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Methamphetamine Inhibits β -Chemokines and Co-Stimulatory Molecule Expression by Dendritic Cells

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Abstract: The US is currently experiencing a serious epidemic of methamphetamine (Meth) use entangled with HIV-1 infection. Blood monocyte derived dendritic cells (DC) are the first line of defense against HIV-1 infection and are the initial target of HIV-1 in injection drug users. Chemokines are known to be HIV-1 suppressing molecules and are positively associated with non- progression of HIV disease. Co-stimulatory molecules are necessary for DC maturation, effective antigen presentation, cell migration, and T cell proliferation. Although previous studies suggest that Meth deregulates various immune responses, the role of Meth on gene expression and production of β -chemokines and co-stimulatory molecules by DC has not been studied. We hypothesize that Meth induced immune defects may be mediated by dysregulation of β -chemokines (MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5), co-stimulatory and maturation molecules (CD83 and CCR7) by DC. Our results show that Meth significantly downregulates the gene expression and production of β -chemokines and co stimulatory molecule by DC from normal subjects. In HIV-1 infected subjects, RANTES variant In1.1c that has been associated with accelerated HIV-1 disease progression was significantly higher compared to normal controls. Further, Meth significantly inhibited total RANTES gene expression with a reciprocal upregulation of RANTES variant In1.1c in a dose dependent manner by both immature DC (IDC) and mature DC (MDC) from normal subjects. These studies report for the first time that Meth deregulates β -chemokines and co-stimulatory molecule expression by DC. The results emanating from these studies may help to support the therapeutic application of chemokines to restore anti-HIV-1 immune responses to prevent or control HIV-1 infection in meth using populations.

Keywords: DC-SIGN,dendritic cell(DC) specific C type ICAM-3 grabbing nonintegrin,RANTES;regulated upon activation T-cell expressed and secreted;IDC immature DC; MDC,mature DC

INTRODUCTION

The US is currently experiencing a grave epidemic of methamphetamine (Meth) use as a recreational drug [1]. US Drug Enforcement Administration reports that as of May 2006, Meth use has surpassed cocaine and heroin use combined as a street or club drug. DC are the first line of defense against HIV-1 infection and are the first cellular components in the immune system to recognize any infection including HIV-1 [2-4]. Immature DC (IDC) are potent antigen presenting cells that engulf microbes and antigens. Upon interaction with antigens, DC become activated or mature and then migrate to regional lymphoid tissues where the processed antigens are presented to naive CD4 T cells subsequently

enabling T cell activation [5-6]. After further maturation, DC express a number of co stimulatory molecules such as CD40, CD80 (B7.1), CD86 (B7.2) and chemokine receptor, CCR7 that are required for efficient antigen presentation by DC. The important role of DC as the first line of defense against HIV-1 infections has recently been recognized. Defective expression or modulation of chemokines and co stimulatory molecules by DC may cause defective antigen presentation that can then lead to defective activation of HIV-1 specific T cells. Chemokines have gained major attention recently because of their specific inhibitory effects on HIV-1 infection [7-8]. β -chemokines such as CCL3 (MIP-1 α), CCL4 (MIP-1 β), and CCL5 (RANTES) are

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natural ligands for the primary human immunodeficiency virus type-1(HIV-1) co receptor CCR5 and are known to block HIV-CCR5 interactions [9]. Decreased production of these chemokines may abrogate anti-HIV-1 effects facilitating HIV-1 infection. Although drugs of abuse are known to affect the immune response in general [10-11] and specifically reduce immune responses against HIV-1 infections [12], the role of Meth on gene expression and production of beta chemokines and co stimulatory molecules by DC from healthy, normal subjects has not been elucidated. Previous studies have shown that blood monocyte derived dendritic cells (MDDC) serve as an effective model to study HIV-1 infection in injection drug users [13-23]. Previous studies show that polymorphism in RANTES chemokine affects HIV-1 disease progression [24]. RANTES-28G mutation increases RANTES expression and delays the progression of the HIV-1 disease [24], while RANTES In1.1C accelerates HIV-1 disease progression [25]. P. An *et al* [26] showed that diminished transcription of RANTES by In1.1C regulatory allele leads to accelerated disease progression. DC produce a number of cytokines and chemokines that are essential for T cell differentiation and clonal expansion [27-28]. While the capacity of DC to migrate is regulated by the expression of a number of cytokines and chemokines, further activation is regulated by various co stimulatory molecules. The co-stimulatory molecule, CD83 which is the hallmark of mature DC (MDC) play a significant role in antigen presentation, stimulation of T cells and anti-HIV-1 immune responses. DC change their phenotypes and functional properties during maturation [29-30,31,32] and CC-chemokine receptor-7(CCR7) is considered as a maturation marker for DC and is critically important for migration of DC to lymph nodes, in vivo function of T regulatory cells [33-34]. β -chemokines have gained major attention recently because of their specific inhibitory effects on HIV-1 infection [7-8]. The present study reports for the first time that Meth significantly inhibits HIV-1 suppressing β -chemokines with a reciprocal upregulation of RANTES variant In1.1c. Further, Meth downregulates maturation molecule, CCR7 and co stimulatory molecule, CD83 in DC.

MATERIALS AND METHODS

Human Subjects: Blood donors were evaluated of this study and consents were obtained consistent with the policies of the National Institutes of Health. Peripheral blood samples from healthy individuals were drawn into a syringe containing heparin (20 units/ml). DC were isolated from peripheral blood mononuclear cells (PBMC) using protocols validated by Dauer *et al*

[35]. Briefly, PBMC were separated on a density gradient and adhered to plastic culture plates in media containing serum. Non-adherent cells were removed after 1 hr at 37°C and adherent cells 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). After 6 days of culture, IDC were removed by gently swirling the plate to resuspend them for use in the experiments. These IDC were allowed to progress to MDC by incubating for five more days with media containing 1000 U/ml of rhGM-CSF and 1000 U/ml of IL-4 as above.

FACS analysis: Phenotypic characterization of IDC and MDC was done by flow cytometry analysis. Both IDC and MDC were washed in FACS buffer (e-Bioscience, San Diego, CA), incubated with nonspecific IgG (20 μ g/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, MDC predominantly express CD83 and CCR7 as described [33-34].

Cell culture: IDC and MDC were cultured at a concentration of 5×10^5 cells/ml with Meth (10 μ M to 100 μ M) in 6 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 ~90%, and it is predominantly *d*-methamphetamine which has greater CNS potency. The concentration of Meth that we have used in the experiments (10-100mM) were within the range that are similar to levels found in the blood, urine or tissue fluid samples of Meth using subjects (11-147mM) [36-37].

RNA extraction: Cytoplasmic RNA was extracted by an acid guanidinium-thiocyanate-phenol-chloroform method as described [38]. Cultured DC were centrifuged and resuspended in a 4M solution of guanidinium thiocyanate. Cells were lysed by repeated pipetting and then phenol-chloroform extracted 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 hr 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 (DEPC) water and the amount of RNA determined using a spectrophotometer at 260 nm. DNA contamination in

the RNA preparation was removed by treating the RNA preparation with DNase (1 IU/ μ g of RNA) for 2 hr at 37°C, followed by proteinase K digestion at 37°C for 15 min and subsequent extraction with phenol/chloroform and NH₄OAc/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 real time PCR. The isolated RNA was stored at -70°C until used.

Real Time, Quantitative RT-PCR: Gene expression was quantitated using real time PCR. Relative abundance of each mRNA species was assessed using the SYBR green master mix from Stratagene (La Jolla, CA) to perform real time semi-quantitative 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^{-delta delta C_T}), calculated using the comparative C_T method [39]. 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.

RESULTS

Meth suppresses the expression of co-stimulatory molecule, CD83 and the maturation marker, CCR7 on DC: Since the co stimulatory molecule CD83, a hallmark of MDC, plays a significant role in antigen presentation, stimulation of T cells and anti-HIV-1 immune responses, we examined the in vitro effect of Meth on the expression of the CD83 molecule on DC by flow cytometry analysis. Data presented in Fig. 1 show that Meth significantly downregulated the expression of CD83 on MDC. The percentage positive cells expressing CD83 at Meth concentrations of 10 and

100 μ M, respectively were 11.3 (p<0.03) and 9.8% (p<0.009) compared to 16.9% in the untreated control culture.

DC change their phenotypes and functional properties during maturation [29-30, 31, 32]. Since CCR7 is a maturation marker for DC, which is critically important for migration of DC to lymph nodes and for in vivo function of T regulatory cells [33-34], we examined the effect of Meth on CCR7 expression by MDC (Fig. 2a). MDC treated in vitro with Meth at a concentration of 100 μ M showed significantly decreased numbers of CCR7 positive MDC (2.6%, p<0.05) compared to 6.9% in the untreated control culture. However, MDC treated with a lower concentration of Meth (10 μ M) did not show any significant change in the phenotypic expression of CCR7 positive cells (5.7%, p<0.06) compared to control culture (6.9%). To rule out the possibility that the immunomodulatory effects induced by Meth were non specific; we examined the effects of Meth (10-100 μ M) on viability as well as on apoptosis by DC. Our results showed that Meth (10-100 μ M) did not induce apoptosis in DC as accessed by Annexin V expression by FACS analysis (2b) as well as evidenced by > 95% viability by trypan blue dye exclusion (data not presented). These results indicate that Meth use decreases the percentage of co-stimulatory molecule positive MDC in HIV-1 infected subjects and these cells likely play a significant role in the progression of HIV-1 disease by preventing the maturation of IDC to MDC.

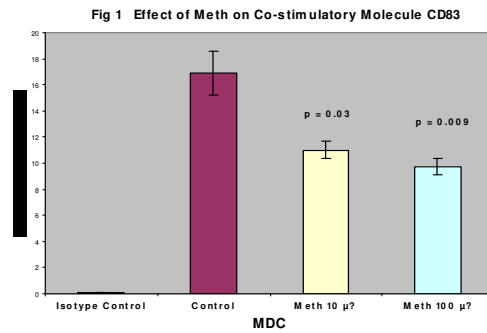


Fig. 1: Meth decreases the expression of co stimulatory molecule, CD83 on MDC. Normal MDC cultures (~ 5X10⁵ cell/ml) were treated with Meth for 24 hr following which FACS analysis was done to determine the percentage of MDC expressing co stimulatory molecule, CD83. Statistical significance was calculated by Students' 't'-test (n=3).

Effect of Meth on RANTES and its variant gene expression in IDCs and MDCs: Since β -chemokines,

the natural ligands for HIV-1 coreceptors are also produced by DC and are known to play a significant role in the prevention of HIV-1 infection [7-8], we investigated whether Meth treatment modulates the gene expression and production of HIV-1 protective β -chemokines, CCL3 (MIP-1 α), CCL4 (MIP-1 β) and CCL5 (RANTES). Data presented in Fig. 3 show a dose response effect of Meth on CCL3 (MIP-1 α) and CCL4 (MIP-1 β) gene expression by MDC. Meth at 10 (TAI = 1.0, p=NS), 25 (TAI = 0.8, p=NS), 50 (TAI = 0.6, p<0.01, 35% decrease), and 100 μ M (TAI = 0.5, p<0.01, 44% decrease) show a dose dependent decrease of CCL3 gene expression compared to the untreated control (TAI = 1.0). Similarly, Meth at 10 (TAI = 1.0, p=NS), 25 (TAI = 0.78, p=0.03, 22% decrease), 50 (TAI = 0.70, p<0.03, 30% decrease), and 100 μ M (TAI = 0.53, p<0.01, 47% decrease)

show a dose dependent inhibition of CCL4 gene expression compared to the untreated control (TAI = 1.0). These results demonstrate selective inhibition of CCL3 and CCL4 genes and rule out the possibility of non-specific toxic effects as expression of the housekeeping gene was not affected by Meth. Further Meth at 10-100 μ M did not induce apoptosis by DC as assessed by Annexin V expression by FACS analysis (Fig. 2b) as well as evidenced by > 95% viability by trypan blue dye exclusion (results not provided).

Since previous studies show that a RANTES (CCL5) variant, In1.1c, has been associated with accelerated HIV-1 disease progression [26,40,24], we examined the in vitro effect of Meth on total RANTES and its variant, In1.1c, gene expression by both IDC and MDC. Data presented in Fig. 4 show that Meth significantly inhibited total RANTES gene expression in a dose dependent manner by both IDC and MDC; whereas RANTES variant In1.1c was significantly upregulated in a dose dependent manner by both IDC and MDC. Using real time PCR, we also quantified the gene expression of total RANTES and its variant In1.1c by MDC from 3 normal subjects and age and sex matched 3 HIV-1 infected subjects (Fig. 5a and 5b). Results (Fig. 5c) show that in HIV-1 infected subjects, the mean RANTES gene expression (TAI = 3.1, p<0.001) was significantly lower (40% suppression) than the normal controls (TAI = 5.2) while the RANTES variant In1.1c gene expression was significantly higher (147% increase) in the HIV-1 infected subjects (TAI = 7.3, p<0.003) compared to the normal controls (TAI = 2.9). Data presented in Fig. 6 show that Meth significantly suppressed the endogenous production of CCL4 and CCL5 by normal MDC in a dose dependent manner.

Fig. 2a Effect of Meth on a DC Maturation Marker, CCR7

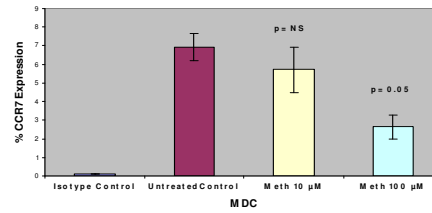


Fig. 2a: Meth inhibits MDC maturation by decreasing the expression of CCR7. 2a: MDC (~ 5X10⁵ cell/ml) were treated with Meth for 24 hr following which FACS analysis was done to determine the percentage of cells expressing CCR7. Statistical significance was calculated by Students' 't'-test (n=3).

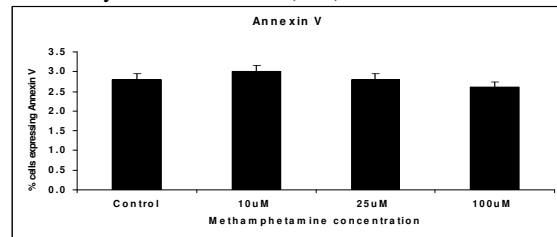


Fig.2b: Effect of Methamphetamine on cell viability using Annexin V staining. 2b: Cells are stained with PE (Phycoerythrin) labeled Annexin V and percentage of cells stained with Annexin V are analyzed using Flow cytometric analysis. The percentage of cells expressing annexin V for the untreated control, 10, 25 and 100 μ M methamphetamine treated cultures are 2.8+ 0.36, 3.0+ 0.10, 2.76+ 0.35, and 2.56+ 0.15 respectively. These data are mean + SD from 3 separate experiments.

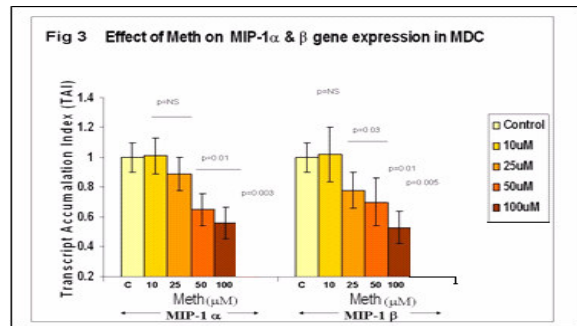


Fig.3: Meth down regulates MIP-1 α and β gene expression in MDC. MDC (~ 5X10⁵ cell/ml) were cultured with and without Meth (10- 100 μ M) for 24 hr, RNA was extracted and reverse transcribed followed by quantitative real time PCR for MIP-1 α and MIP-1 β and housekeeping genes (β -actin) primers. The data represents the mean + SD of 3 independent experiments. Statistical significances between controls and treated samples was calculated by Students' 't'-test (n=3).

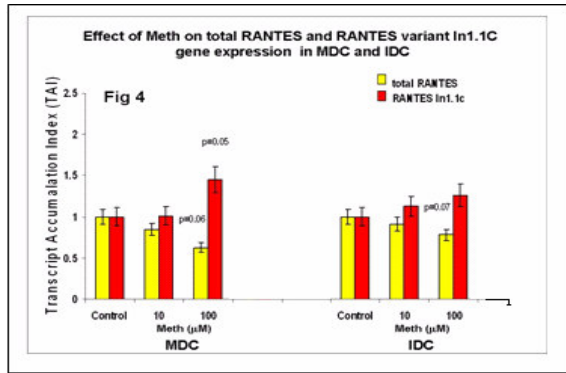


Fig. 4 Meth downregulates total RANTES and upregulates RANTES variant In1.1c gene expression in IDC and MDC. MDC and IDC from normal subjects (~ 5X10⁵ cell/ml) were cultured with Meth (10 & 100 µM) for 24 hr, RNA was extracted and reverse transcribed followed by quantitative real time PCR for total RANTES and RANTES variant In1.1c and house keeping gene β-actin. The data represents the mean + SD of 3 independent experiments. Statistical significance was calculated by Students' 't'-test (n=3).

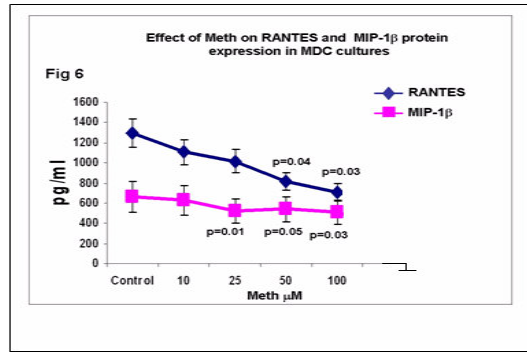


Fig. 6: Meth suppresses endogenous production of RANTES and MIP-1β. MDC from normal subjects were cultured with Meth (10-100 µM) for 72 hr and the RANTES and MIP-1β levels in the culture supernatants were measured by ELISA (n=3) (Biosource Inc, CA). The data represents the mean + SD of 3 independent experiments. Statistical significance was determined by Students' 't'- test.

DISCUSSION

US is currently experiencing a grave epidemic of Meth use as a recreational drug and recent studies show a high prevalence of HIV-1 infection among Meth users [41-45]. The important role of DC as the first line of defense against HIV-1 infection has recently been recognized. β-chemokines have gained major attention recently because of their specific inhibitory effects on HIV-1 infection [7-8]. Previous studies show that DC produce a number of cytokines and chemokines that are essential for T cell differentiation and clonal expansion [27-28]. While the capacity of DC to migrate is regulated by the expression of a number of cytokines and chemokines, further activation is regulated by various co stimulatory molecules. Our previous studies show that the number of MDC expressing co stimulatory molecules (CD86, CD 83 and CD80) is significantly lower in polydrug using HIV-1 infected patients compared to non-using HIV-1 infected subjects [46]. In the present report we demonstrate that Meth downregulates the expression of co stimulatory molecule, CD83 (Fig. 1) and the DC maturation marker, CCR7 (Fig. 2) by DC. Since β-chemokines are natural ligands for HIV-1 co-receptors and play significant role in blocking of HIV-1 infection, our finding that Meth downregulates the HIV-1 suppressing chemokines by DC further support the premise that increased HIV-disease progression in Meth using patients may be via down regulation of these protective chemokines. Further HIV-1 infected patients demonstrate decreased gene expression of the HIV-1 suppressing chemokine

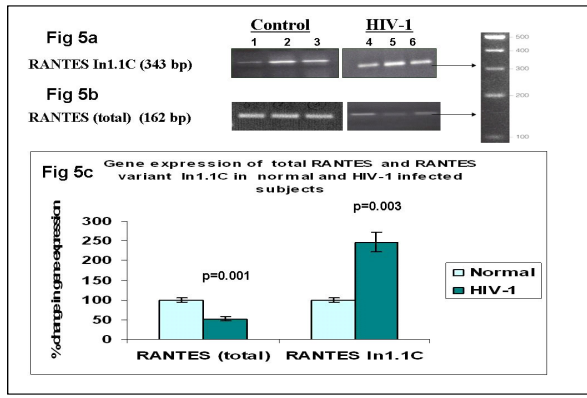


Fig. 5: HIV-1 seropositive patients demonstrate a lower level of total RANTES and a higher level of RANTES variant In1.1c. RNA from the MDC from normal subjects and HIV-1 patients were reverse transcribed followed by quantitative real time PCR against total RANTES and RANTES variant In1.1c primers. The amplified QPCR products were electrophorised on a 1.2% agarose gel containing ethidium bromide to confirm the size of the RANTES variant In1.1c and total RANTES as shown in Fig. 5a and 5b respectively; Lanes 1-3 are normal controls and Lanes 4-6 are HIV-1 infected subjects from a representative experiment. Fig. 5c Shows the mean + SD of data from 9 different subjects. Statistical significance between the expression levels in HIV-1 and normal controls was calculated by Students' 't'-test (n=9).

RANTES, while a genetic variant of RANTES, In1.1C, which is reported to be associated with accelerated disease progression [26,40,24], has been found to be elevated (Fig. 5) compared to control subjects. In our in vitro model using both IDC and MDC, Meth also showed significant inhibition of β -chemokines and a reciprocal upregulation of RANTES variant.

Based on these findings, it is possible that, Meth use by HIV-1 positive patients may suppress costimulatory molecules, DC maturation markers and the production of HIV-1 suppressing chemokines with a reciprocal upregulation of RANTES variant, all of which may lead to progression of HIV-1 disease. The findings that Meth inhibits HIV-1 suppressing chemokines such as CCL3, CCL4, and CCL5 will help to support the therapeutic application of these chemokines to restore anti-HIV-1 immune responses to control HIV-1 infection. These include the replacement of chemokines and co stimulatory molecules downregulated by Meth with recombinant products and use of antisense oligonucleotides and siRNA against RANTES variant. Thus our studies may help to develop novel strategies for preventing and treating HIV-1 infections particularly in high risk populations such as Meth users.

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