represent the mean±SD of three independent experiments and are expressed as picogram per milliliter. Statistical analysis was done using Student's *t* test

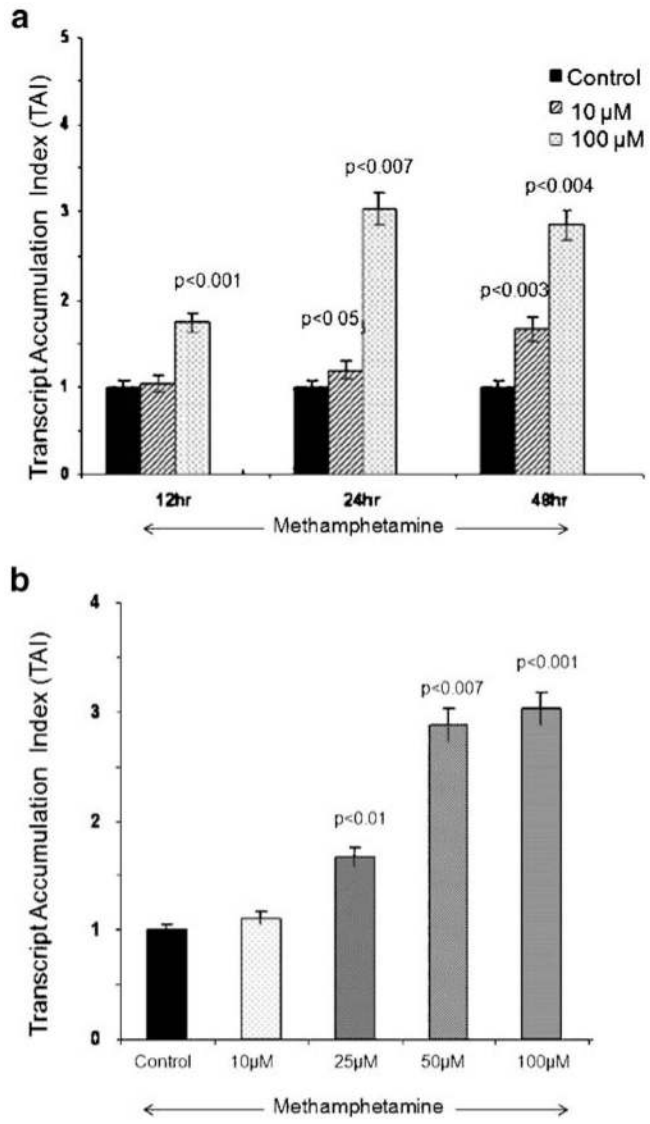
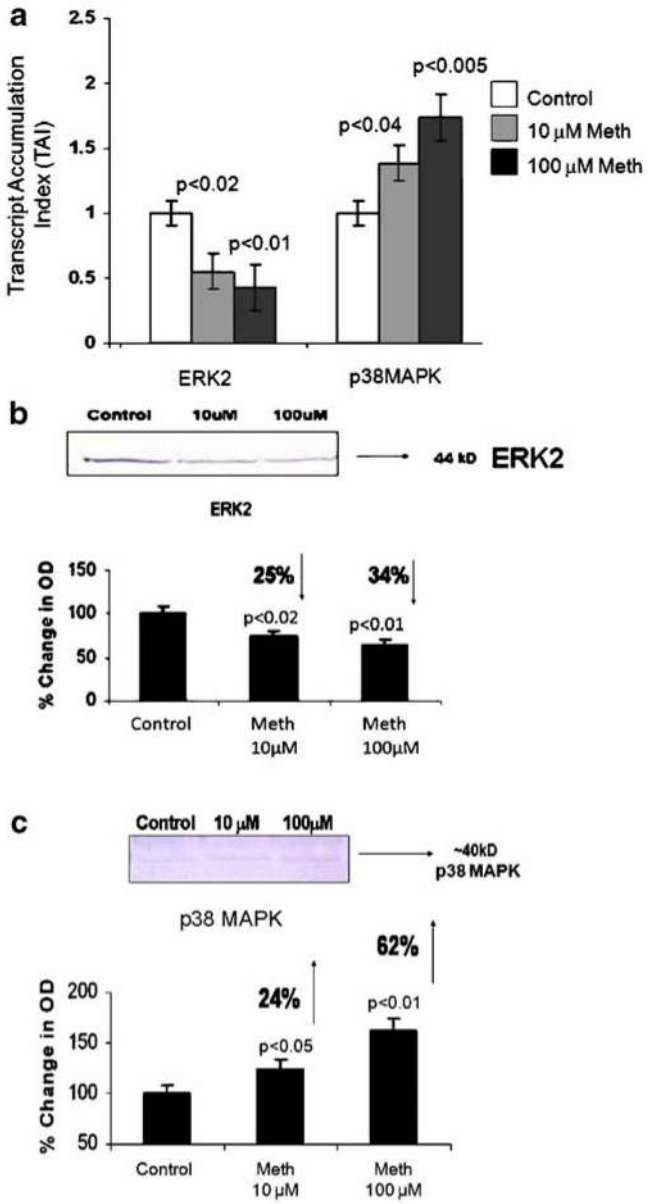


Fig. 2.

a *Meth upregulates CXCR4 gene expression.* MDCs (5×10^5 cells per milliliter) were cultured with Meth (10–100 μM) for 12–48 h; RNA was extracted, reverse-transcribed, and PCR-amplified against a CXCR4 primer using quantitative real-time PCR. The data represent mean±SD of three independent experiments. Statistical analysis was done using Student's *t* test. **b** *Meth upregulates CCR5 gene expression.* MDCs (5×10^5 cells per milliliter) were cultured with Meth (10–100 μM) for 24 h; RNA was extracted, reverse-transcribed, and PCR-amplified against a CCR5 primer using quantitative real-time PCR. The data represent mean±SD of three independent experiments. Statistical analysis was done using Student's *t* test



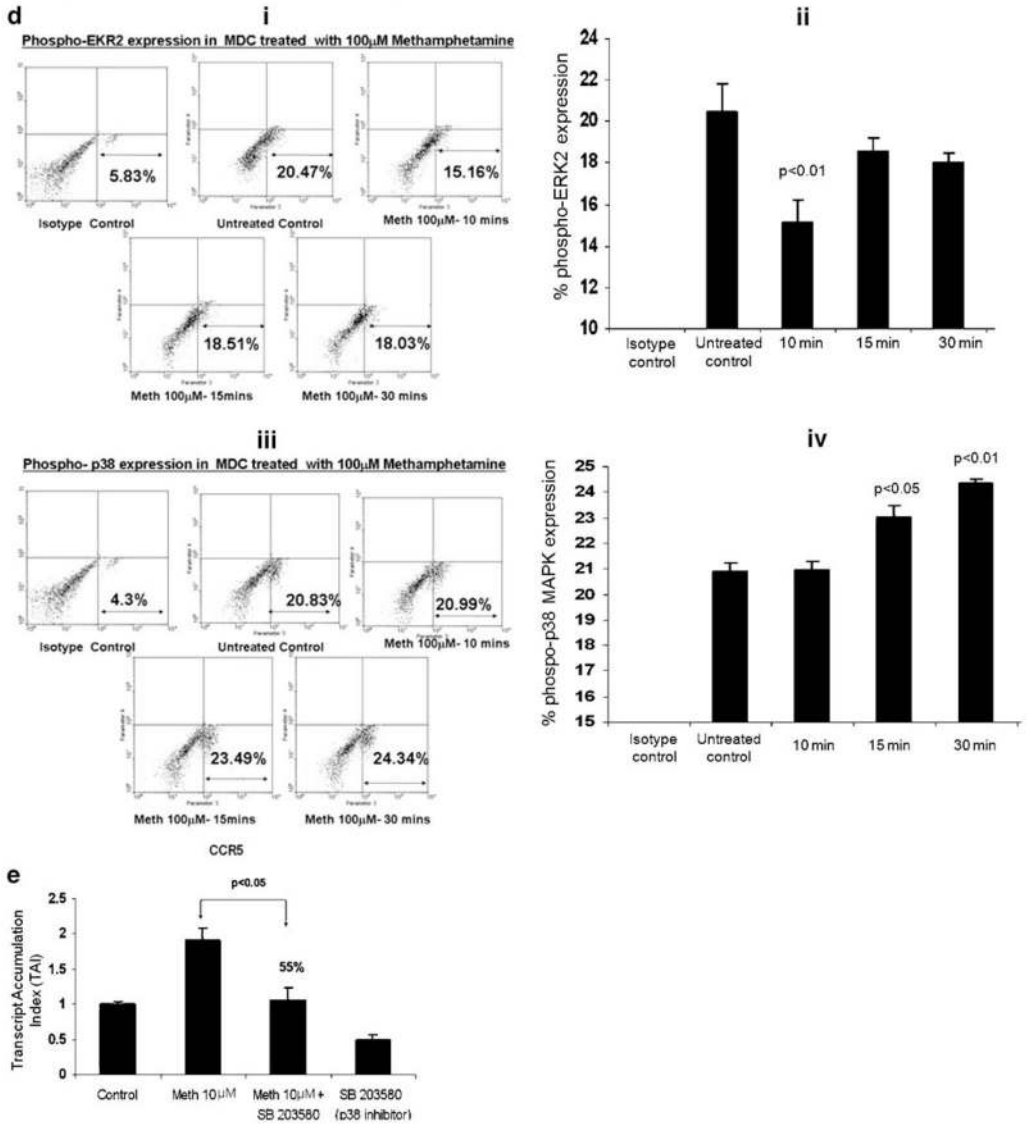


Fig. 3.

a *Meth differentially regulates signal transduction molecules in MDC.* MDCs (5×10^5 cells per milliliter) were cultured for 24 h with and without Meth (10 and 100 μM); RNA was extracted, reverse-transcribed, and QPCR-amplified against various signal transduction molecules ERK2 and p38 MAPK. The data represent mean±SD of three independent experiments. Statistical significance was determined by Student's *t* test. **b** *Meth suppresses ERK2 protein expression in MDC.* MDCs were cultured with Meth (10 and 100 μM) for 72 h; proteins were extracted and ERK2 expression was detected by Western blot analysis using an ERK2-specific antibody (Santacruz Biotech Inc). ERK2 migrates as a 44-kDa band on a 4–20% SDS-PAGE. The graphical representation is of the densitometric quantitation of the protein signal expressed as percent change in OD with respect to the untreated control (n=2). Statistical significance was determined by Student's *t* test. **c** *Meth increased p38 MAPK protein expression in MDC.* MDCs were cultured with Meth (100 μM) for 72 h; proteins were extracted and p38 expression was detected by Western blot analysis using a p38 MAPK-specific antibody (Santacruz Biotech Inc). p38 migrates as a 40-kDa band on a 4–20% SDS-PAGE. The graphical representation is of the densitometric quantitation of the

protein signal expressed as percent change in OD with respect to the untreated control (n=2). Statistical significance was determined by Student's t test. **d** (*i–iv*) *Effect of Meth on the phosphorylation of p38 MAPK and ERK2*. MDCs (~5×10^5 cell per milliliter) were treated with Meth (100 μM) for 10, 15, and 30 min following FACS analysis which was done to determine the percentage of cells expressing phosphorylated ERK2 and p38 MAPK. Statistical significance was calculated by Student's t test. The FACS plots are from a representative experiment, while the histogram is a graphical representation of three independent FACS experiments. **e** *p38 MAPK inhibitor reverses Meth-induced upregulation of CCR5 gene expression*. MDCs (5×10^5 cells per milliliter) were cultured alone or with Meth (10 μM) or the p38 MAPK inhibitor (SB20358; 10 μM) alone and in combination with Meth (10 μM) plus inhibitor (SB20358; 10 μM) for 24 h and CCR5-specific mRNA expression was quantitated using real-time PCR. The data represent mean±SD of two independent experiments. Statistical analysis was done using ANOVA

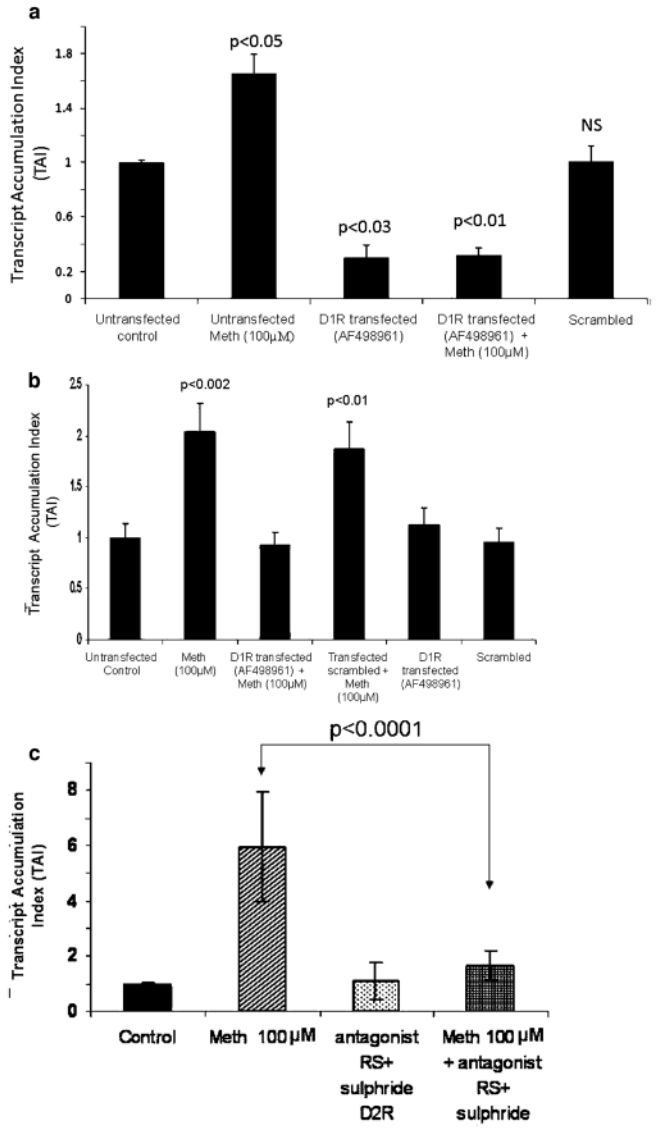


Fig. 4.

a *Effects of D1R-specific siRNA* (*AF498961*) *on D1 receptor gene expression in siRNA-transfected MDC*. siRNA against the D1 receptor was designed using siRNA design software available on the Invitrogen Web site. The D1 receptor gene (accession no. AF498961) siRNA sequences were as follows, AAGUUGGUCACCUUGGACC[dt][dt], and transfection was done using Lipofectamine reagent. Our results show that D1R siRNA transfection (60 pM) was transient and silencing of D1R gene was evident at 24 h (85% inhibition of D1R gene expression) posttransfection. Data represent mean±SD of three separate experiments and statistical analysis was done using Student's *t* test (n=3). **b** *Effects of D1R-specific siRNA on CCR5 gene expression in siRNA-transfected MDC*. MDCs were transfected with siRNA using Lipofectamine reagent. Our results show that Meth-treated D1R siRNA-transfected cells showed a significant reduction in CCR5 gene expression indicating that the effect of Meth may be mediated via the D1 receptor. Data represent mean ±SD of two separate experiments and statistical analysis was done using ANOVA. **c** *D2 receptor antagonist reverses Meth-induced upregulation of CCR5 gene expression.* MDCs were cultured with and without the D2 receptor antagonist RS ± sulpiride (100 μM) alone

and in combination with Meth (10 μM) for 24 h and the CCR5-specific mRNA expression was quantitated using real-time PCR. The data represent mean±SD of three independent experiments. Statistical analysis was done using Student's *t* test